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The influence of the MTHFR C677T genotype and folate status on genomic DNA methylation and uracil misincorporation in the colon of subjects without colorectal neoplasia

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*The influence of the MTHFR C677T genotype and folate status
on genomic DNA methylation and uracil misincorporation in
the colon of subjects without colorectal neoplasia*

A thesis submitted to the University of London for the degree of
Doctor of Philosophy in the Faculty of Science

By Joanna Hanks

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January 2017

To Elizabeth Ann Kennedy and Bernard Joseph Kennedy

ACKNOWLEDGEMENTS

Thank you to the large number of subjects whom I am indebted to for their generosity. You were all kind enough to listen to a lengthy and wordy explanation of this study design. Thank you for considering the research worthwhile, agreeing to share all of the required demographic data, and for donating blood and tissue samples during a stressful clinical procedure

Thank you to my husband Mark Hanks for his unconditional support and patience during this time, for repeatedly telling me this day would arrive, and above all his humour which distracted me through the darker times. To my mother Elizabeth for always believing in me and praising me at the most important moments, for encouraging me to aim higher at every step of my education and for putting herself last, always, in order to provide so many opportunities for me, to my father Bernard for making me question everything and always questioning me, for his everlasting influence, and for making me always tempted by the more interesting choices however difficult they may be. I'm extremely grateful to my uncle Dr Peter Kennedy for being a lasting inspiration and introducing me to the fascination of the medical world, in particular cancer research, the work experiences he arranged for me at 14 ultimately led to a career I love today. My sincere gratitude is reserved for my uncle Paul for being behind this work, the greatest supporter of my subject material and for giving such a large part of himself. Thank you to oncologists and great friends Dr Paul Kennedy Stockman and Dr Martina Puglisi for making medical science fun again after the novelty had worn off temporarily.

Great thanks go to Professor Peter Emery for agreeing to supervise my PhD, for his clarity, ideas, advice, and endless patience throughout this protracted period. Thank you to Dr Maria Pufulete for her excellent study design and for her advice as second supervisor.

Thank you also to Professors Tom Sanders, Andrew Leather, Reyad Al-Ghnaniem, Jeremy Sanderson, and to Professor Paul Sharp for giving me an invaluable piece of advice which I wish I had taken. Thank you to Dr Guy Chung-Faye, Dr Gareth Parkes, Mr Neil Kukreja, Lysetta Purser and all the nurses and physicians in the Endoscopy Departments at King's College Hospital and Guy's and St Thomas' Hospital NHS Foundation Trusts, Tony Wright, Matt Arno,

Robbie Gray at KCL and to Chris Mussell and his colleagues at the Laboratory Government Chemist.

Publication Related to Thesis

Hanks J, Ayed I, Kukreja N, Rogers C, Harris J, Gheorghiu A, Liu CL, Emery P, Pufulete M. The association between MTHFR 677C>T genotype and folate status and genomic and gene-specific DNA methylation in the colon of individuals without colorectal neoplasia. Am J Clin Nutr. 2013 Dec; 98(6):1564-74.

Abbreviations

ACF	aberrant crypt foci
ANOVA	analysis of variance
APC	adenomatous polyposis coli
BMI	body mass index
cDNA	complementary deoxyribonucleic acid
CIMP	CpG island methylator phenotype
CpG	Cytosine-phosphate -guanine dinucleotide sequence
CRC	colorectal cancer
CV	coefficient of variation
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
FAP	familial adenomatous polyposis
FDR	false discovery rate
HNPCC	hereditary non-polyposis colorectal cancer
HPP	hyperplastic polyposis
IBD	inflammatory bowel disease
MLH1	Mut L Homolog 1
MMR	mismatch repair
MS	methionine synthase
MSI	microsatellite instability
MTHFR	methylenetetrahydrofolate reductase
NAT2	N-acetyltransferase 2
PCR	polymerase chain reaction
PPi	inorganic pyrophosphate
RCT	randomised controlled trial
RNA	ribonucleic acid
RNI	reference nutrient intake
SAH	S-adenosylhomocysteine
SAM	S-adenosyl methionine
SD	standard deviation
SNP	single nucleotide polymorphism
SPSS	Statistical Package for Social Sciences
THF	Tetrahydrofolate
TS	thymidylate synthase
TS	tumour suppressor
UC	ulcerative colitis

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1 INTRODUCTION

1.1 Colorectal cancer

Colorectal cancer (CRC) is a malignancy found in either the colon or the rectum, and it is the third most common cancer in the world. With approximately 1.4 million new diagnoses and 700,000 deaths worldwide in 2012, CRC is the fourth leading cause of cancer-related deaths worldwide (Hagggar and Boushey, 2009). There were 41,112 diagnoses and 15,903 deaths due to bowel cancer in the UK in 2013 (CRUK, 2013b). Incidence is 25-fold higher in developed Western countries (including the US, Australia and New Zealand) than in Africa and Asia (WCRF, 2013). Colorectal cancer risk depends on both genetic predisposition and environmental contributions. Of the environmental, or non-genetic, factors, the ageing process is a major risk factor. Between 2011 and 2013, over 60% of all new diagnoses were in people aged 70 years or older. The highest rates were observed in people older than 85 years, and incidence was generally higher in men than in women (CRUK, 2013b). CRC is considered a preventable disease by many specialists in the field, due to numerous modifiable lifestyle risk factors that are reported to be causally linked with the disease. These include low dietary fibre, folate deficiency, high consumption of red and processed meats and fats, smoking, high alcohol intake (WCRF, 2010), obesity, low levels of physical activity and diabetes (Wei et al., 2009). Apart from the lifestyle and dietary factors, chronic bowel disease including ulcerative colitis and Crohn's disease have been associated with a predisposition to CRC (Mattar et al., 2011).

Unfortunately, by the time of diagnosis, over 50% of all colorectal cancers have spread from the colon or the rectum to surrounding tissues (37%) and, in some cases, to distant organs (20%) (Ferlay et al., 2015). The mean five-year survival for people diagnosed with CRC is approximately 40% and is influenced by staging at initial diagnosis (Veigl et al., 1998).

Most CRCs are classified histologically as adenocarcinomas and develop from adenomatous polyps. Between 1 and 10 % of polyps will progress to adenocarcinomas, and this process is strongly influenced by polyp size, villous component and severity of dysplasia. Adenomas are clinically and histologically heterogeneous and are classified as tubular, tubulovillous or

villous (Kufe, 2003). In some cases, adenocarcinomas are thought to develop from hyperplastic polyps via a serrated adenoma intermediate. Hyperplastic polyps, generally measuring between 5 and 10 mm, are usually found in the distal colon and rectum and, though they were previously believed to be benign and clinically insignificant, hyperplastic polyps have been discovered to share molecular similarities with adenomatous polyps (Morimoto et al., 2002). The earliest characterisable lesions are formed when the epithelium from the normal healthy colon hyperproliferates into aberrant crypt foci (ACF) and subsequently progresses to adenoma before transforming into invasive cancer (Kufe, 2003). Adenomas are clonal; they display low-grade dysplasia with hyperchromatic enlargement and elongation of the nuclei along the basement membrane, and they can also display apoptosis. Adenomatous polyps are thought to progress through a “top-down” mechanistic progression. The villous component of tubular adenomas (simple dysplasia with crypt-like glands) is less than 25%. In tubulovillous adenomas (considered intermediate lesions) the villous component ranges from 25-75% and in villous adenomas it comprises more than 75% of the tumour. Large or predominantly villous adenomas are classified as advanced adenomas (Shih et al., 2001).

Surgery remains the initial treatment of choice for CRC, and five year survival after resection is approximately 40% (Scholefield et al., 2002). Patients who do not undergo resection and whose disease progresses metastatically have five-year survival rates of 11% (CRUK, 2013a). Radiation is an additional treatment option but it is normally limited to the rectum. Until the middle of the 1990s, chemotherapy options were restricted to combinations of either leucovorin or fluorouracil and the median overall survival (OS) (from date of diagnosis to death) was only 10-12 months. Between 1996 and 2002, irinotecan and oxaliplatin were approved, and combinations of these cytotoxic drugs improved the median OS to 14-16 months (Gonzalez-Zulueta et al., 1995). Furthermore, the advent of molecular targeted biologics, bevacizumab and cetuximab, administered in combination with standard chemotherapy options, further improved survival (Veigl et al., 1998). Despite advances in chemotherapy combinations and molecular-targeted agents, as well as an emphasis on increased screening and early detection, the outcomes in CRC remain poor for the majority of patients. Those who do undergo chemotherapy and CRC targeted therapy have a high chance of experiencing severe and incapacitating side effects, while in many cases patients

do not respond (Fearon and Vogelstein, 1990). Poor survival has prompted an emphasis on measures to prevent this disease.

Clinical research interest in chemoprevention is increasing, and research indicates that non-steroidal anti-inflammatory drugs (NSAIDs) and cyclooxygenase-2 (COX-2) inhibitors may reduce CRC risk (Cooper et al., 2010). Additionally, a growing body of research into diet-induced CRC risk has led to an increased interest in micronutrients, including folate, vitamin D, calcium, and fibre as chemopreventatives which target multiple pathways in the developmental stages of CRC (Du et al., 2010).

1.2 Heritability of colorectal cancer

Approximately 20-25% of all CRCs occur in patients with a family history of CRC, and 5-6% of cases are due to single-gene defects (CRC genetic syndromes). The two major heritable forms of CRC are familial adenomatous polyposis (FAP) and Lynch syndrome/hereditary non-polyposis colorectal cancer (HNPCC), both of which display autosomal dominant inheritance. FAP arises from mutations in the adenomatous polyposis coli (*APC*) tumour suppressor gene and is characterised by hundreds or even thousands of precancerous polyps. These normally appear by the age of 35 years and will almost certainly cause colorectal cancer if the colon is not removed (Jasperson and Burt, 1993). HNPCC arises from inherited mutations that cause defective DNA mismatch repair and lead to microsatellite instability (MSI). Most HNPCC cases are caused by mutations in the DNA mismatch repair pathway, including the *MLH1* and *MSH2* genes (Yuan et al., 2002).

Various studies, typically employing a case-control design, have identified genetic variants that have small to moderate effects on the odds of developing CRC. These variants include Variable Number Tandem Repeat (VNTR) alleles in the Harvey Rat Sarcoma Virus 1 (*HRAS1*) and a rapid acetylator phenotype caused by N-acetyltransferase 2 (*NAT2*) alleles (Kemp et al., 2004). Other genetic polymorphisms include methionine synthase (*MS A2765G*) and thymidylate synthase (*TS 2R/3R* promoter).

Investigations into polymorphisms specifically in folate genes started over 20 years ago. In

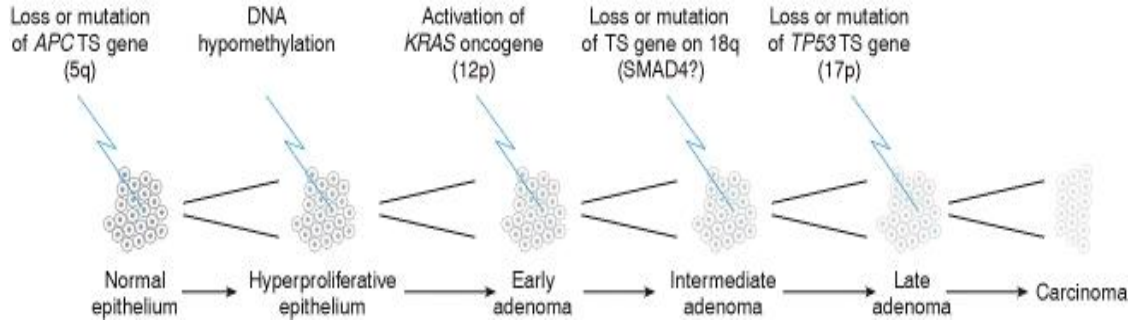
the methyltetrahydrofolate reductase (*MTHFR*) gene, the C677T and A1298C polymorphisms have been of great interest, particularly in terms of their influence on CRC risk, due to their involvement in folate metabolism (Chen et al., 1996, Sharp and Little, 2004). A large meta-analysis concluded that the *MTHFR* C677T is associated with a reduction in CRC risk (Taioli et al., 2009). This is discussed in further detail in **Section 1.4.5**.

1.3 Molecular biology of colorectal cancer

The majority (75-80%) of CRCs arise sporadically from environmental and complex gene-environment interactions, with no obvious links to heredity (Kemp et al., 2004). Twin studies estimate that genetic factors (including gene-environment interactions) contribute to approximately 35% of all CRCs (Lichtenstein et al., 2000). It has been proposed that most of these cases are associated with low-penetrance genetic polymorphisms. Additional risk polymorphisms need to be identified to assess an individual's susceptibility to CRC.

Sporadic neoplasia in the colon was first characterised by Fearon and Vogelstein in 1990 using a multi-step histological model. In this model, mutations in multiple key genes result in the activation of oncogenes and the inactivation of tumour suppressor genes. Together, these multiple hits accumulate, ultimately leading to the development of cancer. (See **Figure 1.1**). Most sporadic cancers derive from adenomas which are intermediate precursor lesions. Adenoma residues are commonly found in colorectal adenocarcinomas (Kinzler and Vogelstein, 1996) and have been useful in investigating the mechanisms involved in disease progression. Over the past two decades, the Fearon and Vogelstein model has developed to include potential epigenetic modifications (accumulative changes in DNA that do not alter the base sequence) in several genes which are now believed to contribute to CRC neoplasia via the following three pathways: chromosomal instability, microsatellite instability and the CpG island methylator phenotype (CIMP). The three pathways are not mutually exclusive, and more than one pathway can be activated within a single tumour (Gonzalez-Zulueta et al., 1995).

Figure 1.1 A multi-step model of colorectal tumorigenesis



(Strachan and Read, 2004)

Chromosomal instability mainly comprises alterations in chromosomal number, sequence changes, chromosomal rearrangements, gene amplification, deletions and loss of heterozygosity. Chromosomal instability is a somatic defect that accounts for more than 80% of colorectal cancers and is seen in specific genes, including the tumour suppressor genes APC, p53 and SMAD. These genes normally oppose malignancy, but when mutated by the loss of a wild-type copy, they play a key role in transforming normal colorectal mucosa into carcinomas (Markowitz and Bertagnolli, 2009).

Microsatellite instability results from mutations in DNA mismatch repair (DNA MMR) such as *MLH1*, *MSH2*, *PMS2*. MSI accounts for approximately 15% of sporadic colorectal cancers (Lawes, 2005). DNA MMR normally rectifies spontaneous errors, such as short insertions or deletions, which tend to arise during DNA replication. Under normal conditions, a protein binds to the mismatch and excises the error, but when there is a defect in DNA MMR, these errors accumulate and novel microsatellites (repeated sequences of 1-6 base pairs) are formed (Iacopetta et al., 2010). In the majority of such tumours, *MLH1* is silenced by DNA methylation (Kane et al., 1997) and it has been suggested that many MSI CRCs are initiated by the epigenetic silencing of DNA repair mechanisms and alternative genes involved in apoptosis (programmed cell death) (Jass, 2007b).

Finally, the CpG island methylator phenotype (CIMP) is increasingly being linked with colorectal cancer risk. Aberrations in normal DNA methylation patterns can potentially

activate oncogenes, silence tumour suppressor genes and influence rates of mutation. Genetic and epigenetic events are now believed to work together to initiate neoplasia and hyperproliferation, subsequently resulting in invasive CRC (Das and Singal, 2004).

1.3.1 DNA methylation

DNA methylation is the addition of methyl groups after DNA replication, typically to CpG dinucleotides. DNA methylation restricts transcription and is one of the mechanisms through which gene expression is regulated. Additionally, DNA methylation influences mutation rates (Jones and Takai, 2001) and is essential to several processes, including genomic imprinting (Li et al., 1993) and x-chromosome inactivation. It is also involved in maintaining DNA structure (Bird et al., 1995). Altered methylation patterns have consistently been reported to exist in cancer cells (Laird, 2005). In early DNA methylation studies, genomic hypomethylation was observed in cancer tissue compared with normal tissue of healthy volunteers (Feinberg and Vogelstein, 1983). Genomic hypomethylation (in regions that are usually methylated) is often found to occur simultaneously with hypermethylation in CpG islands (in regions that are usually unmethylated) (Jones and Laird, 1999, Esteller et al., 2000, Herman and Baylin, 2003).

1.3.2 Genomic DNA methylation in Colorectal Cancer

In tumours, genomic hypomethylation is more frequent than gene-specific hypermethylation, and an overall loss of 5-methylcytosine is often observed in cancer (Ehrlich et al., 2002, Dupont et al., 2004). Genomic hypomethylation has been found in colonic tissue taken from early neoplasia, in benign hyperplastic and adenomatous polyps and in malignant carcinomas (Goelz et al., 1985, Bariol et al., 2003). DNA hypomethylation does not occur solely in neoplastic cells; it is also observed in the normal-appearing colonic mucosa of subjects with adenoma and CRC (Pufulete et al., 2003), which suggests that genomic hypomethylation may play a role in the early stages of colorectal carcinogenesis.

Methylation events on chromosome 18 have been shown to play a particularly important role in the pathogenesis of CRC. In the early stages of the colorectal cancer process, allelic losses on chromosome 18 have been shown in up to 70% of primary colorectal tumours (Cardoso et

al., 2007), and down-regulation of genes primarily on chromosome 18 is also known to take place (Lips et al., 2008). In addition, methylome analysis of colorectal cancer revealed that the highest frequency of methylation occurs on genes located on chromosome 18 (Kim et al., 2011b). Given that genetic alterations, including aberrant DNA methylation (one of the earliest molecular alterations in numerous human cancers (Issa, 1999) occur in concert with specific stages of the adenoma-carcinoma sequence, it is thought that these combined and simultaneous events drive the process of carcinogenesis. For this reason, DNA methylation is potentially useful as a biomarker in colorectal cancer prognosis (Grady, 2005).

DNA hypomethylation can also cause loss of imprinting, a major genetic alteration that drives cellular proliferation and is frequently observed in a number of human cancers. This monoallelic expression ensures that imprinted genes encode proteins for embryonic and placental growth and adult metabolism. Imprinting usually refers to the specific expression of a parental allele in a set of approximately 50-80 genes. When the precision of this expression is altered, loss of imprinting takes place and one of the two alleles is permanently inactivated. An example of loss of imprinting is the result of hypomethylation of a differentially methylated region in IGF2 and the H19 gene in both colorectal tumours and normal-appearing mucosa in the same patient (Cui et al., 1998). Loss of imprinting is also considered an essential tool for the diagnosis, prognosis and treatment of human cancers (Jelinic and Shaw, 2007). The reason for DNA hypomethylation and the mechanism by which it occurs in cancer are yet to be fully elucidated. Dietary factors, including folate status, and common genetic polymorphisms in folate-metabolising enzymes with accompanying reduced enzyme activities in the methylation pathway, have been suggested as potential causes (Pogribny et al., 1997, Stern et al., 2000, Clark and Melki, 2002).

1.3.3 Gene-specific Methylation in Colorectal Cancer

Regional hypermethylation at CpG islands occurs concurrently with genomic hypomethylation in cancer cells (Baylin et al., 1998). CpG islands are clusters of CpG dinucleotides that are between 0.2 and 3kb in length. DNA hypermethylation at CpG islands within the promoter regions of genes is associated with gene silencing. A large amount of research has focused on hypermethylation-induced silencing of tumour suppressor genes (Wong et al., 2006). Since the retinoblastoma gene (RB1) was identified as being silenced by

hypermethylation (Greger et al., 1989), many more tumour suppressor genes, including VHL and APC, have been identified as candidates for this mechanism (Feinberg and Tycko, 2004). Hypermethylation has been detected in normal-appearing colonic mucosa and has been correlated with ageing. It has been suggested that a large proportion of the CpG island methylation in CRC is associated with an accumulation of age-related hypermethylation in normal colonic tissue (Toyota and Issa, 1999). Genes that undergo this age-related methylation include ER, MYOD, N33 and IGF2 (Ahuja et al., 1998). More specifically, in colorectal cancer, the tumour suppressor genes shown to be inactivated by hypermethylation include the Myf-3 gene, adenomatous polyposis coli (*APC*) (Esteller et al., 2000), hMLH1 (Miyakura et al., 2001) and the oestrogen receptor gene (*ER*) (Issa et al., 1994). In addition to hypermethylation-induced tumour suppressor silencing, the hypermethylation of mismatch repair genes and cell cycle regulatory genes is also associated with colorectal cancer (Wong et al., 2006).

An unusually high increase in promoter hypermethylation of CpG islands, in genes such as *MINT1*, *MINT2* and *MLH1*, defines the CpG-induced methylator phenotype (CIMP) in CRC (Issa, 2004). CIMP is associated with microsatellite instability (MSI) (Jass, 2007a) and was first described in detail in 1999 when methylation resulting from the ageing process was distinguished from methylation resulting from cancer; the authors described CIMP in terms of cancer-related methylation. More recently, the role of CIMP has been recognised in CRC, with up to 40% of sporadic MSI CRCs being CIMP positive (Toyota and Issa, 1999). CIMP is usually associated with proximal colonic tumours, hyperplastic polyps and a poor prognosis (Curtin et al., 2007). Two types of CIMP-positive tumours have been postulated: CIMP high (associated with *BRAF* and *MLH1*) (Shen et al., 2007) and CIMP low (associated with *KRAS*) (Ogino et al., 2006).

1.3.4 Uracil misincorporation

In addition to DNA methylation, an alternative pathway through which carcinogenesis may be influenced is uracil misincorporation into DNA. A key factor in this hypothesis is folate deficiency which has been causally linked to strand breakage during replication, hindering the transcription of genes involved in carcinogenesis, but the direct cause of strand breakage has not been confirmed (Duthie et al., 2000a). Uracil misincorporation into DNA is typically quantified by cleaving uracil from DNA and measuring the uracil content in samples (Blount and Ames, 1994). DNA synthesis requires folate for the production of purines and the pyrimidine nucleoside thymidine. Folate deficiency alters the supply of 5,10-methylenetetrahydrofolate (THF), a substrate required for donating a methyl group in the conversion of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (TMP) by thymidylate synthetase (see **Figure 1.2**). Folate deficiency shifts the balance of DNA precursors and subsequently reduces methylation of dUMP, thereby reducing the production of TMP. Raised levels of dUMP lead to increases in the misincorporation of uracil instead of thymine into DNA (Duthie et al., 2000a). Normally uracil residues are excised from DNA by uracil DNA glycosylase. However, if altered deoxyribonucleotide pools (from folate deficiency) persist, repeated repair processes are triggered and transient single-strand breaks can arise. More critically, double strand breaks can occur during the simultaneous repair of two adjacent uracil residues on opposite DNA strands (Choi and Mason, 2000), which can lead to more severe levels of DNA damage and instability.

1.4 Dietary folate and biomarkers of neoplasia (DNA methylation and uracil misincorporation)

Micronutrients such as folates have increasingly become the focus as ‘chemopreventatives’ in CRC due to their involvement in multiple pathways in CRC development and the extent to which they may influence biomarkers of neoplasia such as DNA methylation and uracil misincorporation (Du et al., 2010).

1.4.1 Folate and diet

Folates are a group of B vitamin compounds found in dark green vegetables, including spinach, broccoli and asparagus; fruits, including oranges and strawberries; and offal, particularly liver. Other plant-based sources rich in folate are beans such as kidney, pinto and

chickpeas. Folate compounds are derived from the parent molecule pteroylglutamic acid (also known as folic acid), which is added to fortified foods, including breakfast cereals, yeast extracts and supplements. Each of the folates possess similar biochemical and nutritional properties. Tetrahydrofolate (THF), 5-methylTHF and 5 and 10-formylTHF are the reduced, more labile forms found in foods. These compounds possess methyl or formyl groups for one-carbon substitution. This particular biochemical mechanism, the folate one-carbon metabolism (FOCM), is involved in DNA methylation, DNA synthesis and repair, and cellular proliferation (Gong et al., 2015). As a result, it plays a key role in modulation of oncogenesis including the development of CRC. FOCM is described in detail in **Section 1.4.2**.

Bioavailable folate is defined as the amount of folate that is available to be absorbed, metabolised and stored. Food folates have a bioavailability of approximately 50% (Sauberlich et al., 1987) and in sharp contrast folic acid (the man-made fully oxidised monoglutamate form added to foods and supplements) is 100% bioavailable. Absorption of folate from food is believed to be affected by a number of factors, including cooking methods, insufficient release from the food matrix (cellular structures), inadequate deconjugation of polyglutamate chains to monoglutamates, and folate stability (which may be affected by other nutrients) food constituents and the environment in the gastrointestinal tract. In contrast to food folates, folic acid does not need to be released from the food matrix, and is more stable in the intestinal lumen because it already exists in monoglutamate form (McNulty and Pentieva, 2004).

Monoglutamyl folates are absorbed by humans in the small intestine, in the proximal and distal jejunum as well as in the ileum, where absorption is greatly influenced by pH (Schron, 1991). They are transported across the brush-border, where conjugases break long folate chains into monoglutamates. After absorption, the majority of folates are taken up by the liver via the hepatic portal veins and are either retained or released into the bile or blood. A portion of folates from the liver, in the form of 5-methylTHF, go back into circulation. 5-methylTHF is the major folate compound found in plasma; it is therefore assumed to be the major circulating folate in humans (Pietrzik et al., 2010). A fraction of folates retained by the liver is released into the bile, reabsorbed by the intestine and delivered to the tissue via circulating plasma. This process is known as enterohepatic circulation and it is the mechanism by which plasma folate levels are maintained (Wright et al., 2007).

Adequate folate levels are integral to human health because folate plays an essential role in the synthesis of DNA and RNA and in the maintenance of the DNA methylation cycle. Folate deficiency affects rapidly dividing tissues, including blood, and can cause megaloblastic anaemia. It can also affect the intestinal mucosa, leading to intestinal villous atrophy. Low folate levels appear to contribute to the progression of several disorders in humans, including neural tube defects, cardiovascular disease, cognitive impairment and various cancers, including colorectal cancer (Kim, 2005).

1.4.2 Folate biochemistry

Folate one-carbon metabolism (FOCM) is a complicated system of interrelated reactions that provide an adequate supply of methyl groups available for DNA methylation and DNA synthesis (**Figure 1.2**) (Locasale, 2013).

The form of folate required in methylation reactions is 5-methylTHF, the methyl group donor for the remethylation of homocysteine. Methionine, in its activated form, S-adenosyl methionine (SAM) is the methyl group required in over 100 methylation reactions including DNA methylation (Sibani et al., 2002, Kim, 2007a). Folate deficiency is thought to alter nucleotide levels and interfere with normal DNA methylation patterns (Choi et al., 2005). The essential form of folate in the DNA synthesis cycle is 5,10-methylenetetrahydrofolate (5,10-methyleneTHF). THF is converted to 5,10-methyleneTHF in a reaction catalysed by serine hydroxymethyltransferase.

The activity of the MTHFR enzyme is pivotal in controlling the methyl group supply because it catalyses the conversion of 5,10-methyleneTHF to 5-methylTHF. Reduced enzyme activity affects the flux of the reaction and alters supplies of both 5-methylTHF and 5,10-methyleneTHF. Folate in the 5,10-methyleneTHF form is essential to convert deoxyuridylate to thymidylate. When folate supplies are limited, uracil is misincorporated into DNA, which can lead to breakage of DNA double-strands, chromosomal instability and neoplasia. During folate deficiency, *MTHFR* C677T polymorphism has been associated with thermolability of the enzyme which may increase the risk of CRC by disrupting normal patterns of DNA methylation

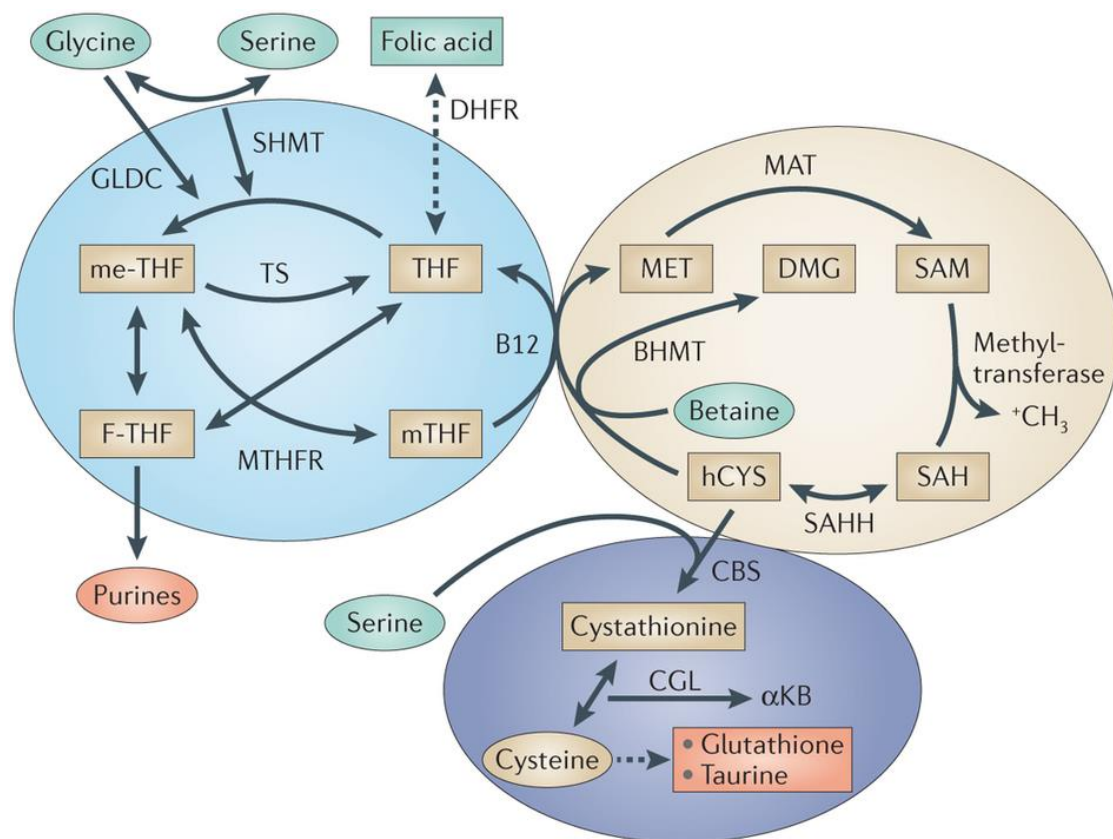
and synthesis (Sharp and Little, 2004).

Tetrahydrofolate (THF) -mediated one-carbon metabolism is a network of interrelated metabolic pathways that take place in the cytoplasm, the mitochondria and also the nucleus. Purine and thymidylate synthesis, as well as remethylation of homocysteine, occurs as a result of one-carbon metabolism in the cytoplasm.

Catabolism of choline, purines, histidine, serine and glycine interconversion, and cytoplasmic metabolism all occur as a result of one-carbon metabolism in the mitochondria.

Anomalies which disrupt folate-mediated one-carbon metabolism are associated with different diseases but the biochemical mechanisms and how they affect the initiation of disease is not well understood (Fox/Stover and Marco, Scotti)

Figure 1.2 Folate metabolism (Locasale, 2015)



THF (tetrahydrofolate), me-THF (5,10-methylene-THF), SHMT (serine hydroxymethyl transferase), mTHF (5-methyltetrahydrofolate), MTHFR (methylenetetrahydrofolate reductase), F-THF (10-formyltetrahydrofolate), hCYS (homocysteine), MET (methionine), MAT (methionine adenytransferase), SAM (S-adenosylmethionine), SAH (S-adenosylhomocysteine), SAHH (S-adenosyl homocysteine hydrolase), CBS (cystathionine synthase), CGL (cystathionine lyase), Akb (α -ketobutyrate), BHMT (betaine hydroxymethyltransferase), DHFR (dihydrofolate reductase), dimethylglycine (DMG), glycine decarboxylase (GLDC) thymidylate synthase (TS)

1.4.2.1 The role of homocysteine

An indirect indicator of folate status is homocysteine, a sulphurated, non-protein amino acid. Homocysteine intersects the two pathways of remethylation and transulphuration, which break the molecule down in the presence of folate, vitamins B₆ and B₁₂. During remethylation homocysteine takes a methyl group from 5-methylTHF. In transulphuration, homocysteine and serine irreversibly form cystathionine. In the absence of sufficient methyl groups from the diet (such as B₁₂ and folate) homocysteine can accumulate and lead to hyperhomocysteinemia, a risk factor for a number of diseases, particularly cardiovascular disease (Cattaneo, 2003).

1.4.2.2 The role of riboflavin

The folate and riboflavin interaction is less well discussed. Riboflavin is a precursor of flavin adenine dinucleotide (FAD), a coenzyme required for 5,10-methylenetetrahydrofolate reductase (MTHFR). FAD-dependent MTHFR catalyzes the reaction that generates 5-methyltetrahydrofolate required to form methionine from homocysteine.

In addition to other B-vitamins, increased riboflavin intakes have been associated with a reduction in plasma homocysteine concentrations (Jacques PF, Bostom AG, Wilson PW, Rich S, Rosenberg IH, Selhub J, Am J Clin Nutr. 2001). Riboflavin has been shown to be most influential in folate metabolism in *MTHFR C677T* individuals, in particular when low folate status is accompanied by increased homocysteine levels, and furthermore riboflavin intake is below optimal levels. However, riboflavin has been shown to directly reduce elevated

homocysteine concentrations in these individuals, which supports the importance of the riboflavin and MTHFR genotype interaction (McNulty H, Dawsey le RC, Strain JJ, et al, Circulation. 2006;113(1):74-80)

1.4.3 Folate and colorectal cancer

Numerous epidemiological studies have reported that foods containing folate confer protection against colorectal cancer. The active compounds found in these foods have not yet been isolated. Nevertheless, a considerable amount of research into the mechanisms by which this protection may occur has been conducted (Terry et al., 2002). Insufficient folate status, leading to DNA strand breaks and impaired DNA methylation, has been proposed as one of the mechanisms through which CRC is caused (Kim, 2003). In seeming contradiction to the evidence on folate deficiency, studies have reported that raised folate levels from supplementation present an increased risk of CRC, though this risk may only exist in the presence of precancerous lesions rather than in disease free individuals (Kim, 2006). This phenomenon has been termed the 'dual modulatory effect' of folate supplementation. Epidemiological studies investigating the link between folate status and CRC risk have generally been based on dietary and supplementary intake, folate in serum and red blood cells, and plasma homocysteine. However, because neoplastic changes occur at a cellular level, there is increasing interest in site-specific folate status and its relation to different cancers (Brockton, 2006, Brockton, 2008). When investigating CRC risk, assessing folate status in the colon may be more useful than simply assessing folate status in the serum or red blood cells. Additionally, lifestyle factors may affect folate levels differently in colonic tissue than in serum and red cell folate.

1.4.3.1 Folate and colorectal cancer – animal studies

Animal studies have identified an inverse association between folate levels and CRC risk. Study designs have included animal models in which chemical carcinogens were administered to rodents genetically predisposed to CRC and maintained on a low folate diet (Kim et al., 1996a). Rats sustained on a moderately folate-deficient diet and injected with 1,2-dimethylhydrazine, a potent carcinogen that induces mutations primarily in the colon (Newell

and Heddle, 2002), were more likely to develop colonic dysplasia and carcinoma (Cravo et al., 1992). Impaired DNA methylation, including global hypomethylation and regional hypermethylation, has been observed in animal colon carcinogenesis where rats developed tumours in direct response to being fed a diet deficient in methyl donors such as folate and vitamin B₁₂ (Wainfan and Poirier, 1992).

When doses of up to 40 mg folate/kg diet – 20 times the dietary requirement of 2 mg folate/kg – were administered to Sprague-Dawley rats, a decrease in macroscopic tumours was observed for doses up to 8 mg folate/kg diet (Kim et al., 1996b). This study suggested a dose-dependent benefit of folate levels up to 4 times the daily dose, beyond which no further benefit was observed. Another study showed that low folate levels delayed peripheral nerve sheath tumour development in transgenic mice carrying the human-T lymphotropic virus (Bills et al., 1992). Finally, a study of intestinal polyps in a murine model found that dietary folate supplementation may suppress polyp growth if administered before genetic alterations in the colonic epithelium have occurred, after which folate supplementation was associated with increased polyp growth (Song et al., 2000b). Physiological doses of folate supplementation in animals have generally been associated with decreased tumour growth (Kim et al., 1996b), which also appears to be the case if administered during the early stages of neoplasia (Song et al., 2000a, Song et al., 2000b). Conversely, folate administered in pharmacological doses after carcinogenesis is established, generally increases tumour growth (Kim et al., 1996b, Song et al., 2000b).

1.4.3.2 Folate and colorectal cancer – human studies

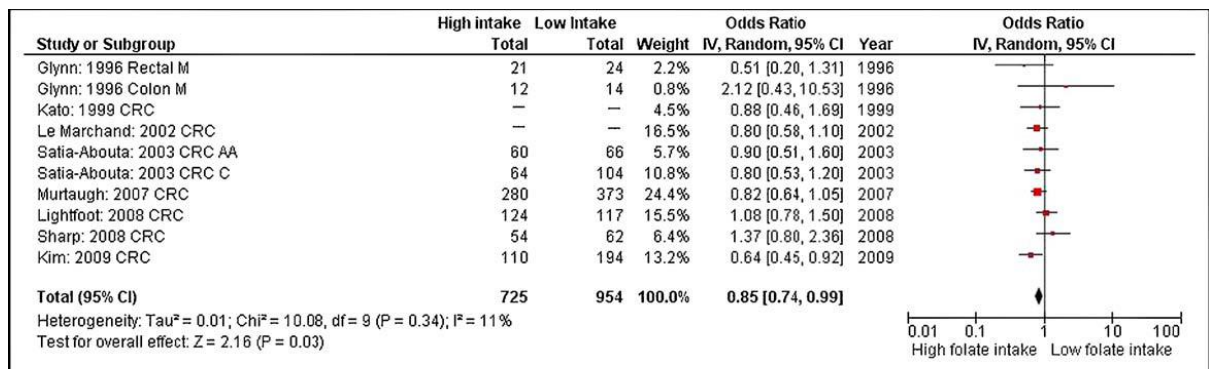
Folate status (based on dietary intake, blood folate and colorectal tissue concentration) has been shown in numerous prospective and case-control studies to be inversely related to colorectal cancer risk in humans (Kim, 2007b).

A 2005 meta-analysis of epidemiological studies that assessed dietary and supplemental folate using food frequency questionnaires, diet records and 24-hour recalls found an overall protective effect from high consumption of folates (Sanjoaquin et al., 2005). In this study, an electronic search of all terms related to folate, colon/rectum and cancer in studies conducted since the 1950s was carried out in PubMed, and studies written in English that included

dietary or total folate (from both food and supplementation) were included. Seven prospective cohort studies showed a 25% lower risk of CRC in subjects with the highest folate intake compared with subjects with the lowest folate intake (RR 0.75, 95%CI 0.64-0.89). The protective effect was observed more when folate was derived from food rather than supplementation. Therefore, potential confounding factors, such as advantage conferred collectively from other nutrients in the diet, should be acknowledged. Case-control studies in the same meta-analysis showed a similar reduction in risk; however, contrary to the group of cohort studies, there was considerable heterogeneity between study designs (Sanjoaquin et al., 2005).

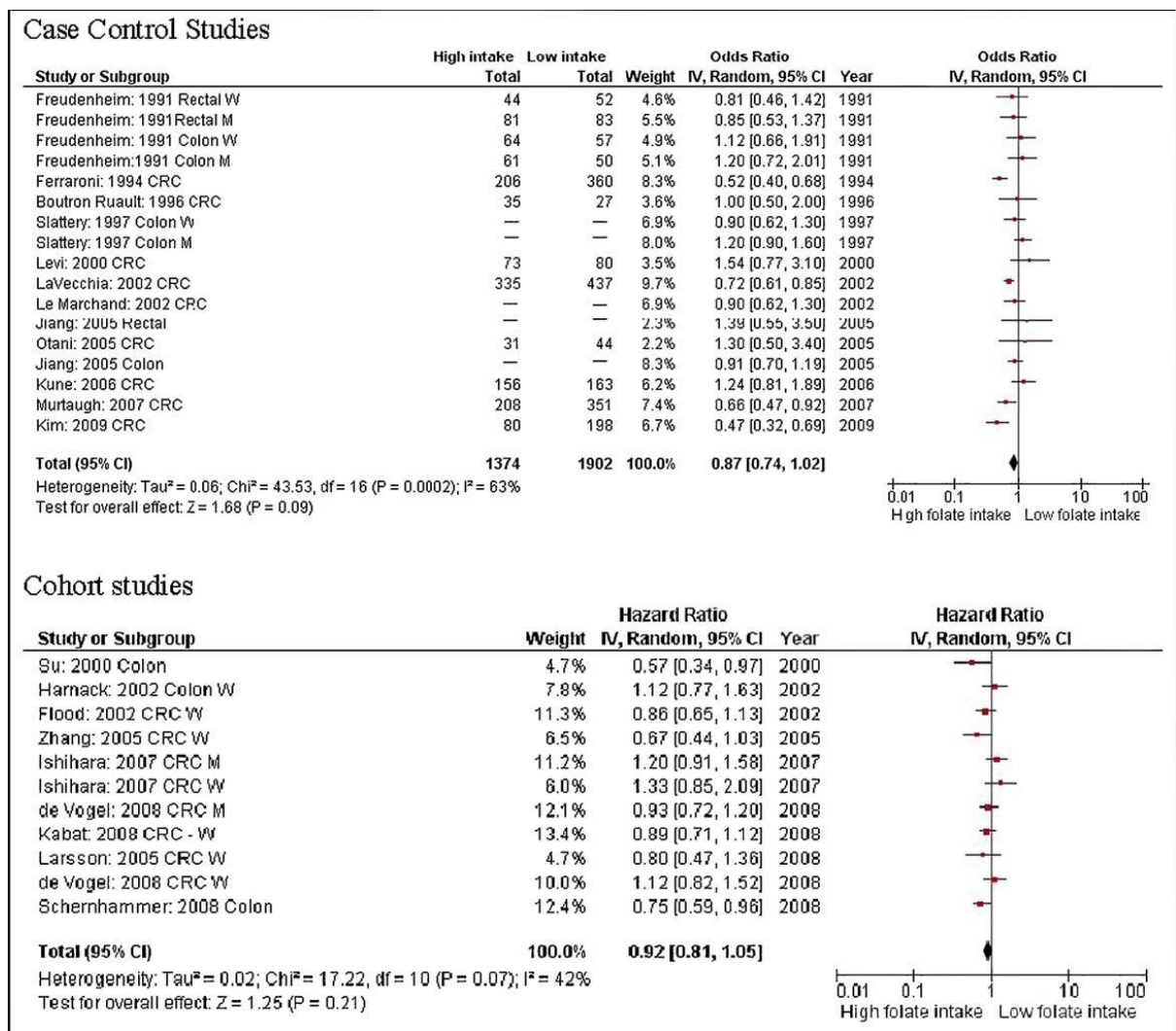
Kennedy *et al*, 2012 performed a meta-analysis and found similar results to those reported in the Sanjoaquin 2005 meta-analysis. In this study, MEDLINE, EMBASE and SCOPUS were searched for all terms related to folate and colorectal cancer in humans between database inception (around the early seventies) and 2009, and studies were only included if at least two measures of folate intake were included. Several studies overlapped between this study and the Sanjoaquin (2005) meta-analysis. Food frequency was captured either by the Food Frequency Questionnaire (FFQ) or the Coronary Artery Risk Development in Young Adults (CARDIA) dietary history questionnaire, as well as dietary recording over 24 hours. In 18 case-control studies, a risk reduction of 13% was observed when high and low folate intake participants were compared (**Figure 1.3**). In the 9 cohort studies, risk was reduced by 18% when those in the highest and lowest folate intake categories were compared (**Figure 1.4**). There were more studies in the Kennedy meta-analysis (18 case-control studies and 9 cohort studies) than in the Sanjoaquin meta-analysis (9 case-control studies and 7 cohort studies); therefore, unsurprisingly, the confidence intervals for the Kennedy meta-analysis for the estimate of risk for folate intake were narrower. The subject characteristics in these studies are heterogeneous, with a variety of ethnicities and ages included in the study populations, however the study still reached statistical significance making this meta-analysis translatable across the population (Kennedy et al., 2012).

Figure 1.3 Total folate intake and risk of CRC - meta-analysis of case-control studies (Kennedy et al., 2012).



Abbreviations: CRC – colorectal cancer, AA - African American, C - Caucasians

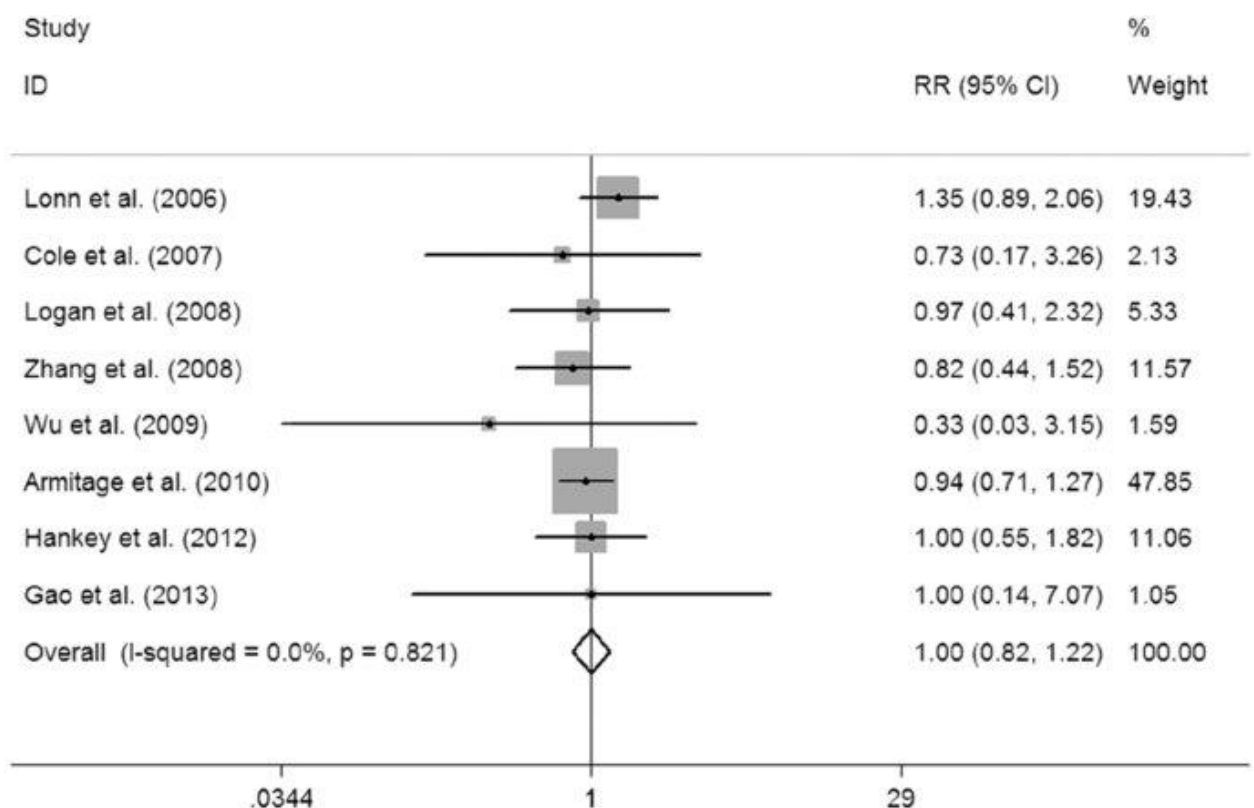
Figure 1.4 Dietary folate intake - case-control studies and cohort studies (Kennedy et al., 2012).



Abbreviations: CRC – Colorectal Cancer, W - women, M - men

A very comprehensive and up-to-date meta-analysis performed in 2015 found no evidence of a relationship between folic acid supplementation and colorectal cancer risk. Out of a total of 1229 studies examined, 72 studies were eligible for further analysis and eventually 8 were finally included. This meta-analysis used a strict inclusion criteria including RCT study design, folic acid dosage, folic acid supplementation and CRC risk and all studies had to be in English. Finally, the authors showed lack of significant heterogeneity amongst populations. Larger studies are needed to validate these findings (Qin et al., 2015)

Figure 1.5 Association between folic acid supplementation and CRC risk (Qin et al., 2015)



1.4.3.3 Folate and colorectal cancer - methods of measuring folate

Associations between folate status in humans and colorectal cancer risk have largely been investigated with blood biomarkers (serum, red cell folate and plasma homocysteine concentrations) (Bird et al., 1995, Kato et al., 1999). In a nested case-control study, 105 women with colorectal cancer had lower serum folate and higher plasma homocysteine concentrations than the 523 controls. After adjustment for potential confounding factors, including smoking and alcohol, the risk of colorectal cancer in subjects in the highest serum folate quartile was half that of the subjects in the lowest serum folate quartile (OR=0.52, 95% CI=0.27-0.97, P=0.04) (Kato et al., 1999). Importantly, in this study the effect of existing CRC on folate cannot be excluded. In a case-control study investigating the correlation of serum and red cell folate and homocysteine levels in adenomatous polyps, there was a 24% reduction in CRC risk in subjects with red cell folate levels above 160ng/mL. (Bird et al., 1995). Folate levels have also been examined in specific tissues in few human and animal studies (Kim et al., 1996b, Kim et al., 1998, Kim et al., 2001, Meenan et al., 1996, Ge et al., 2007). These studies have provided information on the relationship between systemic levels of folate in the body and tissue-specific folate levels in organs such as the colon. This relationship is particularly important for future study design with healthy volunteers because systemic folate markers can provide useful information where site-specific samples are unobtainable (this topic is discussed in more detail in **Chapter 5**). One study demonstrated variations in folate deficiency across different organs. A folate-free diet fed to rats for 25 weeks causes lower folate levels in the liver (60%), kidney (59%) and spleen (67%) when compared with folate-supplemented controls. This effect was not observed in the brain (Varela-Moreiras and Selhub, 1992), suggesting that folate is differentially distributed across different organs. In CRC patients, folate levels were found to be lower in neoplastic tissue in the colon than in the normal tissue of the same subject (Alonso-Aperte et al., 2008, McGlynn et al., 2013).

A potential correlation between blood biomarkers of folate (serum folate and serum homocysteine) and human colonic mucosal folate was assessed as a result of folate supplementation in 20 patients with colonic adenomas over the course of one year. Patients were randomised to receive either 5mg/d folic acid (n=9) or placebo (n=11). Serum and red cell folate, serum homocysteine and colonic mucosal folate concentrations were obtained at baseline, 6 months and one year. In individuals who were not supplemented, there was a direct correlation between colonic mucosal and serum folate at each time point ($r = 0.572$ –

0.845; $P < 0.015$) and a correlation was seen with RBC folate and colonic mucosal folate at 6 months and 1 year ($r = 0.747-0.771$; $P < 0.001$). In the case of serum homocysteine, an inverse correlation was seen with colonic mucosal folate at each time point ($r = 0.622-0.666$; $P < 0.008$). None of the systemic markers of folate correlated with colonic mucosal folate in response to folate supplementation and the authors concluded that at supraphysiological folate quantities an effect is not seen (Kim et al., 2001).

The relationship between human colonocyte folate at different sites (duodenum, caecum and sigmoid colon) within the gastrointestinal tract was investigated for uniformity, and to determine if there was a correlation with serum and red cell folates. An unexpected finding led the authors to report a variation across these sites. Caecum folate was lower than that of duodenum and sigmoid folate, suggesting a variable mechanism for cellular folate transportation and that folate in the colon may not only be derived from blood sources but also could be absorbed from the lumen. In subjects with adequate folate status, colonocyte folate did not correlate with systemic folate (Meenan et al., 1996).

The same group reported that folate levels were lower in gastrointestinal epithelial tumour cells when compared to disease-free cells in the adjacent epithelium ($P < 0.02$). In agreement with their previous study (described above), folate in the normal epithelium of CRC patients was not significantly different from that observed in healthy controls. Blood folates and vitamin B₁₂ were normal across all groups. Therefore, it was proposed that this observation could be due to folate deficiency being localised in the patients with pathologies and that neoplastic cells require greater supplies of folate for cell division and proliferation (Meenan et al., 1996).

In another study, folate levels were compared between subtypes of polyp patients. Levels were 34% lower in the normal mucosa of adenomatous polyp patients than in the normal mucosa of hyperplastic polyps patients ($P=0.04$), suggesting that folate levels may diminish on a global level at some stage in the neoplastic process (Kim et al., 1998). An alternative explanation would be that people with low folate levels are more at risk of developing adenomatous polyps than hyperplastic polyps. However, in this study, there was no polyp-free control group for comparison. The same group concluded that both serum and red blood cell

folate accurately reflect folate concentrations in the colonic mucosa and that the strongest association exists between serum homocysteine and colorectal folate. This finding is in agreement with the proposal that elevated homocysteine levels provide a more sensitive and readily available indication of cellular folate deficiency compared to blood folate (Bailey, 1990).

More recently, the relationship between systemic and colonic folate was examined in response to folic acid supplementation over a period of 8 weeks in 106 polyp patients and 98 healthy volunteers. Increases in plasma folate correlated with colonic tissue folate; however, only plasma folate showed a dose-dependent response. Folate supplementation at a dose of 1,200µg/day did not result in an increase in tissue folate in the colon compared with 400 µg, indicating that the response begins to diminish at the upper folate limit. Furthermore, when folate levels in the colonic mucosa of the polyp group were compared with those in the mucosa of the healthy controls, no differences were detected (Powers et al, 2007).

In conclusion, animal and human studies suggest an inverse association between folate status and CRC risk. Folate deficiency appears to increase the risk of colorectal cancer, while folate supplementation appears to decrease the risk. This has led to folate being considered a potential anti-cancer agent. But for folic acid supplementation to be established as a chemopreventive, the appropriate dosage and timing of treatment requires further investigation (Kim, 2003). Further studies may be useful in clarifying the relationship between localised tissue folate deficiency and systemic folate levels, and whether dietary and supplementary folate can influence this relationship.

1.4.4 Folate and colorectal adenoma

It is important to differentiate adenoma risk from CRC risk because evidence suggests that folate status influences the different stages of colorectal neoplasia differently.

1.4.4.1 Folate and colorectal adenoma – animal studies

In the *APC^{Min}* mouse model, mice were assigned to a spectrum of different dietary folate levels for three months. After three months, dietary intake and serum folate were both inversely

related to the number of small adenomas in the intestine. At the six month time point however, fewer distal adenomas were observed in the mice who were fed a folate deficient diet when compared with the mice on a folate supplemented diet and serum folate correlated positively with the number of distal adenomas (Song et al., 2000a). The authors concluded that folate deficiency inhibited growth of neoplasms which were already established. It is difficult to directly extrapolate findings from animal models to human adenoma because of differences in clinical presentation, histology and molecular biology. In *APC^{Min}* model flat foci of dysplasia develop rather than adenoma and these lesions do not go on to develop into adenocarcinoma because the animals die of intestinal obstruction first .

1.4.4.2 Folate and colorectal adenoma – human studies

Mixed results have been observed for the relationship between folate status and the risk of colorectal adenoma incidence and recurrence in humans.

In the prospective Nurse's Health Study, the role of folate intake in CRC was investigated, particularly with respect to timing, concentration, and the influence on colorectal cancer and adenoma. Long term (12-16 years) and short term (4-8 years) total folate intake, measured by FFQ prior to diagnosis, was associated with a reduced risk of colorectal adenoma. Adenoma risk was strongly inversely associated with folate intake at 4-8 years prior to diagnosis (RR: 0.68 [95% CI: 0.60, 0.78], at doses of ≥ 800 compared with $< 250 \mu\text{g}$ folate/d). This study concluded that folate intake reduces the risk of adenoma but not CRC and that folate intake is therefore only protective in the early stages of neoplasia (Lee et al., 2011).

A recent study investigated the role of folate in the chemoprevention of the primary occurrence of adenoma. The study was carried out over three years in 791 subjects over the age of 50 years who had no history of previous adenomas and who were randomised to receive 1mg/d folic acid or treatment without folic acid. There were 64 adenomas in the folic acid group and 132 adenomas in the control group (unadjusted risk ratio (RR), 0.49; 95% confidence interval (CI), 0.37-0.63; $P < 0.01$), indicating that prevention with 1 mg/d folic acid supplementation could reduce the incidence of colorectal adenomas but this was shown only in patients with low plasma folate levels (Gao et al., 2013).

As part of a randomised trial with a cardiovascular primary endpoint, it was found that a combination of folic acid, vitamin B₆ and vitamin B₁₂ did not significantly affect the risk of colorectal adenoma among women with adequate folate status and at high risk of cardiovascular disease (Song et al., 2012).

Regarding adenoma recurrence, a study by Cole et al demonstrated that 1mg/day of folic acid supplementation for up to 6 years did not reduce risk. However, in this population, folate levels were adequate because of mandatory folate fortification in the US, thus the effect of the supplementation may have been difficult to quantify. A subgroup analysis was carried out for 508 patients who participated in the complete duration of the study. Three or more adenomas were observed in 11 (4.3%) participants in the placebo group versus 27 (10.9%) participants in the folic acid group (RR, 2.52; 95% CI, 1.28-4.98; P=0.008), suggesting an increased adenoma risk for folate-replete patients with previously removed adenomas (Cole et al., 2007). In contrast another group supplemented patients for only 3 years, but at a much higher dose (5 mg/day) and showed that folate prevents adenoma recurrence. A double-blind, placebo-controlled trial of 94 patients with colorectal adenomatous polyps, where all polyps were removed prior to supplementation, one arm was treated with folic acid (n=49) and one arm with placebo (n=45). Subjects were checked for adenoma recurrence at three years, the mean number of polyps observed in the folic acid group was 0.36 polyps (SD 0.69) and the mean number of polyps in the placebo group was 0.82 (SD 1.17), suggesting a greater than two-fold protective effect of folate in preventing adenoma recurrence (Jaszewski et al., 2008).

In a large, double-blind study, patients were randomised to receive 1mg/day folic acid (n=338) or placebo (n=334), and baseline plasma folate levels were recorded. The primary endpoint was the appearance of new adenomas during the study (1996-2004). The secondary endpoints were the total number of recurrent adenomas, the staging and the specific sites of the lesions. Folic acid supplementation failed to protect against the recurrence of at least one adenoma (relative risk RR: 0.82; 95% CI: 0.59,1.13; P=0.22), but an increased risk of neoplasia was not observed. In patients who had low plasma folate concentrations at baseline (<7.5 ng/mL) in combination with high alcohol intake, a significant decrease in recurrent adenomas was observed, suggesting a dose response relationship between folate levels and adenomas.

This study benefited from large numbers, but a key limitation was the lack of endoscopy at baseline to assess current pathology. Additionally, adenomas were not reviewed by a central pathologist for unbiased staging and disease outcome. Similar to the Cole study described above, participants here were subject to mandatory folic acid fortification of food during the course of the study, resulting in a population with an adequate folate status (Wu et al., 2009).

Finally, folate supplementation was not beneficial in protecting against the recurrence of adenomas in a large, double-blind, 2-factorial design trial of 0.5mg/day folic acid or placebo and 300 mg/day aspirin or placebo. However, aspirin was found to reduce adenoma recurrence (Logan et al., 2008).

1.4.5 Genetic polymorphisms involved in folate metabolism

Functional polymorphisms in genes encoding enzymes in folate metabolism is a further consideration in the relationship between folate and CRC risk.

1.4.5.1 The *MTHFR* enzyme

The *MTHFR* enzyme maintains the balance between DNA methylation and DNA synthesis. The DNA methylation and DNA synthesis pathways are both dependent on the availability of methyl groups.

1.4.5.2 The *MTHFR* C677T genotype and colorectal cancer

The *MTHFR* C677T polymorphism, a common mutation in the 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) reductase gene, has been linked to a reduced risk of colorectal cancer risk (Taioli et al., 2009).

The *MTHFR* gene is located on chromosome 1 (1p 36.3), and *MTHFR* C677T is one of several polymorphisms which encode the enzymes involved in folate metabolism that have been extensively investigated in colorectal cancer. The transition from C (cytosine) to T (thymine) is a point mutation occurring at nucleotide 677 in exon 4, and it results in a change from amino acid alanine to valine. The prevalence of this mutation differs with ethnicity. Between 10-12% of Caucasian and Asian populations are homozygous for the T allele, and 50% are

heterozygotes (Frosst et al., 1995, Brattstrom et al., 1998). However, among individuals of African descent, approximately 1-10% of the population carries the TT mutation (Le Marchand et al., 2005).

An inverse association between the *MTHFR* C677T genotype and colorectal cancer has been repeatedly reported, but the precise biological mechanism of this relationship has yet to be established. The *MTHFR* enzyme catalyses the conversion of 5,10-methyleneTHF to 5-methylTHF. The TT genotype increases the thermolability and reduces the activity of the *MTHFR* enzyme, decreasing 5-methylTHF supplies and thus elevating plasma homocysteine levels (Frosst et al., 1995). Limited supplies of 5-methylTHF alter the capacity of methyl group donation and can therefore impair the methylation cycle. Mutations that alter the activity of the *MTHFR* enzyme have been proposed to affect cancer risk through two mechanisms: DNA methylation and uracil misincorporation (Blount et al., 1997, Jia and Guo, 2013).

The CC genotype is referred to as wild-type, and the CT and TT genotypes are referred to respectively as the heterozygous and homozygous variants. Lower levels of enzyme activity relative to the CC genotype are generally observed in the CT and the TT genotypes (30% and 65%, respectively) (Brockton, 2006). In lymphocyte extracts, complementary DNA (cDNA) expression showed that the *MTHFR* C677T mutation is associated with a reduction in enzyme activity and an increase in thermolability (Frosst et al., 1995). It has been proposed that when folate status is sufficient, the methyl group supply is adequate and the reduction in enzyme activity is not enough to have a substantially negative effect on DNA methylation. The reduced *MTHFR* enzyme activity in a folate-adequate environment is thought to increase the pool of 5,10-methyleneTHF (a cofactor in nucleotide synthesis) and improve the efficiency of DNA synthesis and repair. However, when folate status is not sufficient, both DNA methylation and DNA synthesis are thought to be compromised, subsequently increasing the CRC risk (Das and Singal, 2004). A recent literature search performed during the preparation of this thesis did not reveal any studies that have explored the relationship between the *MTHFR* C667T polymorphism and colorectal cancer risk specifically in animals. *Apc*^{min/+} mice are used as an animal model to emulate the *MTHFR* TT mutation because of a characteristic decrease in *MTHFR* activity in comparison to wild-types (Chen et al., 2001). This mouse model has been

explored for adenoma rates in animals with existing tumours or a predisposition to adenomas. This study is described in more detail below **Section 1.4.5.3**.

The *MTHFR* polymorphism and colorectal cancer risk was assessed by a large meta-analysis of over 30,000 subjects stratified by ethnicity (approximately one-third cases and two-thirds controls) and a pooled analysis (approximately 5,000 cases and 8,000 controls), both performed by the same authors (Taioli et al., 2009). The meta-analysis odds ratio reported that the TT genotype was associated with an overall CRC risk of [OR 0.83 (95% CI: 0.77, 0.90)] when compared with the CC genotype (**Figure 1.5**). No overall association was observed between the CT genotype and CRC risk. Of the 27 studies examined, approximately half showed a positive association and half showed negative association with CT and CRC risk. Upon stratification by ethnic group, white and Asian individuals with the TT genotype were both reported to have a lower risk of CRC, but this protective effect was not seen in Latino or black individuals. Smoking and BMI did not influence this interaction in individuals with the TT genotype, but regular consumption of alcohol did have a detrimental effect. Of the 29 studies included in the meta-analysis, 14 were included in the pooled analysis for which data for individuals was extracted from the Genetic Susceptibility to Environmental Carcinogens (GSEC) database (www.gsec.net). The odds ratio for CRC risk with the TT genotypes was similar to the meta-analysis across the three ethnicities (Taioli et al., 2009).

The TT genotype was associated with an overall reduction in the risk of CRC in two earlier meta-analyses (Hubner et al., 2006, Huang et al., 2007). Further analyses that separated patients with cancer of the colon from those with cancer of the rectum determined that this risk reduction was primarily for cancer of the colon. When smoking status was examined, it was noted that those who had smoked more than 14.2 pack years and possessed the TT genotype were at a significantly higher risk of developing CRC than the CC genotype. In addition, alcohol drinkers had a 22% lower risk of developing colorectal cancer if they had the TT genotype compared with the CC genotype. However, in the non-drinker group, no association with CRC risk was observed with the TT genotype (Taioli et al., 2009).

1.4.5.3 The *MTHFR* C677T genotype and colorectal adenoma

The extent to which the *MTHFR* genotype influences adenoma risk has also been extensively studied and some key studies are described in **Table 1.5**.

In humans, a case-control study showed no association between the *MTHFR* C677T genotype and adenoma risk; furthermore, there was no evidence of an interaction between folate status and alcohol habits. This study included 296 cases and 317 controls aged 50-74 years using data from the faecal occult blood test screening program in the UK. None of the participants had any previous malignancies. Dietary folate was assessed by FFQ, which includes information about folate supplementation. The authors suggested that varying folate status in UK studies, in contrast to other studies globally, was the result of mandatory folate supplementation outside of the UK (Lightfoot et al., 2008).

To conclude regarding the role of the *MTHFR* C677T in CRC risk, the TT mutation has consistently been observed to reduce risk, and this reduction is modified by folate status. In contrast, colorectal adenoma risk does not appear to be mediated by the *MTHFR* 677TT genotype and folate metabolism.

Table 1.1 Association between the *MTHFR* C677T genotype and adenoma

Authors	Study design	Folate status	Association between <i>MTHFR</i> C677T and risk of adenoma
Lightfoot <i>et al.</i> , 2008)	296 cases, 317 controls	FFQ	No association
Chen <i>et al.</i> , 1998	379 cases, 726 controls	FFQ	No association
Hirose <i>et al.</i> , 2005	452 cases, 1,050 controls	Food consumption but non-validated	No association
Marugame <i>et al.</i> , 2000	205 cases, 220 controls	FFQ and plasma folate	No association
Levine <i>et al.</i> , 2000	Case-control 471 cases, 510 controls	FFQ, red cell and plasma folate	Increased adenoma risk in TT subjects in lowest quartile for RBC (OR, 2.04 and 95% CI, 0.6 –7.0). Increased adenoma risk in TT subjects in lowest quartile for plasma folate (OR, 1.84 and 95% CI, 0.6 –7.0)
Marugame <i>et al.</i> , 2003	177 cases, 192 controls	Plasma folate	Decreased adenoma risk in TT subjects with high folate compared with CC subjects
Ulvik <i>et al.</i> , 2001	Case-control, 443 cases	Red blood cell	<i>MTHFR</i> 677T and low folate status 'strongly' linked to high-risk adenomas in smokers
Huang <i>et al.</i> , 2007	10,131 TT, 15,362 CC	Various	No association between <i>MTHFR</i> C677T and adenoma, only with CRC.
Boyapati <i>et al.</i> , 2004	177 cases, 228 controls	FFQ	No association
Levine <i>et al.</i> , 2011	665 cases, 695 healthy controls	FFQ and plasma folate	No association
Mitrou <i>et al.</i> , 2006	894 cases, 946 controls	FFQ	No association

1.4.6 Genomic DNA methylation and folate in colorectal cancer

1.4.6.1 Animal studies

Animal studies of methyl group (methionine, folate and B₁₂) depletion have repeatedly shown genome-wide DNA hypomethylation in animal tissues (Kim, 2004). In several animal studies (**Table 1.2**), the effects of folate depletion on DNA methylation in the colon (during timespans ranging from 10 to 26 weeks, with or without exposure to carcinogen) were investigated. The majority of these studies showed no effect of folate depletion on genomic DNA methylation in rodent tissue.

Table 1.2 The influence of folate deficiency on genomic DNA methylation in rodent tissue

Authors	Level of folate depletion	Duration (weeks)	Change in genomic DNA methylation
Kim et al, 1995	Mild	15	No change observed
Kim et al, 1996a	Mild plus carcinogen	20	No change observed
Duthie et al, 2000c	Mild	10	No change observed
Le Leu et al, 2000	Mild plus carcinogen	13/26	No change observed
Davis and Uthis, 2003	Mild plus carcinogen	13	No change observed
Choi et al, 2003	Mild	8	No change observed
Sohn et al, 2003	Severe	5	Increased by 30% after week 3 (P=0.02)
Keyes et al, 2007	Depletion/repletion with folic acid	20	Incremental increase from, depletion to repletion to supplementation (P<0.023)
Liu et al, 2007	Mild	10	No change observed
Uthus et al., 2006	Mild	10	No change observed
Duthie et al, 2010	Mild	24	No change observed
Linhart et al, 2009	Severe	32	Directly correlated with folate increase (P=0.009)

1.4.6.2 Human studies

Human studies investigating the associations between folate status and genomic DNA methylation in leucocytes, lymphocytes and colonic mucosa have produced varying results. *In vitro* studies have shown that folate deficiency can cause modifications of DNA methylation in the colonic epithelium, but this effect is dependent on the cell line (Stempak et al., 2005). Human colon cancer cells cultured in a folate-free medium and analysed by the Comet assay (Quantifies strand breakage caused by enzymatic digestion of hypomethylated DNA) showed an increase in global DNA hypomethylation compared with cells grown in medium containing 3mM/L folic acid after 14 days. This effect was not observed at 7 days and it was reversed when folic acid was reintroduced into the medium (Wasson et al., 2006).

Folate depletion-repletion studies in lymphocytes showed a slight decrease in DNA methylation in response to a low folate diet in healthy post-menopausal women (49 to 63 years). This effect was subsequently reversed by a folate-sufficient diet (Jacob et al., 1998). In a similar study, folate depletion caused marginal DNA hypomethylation, but this was not reversed by folate repletion; however, subjects were older (60-85 years) and age-related methylation factors might have had an effect (Rampersaud et al., 2000). In a study where folate depletion levels and duration were similar to those in the Rampersaud study, hypomethylation was not observed, but in this population subjects were young females (20-30 years) (Shelnutt et al., 2004) (**Table 1.3**).

Table 1.3 The influence of folate depletion on genomic DNA methylation in humans

Authors	Study population	Level of folate depletion	Tissue	Change in genomic DNA methylation
Jacob <i>et al.</i> , 1998	Post-menopausal women (n=8)	56-111 mg/d (9 weeks)	Leucocytes	120% reduction (P<0.05)
Rampersaud <i>et al.</i> , 2000	Post-menopausal women (n=33)	118 mg/d (7 weeks)	Leucocytes	10% reduction (P=0.0012)
Shelnutt <i>et al.</i> , 2004	Young females (n=41)	115 mg/day (7 weeks)	Leucocytes	Reduction (P=0.008)

In observational studies, both case-control and cohort, have consistently shown genomic DNA hypomethylation (in blood and/or tumour tissue) to be associated with CRC, but studies investigating the influence of folate status (intake, dietary or supplementary, or systemic) on genomic DNA methylation have produced mixed results (**Table 1.4**).

Table 1.4 Association between folate status and DNA methylation

Authors	Study design	Tissue	Association with genomic DNA methylation
Fenech <i>et al.</i> , 1998	Cross-sectional young male/female (n=106)	Leucocytes	No correlation with Red cell folate, plasma homocysteine or serum B ₁₂
Pufulete <i>et al.</i> , 2003	Case-control Male/female CRC (n=28), adenoma (n=35), healthy volunteers (n=76)	Colon	Correlated negatively with serum and red cell folate and positively with plasma homocysteine
Pufulete <i>et al.</i> , 2005b	Cross-sectional Male/female healthy volunteers (n=68)	Colon	Correlated negatively with serum folate (r=-0.311, P=0.01), red cell folate (r=-0.356, P=0.03) and positively with plasma tHcy (r=0.256, P=0.04)
Pilsner <i>et al.</i> , 2007	Male/female healthy volunteers (n=294)	Leucocyte	Correlated negatively (P=0.03) but no association after controlling for age, smoking and sex
Figueiredo <i>et al.</i> , 2009	Case-control Male/female (n=388) colorectal adenoma	Colon	No association with dietary folate intake
McGlynn <i>et al.</i> , 2013	Case control male/female (n=109) healthy (53), hyperplastic polyps (16) and adenomatous polyps (40)	Colonocytes	No correlation with blood folates

Furthermore, folate supplementation studies have shown mixed results (**Table 1.5**), with many reporting no change in DNA methylation in response to folate supplementation. Studies that have detected an increase in genomic DNA methylation following folic acid supplementation have generally administered folic acid at pharmacological doses (1-10 mg/day), a dosage 5-50 times that of the UK Reference Nutrient Intake (RNI) of 200µg/day. An increase in genomic DNA methylation in the rectal mucosa of patients receiving 10mg/day folic acid for 6 months was observed but only after the removal of colorectal adenomas (Cravo et al., 1994). Another study by the same group showed increases in DNA methylation in the rectal mucosa after 3 months of folate supplementation at a dose of 5mg/day. However, this increase was only observed in patients possessing a single polyp (Cravo et al., 1998). A study performed by the second supervisor of this PhD project (Dr Maria Pufulete) reported an increase in genomic DNA methylation in leucocytes and colonic mucosa of subjects with adenomas after folic acid supplementation of 400µg/day for 10 weeks and a non-significant increase in genomic DNA methylation in CRC subjects. However, all subjects had neoplasia and this may have influenced DNA methylation (Pufulete et al., 2005a). In two other studies of neoplasia-free volunteers without other colonic pathologies, no changes in DNA methylation were found after supplementation with folic acid at supraphysiological doses of 2mg/day. However, both studies had small populations (63 and 61) and both employed the methyl acceptance assay. The latter has been shown to be less accurate than other methodologies because it estimates genomic DNA methylation from all methylated purified and intact genomic DNA, but this does not include DNA damaged upon sample collection or during preparation (Fenech et al., 1998, Basten et al., 2006). Two larger studies of 216 and 195 healthy volunteers examined DNA methylation in uncoagulated blood, and leucocytes respectively, both employed the LC-ESI-MS/MS method which is described in **Chapter 2**. (Jung et al., 2011, Crider et al., 2011). Neither study showed any association of DNA methylation with supplementation, but in the study by Crider et al., subjects on 4mg/day folic acid for 6 months showed a reduction in methylation when coagulated blood samples were used. One strength of this study was that results in both coagulated and uncoagulated samples were verified using both the methyl acceptance assay and LC-ES MS/MS. Jung's study was the first of its kind to recruit a sample of 216 healthy volunteers for an extended period of time. The participants were given 800µg/day of folic acid for 3 years. However, in this study volunteers with high homocysteine levels (an inverse indicator of folate status) were also included.

Long interspersed nucleotide element (Line-1) methylation pyrosequencing analysis has shown that DNA methylation may be reduced in precursor neoplastic lesions compared with normal colonic mucosa, particularly on the right side of the colon when compared with the left side (Chalitchagorn et al., 2004) (Estecio et al., 2007). Line-1 was used to analyse non-tumour colonic samples in a large study of 388 colorectal adenoma patients in a randomised controlled trial of folic acid at a dose of 1mg/day for 3 years. DNA methylation in the colonic mucosa on the right side was significantly less than on the left side ($P < 0.001$) but no difference was observed as a direct result of supplementation, nor did the results change when adjusted for age, gender, BMI, smoking or alcohol consumption (Figueiredo et al., 2009b).

Table 1.5 Effect of folate supplementation on DNA methylation in humans

Authors	Design	Subjects recruited	Duration	Dosage	Assays	Effect on DNA methylation
Cravo <i>et al.</i> , 1994	RCT	Adenoma (n=12), CRC (n=12), healthy volunteers (n=8)	6 months	10 mg/d	Rectal tissue, [³ H] methyl acceptance	Increase
Cravo <i>et al.</i> , 1998	RCT	Adenoma (n=20)	3 months	5mg/d	Rectal tissue, [³ H] methyl acceptance	No effect
Kim <i>et al.</i> , 2001	RCT	Adenoma (n=20)	1 year	5mg/d	Rectal tissue, [³ H] methyl acceptance	Increase
Pufulete <i>et al.</i> , 2005a	RCT	Adenoma (n=31)	10 weeks	400 µg/d	Leucocytes Rectal tissue [3H] methyl acceptance	Increase: 31% (leucocytes) 25% (rectal tissue)
Figueiredo <i>et al.</i> , 2009	RCT	Adenoma (n=388)	3 years	1mg/d	Rectal tissue LINE-1 analysis, pyrosequencing	No effect
Fenech <i>et al.</i> , 1998	RCT	Healthy volunteers (n=63)	12 weeks	2mg/d	Lymphocytes [[³ H] methyl acceptance	No effect
Basten <i>et al.</i> , 2004	RCT	Healthy volunteers (n=61)	12 weeks	1.2 mg/d	Lymphocytes [[3H] methyl acceptance	No effect
Axume <i>et al.</i> , 2007a	Depletion/repletion	Healthy females (n=43)	14 weeks	800 µg/d	Cytosine extension	No effect
Jung <i>et al.</i> , 2011	RCT	Healthy volunteers with (n=216) elevated tHcy	3 years	800 µg/d	Leucocytes LC-ES MS/MS	No effect
Crider <i>et al.</i> , 2011	RCT	Population-based, women of reproductive age (n=195)	6 months	4mg/d	Coagulated and uncoagulated blood	No effect in uncoagulated samples, decrease in coagulated (-14%; P=0.001)

The studies summarized above show considerable variability in the results reported for the relationship between folate status and genomic DNA methylation. This may be due to a number of factors including study populations, a range of study designs and sample sizes, the types of tissue and tumours analysed and inconsistent assay types. Earlier studies of DNA methylation employed the methyl acceptance assay, which works by enzyme mediated genomic DNA methylation. This is an imprecise technique because DNA may not remain intact during handling, which could lead to irreproducible results (Nephew et al., 2009). Other techniques for investigating genomic DNA methylation have included assessing repetitive element methylation and various restriction sites across the genome. Both techniques have shown inconsistent results (Wu et al., 2011). Furthermore, different regions of the genome have been measured using different assays, which may detect varying levels of upregulation (Nephew et al., 2009).

1.4.6.3 The *MTHFR* C677T genotype

Data on the interaction between folate (intake and status) and the *MTHFR* C677T genotype and their influence on DNA methylation are limited (see **Table 1.6** below). Further studies could help to clarify the mechanism by which folate supplies and the C677T polymorphism interact to affect DNA methylation.

In humans, one study reported that DNA was hypomethylated in the peripheral leucocytes of TT individuals compared with CC individuals ($P < 0.05$) and that DNA methylation was positively associated with systemic folate in red blood cells (Stern et al., 2000). Another study found leucocyte DNA was hypomethylated in TT subjects with inadequate folate status (Friso et al., 2002b).

A folate depletion-repletion study was employed to investigate the influence of folate status on genomic DNA methylation in the leucocytes of young women according to the *MTHFR* C677T genotype. Overall genomic DNA methylation was decreased in all subjects during depletion but, once repleted, women homozygous for the *MTHFR* C677T mutation had a greater increase in DNA methylation than women with the CC genotype. This confirmed a protective effect of the TT mutation (Shelnutt et al., 2004). Another study investigating this

relationship showed that a folate-restricted diet followed by a high folate diet in 18-45-year-old Mexican women for a period of 14 weeks resulted in lower DNA methylation in the TT group than in the CC and CT groups, suggesting an interaction between the mutation, folate metabolism and genomic DNA methylation. The same investigators examined genomic DNA methylation changes in leucocytes in African American women in response to a folate-restricted and a folate-rich diet, but only individuals who possessed the CC genotype were used in this study. During folate treatment, DNA methylation was not modified by differential folate intake and the authors concluded that the 14 week duration of folate supplementation may have been too short a timeframe in which to observe changes in such a young population (Axume et al., 2007a, Axume et al., 2007b). These studies investigated the effects of the *MTHFR* C677T genotype on DNA methylation in leucocytes to determine whether the interactions depended on folate status. A previous pilot study by this group did not show differences in genomic DNA methylation in the colonic mucosa between CC and TT individuals, but sample numbers were small (CC, n=38, CT=24, TT, n=6) (Pufulete et al., 2005b). Larger sample sizes are needed to increase statistical power and detect differences in methylation between individuals with and without the *MTHFR* C677T mutation and to investigate how these interactions are affected by folate status.

Table 1.6 The influence of *MTHFR* C677T genotype on DNA methylation in Neoplasia free volunteers

Authors	<i>MTHFR</i> C677T	Design and folate measured	Tissue for analysis of DNA methylation	Association with DNA methylation
Stern <i>et al.</i> , 2000	9CC, 10TT	Red cell folate	Leucocytes	Methylation reduced in TT compared to CC (P<0.05)
Friso <i>et al.</i> , 2002	187 CC, 105 TT	Red cell folate	Leucocytes	50% Reduction in TT compared with CC (P<0.001)
Shelnutt <i>et al.</i> , 2004	22CC, 19TT	Depletion 115 µg/dDFE *7 weeks, 400 µg/dDFE 7 weeks repletion	Leucocytes	Increased in TT subjects compared with CC during repletion (P=0.03)
Pufulete <i>et al.</i> , 2005b	38CC, 6TT	Serum, red cell folate, plasma tHcy	Rectal	No effect demonstrated
Axume <i>et al.</i> , 2007b	14CC, 17TT	Depletion 135 µg/dDFE *7 weeks, 400/800 µg/dDFE 7 weeks repletion	Leucocytes	Reduced in TT subjects only at week 14 when compared with CC
Elsworthy <i>et al.</i> , 2007	33CC, 33CT, 32TT	Depletion/repletion with 400 µg for 12 weeks	Leucocytes	No effect on depletion or repletion
Jung <i>et al.</i> , 2011	76CC, 70CT, 70TT	0.8 mg/day for 3 years	Leucocytes	No effect
Friso <i>et al.</i> , 2013	116CC, 29TT	Plasma folate	Peripheral blood mononuclear cells (PBMCs)	Reduced in TT subjects with low folate compared with CC (OR 7.04, 95%CI, 1.52-32.63, P=0.013)

1.4.7 Uracil misincorporation and folate in colorectal cancer

1.4.7.1 *In-vitro* studies

In-vitro studies have generally shown that folate deficiency increases strand breaks and uracil misincorporation in DNA. In Chinese hamster ovary cells, folate deficiency exacerbated irradiation-induced strand breaks (Branda and Blickensderfer, 1993). In colonocytes, folate deficiency was reported to increase strand breaks and uracil misincorporation, and these changes were concentration-dependent (1-10ng/mL), corresponding to folate concentrations found in human plasma (Duthie et al., 2000b). Increased uracil misincorporation was also observed in both human myeloid cells (Wickramasinghe and Fida, 1994) and lymphocytes grown in folate-depleted media (Duthie and McMillan, 1997).

1.4.7.2 Animal studies

Animal studies have previously demonstrated that folate deficiency increases uracil misincorporation into DNA in a dose-dependent manner *in vivo*. A surprising finding was that folate supplementation in young mice increased uracil misincorporation (Kim et al., 2011b). However, uracil misincorporation, DNA strand breaks and DNA fragmentation were all observed in hepatocytes of weanling male rats deprived of folate over a 9-week period when compared with controls on a folate-supplemented diet (James et al., 1997).

Folate depletion studies in animal models have shown that uracil misincorporation increases as a result of a folate depleted diet. A folate depletion-repletion and supplementation study investigated the relationship between ageing, folate status, and uracil misincorporation in a rat model. Weanling (n=44) and one-year-old rats (n=44) were given either a folate-free, folate-replete or folate-supplemented diet. The rats were euthanised at 0, 8 or 20 weeks, and folate levels and the amount of uracil that was misincorporated into DNA was measured in the colon. Uracil misincorporation in the colon increased by 50% in the older rats that received the folate-free and folate-replete diets compared with the corresponding younger rats (P=0.05). Furthermore, in the older rats, uracil misincorporation increased stepwise from

the folate-free to the folate-supplemented group ($P=0.05$ for the trend) suggesting a non-favourable effect from folate when combined with the aging process. In younger rats, uracil misincorporation into the DNA was not significantly affected (Choi et al., 2003). Another folate deficiency study was carried out in mice lacking in uracil DNA glycosylase (Ung^{-/-}), which excises uracil residues from DNA. Animals were given either a folate-deficient diet or a control diet for 32 weeks. The folate-deficient diet produced a significant increase in plasma homocysteine and a simultaneous increase in uracil misincorporation; however, these changes were not sufficient to initiate tumour growth (Linhart et al., 2009).

A more recent study was specifically designed to examine the effects of ageing and dietary folate on uracil misincorporation into DNA in the colonic and hepatic tissues of mice (42 male C57BL/6 mice in each arm of the study). Young mice (4 months) in one arm were compared with older mice in the other arm (18 months). For 20 weeks, the mice were given one of four different diets: (1) folate-depleted (2) folate-replete (3) folate-supplemented (8 mg/kg diet) and (4) folate-depleted (0 mg/kg diet) with thymidine supplementation (1.8 mg/kg diet). Unexpectedly, the younger group of folate-supplemented mice at a dose four times the basal requirement showed an increase in uracil misincorporation in the colon when compared with the folate-depleted and folate-replete groups ($P<0.05$). There was no change observed in the older group. In the liver, the opposite effect was observed; in older mice, uracil misincorporation decreased in response to increasing dietary folate, although this effect was not significant. In both old and young mice, uracil misincorporation did not differ between the folate-depleted group and the folate-depleted with thymidine supplementation. The overall effect of age led the authors to speculate that high dose folic acid supplementation can cause uracil misincorporation into colonic DNA and increase CRC risk from a young age. The results of this study are opposed to those of both the Linhart and the Choi study, leading the authors of the Kim study to conclude that mandatory folate fortification and voluntary folate supplementation should be carefully considered in relation to mechanisms for increased colorectal cancer risk (Kim et al., 2011b).

1.4.7.3 Human studies

Folate supplementation studies in humans have shown varying effects on uracil misincorporation across different subpopulations. Supplementing the diet of healthy subjects with folic acid (1.2 mg/day) for 12 weeks caused a 22% reduction in uracil misincorporation ($p < 0.05$) in lymphocyte DNA without reducing other biomarkers of genomic stability, including DNA strand breakage. When these results were split by folate quartiles at baseline, the reduction in uracil misincorporation from supplementation was even more apparent for subjects in the lowest folate quartile (Basten et al., 2006). In a pilot study of 14 individuals, the dietary intake of healthy volunteers was monitored with food diaries for two months. Folate (400 µg/day) was administered from the beginning of the second month. The results showed that adequate folate status (from diet or supplementation) decreased uracil misincorporation in leucocytes, but this effect was observed only in individuals with plasma vitamin B₁₂ concentrations above 400 pg/mL (Kapiszewska et al., 2005). Conversely, a six-month period of high dose folic acid (5 mg/day; 25 times the RNI) administered to patients with a history of colorectal adenoma, caused uracil misincorporation in the rectal mucosa when compared with baseline ($P = 0.02$) but no change was observed when compared with the placebo group ($P = 0.42$). Changes in uracil misincorporation between groups were not significantly different. This study does not support the case for folic acid supplementation as a chemopreventative agent to reduce uracil misincorporation in the colon of subjects with previous history of adenoma (van den Donk et al., 2007a). A more recent study investigated uracil misincorporation in relation to folate status in patients with either hyperplastic ($n = 16$) or adenomatous polyps ($n = 40$) and in disease-free controls ($n = 53$). No association was found between systemic biomarkers of folate status and uracil misincorporation in any group. The relationship between tissue folate and uracil misincorporation in the disease-free group was not reported, but in patients with hyperplastic or adenomatous polyps, tissue folate was lower and the uracil misincorporation higher in the polyp tissue compared with normal-appearing tissue adjacent to the polyp and normal-appearing tissue distal to the polyp (McGlynn et al., 2013).

As part of the Aspirin/Folate Polyp Prevention Study, Hazra *et al.* (2010) evaluated the effect of 3 years of a placebo or folate supplementation (1 mg/day) on uracil misincorporation in both human rectal mucosa (n=92) and white blood cells (n=60) in patients with previous adenomas. Differences between the placebo and the treated groups with regard to measures of plasma and red cell folate, as well as homocysteine, were all in the expected direction after supplementation. However, uracil misincorporation in rectal mucosa after 3 years of supplementation was not significantly associated with red blood cell folate (Spearman's correlation = -0.18, P=0.09) or plasma folate (Spearman correlation = -0.15, P=0.14). Additionally, uracil misincorporation in white blood cells (WBCs) was not significantly associated with red cell folate (Spearman's correlation = 0.003, P=0.97) or plasma folate (Spearman's correlation = 0.006, P=0.97). Folic acid supplementation did not influence either rectal mucosal or WBC uracil misincorporation levels (Hazra et al., 2010).

1.4.7.4 The *MTHFR* C677T genotype

Very few studies have investigated the relationship between the *MTHFR* C677T genotype, folate and uracil misincorporation.

The human HCT116 colonic cancer cell line, a well-established *in vitro* model of the *MTHFR* C677T genotype in humans, was utilised to look at the effect of the mutation on uracil misincorporation. The *MTHFR* C677T mutation was associated with changes in folate cofactors, including decreased *MTHFR* activity, decreased intracellular 5-methylTHF and increased intracellular 5,10-methyleneTHF, increased cellular growth and increased activity of thymidylate synthetase. The *MTHFR* C677T mutation caused a 31% reduction in uracil misincorporation, but this effect did not reach statistical significance (P=0.07) (Sohn et al., 2009).

The effect of 4 weeks of folic acid supplementation on uracil misincorporation into DNA was examined in the leucocytes of young, healthy volunteers. No correlation was observed between plasma folate levels and uracil misincorporation, and when separated out by

genotype, the *MTHFR* C677T polymorphism did not influence uracil misincorporation. This population was small (n=14) and mildly folate deficient; the baseline plasma folate levels were not measured, but the folate intake assessment showed a mean folate dietary intake of 52% RDA. Only one subject consumed 100% of the RDA (Kapiszewska et al., 2005). The relationship between the *MTHFR* C677T genotype, blood folate levels and DNA stability was studied in human lymphocytes from healthy volunteers. Lower plasma folate and higher plasma homocysteine levels were observed in TT individuals, but no significant differences were observed between the CC and TT groups in lymphocyte DNA strand breaks or uracil misincorporation (Narayanan et al., 2004).

In a randomised, double-blind, placebo-controlled trial, the effects of 12 weeks of daily folic acid supplementation (1.2 mg) in a healthy, folate-replete cohort of 15 men and 15 women with a mean age of 42 years was investigated. A significant inverse association was observed between red cell folate and uracil misincorporation into the DNA of lymphocytes, both prior to and after supplementation ($P < 0.005$), whereas DNA strand breakage and global DNA methylation were not influenced by the 3-month intervention with folic acid. The *MTHFR* genotype did not influence uracil misincorporation, but the numbers in the TT group were very small and were not adequate to detect significance (Basten et al., 2006).

Biomarkers of genomic stability need to be investigated in larger populations of healthy subjects in association with the *MTHFR* genotype. Research groups investigating the influence of folate status and supplementation on misincorporation of uracil into DNA report varying results. There have been no large studies looking at the influence of folate status and the *MTHFR* C677T genotype on uracil misincorporation into the DNA in colon cells of volunteers without previous or current colorectal adenomas or cancer.

1.5 Rationale for proposed research in normal neoplasia-free volunteers

There are several gaps in the existing knowledge of folate status and DNA methylation and uracil misincorporation and these are described below (1-5).

1. A limitation of the studies described is that the relationship between folate status and DNA methylation and uracil misincorporation has mostly been reported in subjects with disease and confounding factors of the disease may have affected biomarker measurement. Further investigation is required to determine the extent to which the *MTHFR* C677T genotype folate status influences DNA methylation and uracil misincorporation in normal colonic mucosa of disease free volunteers. Larger samples of subjects free of confounding factors (such as inflammation, early disease or lifestyle factors such as alcoholism) are also needed to shed light on the relationship between these biomarkers of damage and folate status in disease free humans to provide a better understanding of colorectal cancer risk and preventative measures which may be taken to reduce risk.
2. The studies described have assessed CRC risk and folate status and DNA methylation and uracil misincorporation in cell lines, animal models, and human leucocytes and lymphocytes but less so in human colons. Further work is needed to clarify the extent to which mechanisms involving folate status and DNA methylation and uracil misincorporation are affected by site and tissue specificity. Therefore, studies in which these markers are measured in both blood and specific tissues may clarify site-specific risk and also reveal the usefulness of systemic biomarkers as surrogates when it is not possible to obtain tissue samples from the colon.
3. Additionally, many of the studies conducted to date have measured genomic DNA methylation by the [³H]-methyl acceptance assay, which is non-specific and prone to a large degree of variation. More sensitive assays, including LC-MS/MS for genomic

DNA methylation will lead to more accurate quantification.

1.6 Hypothesis

Folate status and the *MTHFR* C677T polymorphism influence intermediary biomarkers of neoplasia (DNA methylation and uracil misincorporation) in the colonic epithelium of human subjects without colorectal adenomas or cancer.

1.6.1 Aims (Study 1)

To investigate the association between *MTHFR* genotype, systemic and colonic folate status, DNA methylation and uracil misincorporation in the colon of subjects without colorectal adenomas or cancer.

1.6.2 Aims (Study 2)

To determine whether increasing folate intake in subjects without colorectal neoplasia modifies DNA methylation and whether the response differs according to the *MTHFR* C677T genotype.

1.6.3 Objectives

- To investigate the influence of systemic and colonic folate biomarkers on DNA methylation.
- To investigate the influence of systemic and colonic folate biomarkers on uracil misincorporation.
- To investigate the influence of *MTHFR* genotype on DNA methylation in the colon.
- To investigate the influence of *MTHFR* genotype on uracil misincorporation in the colon.
- To investigate the influence of *MTHFR* genotype on systemic and colonic folate biomarkers.

- To investigate whether systemic folate biomarkers can predict colonic tissue folate.
- To investigate the influence of demographic and lifestyle characteristics on DNA methylation, uracil misincorporation and colonic mucosal folate level in the colon.
- To determine whether folate supplementation modifies DNA methylation, and systemic and colonic folate levels, and whether the response differs according to the *MTHFR* C677T genotype.

2 Methods

2.1 Study Background

The FOLGEN Study was a continuation of work undertaken in 2000–2002, by the second supervisor of this PhD, where a case-control study investigated genomic DNA methylation in the colonic mucosa of subjects with colorectal cancer or adenoma, and also subjects without neoplasia. Genomic DNA hypomethylation in normal-appearing colonic mucosa was associated with low folate status and a risk of neoplasia (Pufulete et al., 2003). Additionally, an intervention study in subjects with colorectal adenoma suggested that short-term folate supplementation (400 µg/day) increased genomic DNA methylation in leucocytes and colorectal mucosa when compared with controls (Pufulete et al., 2005b). During this study a questionnaire was developed and validated to assess dietary folate intake (Pufulete et al., 2002). These two studies supported a link between folate status and genomic DNA methylation in the colon, but the sample size was small, which limited detection of the influence of specific genetic polymorphisms on colonic DNA methylation. The current project followed on from this work and aimed to assess the impact of folate status (systemic and colonic) and the *MTHFR* C677T mutation on DNA methylation and uracil misincorporation in the colon of volunteers without colorectal adenoma or cancer.

2.1.1 Funding and Ethical Approval

This project was funded by the Biotechnology and Biological Sciences Research Council (BBSRC). All aspects of the study were approved by the Research Ethics Committees (REC) at King's College Hospital NHS Trust and Guys and St Thomas' NHS Foundation Trust (REC number: 06Q0703/29).

2.1.2 FOLGEN subject population and study overview

Subjects for this study were recruited sequentially from a series of subjects attending the Endoscopy Department at King's College Hospital NHS Trust. A referral for a clinically indicated colonoscopy was made in response to complaints such as persistent diarrhea or constipation, bloating, abdominal pain or repeated bleeding on opening of the bowel. Subjects were usually referred by their GP or by a clinician on the ward.

2.2 Study design

FOLGEN Study 1 was a cross-sectional study designed with the aim of recruiting approximately 400 subjects with no abnormal findings on colonoscopy. Primary endpoints were differences in *MTHFR* C677T genotypes (CC, CT and TT) for both genomic methylation and uracil misincorporation in the colon. At the time of study design there was no data available on differences in uracil misincorporation for these genotypes. However, it was possible to calculate sample size from an observational study in which leucocyte DNA methylation was measured in healthy subjects (Friso et al., 2002b). This showed that 32 TT individuals and 128 CC individuals would be sufficient to detect a 50% difference (0.5SD, 30ng mCyt/ μ g DNA) in genomic DNA methylation between CC and TT individuals (5% significance level, two tailed) for the *MTHFR* C677T mutation. In order to recruit approximately 40 TT subjects an upper limit of 400 was proposed because the frequency of the TT genotype in the general population is approximately 10% (Frosst et al., 1995).

FOLGEN Study 2 was a randomised, double-blind, placebo-controlled parallel-design intervention study to investigate the effect of folic acid supplementation on DNA methylation and uracil misincorporation. Sample size was calculated from a previous folate intervention study in which genomic DNA methylation in colonic mucosa was an endpoint. Subjects were assigned to receive either a 400 μ g folic acid supplement or placebo for 3 months. Blood and colorectal tissue samples were obtained at baseline (at the end of colonoscopy) and at post intervention (using sigmoidoscopy).

2.2.1 Subject recruitment methods – Folgen 1

Medical records of all subjects referred to the Endoscopy Departments at King's College Hospital and Guy's and St Thomas' Hospital NHS Foundation Trusts between December 2006 and February 2009 were screened according to study criteria (described below) and subjects who satisfied the inclusion criteria were approached to take part in the study. Prior to any study-specific procedures taking place, an Informed Consent Form (ICF) was signed by each subject who had agreed to participate (**See Appendix 1**).

2.2.1.1 Inclusion criteria:

At colonoscopy:

- Subjects who had a normal colonoscopy (to the ileocaecal valve).
- Subjects with no macroscopic evidence of malignancy, adenomatous or hyperplastic polyps, or inflammation.

2.2.1.2 Exclusion criteria:

At Screening:

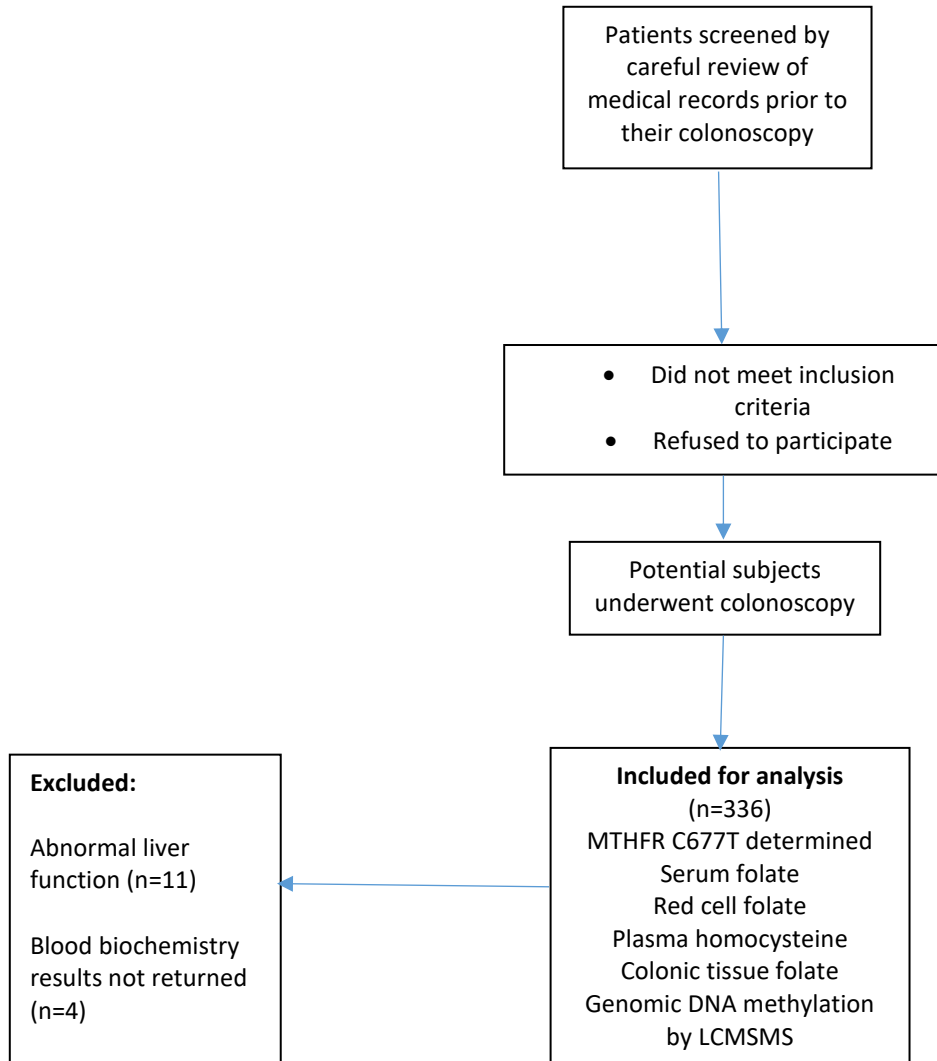
- Subjects with a previous history of colorectal cancer/polyps.
- Subjects with a strong family history of colorectal cancer or adenomatous polyposis coli (according to the Amsterdam Criteria: one first degree relative under age 40 or two first degree relatives of any age diagnosed with colorectal cancer).
- Subjects with inflammatory bowel disease or current/past history of gluten-sensitive enteropathy.
- Family history of endometrial cancer.
- Documented renal or liver disease.
- Pregnancy, epilepsy, alcoholism or pernicious anaemia.
- Use of anti-folate (e.g., methotrexate) medication or other folate antagonists.

At colonoscopy:

- Evidence of tumours/polyps/inflammation at colonoscopy.

Figure 2.1 depicts the flow of sequential events in Study 1. After consent and prior to colonoscopy, newly recruited subjects were questioned further to check for exclusions that may not have been detailed in the hospital notes. If the subject consented, they were taken through the study Health Questionnaire (see **Appendix 2**) for the collection of demographic data on height and weight (for calculation of BMI), ethnicity, smoking habits, alcohol intake, Marmite consumption, current medication (including confirmation of no folate antagonists), folic acid supplement usage in the past three months, allergies, medical problems, and physical activity levels. A cannula was then put into the arm of the subject for sedation, pain relief and bowel relaxant to make the procedure more comfortable. At the point of cannulation, five blood samples were taken for the study. The subject then entered the procedure room for colonoscopy. The procedure was monitored closely throughout the colonoscopy, and subsequent enrolment into the study only happened if the inclusion/exclusion criteria was satisfied. Under no circumstances were subjects enrolled if there were any signs of redness or inflammation observed in the large bowel. Additionally, if the colonoscopy had to be terminated early for any reason, such as pain or lack of compliance with bowel preparation (colonic lavage solution – KleanPrep[®], Norgine, Harefield, UK), subjects were immediately excluded because pathology or inflammation could not be ruled out in parts of the colon that had not been visible, including the ileocaecal valve. If there was no evidence of pathology or inflammation to explain the subject's symptoms, and if the colonoscopy was successfully completed to the ileocaecal valve, six tissue biopsies were taken on withdrawal of the colonoscope, and the subject was included in the study. Blood samples were sent to the Departments of Clinical Biochemistry and Haematology for systemic folate biomarker analysis and tissues samples were transported and stored (according to the Human Tissues Act) for primary and secondary endpoint analysis. All of these methods are described in more detail below (Sections **2.4-2.7**). A previously validated FFQ was given to each subject to complete at home and return in a pre-stamped and addressed envelope.

Figure 2.1 Patient Recruitment Schema Study 1



2.2.2 Subject recruitment – Study 2

Study 2 was conducted in a subgroup of subjects who were recruited into Study 1. After participation in Study 1 and determination of the *MTHFR* C677T genotype using restriction fragment length polymorphism (RFLP) analysis (**see section 2.4.5**), subjects with CC and TT genotypes and who met the inclusion criteria (described below) were approached by telephone call and asked if they would be willing to take part in the 12-week study. The consent was signed and the FFQ was completed if it had not been returned through the post.

2.2.2.1 Inclusion criteria:

- Subjects with a normal colonoscopy (to the ileocaecal valve)
- Subjects with no adenomas or polyps (apart from very small polyps sent to histology and no neoplasia confirmed)
- Subjects with no macroscopic evidence of malignancy, adenomatous or hyperplastic polyps, or inflammation
- Subjects from Study 1 with either the *MHTFR* CC or TT genotype
- Subjects who agreed to attend the hospital for a repeat biopsy by rigid sigmoidoscopy

2.2.2.2 Exclusion criteria:

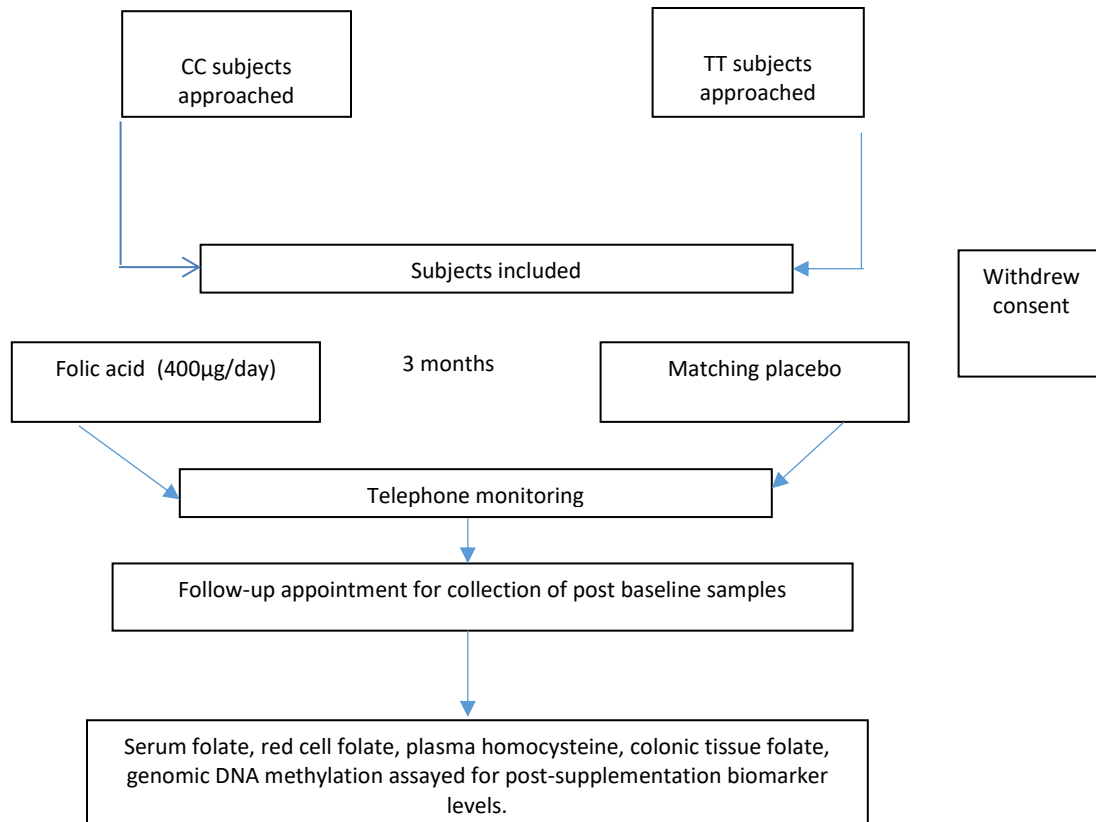
- Use of anti-folate (e.g., methotrexate) medication or other folate antagonists
- Subjects whose biopsies results showed inflammation or any sign of neoplasia
- Subjects from Study 1 with the *MHTFR* CT genotype

Figure 2.2 depicts the flow of sequential events in Study 2. The aim of the study was for patients to be randomised to either folic acid (400 µg/day) or an identically matched placebo (30 subjects in each arm) and stratified according to the *MTHFR* C677T genotype for a three-month intervention period. After several months, recruitment remained extremely slow and

it became evident that final recruitment would be far lower than expected. The decision was made to remove the blind. At this point folic acid or placebo was deliberately assigned to subjects with the *MTHFR* CC and TT genotypes in as balanced a way as possible to try and enable a meaningful comparison in the analysis.

During this time, progress and compliance was monitored by telephone every 2 weeks. At the end of the intervention, subjects attended the Outpatient Department for post-intervention follow-up. Unused tablets were returned and counted (so that compliance could be verified), post-intervention fasting venous blood samples were drawn and rectal biopsies were collected with rigid sigmoidoscopy. Blood and tissue samples from Study 2 were compared with matching baseline samples from Study 1 for primary and secondary endpoint analysis. The study aimed to recruit 60 subjects in total – 30 subjects in the folic acid arm and 30 subjects in the placebo arm. This number was based on a previous folate intervention study carried out by this group, which also assessed genomic DNA methylation in colonic mucosa as an endpoint. This trial suggested that 23 subjects in each arm (folic acid and placebo) would be adequate to detect a 136Bq/μg/DNA (0.8SD) change in genomic DNA methylation as a result of supplementing with folic acid with $P < 0.05$ (80% power). The decision to recruit 30 subjects in each arm (a total of 60) was reached in order to allow for withdrawal of consent.

Figure 2.2 Patient Recruitment Schema Study 2



2.3 Demographic and dietary data, and biomarker sample collection

2.3.1 Health Questionnaire (HQ)

As described above, the Health Questionnaire (see **Appendix 2**) was used to collect demographic and lifestyle data as well as medication, supplementation usage and activity levels. Data for ethnicity were recorded into four ethnic subgroups (white, black, Asian and other) but for the purpose of the analysis Asian and other were combined and reported as 'other' due to small numbers in both of these groups.

2.3.2 Food Frequency Questionnaire

On leaving the hospital, all subjects were given a Food Frequency Questionnaire to complete in order to be able to assess dietary folate intake (previously validated in healthy volunteers recruited from the public) (Pufulete et al., 2002). Subjects were invited to a non-compulsory research follow-up and they were also given a stamped addressed envelope and asked if they would mind posting their questionnaires should they be unable to attend their study follow-up appointment.

2.3.3 Blood sample collection

Prior to colonoscopy, fasting venous blood samples were collected from the cannula using the vacutainer technique. Blood was collected into EDTA vacutainers for full blood count, plasma homocysteine, red cell folate and leucocyte DNA extraction, and into vacutainers containing no anticoagulant for determination of serum folate and vitamin B₁₂. The blood samples for plasma homocysteine and DNA extraction were immediately chilled on ice. The plasma homocysteine sample was centrifuged and the plasma was collected within 2 hours, both the whole blood and the plasma extract were then frozen at -70°C until use.

2.3.4 Tissue biopsy sample collection

If there were no macroscopic abnormalities (inflammation) found at the end of colonoscopy, up to six biopsies (5-64mg) were removed from the rectum. Each tissue sample was placed

into a sterile, DNase-, RNase-free cryovial (Greiner Labortechnik) and instantly snap frozen in liquid nitrogen before storage at -70°C for analysis of three endpoints: tissue folate concentration, genomic DNA methylation and uracil misincorporation.

2.4 Blood Biomarker analysis

2.4.1 Determination of blood folates, serum B₁₂ and plasma homocysteine

Folate status (serum and red cell folate and plasma homocysteine), serum vitamin B₁₂ and liver function were all analysed in the Departments of Clinical Biochemistry and Haematology at King's College Hospital NHS Trust London.

Serum and red cell folate, serum vitamin B₁₂ and homocysteine were all determined using the Bayer Advia Centaur® assay (Siemens Healthcare Diagnostics, US), a competitive immunoassay which utilizes direct, chemiluminescent technology. Within batch CV was <2.2%, and between batch CV was <5.2% for serum and red cell folate, as well as the homocysteine assay.

2.4.1.1 Serum Folate Assay – A brief Description

The serum sample was pretreated to release the folate from endogenous binding proteins in the sample. Folate in the sample competes with acridinium ester-labelled folate in the Lite Reagent (Siemens Healthcare Diagnostics Ltd) for biotin-labelled folate binding protein; this binds to avidin, which is covalently bound to paramagnetic particles in the Solid Phase. The amount of folate present is inverse to the amount of relative light units (RLUs) detected by the system.

2.4.1.2 Red Cell Folate Assay – A brief Description

Folate in the sample competes with acridinium ester-labelled folate in the Lite Reagent for a biotin-labelled folate binding protein. This is bound to avidin, which is covalently coupled to paramagnetic particles in the Solid Phase. The Advia Centaur folate assay requires the sample to be pre-treated to release folate from endogenous binding proteins. The amount of folate present is inverse to the number of relative light units (RLUs) detected by the system.

2.4.1.3 Vitamin B₁₂ Assay – A brief Description

Vitamin B₁₂ in the sample competes with acridinium ester-labelled vitamin for purified intrinsic factor; this is covalently coupled to paramagnetic particles in the Solid Phase. A sodium hydroxide releasing agent and dithiothreitol DTT release the vitamin B₁₂ from the endogenous binding proteins in the sample. Vitamin B₁₂ levels are inversely related to the amount of relative light units (RLUs) detected by the system.

2.4.1.4 Homocysteine Assay – A brief Description

Homocysteine in the sample was reduced using a reducing agent to free homocysteine and converted to S-adenosylhomocysteine (SAH) with a homocysteine enzyme reagent (supplied by Siemens Healthcare Diagnostics Ltd). SAH covalently linked to paramagnetic particles in the solid phase competes with converted SAH from the subject sample for acridinium ester-labelled anti-SAH in the lite reagent. An inverse relationship exists between the amount of homocysteine present in the sample and the amount of relative light units (RLUs) detected by the system.

2.5 Determination of folates in colorectal mucosa - method development

Tissue folate concentration for the 336 subjects was determined in 1-2 colorectal biopsies by the microbiological assay (MBA) using *Lactobacillus Rhamnosus* (ATCC 7469): The method was refined from a well-established assay that had been used in the department for some years. The assay was first established from a method kindly supplied by Anthony Wright from the Institute of Food Research. The assay was originally developed by the current author for a MSc project investigating folate bioavailability in food (Kennedy, 2004). The standard microbiological assay is routinely employed for folate quantification in food such as broccoli and in human samples – usually whole blood. The assay was particularly suitable for this study because it measures the total content of the different folate molecules rather than the individual folate values as measured using other methods. The bacterium *Lactobacillus Rhamnosus* is highly sensitive to the presence of folate which was ideal in this study because available biopsies were small (5-64mg).

2.5.1 Assay Development

In any folate quantification method, folate must first be extracted from the sample by careful disruption and homogenisation of the samples in extraction buffer to release the folate from the cells (Gregory, 1989).

Next, the folate must be deconjugated into shorter chains (mono- and diglutamates) as the molecule exists in tissue as polyglutamate chains. *Lactobacillus Rhamnosus* produces a signal far more reliably in response to smaller mono and diglutamate folate units. This conversion requires a deconjugase such as chicken pancreas or rat plasma (Shrestha et al., 2000).

Biopsies in this study were small. Large tissue samples were not available as biopsies were collected from healthy volunteers who had not undergone resection for previous disease. To quantify tissue folate from small colonic biopsies of 5–64mg, the food protocol described above required considerable method development to adapt the previous microbiological assay (MBA) protocol for much smaller samples (Kim et al., 1998, Kim et al., 2001). Where extraction of folates in the food protocol was straightforward using an Ultra Turrax probe, it was much more challenging to physically disrupt the cells in very small pieces of mucosa while optimising and preserving the total folate released. An Ultra Turrax probe, sonication (bath and probe) and a reusable Teflon pestle in a 1.5mL Eppendorf tube with liquid nitrogen were three methods that were all tested in an attempt to pulverise the biopsies.

It became apparent that homogenising the tissue with the Ultra Turrax was not possible because the tissue was so small that it ended up lodged on the blade and there was a risk of losing the tissue altogether. In the same vein, sonication was difficult to achieve and cells were left largely intact. Ultimately, after testing all techniques and measuring folate released from similar sized biopsies, the pestle technique resulted in superior mechanical disruption of the cells. The Teflon pestle fitted tightly into the 1.5mL Eppendorf tube, which enabled samples to be forcibly crushed and sheared against the wall of the Eppendorf tube until there was nothing visible. Additionally, this option prevented samples from thawing prior to the addition of the extraction buffer, which was essential to prevent folate degradation by cellular enzymes.

2.5.1 Extraction and deconjugation of folate from Colonic tissue

1M KPO₄ Stock Buffer was made up by dissolving 68.045g KH₂PO₄ and 87.087g K₂HPO₄ in 1L AR water and storing at 2-8°C.

Reagents:

Potassium Dihydrogen Orthophosphate (KH₂PO₄) (Sigma Aldrich)
Dipotassium Hydrogen Orthophosphate (K₂HPO₄) (Sigma Aldrich)
β-mercaptoethanol (Sigma Aldrich)
Bis (2-hydroxyethyl)imino-tris(hydroxymethyl)methane (Bis-triz)
Sodium Ascorbate (Sigma Aldrich)
Analytical Reagent (AR) grade water (Thermoscientific)
Desiccated Chicken Pancreas (BD Biosciences Codified Cat. No. 245910)

2.5.1.1 Extraction of folate from 5-64mg samples of colorectal mucosa

After removal from the -70°C freezer, colonic biopsies were weighed and placed back on ice to ensure that the folates remained intact until extraction. Each biopsy sample was then crushed and sheared against a 1.5mL Eppendorf tube with a Teflon pestle over dry ice until only minute pieces of sample remained. However, the texture of the tissue was very tough and, therefore, difficult to completely eliminate in some cases.

200μL freshly prepared folate extraction buffer [5 mmol β-mercaptoethanol/L and 0.1mol sodium ascorbate/L in 0.1mol bis (2-hydroxyethyl)imino tris(hydroxymethyl)methane/L, pH 7.85] were added to each sample, and the pestle was rinsed in it to remove all of the homogenate. Eppendorf tubes were placed in a hot water bath at >90°C for 20 minutes before cooling in an iced water bath for 10 minutes. Samples were then centrifuged at 13,500 rpm for 30 minutes, and the supernatant was pipetted off and stored in an Eppendorf tube at -70°C. At the end of each extraction, pestles were sterilised for the next use.

2.5.1.2 Deconjugation of folate previously removed from colorectal tissue samples

A 5mg/mL stock chicken pancreas solution was prepared by adding 60mg chicken pancreas to 12mL AR water in a Falcon tube. The solution was carefully and gently mixed for 10 minutes

and centrifuged for a further 10 minutes at 1250 x g, 2000 rpm at 15°C. A working buffer 0.1M KPO₄ containing 10mg/mL sodium ascorbate solution – was also prepared. The following deconjugation systems were constructed:

- 100µL folate sample (biopsy extract described above)
- 10µL chicken pancreas supernatant
- 90µL 0.1 M NaPO₄ buffer pH 7

Ten blanks were constructed by replacing the folate extracts with KPO₄ buffer to obtain a total buffer volume of 190µL and combining with 10µL chicken pancreas solution. The tubes were incubated at 37°C for 2 hours, after which the process was halted by freezing. Samples were stored at -70°C prior to assay.

2.5.2 Quality Control Stock Sample

One large colonic mucosal sample (left over from a resection in a previous study) was weighed (662.5mg) and placed into a sterile 100mL Duran Bottle with freshly prepared folate extraction buffer (66.3mL). The mixture was homogenised with a Turrax probe to mechanically disrupt the tissue cells (this method worked well with a much larger piece of tissue). The mixture was then split between two sterile 50mL Falcon tubes, which were placed in a water bath at 90°C for 20 minutes. After heating, the tubes were cooled in an iced water bath for 10 minutes. The Falcon tubes were then centrifuged at 13,500 rpm for 30 minutes. The quality control samples were incubated at 37°C for 2 hours. Incubation was halted by freezing at -70°C prior to analysis.

The QC deconjugation system comprised 100µL of tissue sample extract, 10µL of chicken pancreas solution and 90µL of 0.1 M KPO₄ containing 10mg/mL of sodium ascorbate solution. The tubes were incubated at 37°C for 2 hours, after which the process was halted by freezing. Samples were stored at -70°C prior to analysis.

Preparation of the calibration dilutions, the assay medium and the folic acid standards is described below.

2.5.3 Assay Plate Preparation

The subject sample, the blank and the QC were diluted 25-fold in two steps:

- 1 100µL of the subject sample was diluted with 400µL of 0.5% sodium ascorbate solution.
- 2 200µL of the above sample was diluted with 800µL of 0.5% sodium ascorbate solution.

Dilutions were made according to previous concentrations described in the literature (Kim et al., 1998).

2.5.4 Preparation of Folic acid standards

Reagents

- Folic acid (Sigma Aldrich)
- Sodium hydroxide (NaOH) (Sigma Aldrich)
- Sodium ascorbate (Sigma Aldrich)

Solution A: Folic acid stock solution (200 µg/mL)

Folic acid (20mg) was weighed and added to a volumetric flask with 0.1 M NaOH (4mL) solution and made up to 100mL with AR water to obtain a 200 µg/mL solution.

Solution B: Folic acid intermediate solution (10 µg/mL)

Solution A (10mL) was added to 200mL 0.1 M NaPO₄ buffer (20mL stock buffer 1 M NaPO₄ diluted by 180mL AR water) to obtain a 10 µg/mL intermediate folic acid solution. Adjustment to pH 7 was obtained with dropwise addition of concentrated NaOH and monitored with a spectrophotometer (E_{max} 1 cm 1% 10 µg/mL at 282 nm=0.625).

Solution C: Folic acid working standard (100 ng/mL)

Solution B (2.5mL) was diluted with 250mL 0.5% sodium ascorbate solution (1.25g sodium ascorbate in 250mL AR water) in a volumetric flask. Aliquots of 1mL were dispensed into Nunc tubes and stored at -70°C.

Solution D: Folic acid calibration curve standard (1 ng/mL)

Solution D was made up on the day of the assay with the addition of 0.5mL of Solution C made up to 50mL with freshly prepared 0.5% sodium ascorbate solution in a volumetric flask to obtain a 1 ng/mL concentration.

2.5.5 Preparation of Folic Acid Casei Medium (FACM)

Reagents:

- Folic acid casei medium (FACM) (Biosciences Cat. No. 282210)
- Chloramphenicol (Fisher Scientific)
- Manganese sulphate ($\text{MnSO}_4\text{H}_2\text{O}$) (Sigma Aldrich)
- Ethanol
- Analytical Reagent (AR) grade water

The assay medium was prepared by weighing out 4 x 11.75 g FACM and placing each in a 250mL Duran bottle to be made up to 200mL with AR water. Each solution was shaken vigorously to dissolve the FACM thoroughly. 40mg chloramphenicol was dissolved in 400 μ L ethanol and made up to 20mL with AR water. 3.75mL of the chloramphenicol solution was added to each bottle of medium. Manganese sulphate (878.4mg) was dissolved in 40mL AR water, and 5mL was added to each bottle before the 4 bottles were brought to the boil for 1 to 2 minutes, cooled down and made up to 250mL with AR water. The media were decanted into 50mL Duran bottles, and each bottle was autoclaved at 121°C for 5 minutes before being left to cool and then refrigerated at 2-8°C until the day of the assay.

2.5.6 Preparation of calibration dilutions

Nunc tubes containing **Solution D** were taken from the -70°C freezer on the day of assay and further diluted into 9 calibration series with 0.5% sodium ascorbate solution as shown below.

Table 2.1 Calibration series dilutions 1

Tube	Solution D (mL)	0.5% sodium ascorbate solution (mL)	Folic acid concentration (pg/mL)
1	0	2.0	0
2	0.05	1.95	2.5
3	0.1	1.9	5.0
4	0.15	1.85	7.5
5	0.2	1.8	10.0
6	0.3	1.7	15.0
7	0.4	1.6	20.0
8	0.5	1.5	25.0
9	0.6	1.4	30.0

2.5.7 Preparation of calibration and assay plates

Lactobacillus Rhamnosus aliquots were prepared previously during an Msc project from freeze dried organisms by the current author (Kennedy, 2004). Briefly 3ng folic acid (30 µl) was added to 20mL growth and 20mg ascorbic acid. This was incubated at 37°C until the organism was growing freely, a process which took approximately 5 or 6 days to achieve. Following this, the log phase could be pre-determined. At the beginning of the day, 4 x 20mL growth medium (with added folic acid + ascorbic acid) and different concentrations of subculture (100 µl, 500 µl and 2mL) were incubated at 37 °C. The absorbance of the three cultures were followed hourly at 600nm and plotted against time for up to 48h to find an optimum time and concentration in which to use the culture for the assay.

On the day of the assay, the cryopreserved organism was removed from the freezer and defrosted. Each 50mL Duran bottle of assay medium was inoculated with 200µL of the organism, and 50mg of ascorbic acid was also added. The FACM was carefully shaken to homogenise with a minimum of air bubbles created.

Aliquots of 200µL of inoculated medium were then added to every well (using a multi-channel pipette) in both the calibration plate and the assay plate.

2.5.7.1 Calibration Plate

The nine calibration series described above were added to the calibration plate; 8 wells in each column were filled with one dilution standard (i.e. 100µL of each standard in a full column of 8 wells across 9 columns) as shown in **Figure 2.3**.

2.5.7.2 Assay plate

There were 8 wells (one column per subject) in the assay plate. 100µL diluted folate extract was added to the 200µL inoculated medium in each well for each of the 336 subjects. The assay plate blanks and the deconjugation blanks each made up one column of the plate. **See Figure 2.4**

Plates were firmly sealed using a Mylar acetate plate sealer and roller (ICN pharmaceuticals); the plates were then carefully vortexed to ensure homogenous mixtures. They were then incubated at 37°C for 42 hours.

After incubation, the plates were inverted to mix the liquid, and the optical density in each well was determined on a microplate reader (Anthos 2001) at 620nm in order to calculate the folate content in each well from the calibration curve.

Figure 2.3 Calibration Plate Layout

[illegible]

Figure 2.4 Folate Sample Plate Layout

[illegible]

2.6 The *MTHFR* C677T genotype

2.6.1 Genomic DNA extraction

DNA was extracted from leucocytes in the blood samples using the GenElute™ Blood Genomic DNA Kit (Sigma).

Laboratory Equipment required:

- Water bath @ 55°C
- Filter barrier pipette tips
- Microcentrifuge tubes (1.5mL)
- Microcentrifuge (Micro Centaur MSE)

GenElute™ Blood Genomic DNA Kit components (product code NA 2010, Sigma):

- Resuspension Solution
- Lysis Solution
- Column Preparation Solution
- Wash Solution Concentrate
- Elution Solution (10mM Tris-HCl, 0.5mM EDTA, pH 9.0)
- Proteinase K
- RNase A Solution
- GenElute Miniprep Binding Columns
- Collection Tubes, 2.0mL
- Molecular biology grade ethanol (product code E 7148, Sigma)
- Molecular biology reagent grade water (Nuclease free water, Ambion)

Reagent preparation:

Prewash Solution:

The Prewash Solution Concentrate was diluted with 27.5mL molecular biology grade ethanol (100%).

Wash Solution:

The Wash Solution Concentrate was diluted with 80mL molecular biology grade ethanol (100%).

Proteinase K:

Proteinase K powder was dissolved in molecular biology reagent water to yield a 20mg/mL stock solution.

Protocol:

- 200µL room temperature-equilibrated whole blood was added to a 1.5mL Eppendorf tube containing 20µL Proteinase K solution. This was vortexed to thoroughly mix the enzyme.
- 20µL RNase A Solution was added to the sample before vortexing again for 15 seconds and then incubated for 2 minutes at room temperature. This step was carried out to obtain RNA-free genomic DNA.
- 200µL Lysis Solution C, a chaotropic salt-containing solution, was added to the sample before vortexing for 15 seconds before being incubated at 55°C in a water-bath for 10 minutes.
- 200µL 100% ethanol was added to the lysate and vortexed for 5-10 seconds to obtain a homogeneous solution.
- To maximise consistent binding of DNA to the silica membrane, 500µL column preparation solution was added to each pre-assembled GenElute Miniprep Binding Column and centrifuged at high speed ($12,000 \times g$) for 1 minute. The flow-through liquid was discarded.
- The lysate was carefully loaded into the pre-treated GenElute Miniprep Binding Column and centrifuged at low speed ($\geq 6,500 \times g$) for 1 minute. The collection tube containing the flow-through liquid was discarded, and the column was placed into a new 2mL collection tube.
- To remove potential contaminants, 500µL Prewash Solution was added to the column, which was centrifuged at low speed ($\geq 6,500 \times g$) for 1 minute. The collection tube containing the flow-through liquid was discarded, and the column was placed into a new 2mL collection tube.
- To ensure the column was free of ethanol (known to interfere with the Polymerase Chain Reaction (PCR)), 500µL Wash Solution was added to the column and centrifuged at high speed ($12,000 \times g$) for 3 minutes until the column was dry. The flow-through liquid was discarded, and the collection tube was used to collect further flow-through liquid during an additional centrifugation step at high speed ($12,000 \times g$) for 1 minute.

The collection tube containing the flow-through liquid was discarded, and the column was placed into a new 2mL collection tube.

- 100µL Elution Solution was added directly into the centre of the column, and after incubation for 5 minutes at room temperature, it was centrifuged at low speed ($\geq 6,500 \times g$) for 1 min. This step was repeated, and both eluates were collected into a 1.5mL Eppendorf tube.
- The combined eluate containing pure genomic DNA was stored at 4°C on a short-term basis.

2.6.2 DNA Quantitation

DNA concentration was quantified by a Nano Drop® ND-1000 UV spectrophotometer, and agarose gel electrophoresis (**Figure 2.5 below**) was used to confirm the presence of a pure genomic DNA product that could be used for genotyping (**Figure 2.7**).

Equipment

Nano Drop® ND-1000 UV spectrophotometer

Pipette and pipette tips with filter barrier

Molecular biology reagent water (nuclease free water, Ambion)

The Nano Drop spectrophotometer was switched on, the software was set to 'measure DNA', and initialisation was achieved by blanking with 1.5µL nuclease-free water pipetted onto the measurement surface. The surface was carefully cleaned and dried before 1.5µL DNA solution was added and the measurement was made. Each sample was measured in duplicate to optimise accuracy. The Nano Drop® output provided the concentration in ng/µL (the peak should be at 260nm for DNA with a 280/260nm ratio ideally between 1.7 and 2.0; below 1.7 suggested high concentrations of protein were still present, and above 2.0 indicated poor quality DNA product). If the ratios were outside of these limits the dilutions were repeated and samples were rerun.

2.6.3 Gel Electrophoresis

Laboratory Equipment required:

Horizontal electrophoresis tank

Horizontal electrophoresis gel tray

Power supply (Apelex, PS 3002)
UV transilluminator (Alpha Imager, Alpha Innotech Corporation 2000)
Gel combs (16 well)
Eppendorf tubes (0.5mL)
Pipettes and pipette tips (Gilson: 10, 20, 100 µL)
Autoclave tape

Reagents required:

Routine use agarose (product code A9539)
Ethidium bromide (product code E-7637, Sigma)
Trizma base (product code T-6066, Sigma)
Boric Acid (product code B-6768, Sigma)
EDTA (product code E-5134, Sigma)
DNA ladder (Hyperladder V and VI, Bioline)
Deionized water
Loading buffer (50mg BB, 3mL 10×TBE, 7mL glycerol)
Molecular biology reagent water (nuclease free water, Ambion)

10 × TBE buffer (1 L)

108g Trizma base, 55g boric, acid and 7.45g EDTA were dissolved in deionised water and diluted by a factor of 10.

Agarose gel preparation (0.7, 1.5, 3.0% w/v)

- 0.7, 1.5, or 3.0g of agarose powder was dissolved in 100mL 1 × TBE buffer in a conical flask and heated in a microwave.
- The gel was left to cool, and 10µL ethidium bromide (5mg/mL) was added.
- The mixture was swirled for homogeneity and poured into the gel tray with the comb placed in the well position and left to set for approximately 20 min.

DNA preparation

- Genomic DNA: 7 µL molecular biology grade water, 2µL loading buffer, and 1 µL pure genomic DNA were added to a 0.5mL Eppendorf tube, and a 0.7% w/v agarose gel was used.

- PCR product: 2µL loading buffer and 15µL PCR product were added to a 0.5mL Eppendorf tube, and a 1.5% w/v agarose gel was used.
- Digested PCR product: 2µL loading buffer and 20µL digested PCR product were added to a 0.5mL Eppendorf tube, and a 3% w/v agarose gel was used.

Gel electrophoresis

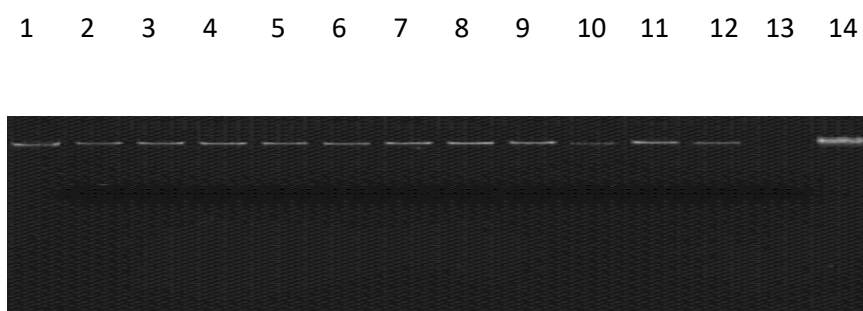
- Approximately 500mL 1 × TBE buffer was poured into the electrophoresis tank.
- The autoclave tape and comb were removed from the set gel, and the gel tray was inserted into the tank.

For a 0.7% w/v agarose gel, 2 µL Hyperladder VI was loaded into one of the outside wells, and 10µL of the DNA solution was added to the remaining wells (**Figure 2.5**).

Figure 2.5: 0.7% Electrophoresis gel

To confirm the presence of genomic DNA from blood samples in 351 subjects in order to be able to ascertain *MTHFR* genotype for Study 1 analysis, and also to screen for Study 2

Lanes 1-11:	10µL genomic DNA solution (1µL DNA)
Lane 12:	10µL control sample (pooled DNA taken from student blood samples)
Lane 13:	Buffer (control)
Lane 14:	2µL Hyperladder VI (Size Range 100 to 1000bp)



Size BP
48,500

Figure 2.6: 1.5% Electrophoresis gel

For a 1.5% w/v agarose gel, 3 μ L DNA Hyperladder V (Size Range 25 to 500bp) was loaded into one of the outside wells, and 17 μ L PCR product was loaded into the remaining wells.

Electrophoresis run on a 1.5% agarose gel to confirm presence of PCR products of the *MTHFR* gene in 351 subjects to ascertain MTHFR genotype for Study 1 analysis and also screen for Study 2

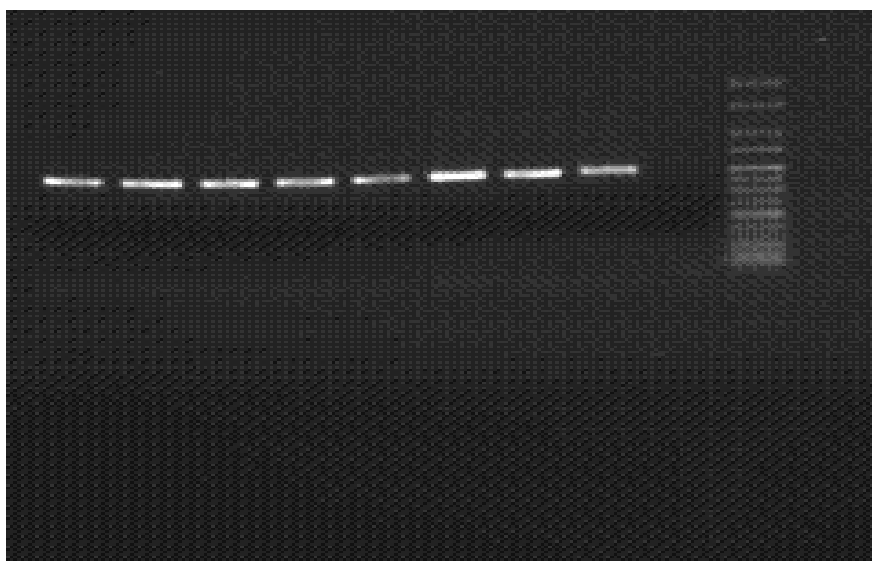
Lanes 1-7: 15 μ L *MTHFR* gene PCR product

Lane 8: 15 μ L control sample - pooled DNA taken from student blood samples

Lane 9: Buffer (control

Lane 10: 3 μ L DNA Hyperladder V (Size Range 25 to 500bp)

1 2 3 4 5 6 7 8 9 10



2.6.4 Determination of the MTHFR mutation

Genotype:

MTHFR C677T genotype was determined by Restriction Fragment Length Polymorphism (RFLP) analysis using a previously developed protocol (Frosst et al., 1995) that comprised PCR followed by Hinf I digestion.

Reagents:

MTHFR primers (MWG-Biotech AG)

HotStar Taq polymerase (Biotage)

dNTPs (Amersham Pharmacia Biotech)

Amplitaq Gold (Perkin-Elmer)

Restriction enzymes Hinf I (Invitrogen)

Hinf I H Buffer (Invitrogen)

Molecular biology reagent grade water (nuclease free water, Ambion)

Hyperladder V (Bioline)

TrackIt ladder 100bp (Invitrogen)

PCR for the *MTHFR* gene:

Forward and reverse primer sequences for the methylenetetrahydrofolate reductase (*MTHFR*) gene were as follows:

Forward primer (5'→ 3')	T	G	A	A	G	G	A	G	A	A	G	G	T	G	T	C	T	G	C	G	G	G	A
Reverse primer (5'→ 3')	A	G	G	A	C	G	G	T	G	C	G	G	T	G	A	G	A	G	T	G			

Both forward and reverse primers were supplied at a concentration of 100µM and diluted to a 20µM stock solution.

Nucleotides:

- 25µL of all four individual 100 mM dNTP stocks were mixed together to yield a 100 µL solution of 25 mM mixed dNTPs. The mixed dNTPs were then diluted in a

1:5 ratio (100 μ L dNTPs in 400 μ L water) to obtain a 5mM solution and were aliquoted into 3 tubes.

- 2 μ L dNTP mix was used per 50 μ L PCR reaction volume to reach a concentration of 200 μ M for each dNTP.

Reaction mix

10 $\times$ PCR buffer	2.5	1.5 mM MgCl ₂
dNTPs	1.0	200 μ M
Forward primer	0.5	0.1 μ M
Reverse primer	0.5	0.1 μ M
Water	19.375	
HotStar Taq polymerase	0.125	1.25 U

Total	24.0 μ L	
-------	--------------	--

- 1.0 μ L purified genomic DNA solution was added to 24 μ L reaction mix, giving a final volume of 25 μ L in each well.
- PCR reaction programme:

95°C 15 min

95°C 1 min, 55°C 1 min, 72°C 1 min; for 35 cycles

72°C 10 min

4°C ∞

- After PCR, purity was investigated on a 1.5% agarose gel (**Figure 2.6**), and the success of the amplification process was confirmed with regard to the MTHFR gene as explained above.

Restriction Fragment Length Polymorphism (RFLP)

The restriction enzyme Hinf I recognises the G*ANTC sequence and cleaves after G. After Hinf I digestion, an individual homozygous for the wild-type *MTHFR* C677 polymorphism possesses one 198 bp fragment; an individual heterozygous for the *MTHFR* C677T polymorphism possesses 3 fragments of 198 bp, 175 bp and 23 bp; and an individual homozygous for the mutant *MTHFR* C677T polymorphism has 2 fragments of 175 bp and 23 bp as follows:

<u>Genotype</u>	<u>After Digestion</u>
CC	198 bp
TT	175 bp + 23 bp
CT	198 bp + 175 bp + 23 bp

10 µL restriction digest was prepared as follows and scaled up accordingly:

Reaction Mix	× 1
10 × buffer (H buffer)	2.0
Water	7.5
Restriction enzyme (Hinf I)	0.5
Total	10µL

- 10µL PCR product was added to the reaction mix.

- Samples were incubated overnight at 37°C on a thermal cycler.
- The digested PCR products were then analysed on a 3.0% agarose gel (**Figure 2.7**), and the genotype for the *MTHFR* C677T polymorphism was determined.
- The power was set to 150V for 45 minutes for a 0.7 and 1.5% w/v agarose gel and 100V for 1 hour 30 minutes for a 3.0% w/v agarose gel.
- After the gel was run, it was removed from the electrophoresis tank and photographed under a UV illuminator.

For a 3.0% w/v agarose gel, 3 µL DNA HyperladderV and 4 µL 10 bp TrackIt ladder were loaded into one of the outside wells and 2 µL sample DNA was loaded into the remaining wells.

In **Figure 2.7**, homozygous wild-type (CC) samples are seen in lanes 2, 4, 7, 12, and 13; heterozygous (CT) samples are in lanes 3, 5, and 8-11; and a homozygous mutant for the *MTHFR* C677T polymorphism sample can be seen in lane 6.

Hinf I digested PCR product of the *MTHFR* gene run on a 3.0% agarose gel from samples of 351 subjects to ascertain *MTHFR* genotype for Study 1 analysis and also screen for Study 2.

Lane 1: 4 µL Track It 10 bp DNA Ladder

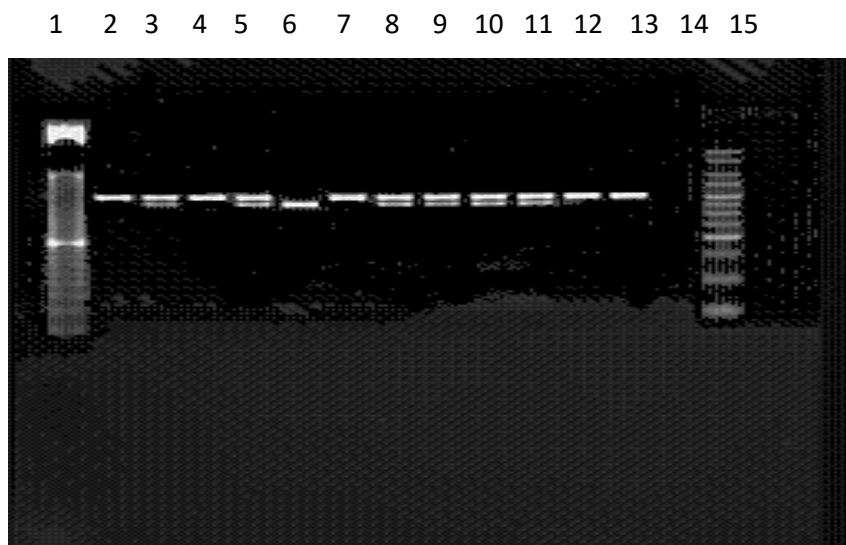
Lanes 2-12: Digested PCR product

Lane 13: control sample

Lane 14: Buffer (control)

Lane 15: 3 µL DNA Hyperladder V

Figure 2.7 3.0% electrophoresis gel



2.7 Genomic DNA Methylation

Genomic DNA methylation in colorectal mucosa was quantitatively determined by liquid chromatography/electrospray ionisation mass spectrometry (LC-ESI-MS/MS). The method, which was selected for precision, sensitivity, selectivity, and speed was adapted from a previously reported protocol using the LC-ESI-MS/MS method (Friso et al., 2002b).

Genomic methylation was assessed firstly by heating to denature the DNA and adding the stable isotope [^{13}C , $^{15}\text{N}_2$] 2'-deoxycytidine. Then enzymatic hydrolysis was carried out by sequential digestion, and the whole solution was injected onto an analytical column. The four DNA bases were separated and 5-methylcytosine was identified using LCMS in the Multiple Reaction Monitoring (MRM) mode.

The precursor/product ion pair of m/z 228/112 was monitored for 2'-deoxycytidine and m/z 242/126 for 5-methyl-2'-deoxycytidine, with a dwell time of 500ms for each pair. The chromatographic peaks for 2'-deoxycytidine and 5-methyl-2'-deoxycytidine eluted at 5.0 and 6.5 minutes, respectively. Quantification was achieved by comparison to known

concentrations of internal standard. Chromatograms were analysed using the Quanlynx v 4.1 software package (Manchester, UK).

Cytosine and 5-methylcytosine content in DNA was calculated from calibration curves where the area ratios of 2'-deoxycytidine or 5-methyl-2'-deoxycytidine to the internal standard were determined.

2.7.1. Reagents:

S1 nuclease (deoxyribonuclease P1, endonuclease P1) Sigma N8630

Shrimp alkaline phosphatase GE Healthcare e70092Y

Snake Venom Phosphodiesterase (SVP) USB Europe 20240Y

[¹³C, ¹⁵N₂] 2'-deoxycytidine (TRC Toronto Research Chemicals Inc., Ontario, Canada)

2.7.2. Equipment:

Heating block

Filter barrier pipette tips

Microcentrifuge tubes (0.5mL)

Microcentrifuge (Micro Centaur MSE)

Phenomenex Synergi Polar RP column

2.7.3. Solution preparation:

S1 nuclease (deoxyribonuclease P1, endonuclease P1) (2 units/μL)

The certificate of analysis was consulted for the S1 nuclease (deoxyribonuclease P1, endonuclease P1) vials, which contained 205 units/vial. As instructed, 152.5μL 0.05M ammonium acetate, pH 5.3 was added to obtain a final concentration of 2 units/μL (Crain, PF methods in enzymology vol 193, 1990). The diluted solution was divided into aliquots and stored at -20°C until use.

SV Phosphodiesterase (0.002 units/μL)

The SVP vial containing 100 units was re-suspended in 500μL re-suspension buffer provided to produce a solution of 0.2 units/μL. This was divided into 50 aliquots of 10μL

each. To each aliquot, 90µL re-suspension buffer was added to make a concentration of 0.02 units/µL. This was stored at -20°C until use. Before use, a further dilution was carried out by transferring 10µL of the 0.02 units/µL solution into another 0.5mL and adding 90µL re-suspension buffer to make a final concentration of 0.002 units/µL.

2.7.4 Shrimp alkaline phosphatase 1 unit/µL

A 500µL vial had was divided into aliquots and stored at -20°C until used.

2.7.5 DNA Digestion

For each subject, 20µL of DNA was aliquoted into a 0.5mL Eppendorf tube and placed in a heating block at 100°C to denature it. After exactly 5 minutes, each tube was immediately chilled on ice, and 10µL labeled deoxycytidine was added to each Eppendorf tube along with 3µL 0.1M ammonium acetate pH 5.3 and 2µL Nuclease S1 (4 units). The Eppendorf tubes were placed back on the heating block for two hours at 45°C. At the end of the two hours, 3.5mL 1M ammonium bicarbonate + 2µL SVP (0.004 units) were added, and the Eppendorf tubes were heated for a further two hours at 37°C. Finally, in the third stage of the trienzymatic digestion, 4.05mL SAP reaction buffer + 1µL SAP (1 unit) was added before heating again at 37°C for one hour. At the end of the hour, the heat was increased to 65°C for a further 15 minutes. Next 200µL of the mobile phase was added, and the digested DNA solution was added to a glass vial prior to LC/ESI/MS analysis.

2.7.6 Genomic DNA methylation analysis

For quantitation of genomic DNA methylation by LC-ESI-MS/MS, chromatography was performed by injecting 40µL of the digested DNA/isocratic mobile phase consisting of 10mM ammonium formate solution + 0.1 % formic acid:methanol (95:5) onto a Phenomenex Synergi Polar RP column, 250 × 2 mm, 4 µm particle size, 80 Å pore size (Cheshire, UK), coupled to a Column tandem mass spectrometer (Micromass Quattro Ultima) operating in the electrospray ionisation (ESI positive) mode.

For mass spectrometry, the experimental conditions were as follows: column temperature, 30°C; sample rack temperature, 4°C; source block temperature, 120°C; desolvation temperature, 450°C; cone gas (N₂) flow, ~100 L/h; desolvation gas (N₂) flow; ~650 L/h; collision gas (Ar) pressure; ~2.7 x 10⁻³ mbar; capillary voltage, 2.5 KV; and cone voltage, 15 V.

Unit mass resolution (LM & HM) 1&2 was set to 13, and collision energy voltage and multiplier voltages were 12V and 650V, respectively.

2'-deoxycytidine and 5-methyl-2'-deoxycytidine (Sigma-Aldrich, USA) were used as external standards, and the chromatographic peaks were eluted at 5.0 and 6.5 minutes, respectively (**Figure 2.8**).

As described above, quantification was carried out in the multiple reaction monitoring (MRM) mode by monitoring the precursor/product ion pair of m/z 228/112 for 2'-deoxycytidine and m/z 242/126 for 5-methyl-2'-deoxycytidine, with a dwell time of 500ms for each pair.

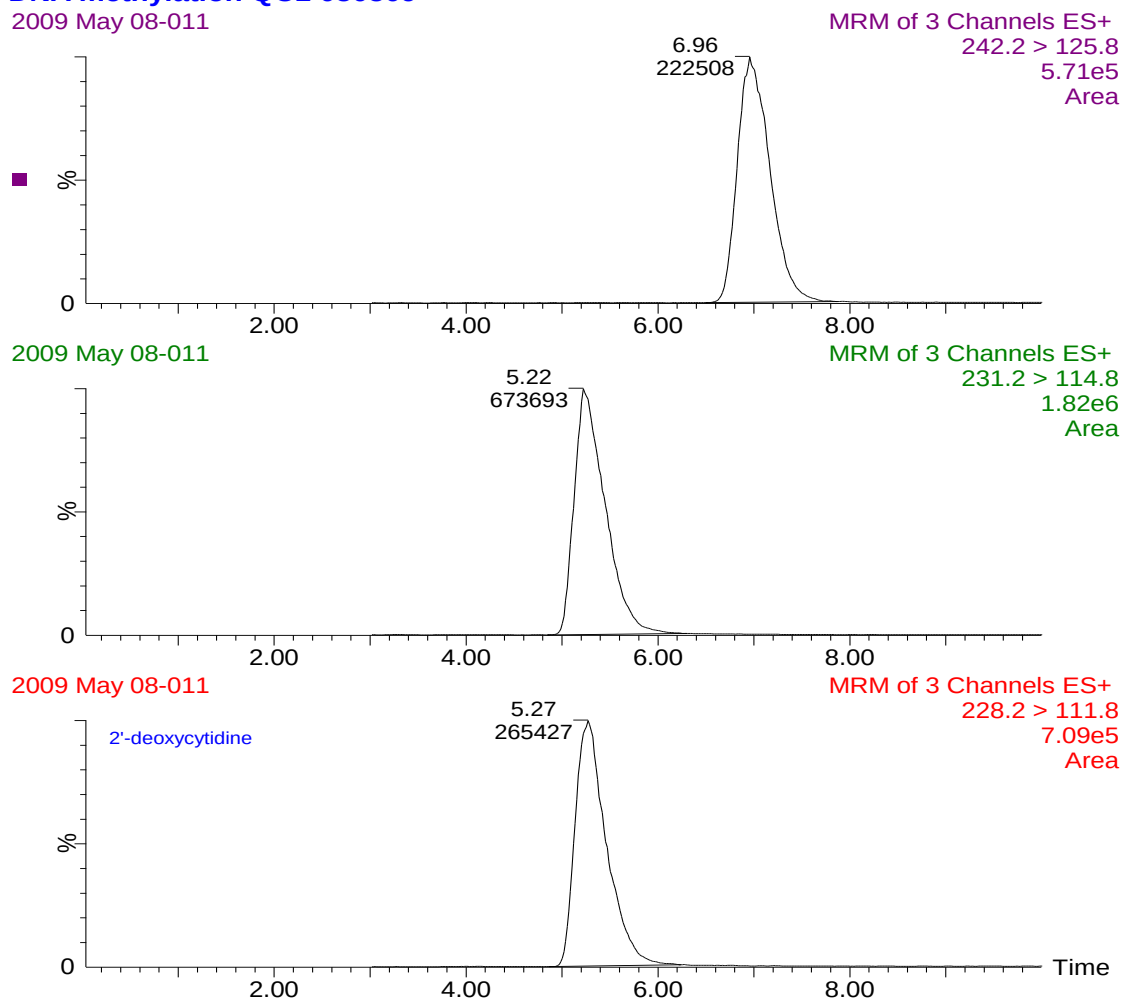
The Quanlynx v 4.1 software package (Manchester, UK) was used to analyse the chromatograms. DNA methylation status was calculated from the area ratios of 2'-deoxycytidine or 5-methyl-2'-deoxycytidine to internal standard (labelled [^{13}C , $^{15}\text{N}_2$] 2'-deoxycytidine) under the curve, which were plotted against known concentrations of 2'-deoxycytidine or 5-methyl-2'-deoxycytidine. Genomic DNA methylation status was expressed as the relative amount of 5-methyl-2'-deoxycytidine compared to total deoxycytidine residues.

Pooled DNA extracts from study subjects were assayed for the control samples. The within-assay and between-assay coefficients of variations were 3.9% and 10.0%, respectively.

Figure 2.8 Example Sample Chromatogram

DNA methylation QC1 050509

2009 May 08-011



2.8 Quantification of Uracil content in DNA

Uracil Misincorporation was determined by measuring uracil content in DNA samples using an adaptation of a previously described method (Blount et al., 1994) using gas chromatography and negative chemical ionization-MS.

2.8.1 Reagents

Uracil (Sigma Aldrich)

Uracil internal standard (U-13C4, 99%; U-15N2, 98%) (Cambridge Isotope Laboratories, Inc.)

Uracil DNA glycosylase (UDG) (Invitrogen)

1 μ L 3,5-bis(trifluoromethyl)benzyl bromide (BTFMBzBR) (SigmaAldrich)

EDTA Na salt (EDTA (product code E-5134, Sigma)

Trizma base (product code T-6066, Sigma)

Acetonitrile (271004 Sigma-Aldrich)

Triethylamine (T0886 Sigma-Aldrich)

Isooctane (540-84-1) Fisher Scientific

2.8.2 Equipment

G24 incubator shaker

Agilent Technologies 6890N network Gas Chromatograph system

Agilent Enhanced MSD Chem Station running on Windows XP Professional.

SpeedVac container

Eppendorf tube

Milli-Q purification system water

2.8.3 Solution preparation

Stock 1 M Tris HCL pH8

Trizma base (12.1 g) was added to 100mL AR water and mixed until dissolved; pH was adjusted to 8 with concentrated HCl. The solution was then autoclaved.

Stock 0.2 M EDTA Na salt pH8

EDTA Na salt (14.888 g) was dissolved into 200mL AR water, and the pH was adjusted to 8 with NaOH, after which the solution was autoclaved.

TE Buffer

TE buffer was prepared by combining Tris HCL, pH 8 and stock EDTA Na Salt (1 M Tris HCL, pH 8 was prepared from 12.1 g Trizma base added to 100mL AR water in a volumetric flask, and the pH was adjusted to 8 with concentrated HCl before autoclaving. Stock EDTA Na Salt 0.2 M, pH 8 was made by adding 14.888 g EDTA Na salt to 200mL AR water, and the pH was adjusted to 8 with NaOH before autoclaving at 120°C).

Uracil standards

Uracil (Sigma Aldrich) and the uracil internal standard (U-13C4, 99%; U-15N2, 98%) (Cambridge Isotope Laboratories, Inc.) were both weighed out (10mg) and made up to 100mL with Milli-Q purification system water in a volumetric flask to give a 100 pg/μl stock supply of each solution from which a calibration series of uracil standards could be made as follows:

Uracil Standard and Uracil Internal standard Solutions

100ng/μl - Master Standard or Master Internal Standard:

10mg uracil or uracil internal standard (U-13C4, 99%; U-15N2, 98%) was weighed out and made up to 100mL with Milli-Q purification system water in a volumetric flask.

100 pg/μl - Stock Standard or Stock Internal Standard:

10μL Master Stock Solution above was made up to 100mL with Milli-Q purification system water.

0.2 pg/μl Working Standard:

200μL Stock Solution was made up to 100mL with Milli-Q purification system water.

2.5 pg/μl Internal Working Standard:

2.5mL Stock Internal Solution was made up to 100mL with Milli-Q purification system water.

Calibration Series

A dilution series were constructed in 2.0mL tubes (**Table 2.2**)

Table 2.2 Uracil calibration series

Tube	Weight uracil in each vial (pg)	Volume of working solution (μl)	Weight of internal standard (pg)	Volume internal standard (μl)
1	0	0	0	0
2	0	0	50	20
3	50	250	50	20
4	0.6	3	50	20
5	1.3	6.5	50	20
6	2.5	12.5	50	20
7	5	25	50	20
8	10	50	50	20
9	30	150	50	20
10	50	250	50	20
11	100	500	50	20
12	150	750	50	20

Next 50µL TE buffer and 2 units uracil DNA glycosylase (UDG) (Invitrogen) were added to each DNA sample (1-100µL) of known individual concentration before incubation at 37°C for 1 hour to remove the uracil, after which 20µL (50pg) (U-13C4, 99%; U-15N2, 98%) uracil internal standard was added. Solutions of standards and DNA samples were then dried for 4 hours in a SpeedVac container. The residue in each Eppendorf tube was re-suspended in the following: 50µL acetonitrile, 10µL triethylamine, 1µL 3,5-bis(trifluoromethyl)benzyl bromide (BTFMBzBR) (SigmaAldrich). Samples were shaken at 500 rpm in a G24 incubator shaker at 30°C to form N₁N₃-(3,5-bis[trifluoromethyl]benzyl)uracil (ura-diBTFMBz), and 50µL water was added to improve extractability of ura-diBTFMBz. The derivatised uracil was extracted into 100µL isooctane by vortexing for 30s and then minicentrifuged at full speed for 30s. The top phase (isooctane) was collected and analysed by GCMS.

2.8.4 Determination of uracil content in DNA

Uracil content was measured using the Agilent Technologies 6890N network Gas Chromatograph system equipped with a 7683 series autoinjector, a PTV (Gerstel) Inlet and a 5673 inert mass selective detector with a chemical ionization module, and the software was Agilent Enhanced MSD Chem Station running on Windows XP Professional. Samples were introduced onto the column via a Programmable Temperature Vaporisation (PTV) inlet in solvent mode with carrier gas: helium (BOC products GC grade), and the operating parameters were optimised using Agilent technologies PTV flow calculator software.

GCMS operating parameters

Parameter	Specification
Column type	Agilent technologies HP-5 MS capillary
Column dimensions	30 m length x 250 mm internal diameter x 0.25 mm film thickness
Injection liner	Agilent technologies open baffle swirl hole design
Column flow	1.5mL/min (constant flow mode)
Injection mode	solvent vent, inlet temperature program
Pneumatics control	vent pressure 1.3 psi, vent flow 100mL/min, vent end time 0.9 min. purge time 1.5 min.
Injection volume	6 ml total as 3x 2 ml, 1 s delay time.
Oven program	initial temp. 100°C, holding for 1 min and ramping to 240°C by 25°C/min, then to 280°C, hold 2 min, then 40°C/min to 320, hold 2 min.
Run time	13.6 min. Interface/Source/Quad Temperatures: 250°C/106°C/150°C. EM Offset: 400 above autotune.
Solvent Delay	6 min. Tune file: ncich4.u
Acquisition mode	negative chemical ionization with selected ion monitoring
Resolution	low
Dwell time per ion	120 ms
Ion masses	337, 343 corresponding to amidic anion (M-227) via a dissociative electron capture mechanism in a thermalised electronic methanic environment.

Calculation of uracil in DNA

Selected ion chromatograms were extracted, and the peak areas (integrated detector responses) were tabulated in Excel. A calibration curve was constructed for uracil by plotting the ratio of the 337 ion signal (native uracil) to that of the 343 ion (internal standard) against the molecular mass of uracil. Uracil concentrations were derived using the equation from the calibration curve.

Within- and between-assay variation was 6% and 7%, respectively.

2.9 Statistical analysis

All data were analysed using the Statistical Package for Social Sciences (SPSS) version 21.0.

Statistical analysis is described in each of the separate chapters.

3 The association between the MTHFR C677T genotype and folate status and genomic DNA methylation in the colon of subjects without colorectal adenoma or cancer.

3.1 Introduction

Disruptions in DNA methylation patterns can occur both on a genomic and gene-specific level in colorectal carcinogenesis (Issa et al., 2004). Reduction in genomic DNA methylation (hypomethylation) normally occurs with an increase in gene-specific DNA methylation (hypermethylation). Genomic DNA methylation in blood cells has been associated with serum folate and red cell folate in CRC patients and healthy volunteers (Pufulete et al., 2003, Pufulete et al., 2005b). The MTHFR C677T genotype is an additional factor which may influence the interaction between DNA methylation and folate status in leukocytes (Friso et al., 2002b, Axume et al., 2007b). This relationship has not been fully elucidated in the colon and at the time the current study was carried out, there were no reports of this relationship in subjects without colorectal cancer.

The aim of this study was to investigate the association of biomarkers of folate status and the *MTHFR* C677T polymorphism on genomic DNA methylation in the colon of human subjects without colorectal adenoma or cancer.

3.1.1 Hypothesis

Folate status and the *MTHFR* C677T mutation are associated with DNA methylation in the colon of subjects without colorectal adenoma or cancer

3.1.2 Aims

1. To investigate the association between *MTHFR* C667T genotype and genomic DNA methylation in the colon of subjects without colorectal adenoma or cancer.
2. To investigate the association between folate status and genomic DNA methylation in the colon of subjects without colorectal adenoma or cancer.

3.1.3 Objectives

1. To determine the *MTHFR* genotype using restriction fragment length polymorphism (RFLP) analysis in blood cells of all study subjects
2. To determine serum and red cell folate, vitamin B₁₂ and homocysteine status in blood using the competitive immunoassay and folate status in colonic mucosa using a microbiological assay.
3. To quantitatively determine genomic DNA methylation in colonic mucosa by liquid chromatography/electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS) and investigate associations with the *MTHFR* C677T genotype and with these folate biomarkers.

3.2 Methods

3.2.1 Subject recruitment

Subject recruitment, inclusion and exclusion criteria, demographic data collection and tissue sample collection are all described in **Chapter 2, Sections 2.1-2.3**. All subjects provided blood samples for determination of the *MTHFR* C677T genotype, serum and red cell folate, colon tissue folate, serum vitamin B₁₂ and plasma homocysteine, as well as mucosal biopsies for the determination of genomic DNA methylation.

3.2.2 Laboratory Methods

Methods for the extraction of DNA, and for the determination of genomic DNA methylation and biomarkers of folate and vitamin B₁₂ status are described in detail in **Chapter 2, Sections 2.4-2.7.1**.

3.2.3 Statistical analysis

The primary endpoint of this current study was to look for differences in genomic DNA methylation between the *MTHFR* 677 CC and TT genotypic subgroups. Target sample size (n=400) was calculated using data from an observational study which measured DNA methylation in the leukocytes of healthy volunteers with the CC and TT genotypes (Friso et al., 2002a). The study was powered to detect a 50% difference (0.5 SD, 30 ng mCyt/mg DNA) in genomic DNA methylation between the CC and TT genotypes (5% significance level, 2 tailed, and 80% power; with a ratio of CC to TT as 4:1).

Subject characteristics and dietary and lifestyle data are displayed as mean (SD) and frequency (%) apart from daily alcohol consumption and smoking, which are displayed as median (IQR) due to being very skewed. Differences in subject characteristics between genotypes were tested using ANOVA for continuous variables (age, BMI, folate intake), Chi-squared tests for categorical variables (gender, ethnicity and nutritional supplement use). The Kruskal-Wallis test was used for alcohol intake and smoking, this was because variables were not normally distributed and did not meet the assumptions of ANOVA.

Biomarkers are displayed as median and inter-quartile Range (IQR) (the range between the first and the third quartile) because they were not normally distributed. Differences in single continuous variables (serum and red cell folate, colonic tissue folate, serum vitamin B₁₂, plasma homocysteine and genomic DNA methylation) between genotypes were compared using the Kruskal-Wallis test because they were not normally distributed and did not meet the assumptions of ANOVA.

Spearman's rank correlation coefficients were used to investigate associations between DNA methylation and folate biomarkers because data were not normally distributed.

Spearman's rank correlation coefficients were also used to investigate associations between DNA methylation and continuous demographic variables age, BMI, dietary folate intake, total folate intake, number of cigarettes smoked and amount of alcohol consumed.

Differences in genomic DNA methylation between demographic and lifestyle factor sub-groups were assessed using the Mann-Whitney test (gender, smoking status, drinking status and nutritional and folic acid supplementation) and the Kruskal-Wallis test (ethnicity) as these variables were not normally distributed and did not meet the assumptions of ANOVA.

Multiple linear regression was used to assess whether DNA methylation could be predicted from any of the independent variables. Forced Entry, or 'Enter' in SPSS, was selected as the method of Multiple Linear Regression. All predictors are entered into the model at the same time and in no particular order. Ranked variable DNA methylation was added to the model as the dependent variable. The *MTHFR C677T* genotype and ranked

variables (tissue folate, serum and red cell folate, plasma homocysteine) were all added as predictors.

In a second model, additional variables were added to the model, these were age (continuous), gender (male or female), ethnic group (white, black, other), nutritional supplement use (user or non-user) and serum vitamin B₁₂ (continuous). This method relies on theoretical justification for the selection of predictor variables. Adjustments were made for age, gender and ethnicity because there is evidence that DNA methylation differs by these variables (Jones et al., 2015, Zhang et al., 2011).

3.3 Results

3.3.1 Study recruitment

A total of the 3112 subjects were screened for the current study, 2466 did not meet the inclusion criteria and 295 declined to participate. A total of 351 subjects were included and screened. Of those, 4 subjects were excluded because their biochemistry results were reported to be missing by the laboratory and 11 were excluded due to abnormal liver function tests (an exclusion criterion). Overall 336 (96%) patients were included in the main analysis.

3.3.2 Subject characteristics and dietary and lifestyle data

Table 3.1 shows the subject characteristics in this study. The average age of the subjects was 57 years. Overall, there were more women (62%) than men, the majority of subjects were Caucasian (67%), and there was a large proportion of African Caribbean Black subjects (21%), 5% of the study were Asian and the remaining 3% were classified as other. Mean BMI was 26.9 kg/m². Approximately 23% of the population were smokers with a median consumption of 12 cigarettes per day. Approximately 56% of the subjects reported drinking alcohol with a median consumption of 11g per day. 31% were nutritional supplement users (sources were variable and data was not available on micronutrient content across the study), and 14% consumed folic acid containing supplements (sources were variable, the majority of which contained 400 µg) on a daily basis. Of the 60 subjects who provided data on dietary folate intake, the average daily intake was 348 µg/day, and when combined with folate from supplementation, the mean total daily intake was 373 µg/day. It was not possible to complete health questionnaires

for 28 subjects due to time pressure when subjects were required to go in for their colonoscopy immediately after signing their consent form.

Figure 3.1 Patient recruitment schema

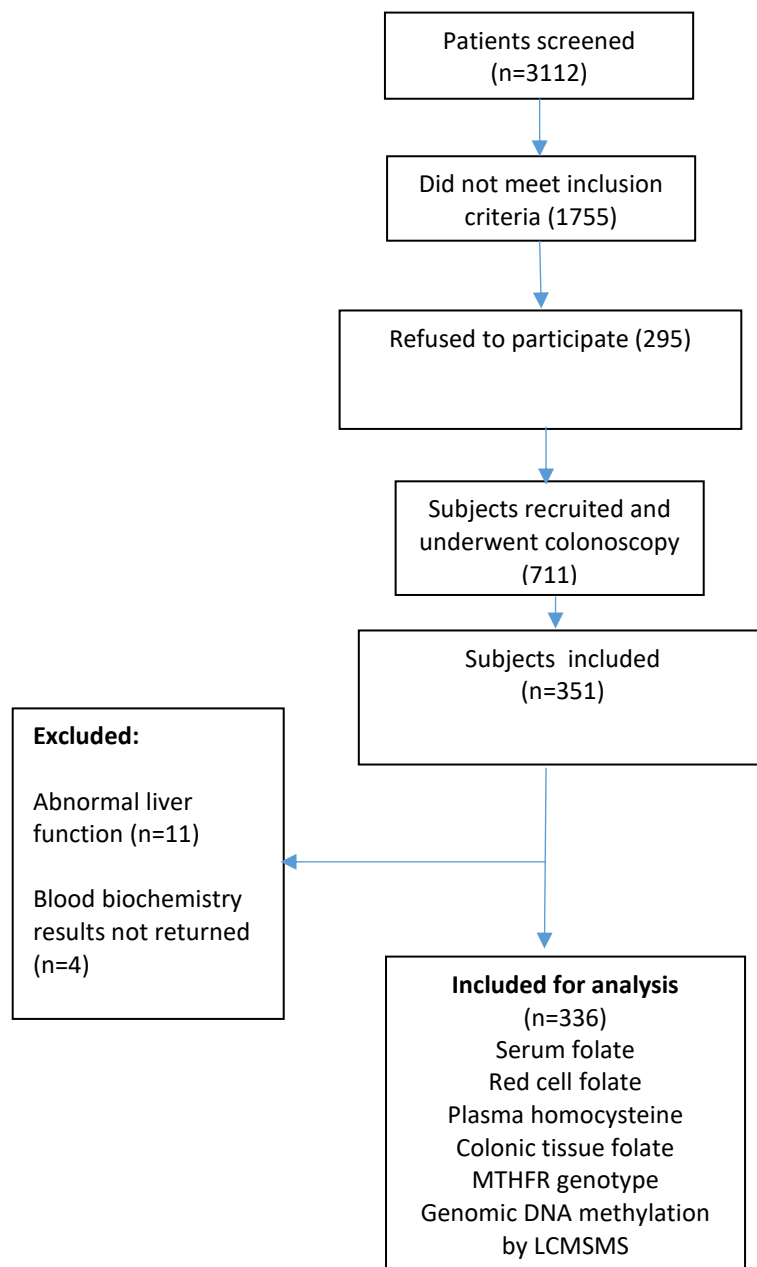


Table 3.1 Subject characteristics and dietary and lifestyle data

	n		
Age (Years)	336		57 (16)
Gender [n (%)]	336	Male	128 (38)
		Female	208 (62)
Ethnic group [n (%)]	324	Caucasian	226 (67)
		African Caribbean	69 (21)
		Asian	18 (5)
		Other	11 (3)
BMI (kg/m ²)	308		26.9 (6)
Nutritional supplement use [n (%)]	309	Non-user	214 (69)
		User	95 (31)
Folate containing supplement use [n (%)]	308	Non-user	264 (86)
		User	44 (14)
Dietary folate intake (µg/day)	60		348 (115)
Total folate intake (µg/day) (diet + supplements)	60		373 (140)
Drinking [n (%)]	308	Non-drinker	136(44)
		Drinker	172(56)
Alcohol Intake (g/day)	172		11.0 (4-22)
Smoking [n (%)]	314	Non-smoker	243 (77)
		Smoker	71 (23)
Cigarettes/day	71		12 (5-20)

Data are mean (SD) or frequency (%)

Alcohol intake and cigarettes smoked are displayed as Median (IQR)

3.3.3 Subject characteristics by genotype

Table 3.2 shows subject characteristics by *MTHFR* C677T genotype for the 336 subjects included in the final analysis, 55% were homozygous for the wild-type allele (CC), 35% carried one mutant allele (CT) and 10% were homozygous for the mutant allele (TT). There were no significant differences between genotypes in age, gender, BMI, smoking status, drinking status, nutritional supplement use, or folic acid supplement use. However, there was an association between genotype and ethnicity. In order to test for differences between the distributions of genotypes between ethnic groups it was necessary to combine the Asian and other ethnic sub-groups. The Chi-squared test showed that there were fewer TT subjects in the black subgroup than expected ($P=0.03$).

Table 3.2 Demographic and lifestyle characteristics according to the *MTHFR* C677T genotype

	n		CC (n=185)	CT n=119)	TT (n=32)	P
Age (yr)	336		57 (15)	57 (16)	54 (17)	0.58
Gender [n (% within gender)]	336	Male	67 (52)	51 (40)	10 (8)	0.36
		Female	118 (57)	68 (32)	22 (11)	
Ethnic group n (% within ethnic group)	324	White	114 (50)	87 (38)	25 (12)	0.03
		Black	50 (72)	16 (23)	3 (4)	
		Other	16 (55)	10 (34)	3 (10)	
BMI (kg/m ²)	308		26.9 (6.7)	26.7 (5.2)	25.6 (6.3)	0.8
Nutritional supplement use n (% within groups)	308	Non- user	114 (53)	78 (36)	22 (10)	0.8
		User	54 (57)	33 (35)	8 (9)	
Folate containing supplement use [n (% within groups)]	308	Non- user	145 (55)	94 (35)	26 (10)	0.92
		User	23 (52)	17 (38)	4 (9)	
Dietary folate intake (µg/day)	60		327 (112)	376 (120)	370 (111)	0.28
Total folate intake (µg/day) (diet + supplements)	60		345 (135)	415 (161)	392 (91)	0.21
Alcohol Intake (g/day)	172		10.6 (3.2-21.7)	12.0 (4.1-24.0)	9.6 (6.2-22.3)	0.55
Cigarettes/day	71		15 (5-20)	11 (5.8-20)	10 (5.3-18.8)	0.88

Date are mean (SD) (Age, BMI and folate intake), median (IQR) (alcohol intake and cigarettes smoked) and frequency (%) (gender, ethnicity and nutritional supplement use)

Differences between genotypes were assessed using Chi squared tests (gender, ethnicity, nutritional supplement use, and folic acid supplement use) and ANOVA (age, BMI, folate intake) and the Kruskal-Wallis test (number of cigarettes and alcohol intake).

3.3.4 Biomarkers of folate status, vitamin B₁₂ and genomic DNA methylation by *MTHFR* C667T genotype

None of the biomarkers of folate status, serum vitamin B₁₂, or DNA methylation differed by *MTHFR* C667T genotype (**Table 3.3**).

Table 3.3 DNA methylation, tissue folate, systemic folate biomarkers and vitamin B₁₂ by the *MTHFR* genotype

	n	CC (n=185)		CT (n=119)		TT (n=32)		P
		Median	IQR	Median	IQR	Median	IQR	
DNA Methylation (% methylcytosine)	335	4.30	3.90-4.78	4.23	3.80-4.60	4.22	3.65-4.70	0.54
Serum folate (nmol/l)	332	26.1	17.9-38.5	24.5	16.4-38.3	25.4	15.7-33.5	0.40
Red cell folate (nmol/l)	330	977	773-1133	927	747-1124	969	840-1238	0.40
Colonic tissue folate (nmol/g tissue)	335	0.89	0.59-1.26	0.88	0.56-1.21	0.90	0.52-1.36	0.91
Serum vitamin B ₁₂ (pmol/l)	332	314	237-459	315	241-413	285	247-285	0.50
Plasma homocysteine (μmol/l)	330	15.6	12.3-21.0	16.2	13.5-16.2	17.8	9.80-11.6	0.30

Data are median (IQR). The Kruskal-Wallis test was used to test for differences between genotypes

3.3.5 Association between demographic variables and biomarkers of folate status, and genomic DNA methylation

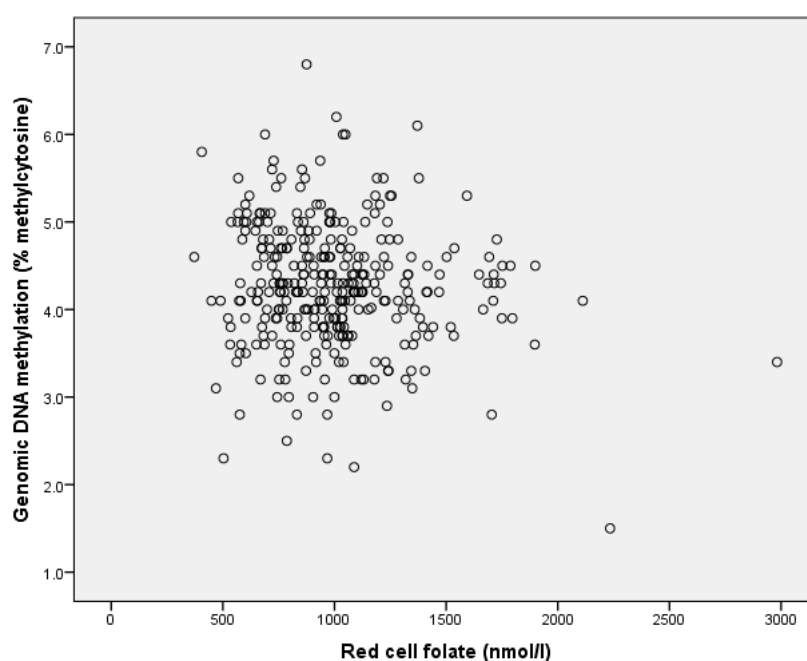
There were no significant relationships between genomic DNA methylation and colonic tissue folate, serum or red cell folate, serum vitamin B₁₂ and plasma homocysteine. Red cell folate was tending towards significance but weakly and non-significantly (**Table 3.4** and **Figure 3.2**).

Table 3.4 Association between genomic DNA methylation and biomarkers of folate status

	n	Correlation coefficient	P
Colonic tissue folate	335	0.03	0.61
Serum folate	332	-0.001	0.98
Red cell folate	330	-0.10	0.08
Serum vitamin B ₁₂	332	-0.03	0.57
Plasma homocysteine	330	0.04	0.49

Data are Spearman's Rank correlation coefficient

Figure 3.2 Genomic DNA methylation versus red cell folate ($\rho = -0.10$, $P=0.08$, $n=329$)



There were no associations between genomic DNA methylation and any of the continuous demographic variables (Table 3.5).

Table 3.5 Association between genomic DNA methylation and demographic and lifestyle characteristics

	n	Correlation coefficient	P
Age	335	-0.01	0.84
BMI	307	-0.05	0.37
Alcohol intake	172	-0.02	0.78
Cigarettes smoked	71	0.01	0.93
Dietary folate	60	0.10	0.45
Total folate intake	60	0.20	0.13

Data are Spearman's rank correlation coefficient

Correlations with cigarettes and alcohol intake were tested in drinkers and smokers only

3.3.6 DNA methylation by demographic and lifestyle subgroups

There were no differences in DNA methylation in the colon between men and women or between ethnic sub-groups (**Table 3.6**).

Table 3.6 Genomic DNA methylation by gender and ethnic group

		DNA Methylation (% methylcytosine)			
	n		Median	IQR	P
Gender	128	Male	4.30	3.90-4.80	0.35
	207	Female	4.20	3.80-4.70	
Ethnicity	225	White	4.20	3.80-4.70	0.48
	69	Black	4.30	3.90-4.80	
	29	Other	4.30	3.90-4.70	

Data are median (IQR). The Mann-Whitney test was used to assess differences between males and females and the Kruskal-Wallis test was used to assess differences in ethnic groups.

Additionally, there were no differences in DNA methylation between smokers and non-smokers, drinkers and non-drinkers, supplementation users and non-users (**Table 3.7**).

Table 3.7 Genomic DNA methylation by smoking, drinking and supplement use

	n		DNA Methylation (% methylcytosine)		P
			Median	IQR	
Smoking	243	Non smoker	4.30	3.90-4.70	0.65
	71	Smoker	4.30	3.80-4.70	
Drinking	136	Non drinker	4.30	3.90-4.70	0.64
	172	Drinker	4.20	3.80-4.70	
Nutritional supplement use	214	Non-user	4.20	3.90-4.70	0.94
	95	User	4.30	3.90-4.70	
Folic acid supplement use	264	Non user	4.40	3.90-4.90	0.49
	44	User	4.30	3.80-4.70	

Data are median (IQR). The Mann-Whitney test was used to assess differences in genomic DNA methylation between groups

3.3.7 Multiple linear regression

Multiple linear regression was used to assess the relationship between DNA methylation in the colonic mucosa (which was added as the dependent variable) and the predictor variables MTHFR C677T genotype (CT or TT with CC as the reference variable), and biomarkers of folate status which were ranked (**Table 3.8a**). DNA methylation was not associated with any of the predictor variables apart from red cell folate where there was a negative, non-significant association ($\beta = -0.001$, $P=0.08$).

Table 3.8a ANCOVA model with genomic DNA methylation as the dependent variable and the MTHFR C677T genotype, biomarkers of folate status and serum B₁₂ as the predictors (n=336)

	β (unstandardised coefficient)	P value
MTHFR C677T		
CC (reference)		
CT	-0.03	0.79
TT	-0.12	0.43
Serum folate	0.001	0.44
Red cell folate	-0.001	0.08
Tissue folate	0.0002	0.83
Plasma homocysteine	0.0003	0.66

Multiple linear regression model (Forced Entry).

All folate biomarkers are ranked variables.

Model summary: Adjusted R square = -0.007, F= 0.75, P=0.61

The same relationship was investigated with additional adjustments made for age, gender, ethnicity (black or other, with white as a reference), supplement use (user or non-user), and serum vitamin B₁₂ (**Table 3.8b**). The relationship between DNA methylation and red cell folate described in the above model was no longer observed after adjustment with the additional variables.

Table 3.8b ANCOVA model with genomic DNA methylation in colonic mucosa as the dependent variable and the MTHFR C677T genotype, biomarkers of folate status and serum B₁₂ as the predictors (n=336)

	β (unstandardised coefficient)	P value
MTHFR C677T		
CC (reference)		
CT	-0.04	0.68
TT	-0.18	0.27
Serum folate	0.001	0.52
Red cell folate	0.00	0.43
Tissue folate	0.001	0.47
Plasma homocysteine	0.001	0.44
Age	-0.01	0.12
Gender	0.03	0.80
White ethnic group (reference)		
Black ethnic group	0.01	0.89
Other ethnic group	-0.02	0.87
Supplement user	-0.05	0.63
Multiple linear regression model (Forced Entry). All folate biomarkers are ranked variables. Model summary: Adjusted R square = -0.02, F= 0.59, P=0.84		

3.4 Discussion

The aim of this cross-sectional study was to investigate the influence of folate and the *MTHFR* C677T polymorphism on genomic DNA methylation in the colon. Volunteers without neoplasia were selected for inclusion with the aim of excluding potentially confounding effects of cancer on DNA methylation in the colon. DNA methylation was quantified using a sensitive LC-ESI-MS/MS approach and both colonic tissue and systemic folate were measured for comprehensive characterization of folate status.

There were three main findings in the present study which are described below (1-3):

1. DNA methylation, an intermediate marker of colorectal cancer, did not differ by *MTHFR* C677T genotype.
2. There were no significant associations observed between DNA methylation and any of the biomarkers of folate status (in blood or colonic mucosa), or dietary intake or folate supplementation. DNA methylation in colonic tissue was non-

significantly and inversely associated with red cell folate (DNA hypomethylation was associated with increases in red cell folate).

3. DNA methylation in the colon was not associated with demographic variables and lifestyle variables (age, BMI, cigarettes smoked, alcohol intake, dietary and total folate intake).

The only association observed with DNA methylation in the current study was an inverse relationship with red blood cell folate. This is contradictory to previous work in this group where a non-significant weak, positive association was seen with DNA methylation and both serum and red cell folate ($P = 0.07$ and $P = 0.08$, respectively) in subjects without neoplasia (Pufulete et al., 2005b). The important difference between the two studies is the assay that was utilised. The previous study used the methyl acceptance assay to measure DNA methylation, while the current study used LC-ESI-MS/MS. A parallel study led by another student demonstrated that gene-specific DNA methylation was also unaffected by the MTHFR C667T genotype in the same population (Hanks et al., 2013). Similar findings have also been reported in patients with resected colorectal adenomas (Figueiredo et al., 2009b).

Associations between folate status and DNA methylation have been observed with the methyl acceptance assay (Cravo et al., 1994, Cravo et al., 1998, Pufulete et al., 2005a) but these findings have not been replicated using other global DNA methylation

The influence of the MTHFR C677T genotype and folate on DNA methylation in the colonic mucosa has previously been examined in subjects with a history of neoplasia. Figueiredo et al., (2009) studied 388 subjects participating in a trial testing the efficacy of aspirin and folic acid in the prevention of colorectal adenomas. They measured DNA methylation by pyrosequencing for LINE-1 repetitive elements (surrogate measure of global DNA methylation) on normal-appearing colonoscopy specimens from the left and right colon. In agreement with findings in this chapter, global DNA methylation was not influenced by the MTHFR genotype and showed no association with demographic or lifestyle characteristics, plasma B₆ and dietary folate. Interestingly, DNA methylation differed significantly between the left and right side of the colon which suggested site-specific differences in DNA methylation of the colonic mucosa. It is important to note that though normal colonic tissue was collected in the Figueiredo study, these subjects had previously

resected adenomas and neoplasia could have had a significant effect on the DNA methylation status of the remaining normal colonic tissue (Esteller et al., 2000).

In another study, methylation of LINE-1 repetitive elements was investigated in paired samples of tumour tissue and normal mucosa in a cohort of 178 subjects. Lower levels of LINE-1 DNA methylation were observed in patients with the *MTHFR* C677T and A1298C polymorphism haplotype (low activity genotype) than in the wild types (~5%; $P=0.033$) (Iacopetta et al., 2007).

A lack of association between the *MTHFR* TT genotype and DNA methylation in healthy subjects has been previously reported by our group (described above) in a much smaller cross-sectional study of folate-replete subjects ($n=68$) without colorectal polyps or cancer (Pufulete et al., 2005b) in this study a non-significant, weak positive association was found between genomic DNA methylation and the *MTHFR* C677T genotype. In contrast, a non-significant negative correlation ($P=0.08$) between red cell folate and genomic DNA methylation was observed in the present study (**Table 3.8a**), suggesting that DNA might be hypomethylated as red cell folate concentration increases. In the present study, genomic DNA methylation was quantified by LC-ESI-MS/MS, a method which has demonstrated superior sensitivity, precision and reproducibility (Kok et al., 2007, Friso et al., 2002a) over the methyl acceptance assay (Nephew et al., 2009) and the LINE-1 approach which quantifies repetitive element DNA methylation (Figueiredo et al., 2009b, Wallace et al., 2010).

In general, it has been reported that there is no association between the *MTHFR* C677T genotype and DNA methylation but the influence of folate on this relationship has produced conflicting results.

In the present study, biomarkers of folate status were not found to influence genomic DNA methylation. Additionally, neither folate intake from diet or supplementation affected DNA methylation. However, availability of data on folate intake was disappointingly low due to FFQ completion by only 60 subjects (18%). The reason for this was that on the day of colonoscopy it was inappropriate to expect subjects to complete a long questionnaire prior to their procedure as they were often nervous and anxious, while after their procedure many patients were still affected by sedation from the procedure.

Patients who had not been sedated were asked to complete the questionnaire before leaving the hospital. If this was not possible it was difficult to find an opportunity where patients could complete the questionnaires at a later date because most did not have to return to the hospital for further follow-up.

A possible limitation of the present study is that although these patients were deemed neoplasia free, they had all been referred for colonoscopy because of gastrointestinal problems and colonic symptoms such as bloating, bleeding or change in bowel habit (usually diarrhea or constipation). Though there were no obvious signs of clinical pathology on colonoscopy or histology, inflammatory bowel diseases are a known risk factor for CRC (World Cancer Report, 2014). Therefore it cannot be ruled out that the underlying causes of these symptoms did not in some way affect DNA methylation in the colon, nor that these causes could be associated with folate status. Additionally, in Chapter 2, an overview of colorectal mucosal biopsy collection and a range of biopsy sizes is described (5-64mg) and this considerable heterogeneity in sample size is likely to have influenced the proportion of cell types contained in each sample which may have in turn affected molecular measurements made on these biopsies due to different cell types having different levels of DNA methylation and uracil misincorporation. As described in Chapter 1, the range of methodology in the literature indicates variation in these endpoints depending on the tissue assayed i.e. between lymphocytes, leucocytes, and colonic biopsies so it is assumed that this wide variability in size range would add to the variability of the readings and potentially introduce less reliability.

As discussed above, findings on the influence of the MTHFR C677T genotype and biomarkers of folate status on global DNA methylation have been inconsistent. This is not surprising given the variation in study designs and the populations studied. Neoplasia free and cancer patients have been studied with vastly different sample sizes and demographic variables while different methodologies have been used to quantify DNA methylation. In addition, colonic samples have been collected from variable anatomical sites and different peripheral or tissue-derived cell types have been analysed. More work is needed to explain the relationship between folate and DNA methylation when other polymorphisms are considered (in addition to MTHFR C677T) and with contributions from other 1-carbon sources such as choline and betaine, in the methylation reaction which make this interaction complex. These complexities may be addressed in part by genome-wide analysis of DNA methylation which enables analysis of multiple polymorphisms and

mutations in candidate genes, and by taking into account the potential effects of other vitamins to obtain a more comprehensive picture (Crider et al., 2012, Cheng et al., 2015).

In summary, this study found that the MTHFR C667T genotype and folate status did not influence genomic DNA methylation in the colon of this cohort. Inclusion of well-matched neoplasia-free subjects was important for minimizing the potential effects of cancer on DNA methylation in the colon. The subjects included in this study all possessed normal or high folate levels and folate biomarkers did not differ between genotypes. Further work is needed to verify the protective mechanism of the MTHFR C667T genotype on DNA methylation when folate status is low.

4 The association between the MTHFR C677T genotype and folate status with uracil misincorporation in DNA in the colon of subjects without colorectal adenoma or cancer.

4.1 Introduction

Folate is essential for the conversion of deoxyuridylate to thymidylate but when folate supply is limited, thymidylate levels are depleted. As a result, uracil can be misincorporated into the DNA. Uracil misincorporation may cause DNA double-strands breaks, chromosomal instability and eventually neoplasia (Sharp and Little, 2004).

There have been no large studies looking at the influence of folate status and the *MTHFR* C677T genotype on uracil misincorporation into DNA in colonic cells of humans without previous or current colorectal adenomas or cancer. Uracil misincorporation can be measured by simply measuring the uracil content of DNA since DNA should not normally contain uracil.

4.1.1 Hypothesis

Folate status and the MTHFR C677T mutation are associated with uracil misincorporation in the colon of subjects without colorectal adenoma or cancer.

4.1.2 Aims

1. To investigate the association between the MTHFR C667T genotype and uracil content of DNA in the colon of subjects without colorectal adenoma or cancer.
2. To investigate the association between folate status and uracil content of DNA in the colon of subjects without colorectal adenoma or cancer.

4.1.3 Objectives

1. To determine the MTHFR genotype using restriction fragment length polymorphism (RFLP) analysis in blood cells of all study subjects.
2. To determine serum and red cell folate, vitamin B₁₂ and homocysteine status in blood using competitive immunoassay status and folate status in colonic mucosa using a microbiological assay.

3. To quantitatively determine uracil content of DNA in colorectal mucosa by gas chromatography mass spectrometry (GCMS) and investigate associations with the MTHFR C677T genotype and with folate biomarkers.

4.2 Methods

4.2.1 Subject recruitment

A total of 336 study participants were included, all of whom provided blood samples for the analysis of systemic folate biomarkers and mucosal biopsies for analysis of tissue folate levels and DNA uracil content. Due to time constraints, it was only possible to determine uracil content in the DNA of 236 subjects. Subject recruitment, inclusion and exclusion criteria, demographic data collection, and dietary folate data, blood and tissue sample collection are described in detail in **Chapter 2, Sections 2.1-2.5**.

4.2.2 Laboratory Methods

In brief, uracil content in DNA was determined by extraction of DNA followed by gas chromatography mass spectrometry (GCMS). Details on blood assays, MTHFR genotyping and the determination of tissue folate and uracil content in DNA can be found in methods detailed in **Chapter 2, Section 2.7**.

4.2.3 Statistical analysis

All data were analysed by using IBM® SPSS® version 21. Subject characteristics and dietary and lifestyle data are displayed as mean (SD) and frequency (%).

Systemic folate biomarkers, vitamin B₁₂ and colonic folate in the uracil analysis group (n=236) were compared with the group for whom uracil was not analysed (n=100). This was to investigate whether the uracil sub-group was representative of the whole sample.

Balance between the group who had uracil content results (n=236) and the group for whom uracil could not be analysed (n=100) was compared using the unpaired t-test for subject characteristics and dietary and lifestyle variables. Biomarkers are displayed as median (interquartile range) as they were not normally distributed and differences

between the groups with and without uracil results were tested using the Mann Whitney test.

Differences in uracil content between genotypes were compared using the Kruskal-Wallis test. Spearman's rank correlation coefficients were used to look for associations between uracil misincorporation and folate biomarkers because data were not normally distributed.

Spearman's rank correlation coefficients were also used to test for associations between uracil content and demographic variables (age, BMI, dietary folate intake, total folate intake, number of cigarettes smoked and amount of alcohol consumed).

Differences in uracil content between groups were assessed using the Mann Whitney test (gender, smoking status, drinking status and nutritional and folic acid supplementation) and the Kruskal Wallis test (ethnicity).

Multiple linear regression was used to assess whether uracil content could be predicted from any of the independent variables. Forced Entry, or 'Enter' in SPSS, was selected as the method of Multiple Linear Regression. This method relies on theoretical justification for the selection of predictor variables. All predictors are entered into the model at the same time and in no particular order. Uracil content (ranked) was added to the model as the dependent variable and the following variables were added to the model as the independent predictor variables: MTHFR C677T genotype, tissue folate, serum and red cell folate, plasma homocysteine and serum vitamin B₁₂ (ranked). In a second model, adjustments were made for demographic variables (age, sex, ethnic group, BMI, smoking, nutritional supplement use, and alcohol intake). For the categorical predictor variables, MTHFR C677T, CT and TT were tested against CC as the reference group and for ethnicity as a categorical predictor variable, black and other were tested with 'white' as the reference group.

4.3 Results

4.3.1 Characteristics and habitual dietary and lifestyle data

General demographic and folate characteristics of the 236 subjects in the uracil test group are shown next to the group for whom there were no uracil results (**Table 4.1**). Statistical analysis showed that there were no differences in demographic variables between subjects in the two groups. The majority of subjects were white (67%) followed by black subjects (21%) reflecting the local population in South East London. Approximately 75% of the population were non-smokers and 45% were non-drinkers. Only 27% of subjects took nutritional supplements (multivitamins from various companies) and 14% took daily folic acid supplements. Data on dietary folate intake was only available for 33 subjects (14%) because Food Frequency Questionnaires were only completed by this number of subjects.

Table 4.1 Subject characteristics, dietary and lifestyle characteristics in subjects with uracil results and those without uracil results

	n		Uracil results obtained (n=236)	n	Uracil result <u>not</u> obtained (n=100)	P
Age (Years)	236		56 (16)	100	57 (16)	0.62
Gender [n (%)]	236	Male	98 (42)	100	30 (30)	0.06
		Female	138 (58)		70 (69)	
Ethnic group [n (%)]	228	White	159 (70)	95	66 (69)	0.90
		Black	50 (22)		19 (20)	
		Other	19 (8)		10 (11)	
BMI (kg/m ²)	236		27.2 (6)	100	26.3 (6)	0.23
Supplement use [n (%)]	219	Non-user	159 (73)	89	55 (61)	0.07
		User	60 (27)		34 (39)	
Folic acid supplement use [n (%)]	219	Non-user	189 (86)	89	76 (85)	0.90
		User	30 (14)		13 (15)	
Dietary folate intake (µg/day)	33		360 (109)	27	338 (121)	0.17
Total folate intake (diet + supplements) (µg/day)	33		379 (128)	27	365 (155)	0.56
Drinking [n (%)]	222	Non-drinker	99 (45)	93	44 (47)	0.13
		Drinker	123 (55)		49 (53)	
Smoking [n (%)]	221	Non-smoker	171 (77)	93	72 (77)	0.38
		Smoker	50 (23)		21 (23)	
MTHFR genotype	236	CC	130 (55)	100	55 (55)	0.17
		CT	80 (34)		39 (39)	
		TT	26 (11)		6 (6)	

Data are mean (SD) (Age, BMI and folate intake), median (IQR) (alcohol intake and number of cigarettes smoked) and frequency (%) (gender, ethnicity and nutritional supplement use). Differences between the two groups were assessed using t-tests for continuous variables (age, BMI, folate intake, cigarettes smoked) and Chi Squared tests for categorical data (gender, ethnicity, supplement use, drinking, smoking and MTHFR genotype) and the Mann-Whitney test for cigarettes smoked and alcohol intake.

4.3.2 Biomarkers of folate status and serum B₁₂

Systemic and colonic folate biomarkers in the uracil analysis group (n=236) were compared with the group for whom uracil was not analysed (n=100) (**Table 4.2**). Biomarkers in the group for whom uracil was reported were broadly representative of the group where uracil content was not analysed, except plasma homocysteine which was significantly higher in the uracil-tested group (P=0.02).

Table 4.2 Folate biomarkers: a comparison of subjects with uracil results and those without uracil results

	n	Uracil reported	n	Uracil not reported	P
Serum folate (nmol/l)	233	25.40 (7.5-54.4)	98	25.60 (16.8-40.1)	0.97
Red cell folate (nmol/l)	232	954 (758-1126)	100	1004 (832-1186)	0.16
Colonic tissue folate (nmol/g tissue)	235	0.88 (0.61-1.27)	100	0.89 (0.55-1162)	0.19
Serum vitamin B ₁₂ (pmol/l)	232	315 (233-417)	100	310 (253-462)	0.50
Plasma homocysteine (mmol/l)	232	17.0 (13.1-21.5)	100	14.6 (11.8-20.6)	0.02

Data are median (IQR). Differences between groups were assessed using the Mann-Whitney test.

4.3.3 Uracil content by MTHFR C677T Genotype

Median uracil content in DNA (taken from colonic mucosa) for the cohort was 5.1pg/μg (IQR 3.2-8.5) Uracil content by genotype is displayed in **Table 4.3** where it can be seen that there were no differences between the three *MTHFR C677T* genotypes.

Table 4.3 Uracil content according to MTHFR C677T Genotype

MTHFR C677T	Uracil (pg/μg)			
	n	Median	IQR	P value
CC	130	5.00	3.35-8.35	0.99
CT	80	5.05	2.73-8.80	
TT	26	5.30	3.63-8.05	

Data are median (IQR). Differences between the MTHFR C677T genotypes were tested using the Kruskal-Wallis test

4.3.4 Association between uracil content and DNA methylation and biomarkers of folate status and vitamin B₁₂ in the colon

Next, uracil content in DNA was tested for associations with DNA methylation, and then for associations with folate biomarkers. No association was observed with uracil content and DNA methylation, or with colonic tissue folate, homocysteine, serum vitamin B₁₂ (**Table 4.4**). The exception was with serum folate which showed a positive association with uracil content in DNA ($p=0.20$; $P=0.02$), this relationship is also depicted in **Figure 4.1** and a non-significant, positive association was observed with red cell folate ($p=0.13$; $P=0.06$) (**Figure 4.2**).

Table 4.4 Association between uracil content in DNA and genomic DNA methylation and biomarkers of folate status

	n	Correlation coefficient	P value
DNA Methylation (% methylcytosine)	236	-0.03	0.70
Colonic tissue folate (nmol/g)	235	0.08	0.20
Serum folate (nmol/l)	233	0.20	0.02
Red cell folate (nmol/l)	230	0.13	0.06
Serum vitamin B ₁₂ (pmol/l)	232	0.04	0.56
Plasma homocysteine (mmol/l)	233	-0.004	0.95

Data are Spearman's Rank correlation coefficient

Figure 4.1 Uracil content versus serum folate ($\rho = 0.20$, $P=0.02$, $n=233$)

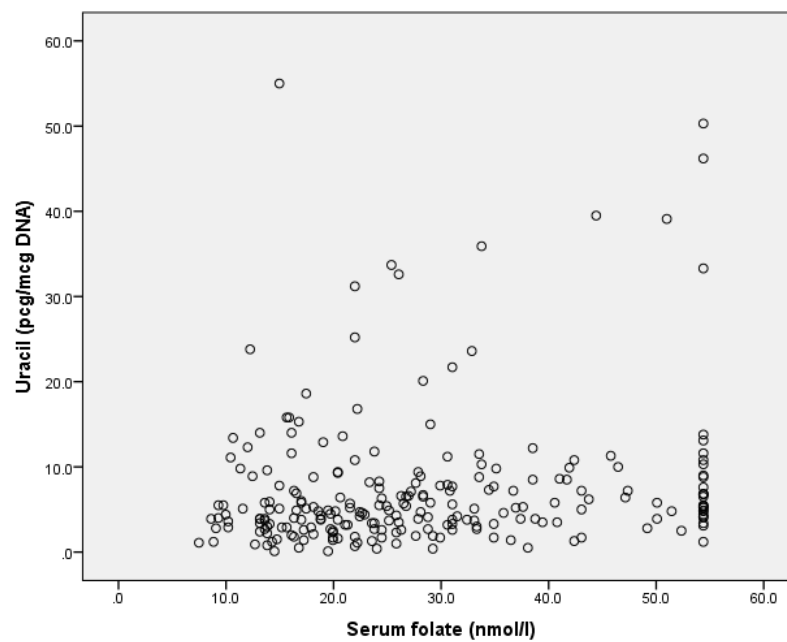
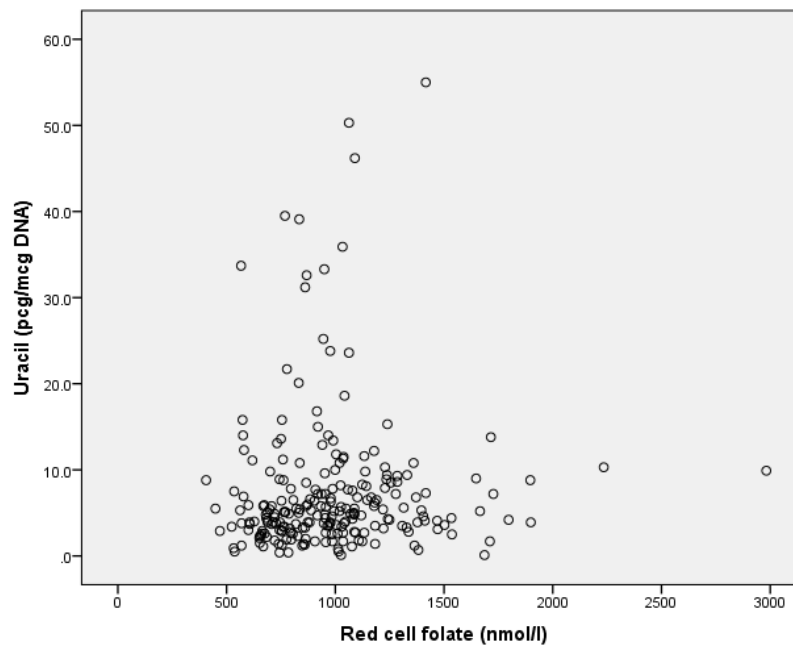


Figure 4.2 Uracil content versus red cell folate ($p = 0.13$ $P=0.06$, $n=233$)



4.3.5 Differences in uracil content in the colon between demographic and lifestyle subgroups

There were no differences in uracil content between men and women, or between ethnic groups (**Table 4.5**).

Table 4.5 Uracil Content in DNA by gender and ethnic subgroups

			Uracil (pg/ μ g)		
	n		Median	IQR	P
Gender	98	Male	5.00	3.50-9.45	0.37
	138	Female	5.10	2.95-7.85	
Ethnicity	159	White	5.05	3.00-8.80	0.66
	50	Black	4.80	3.20-7.13	
	19	Other	5.50	3.80-9.30	

Data are median (IQR). Differences in uracil content in DNA were assessed using the Mann-Whitney test for gender and the Kruskal-Wallis test for ethnic groups

Uracil content was significantly higher (6.10 pg/ μ g) in the DNA of nutritional supplementation users than in non-users (4.80 pg/ μ g) ($P=0.01$) (**Table 4.6, Figure 4.3**).

Additionally, there were no differences in uracil content between smokers and non-smokers or drinkers and non-drinkers.

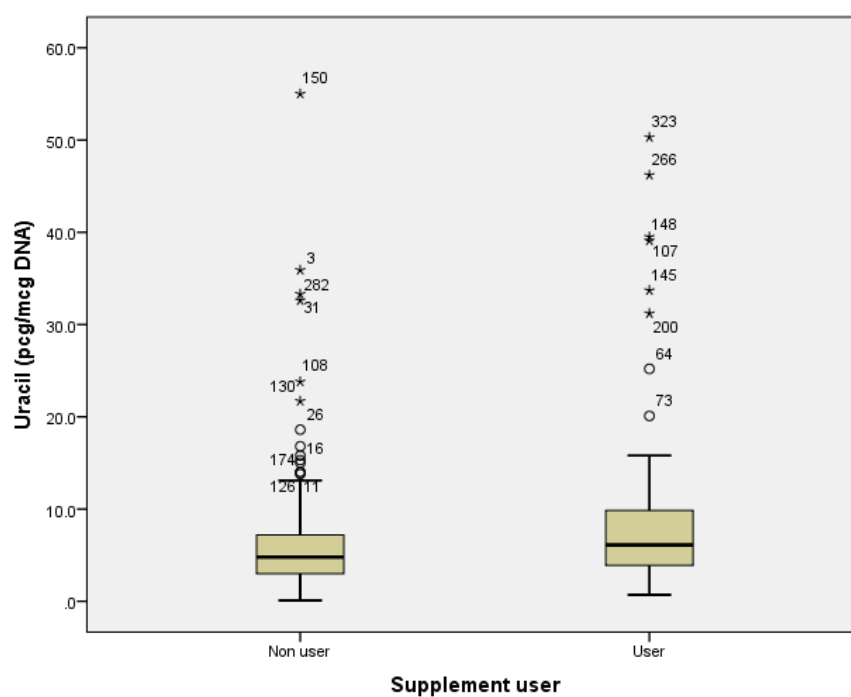
Table 4.6 Uracil content in DNA by smoking, drinking and supplement use subgroups

	n		Uracil (pg/μg)		P
			Median	IQR	
Drinking	123	Non-drinker	5.50	3.40-8.80	0.27
	99	Drinker	4.85	2.98-7.80	
Smoking	171	Non-smoker	5.05	3.30-8.80	0.72
	50	Smoker	5.25	3.13-7.35	
Nutritional supplement use	158	Non-user	4.80	2.98-7.20	0.01
	60	User	6.10	3.90-9.88	
Folic acid supplement use	188	Non-user	4.90	3.20-7.68	0.06
	30	User	6.70	3.20-10.9	

Uracil content is displayed as median and IQR. The Mann-Whitney test was used to assess differences in uracil content between groups.

The significant difference in uracil content in DNA between nutritional supplement users and non-users is also depicted below in a boxplot. Circles represent outliers and stars represent extreme outliers (**Figure 4.3**).

Figure 4.3 Uracil content in DNA by supplement users and non-users, P=0.01



4.3.6 Associations between uracil content in DNA in the colon and demographic and lifestyle characteristics

Spearman's Rank correlation showed no associations observed between uracil content in DNA and age, BMI, dietary or total folate intake, alcohol quantity consumed, or number of cigarettes smoked (**Table 4.7**).

Table 4.7 Association between uracil content in DNA and demographic and lifestyle characteristics

	n	Correlation coefficient	P value
Age	236	-0.02	0.72
BMI	236	0.07	0.28
Dietary folate intake	33	0.001	1.0
Total folate intake	33	0.08	0.68
Alcohol intake	123	-0.02	0.82
Cigarettes smoked	50	-0.04	0.53

Data are Spearman's rank correlation coefficient

Correlations with cigarettes and alcohol intake were tested in drinkers and smokers only

4.3.7 Multiple linear regression

Multiple linear regression (Forced Entry method) was used to assess the relationship between ranked variable uracil content as the dependent variable, the MTHFR C677T genotype (CT or TT with CC as the reference variable) and each of the biomarkers of folate status and vitamin B₁₂ which were all ranked. The weak association with red cell folate disappeared. As seen with Spearman's correlation, serum folate was positively associated with uracil content ($\beta=0.19$, $P=0.03$) however the overall model was not significant (**Table 4.8a**).

Table 4.8a ANCOVA model with uracil content in DNA as the dependent variable and the MTHFR C677T genotype and biomarkers of folate status as the predictors (n=236)

	Unadjusted B (unstandardised coefficient)	P value
MTHFR C677T		
CC (reference)		
CT	0.60	0.95
TT	-6.16	0.69
Serum folate (nmol/l)	0.19	0.03
Red cell folate (nmol/l)	0.05	0.55
Colonic tissue folate (nmol/g tissue)	0.01	0.93
Serum vitamin B ₁₂ (pmol/l)	0.005	0.95
Plasma homocysteine (μ mol/l)	0.06	0.44

Multiple linear regression model (Forced Entry).

All folate biomarkers are ranked variables.

Model summary: Adjusted R square = 0.01, F= 1.37, P=0.22

In a second model, further adjustment was made for DNA methylation, age, gender, ethnicity (black or other, with white as a reference) and supplement use (user or non-user) whilst excluding each of the folate biomarkers to see if it affected the model. With additional adjustment for these demographic and lifestyle variables and with plasma homocysteine removed, the positive association between uracil content and serum folate became more significant ($\beta=0.22$, $P=0.02$). A positive association with supplement use was observed ($\beta =27.3$ $P=0.01$) and the overall model was significant ($P=0.09$) (**Table 4.8b**).

Table 4.8b ANCOVA model with uracil content in DNA as the dependent variable and the MTHFR C677T genotype, biomarkers of folate status, demographic and lifestyle variables as the predictors (n=236)

	Adjusted B (unstandardised coefficient)	P value
MTHFR C677T		
CC (reference)		
CT	1.08	0.92
TT	-4.57	0.77
Genomic DNA methylation (% methylcytosine)	-0.02	0.82
Serum folate (nmol/l)	0.22	0.02
Red cell folate (nmol/l)	0.05	0.61
Tissue folate (nmol/g tissue)	0.03	0.73
Serum vitamin B ₁₂ (pmol/l)	-0.05	0.57
Plasma homocysteine (μmol/l)	0.09	0.31
Age (years)	-0.39	0.27
Gender	-15.0	0.14
Ethnicity White (reference)		
Black ethnic group	1.34	0.89
Other' ethnic group	-5.20	0.63
Supplement user	27.3	0.01

Multiple linear regression model (Forced Entry).

All folate biomarkers are ranked variables.

Model summary: Adjusted R square = 0.04, F= 1.59, P=0.09

4.4 Discussion

The aim of this chapter was to examine whether folate status and the MTHFR C667T polymorphism influenced uracil DNA misincorporation in the colonic mucosa and to examine possible associations between uracil content and demographic and lifestyle variables. Uracil content in DNA was determined using gas chromatography mass spectrometry (GCMS) and both colonic tissue and systemic folate were measured for comprehensive characterisation of folate status.

There were four main findings in the present study which are described below (1-4):

1. Uracil content did not differ by MTHFR C677T genotype.
2. Uracil content in colonic tissue was positively associated with serum folate and red cell folate (non-significantly) but there were no associations with the other folate biomarkers or serum B₁₂.
3. Uracil content in colonic mucosa was higher in nutritional supplement users than in non-users.
4. There were no associations between uracil content and all other demographic variables (age, BMI, cigarettes smoked, alcohol intake, and dietary and total folate intake).

Uracil analysis was performed on 236 subjects from the original cohort of 336 healthy participants. Colonic DNA was previously used for quantitation of DNA methylation, genotyping and for the determination of tissue folate. 100 individuals could not be included due to lack of sufficient DNA remaining for uracil analysis. Nevertheless, the uracil cohort was broadly representative of the larger cohort with no differences observed between demographic and lifestyle characteristics or biomarkers apart from plasma homocysteine which was significantly higher in the uracil-tested group.

Median uracil content in DNA from colonic mucosa for the entire cohort was 5.1 pg/μg (IQR 3.2-8.5). When tested by the MTHFR C667T genotype there was no effect, suggesting that the MTHFR C667T polymorphism conferred no significant protective effect on uracil misincorporation in pathology-free subjects with adequate folate status (**Table 4.3**). A similar observation was previously reported by Narayanan *et al.* (2004), who examined lymphocytes and showed that markers of DNA stability (including strand breaks,

misincorporated uracil and global DNA methylation) did not differ between the three MTHFR C667T variants (Narayanan *et al.*, 2004). In line with these findings, there was no relationship observed between the two primary endpoints, genomic DNA methylation (tested as genomic DNA hypomethylation) and uracil misincorporation (tested as uracil content), in the colonic mucosa of this cohort.

In this chapter a significant positive correlation was found between serum folate and uracil content in DNA). Uracil content in the colon was higher in nutritional supplement users than in non-users but no differences were seen between folic acid users and non-users and no associations were seen with dietary folate alone or total folate intake (dietary plus supplements). Thus, the data suggests that in this cohort of subjects with adequate folate status, who were free of colorectal neoplasia or other obvious colonic pathologies, uracil misincorporation into DNA in the colon is positively associated with systemic folate and increased in subjects who take folate containing daily nutritional supplements. Whether this increase in uracil content in nutritional supplement users reflects confounding effects of other micronutrients (such as vitamin B₁₂ which also plays a prominent role in 1-carbon metabolism) contained in the nutritional supplements is not known and would require further investigation. These findings are intriguing given that folate deficiency is generally associated with DNA hypomethylation and increased uracil misincorporation into DNA. ((Choi and Mason, 2000, Linhart *et al.*, 2009).

More specifically, older *in vitro* studies using different cell types have indicated an inverse relationship between folate levels and uracil misincorporation (Libbus *et al.*, 1990, Branda *et al.*, 1997, James *et al.*, 1994, Wickramasinghe SN, 2009, Melnyk S1, 1999) and animal studies have reported elevated uracil misincorporation following depletion of dietary folate (James and Yin, 1989, Duthie *et al.*, 2000a, Linhart *et al.*, 2009). In humans, McGlynn *et al.* (2013) compared folate levels and uracil misincorporation in cultured colonocytes isolated from biopsy specimens from specific regions of the colon in patients with either adenomatous or hyperplastic polyps (n=56) and disease-free controls (n=53). They reported a significant inverse relationship between folate levels and uracil misincorporation in polyps but this was not observed in cells derived from normal mucosa. They also found that folate levels in the normal mucosa of subjects with adenoma were significantly lower than those in anatomically matching colonic segments taken from healthy individuals. The authors proposed that localised folate deficiency in specific regions of the colon might be carcinogenic due to elevated uracil misincorporation and

DNA hypomethylation. Folate deficiency could promote carcinogenesis while tumours might also deplete folate from the surrounding tissue (McGlynn *et al.*, 2013).

In a very recent study of patients with adenomatous polyps, who were assigned to receive 600µg/day folic acid or placebo for six months, an increase in colonic mucosal folate was observed with a decrease in uracil misincorporation in the mucosa adjacent to the polyp ($P=0.05$). These results led the authors to conclude that site specific folate depletion (adjacent to the adenomatous polyp) may have become replete in response to supplementation (O'Reilly *et al.*, 2016).

Hazra *et al.* (2010) found that folic acid supplementation for 3 years (1 mg/day) combined with aspirin, conferred no influence on uracil misincorporation in the rectal mucosa or in white blood cells in subjects who were replete in folate and with a history of colorectal adenoma (Hazra *et al.*, 2010). Importantly, these authors suggested that additional large-scale studies are needed to clarify if uracil misincorporation is an important risk factor in CRC.

Moreover, previous studies in animal models have suggested that folate can promote carcinogenesis once a neoplasm has been established (Kim *et al.*, 1996b, Song *et al.*, 2000b). Kim *et al.* (2011) suggested that uracil misincorporation might be influenced by age and found that young mice showed an increase in uracil misincorporation into DNA when folate intake was increased ($P=0.03$) but, in contrast, this positive association was not seen in older mice at the upper limit of age (22 months) (Kim *et al.*, 2011a).

In human subjects with a history of colorectal adenomas, high dose folic acid (5mg/d) administered for six months together with vitamin B₁₂ (1.25mg/day) induced an increase in uracil misincorporation in the rectal mucosa as a result of folic acid supplementation though this was not significant when compared with increases seen in the placebo group (van den Donk, 2007). This appears to be the only study in humans which is in agreement with the results of the current study.

It is important to note that, although neoplasia-free, subjects were admitted to hospital as day patients because they had experienced symptoms of colonic pathology and underwent diagnostic sigmoidoscopy. Thus, other underlying pathology of the colonic mucosa which might influence uracil misincorporation cannot be completely excluded.

In summary, uracil misincorporation into DNA in the colonic mucosa of healthy subjects with adequate folate levels was influenced by serum and red cell folate (marginally) as well as nutritional supplementation but was not influenced by the MTHFR C667T polymorphism or demographic and lifestyle characteristics. To the best of the author's knowledge, this is the first time a positive correlation between folate levels and uracil misincorporation in the colonic mucosal DNA of neoplasia-free subjects with adequate folate status has been observed. This unexpected observation may reflect localised metabolic mechanisms in the colon but this would require further investigation.

5 Colonic tissue folate measurements and whether they are predicted by systemic markers of folate status, and the influence of dietary and lifestyle variables on folate status.

5.1 Introduction

Folate has long been associated with colorectal cancer risk. Low folate status and dietary folate intake has generally been associated with an increased risk of colorectal cancer in meta-analyses (Sanjoaquin et al., 2005, Kennedy et al., 2012). Reports on the relationship between systemic folate levels and colorectal cancer risk have been less consistent. This might be due to the sensitivity and precision of the assays because small changes in folate levels may not be detected in routine blood analysis (Kim et al., 1998). Thus, the precise relationship between folate and cancer risk may not have been fully assessed. It has also long been established that vulnerability to folate depletion is tissue specific (Varela-Moreiras and Selhub, 1992) and it is not clear whether a relationship exists between systemic folate concentrations and folate levels in the colonic mucosa, the site where colonic carcinogenesis is initiated. Additionally, folate in the colonic mucosa is not exclusively derived from the blood and may also come from intestinal luminal folates in the colorectum (Camilo et al., 1996).

In a study of women taking pharmacological doses of folate depleting contraceptive pill, subjects were observed to have megaloblastosis in the cervico-vaginal cells whereas the controls were not seen to have this change. This is an abnormality caused by impaired DNA synthesis, often arising from folate and vitamin B₁₂ deficiencies. Perhaps surprisingly, these cellular changes did not correlate with serum folate and vitamin B₁₂ depletion, and they returned to normal once the contraception was ceased. The authors of this study concluded that folate metabolism is organ specific (Whitehead et al., 1973).

The issue of whether systemic folate status may predict folate levels in human colonic mucosa has been much debated. Folate status has been significantly and positively associated with dietary intake and systemic folate markers (serum, red cell folate, plasma homocysteine) (Kim et al., 1998, Kim et al., 1996b, Kim et al., 2000) whereas in other studies no correlation was observed (Meenan et al., 1996).

Reports on folate status specifically in the colon have largely been derived from studies of subjects with CRC, including polyps and adenoma, which may confound results due to

complicated mechanisms involved in cancer development and progression, and the involvement of folic acid in 1-carbon metabolism (Kim et al., 2011b).

There were two aims in this chapter. Firstly to investigate whether systemic markers of folate status (serum, red cell folate, plasma homocysteine) and serum vitamin B₁₂ reflect colonic mucosal folate levels. A microbiological assay already present in this laboratory from a previous experiment carried out by the current investigator for an MSc project (Kim et al., 1998, Kim et al., 2001) was modified. The concentration of folate in small biopsies of colonic mucosa obtained from neoplasia-free subjects was measured and compared with folate in their blood samples. The second aim was to investigate whether the MTHFR C677T genotype and demographic and lifestyle factors influence folate levels in the colonic mucosa.

5.1.1 Hypothesis

1. Folate status in the colon is influenced by the *MTHFR* C677T genotype, demographic and lifestyle factors.
2. Systemic markers of folate status predict folate status in the colon of healthy volunteers.

5.1.2 Aims

1. To determine whether systemic markers of folate status reflect colonic folate status.
2. To determine the influence of the *MTHFR* C677T genotype, demographic and lifestyle factors on colonic folate levels.

5.1.3 Objectives

1. To quantify folate levels in colonic mucosal biopsies using a microbiological assay (MBA).
2. To compare colonic folate levels with systemic markers of folate status.
3. To investigate the influence of MTHFR C677T genotype, demographic and lifestyle variables on colonic folate levels.

5.2 Methods

5.2.1 Subject recruitment

Folate analysis in this chapter was performed on the same cohort of 336 healthy subjects participating in the cross-sectional Study 1 (detailed in Chapter 3). All patients provided blood samples for determination of systemic markers of folate status and vitamin B₁₂, and mucosal biopsies for determination of colonic mucosal folate. Subject recruitment, inclusion and exclusion criteria, demographic data collection, blood and tissue sample collection for measurement of colonic and systemic folate and the *MTHFR* C677T genotype are fully described in **Chapter 2**.

5.2.2 Laboratory Methods

Methodology for determination of colonic tissue folate and systemic markers of folate status and vitamin B₁₂ are described in **Chapter 2**, Section 2.4 and 2.5, pages 77-87. Serum and red cell folate, serum vitamin B₁₂ and homocysteine were all determined using the Bayer Advia Centaur® assay (Siemens Healthcare Diagnostics, US). Tissue folate was determined using an in-house microbiological assay which was developed for a previous study on folate bioavailability in food. The assay was modified to measure total folate in small tissue biopsies (5-64mg) using a method developed by Kim. This method is described previously in **Section 2.5.1** (Kim et al., 1998, Kim et al., 2001)

5.2.3 Statistical Analysis

A power calculation was originally performed for Study 1 (see **Statistical Analysis, Chapter 3**) and the same cohort of 336 healthy subjects were included in this study.

All data were analysed using IBM® SPSS® version 21.

Folate biomarkers were not normally distributed and therefore displayed as median (IQR), the range between the first and the third quartile.

Differences in colonic tissue folate between two groups (gender, smoking status, drinking status and nutritional and folic acid supplementation) were assessed using the Mann Whitney test; and the Kruskal-Wallis test was used between three groups for ethnicity.

Data were not normally distributed, so Spearman's rank correlation coefficient was used to test for associations between colonic tissue folate and systemic markers of folate status. Spearman's rank correlation coefficient was also used to test the relationship between colonic tissue folate and the continuous demographic variables age, BMI, dietary folate intake, total folate intake, number of cigarettes smoked and amount of alcohol consumed. Where correlations were seen with tissue folate, additional analyses were performed to investigate a possible relationship with the systemic markers of folate status and serum B₁₂.

Multiple linear regression was used to assess whether tissue folate could be predicted from any of the independent variables. Forced Entry, or 'Enter' in SPSS, was selected as the method of Multiple Linear Regression. All predictors are entered into the model at the same time and in no particular order. Tissue folate (ranked) was added to the model as the dependent variable and the following variables were added as the predictor variables: *MTHFR* C677T genotype, serum and red cell folate, plasma homocysteine, and serum vitamin B₁₂. The Forced Entry method relies on theoretical justification for the selection of predictor variables. In a second model, age (continuous), gender (male or female), ethnic group (white, black, other), cigarettes smoked and nutritional supplement use (user or non-user) were all added because preliminary tests suggested there was a relationship. For the categorical predictor variables, *MTHFR* C677T, CT and TT were tested against CC as the reference group and for ethnicity as a categorical predictor variable, black and other were tested with white as the reference group.

5.3 Results

5.3.1 Colonic tissue folate and systemic markers of folate status

Median folate concentration in the colonic mucosa for all subjects (n=336) was 0.88 (IQR: 0.59-1.23) nmol/g tissue, which is comparable to values previously described in the literature (range of 0.50-1.50 nmol/g) (Kim et al., 1998). The in-house folate microbiological assay enabled reproducible measurement of folate in 336 small tissue specimens. Within and between-days coefficients of variation were 3.2% and 6.8%, respectively.

In addition to tissue folate, median (IQR) for serum and red cell folate, serum vitamin B₁₂ and plasma homocysteine in all subjects are displayed in **Table 5.1**.

Table 5.1 Folate biomarkers and vitamin B₁₂ for the whole sample

	n	Median	IQR
Colonic tissue folate (nmol/g)	336	0.88	0.59-1.20
Serum Folate (nmol/l)	332	25.5	17.1-38.0
Red cell folate (nmol/l)	330	965	765-1139
Serum B ₁₂ (pmol/l)	332	313	239-428
Plasma homocysteine (μmol/l)	330	16.1	12.7-21.1
Data are median (IQR)			

5.3.2 Blood folates as predictors of colonic mucosal folate

As shown in **Table 5.2** and **Figures 5.1-5.4**, folate in the colonic mucosa correlated positively with serum folate and red cell folate, serum vitamin B₁₂ and negatively with plasma homocysteine.

Table 5.2 Association between colonic tissue folate and systemic markers of folate status and vitamin B₁₂

	n	Correlation coefficient	P value
Serum folate	331	0.46	<0.001
Red cell folate	329	0.29	<0.001
Serum B ₁₂	329	0.17	0.002
Plasma homocysteine	331	-0.14	0.01
Data are Spearman's Rank Correlation Coefficient			

Figure 5.1 Tissue folate versus serum folate ($\rho = 0.46$, $P < 0.001$, $n = 331$)

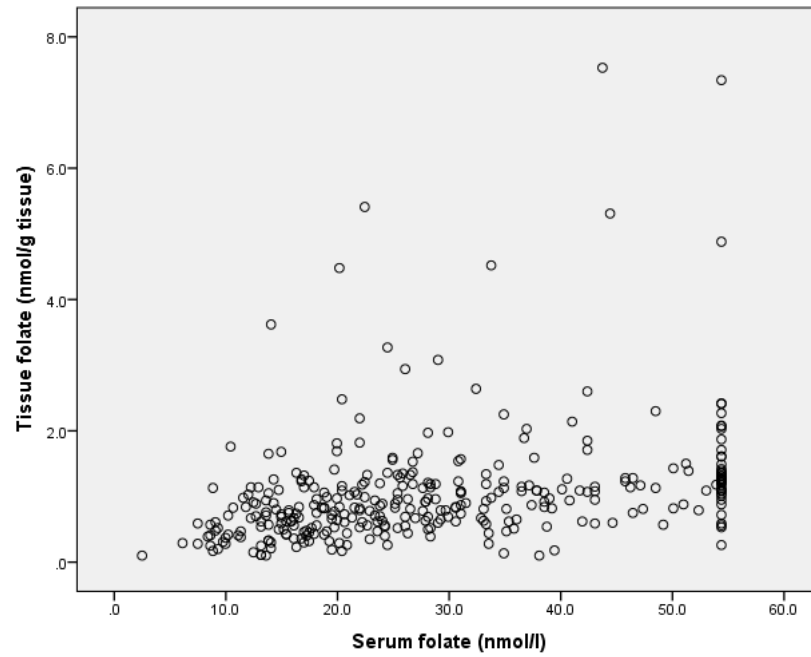


Figure 5.2 Tissue folate versus red cell folate ($\rho = 0.29$, $P < 0.001$, $n = 329$)

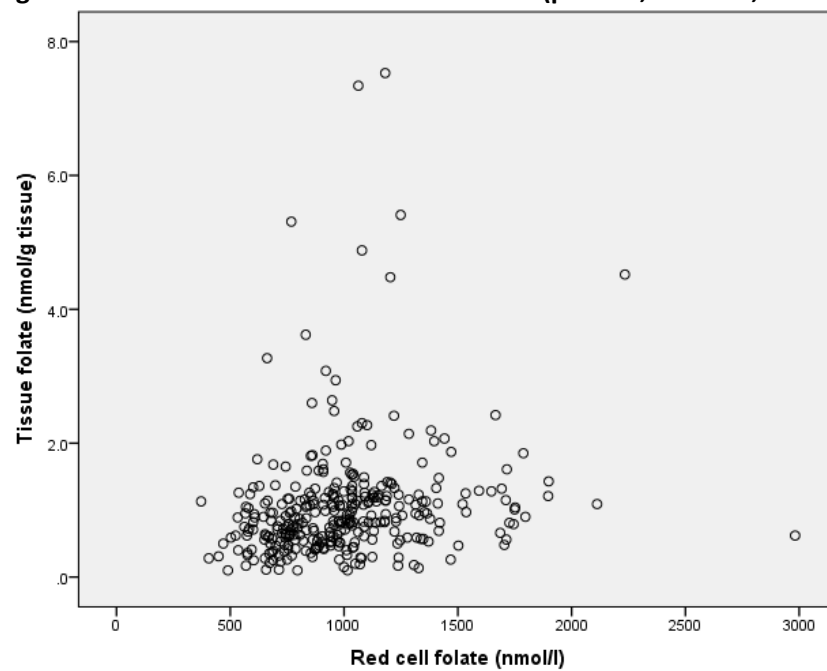


Figure 5.3 Tissue folate versus serum B₁₂ ($\rho = 0.17$, $P < 0.002$, $n = 329$)

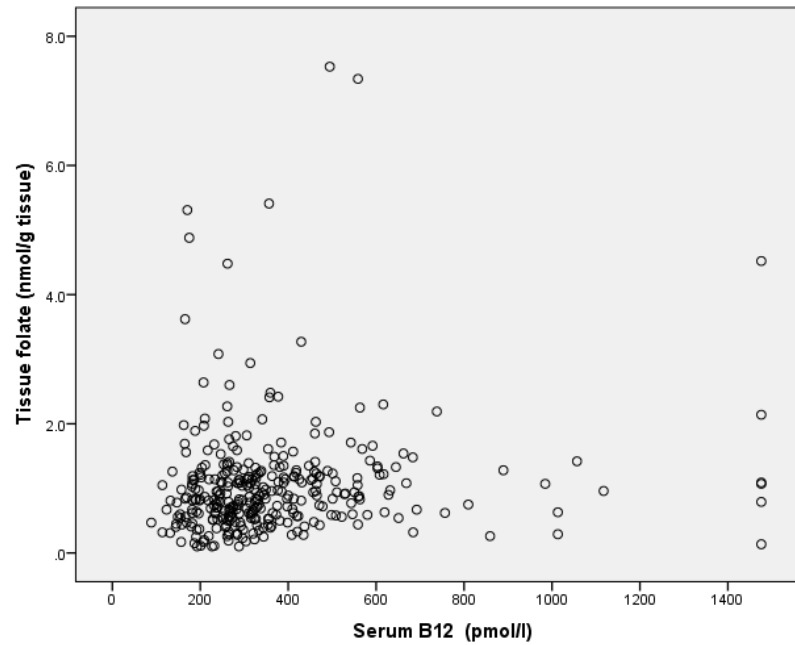
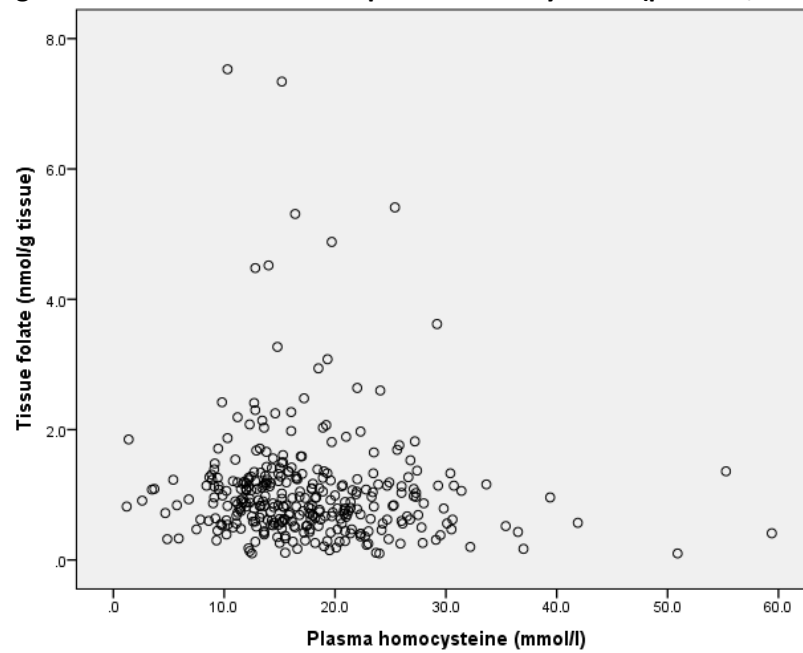


Figure 5.4 Tissue folate versus plasma homocysteine ($\rho = -0.14$, $P = 0.01$, $n = 331$)



5.3.3 Colonic tissue folate, systemic markers of folate status and serum B₁₂, by demographic and lifestyle subgroups

Diet and lifestyle characteristics for the whole cohort in the current study are described in **Chapter 3 (Table 3.1)**. Colonic tissue folate was shown by the *MTHFR* C677T genotype and no differences were seen in tissue folate levels across the three genotypes (**Chapter 3, Table 3.3**).

In this chapter, colonic tissue folate was assessed by demographic and lifestyle subgroups. Tissue folate did not differ between genders, but serum folate, red cell folate and vitamin B₁₂ were all higher in women than in men. Plasma homocysteine was lower in women than in men (**Table 5.3**).

Table 5.3 Biomarkers of folate status by gender

	Male			Female			
	n	Median	IQR	n	Median	IQR	P
Colonic tissue folate (nmol/g)	128	0.80	0.60-1.20	207	0.92	0.60-1.30	0.60
Serum Folate (nmol/l)	125	23.6	16.1-32.1	207	27.20	12.4-41.0	0.01
Red cell folate (nmol/l)	124	911	744-1049	206	994	777-1205	0.01
Serum B ₁₂ (pmol/l)	332	287	233-408	208	327	250-459	0.02
Plasma homocysteine (μmol/l)	125	18.5	14.3-29.2	205	15.20	12.1-19.7	<0.001

Biomarkers are displayed as median (IQR). The Mann Whitney test was used to assess differences in folate biomarkers and serum B₁₂ between males and females

There were no differences in colonic tissue folate, serum folate or plasma homocysteine between ethnic subgroups but there was a significant difference between groups for red cell folate and vitamin B₁₂. Further testing between two groups using the Mann-Whitney test revealed red cell folate was higher in the other group than in both the white and the black groups. Further testing revealed that serum B₁₂ was higher in the black than the white group and higher in the white than the other group (**Table 5.4**).

Table 5.4 Biomarkers of folate status by ethnic group

	White			Black			Other			
	n	Median	IQR	n	Median	IQR	n	Median	IQR	P
Colonic tissue folate (nmol/g)	226	0.89	0.57-1.20	68	0.82	0.62-1.20	29	1.1	0.59-1.50	0.44
Serum Folate (nmol/l)	224	25.4	17.1-38.4	67	22.4	16.3-35.4	29	32.4	20.1-49.4	0.36
Red cell folate (nmol/l)	223	979	768-1133	67	866	739-1079	29	1063	893-1261	0.03
Serum B ₁₂ (pmol/l)	224	287	224-366	67	410	311-559	29	368	262-533	<0.001
Plasma homocysteine (μmol/l)	221	16.5	12.9-22.2	69	15.8	12.3-19.6	28	14.9	11.6-18.2	0.20

Biomarkers are displayed as median and IQR. The Kruskal Wallis test was used to assess differences in folate biomarkers between ethnic groups

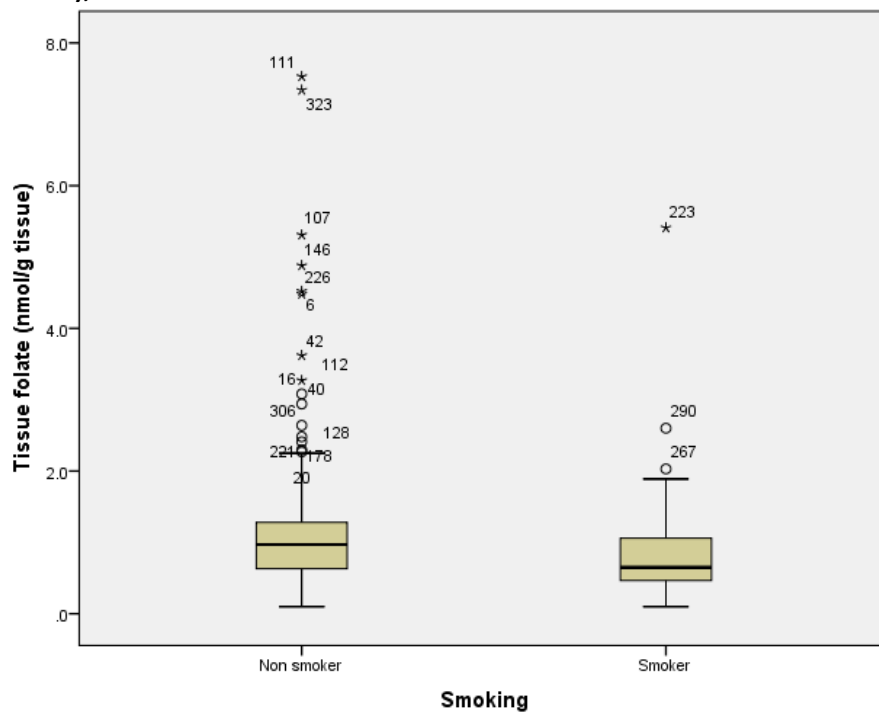
Tissue folate was significantly higher in non-smokers than in smokers (0.97nmol/g versus 0.65nmol/g, $P<0.001$). Serum and red cell folate and serum B₁₂ were significantly higher in non-smokers than in smokers but there were no significant differences in plasma homocysteine levels between smokers and non-smokers (**Table 5.5** and **Figure 5.5**).

Table 5.5 Biomarkers of folate status by smoking

	Smoker			Non-smoker			P
	n	Median	IQR	n	Median	IQR	
Colonic tissue folate (nmol/g tissue)	71	0.65	0.46-1.10	242	0.97	0.60-1.28	<0.001
Serum folate (nmol/l)	69	20.4	13.9-30.8	242	27.6	18.0-40.6	<0.001
Red cell folate (nmol/l)	70	903	705-1076	241	981	786-1178	0.02
Serum B ₁₂ (pmol/l)	69	283	199-359	242	323	249-457	0.002
plasma homocysteine (μmol/L)	70	18.0	13.7-22.4	238	15.8	12.8-20.9	0.14

Biomarkers are displayed as median and IQR. The Mann Whitney test was used to assess differences in folate biomarkers between smokers and non-smokers

Figure 5.5 Tissue folate by smokers 0.65nmol/g (n=71) and non-smokers 0.97nmol/g (n=242), P <0.001



There were no differences in any of the folate biomarkers between drinkers and non-drinkers (**Table 5.6**).

Table 5.6 Biomarkers of folate status methylation by drinking habits

	Drinker			Non-drinker			P
	n	Median	IQR	n	Median	IQR	
Colonic tissue folate (nmol/g)	171	0.90	0.61-1.30	143	0.87	0.57-1.20	0.40
Serum Folate (nmol/l)	169	26.5	18.1-39.8	143	22.9	16.3-37.4	0.10
Red cell folate (nmol/l)	172	960	766-1117	140	977	773-1183	0.30
Serum B ₁₂ (pmol/l)	169	306	238-412	143	327	246-461	0.09
Plasma homocysteine (μmol/l)	168	16.0	12.9-21.1	141	17.0	12.6-22.0	0.60

Biomarkers are displayed as median and IQR. The Mann Whitney test was used to assess differences in folate biomarkers between drinkers and non-drinkers

Colonic tissue folate was higher in the folic acid supplement user group than in the non-user group but no differences were observed in colonic tissue folate between nutritional supplement users and non-users. Serum and red cell folate and vitamin B₁₂ levels were higher, and plasma homocysteine was lower, in both nutritional and folic acid supplement users than in non-users. (**Table 5.7** and **5.8**).

Table 5.7 Biomarkers of folate status by nutritional supplement use

	Nutritional supplement user			Non -nutritional supplement user			P
	n	Median	IQR	n	Median	IQR	
Colonic tissue folate (nmol/g tissue)	95	1.0	0.60-1.30	213	0.87	0.60-1.20	0.30
Serum folate (nmol/l)	94	33.50	20.3-54.4	212	23.80	16.3-33.1	<0.001
Red cell folate (nmol/l)	93	1038	844-1236	213	938	755-1085	0.01

Serum B ₁₂ (pmol/l)	95	354	287-493	211	288	227-398	<0.001
plasma homocysteine (μmol/L)	95	15.0	12.0- 19.80	208	17.40	13.30- 22.0	0.02

Biomarkers are displayed as median and IQR. The Mann Whitney test was used to assess differences in folate biomarkers between nutritional supplement users and non-users

Table 5.8 Biomarkers of folate status by folic acid supplement use

	FA supplement user			Non -FA supplement user			P
	n		IQR	n		IQR	
Colonic tissue folate (nmol/g tissue)	44	1.0	0.60-1.20	264	0.90	0.60-1.20	0.03
Serum folate (nmol/l)	43	35.10	22.0-51.0	263	24.20	16.5-36.5	<0.001
Red cell folate (nmol/l)	43	1045	795-1278	263	952	759-1108	0.08
Serum B ₁₂ (pmol/l)	44	387	271-530	262	306	234-411	0.002
plasma homocysteine (μmol/L)	44	13.70	10.90- 16.90	259	17.20	13.30-22.0	<0.001

Biomarkers are displayed as median and IQR. The Mann Whitney test was used to assess differences in folate biomarkers between folic acid users and non-users

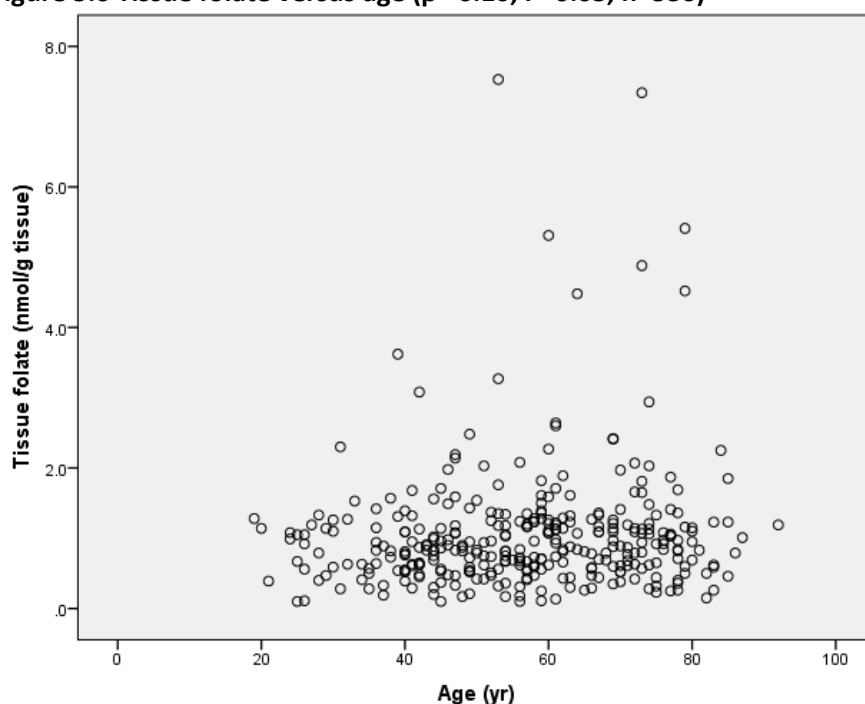
Spearman's rank correlation coefficient was used to investigate associations between colonic tissue folate and continuous demographic and lifestyle variables. Colonic tissue folate correlated positively with age but only marginally ($\rho=0.1$, $P=0.05$) (**Table 5.9** and **Figure 5.6**).

Table 5.9 Association between colonic tissue folate concentration and demographic and lifestyle characteristics

	n	Correlation coefficient	P value
Age	336	0.10	0.05
BMI	307	-0.03	0.60
Alcohol intake	171	0.06	0.31
Number of cigarettes smoked	71	0.09	0.47
Dietary folate	60	0.07	0.56
Total folate intake	60	0.14	0.29

Data are Spearman's Rank Correlation Coefficient

Figure 5.6 Tissue folate versus age ($\rho = 0.10$, $P < 0.05$, $n = 336$)



Due to the correlation found between colonic tissue folate and age in **Table 5.9**, age was further investigated for associations with the other folate biomarkers and serum B₁₂. Serum and red cell folate were positively correlated with age and negatively correlated with plasma homocysteine (Table 5.10).

Table 5.10 Association between age and folate biomarkers

	n	Correlation coefficient	P value
Colonic Tissue Folate	336	0.10	0.05
Serum folate	332	0.15	0.01
Red cell folate	330	0.20	<0.001
Serum B ₁₂	332	0.07	0.2
Plasma homocysteine	330	-0.30	<0.001

Data are Spearman's Rank Correlation Coefficient with all folate biomarkers and serum B₁₂

Finally, multiple linear regression analysis was used to assess and validate associations with between tissue folate and systemic markers of folate status and serum B₁₂ with adjustment for the MTHFR genotypes. The model revealed a positive association between colonic tissue folate with serum folate ($\beta=0.37$; $P<0.001$) (**Table 5.11a**).

Table 5.11a ANCOVA model with colonic tissue folate as the dependent variable, and the MTHFR C677T genotype, systemic markers of folate status as the predictors

	Unadjusted β (unstandardised coefficient)	P value
MTHFR C677T		
CC (reference)		
CT	-3.54	0.71
TT	3.54	0.80
Serum folate (nmol/l)	0.36	<0.001
Red cell folate (nmol/l)	0.04	0.64
Serum B ₁₂ (pmol/l)	0.12	0.09
Plasma homocysteine (μ mol/l)	0.06	0.43
Multiple linear regression model (Forced Entry)		
All folate biomarkers are ranked variables		
Model summary: Adjusted R square = 0.15, F= 7.55, $P<0.001$		

The same model was run again with additional adjustments made for age, gender, ethnicity, smoking and supplementation. A positive association between colonic tissue folate with serum folate remained ($\beta=0.36$; $P<0.001$) and a non-significant negative association with smoking remained ($\beta=-20.43$; $P=0.07$) (**Table 5.11b**)

Table 5.11b ANCOVA model with colonic tissue folate as the dependent variable, and the MTHFR C677T genotype, systemic markers of folate status, and demographic and lifestyles variables as the predictors (n=336)

	Adjusted β (unstandardised coefficient)	P value
MTHFR C677T		
CC (reference)		
CT	-5.04	0.60
TT	0.68	0.96
Serum folate (nmol/l)	0.36	<0.001
Red cell folate (nmol/l)	0.03	0.73
Serum B12 (pmol/l)	0.11	0.16
Plasma homocysteine (μ mol/l)	0.06	0.47
Age (years)	0.19	0.56
Gender	-5.77	0.55
Ethnicity White (reference)		
Black ethnic group	0.12	0.99
Other ethnic group	13.2	0.19
Supplement user	-9.24	0.37
Smoking	-20.43	0.07
Multiple linear regression model (Forced Entry)		
All folate biomarkers are ranked variables		
Model summary: Adjusted R square = 0.17, F= 4.36, P<0.001		

5.4 Discussion

The key aim of this study was to assess the relationship between folate levels in the colonic mucosa and systemic markers of folate status in a neoplasia-free population with adequate folate status. The influence of the MTHFR C677T genotype, demographic and lifestyle factors on colonic folate levels was also assessed. At the time that this study was conducted no updates of a similar kind had been reported. It was the largest prospective investigation of these associations, using a cohort who presented with no evidence of any pathology in the colon after colonoscopy. Subjects were recruited if they were free from colonic adenoma, CRC or any inflammation in the mucosa.

There were six main findings in the current study:

1. Colonic tissue folate was positively correlated with serum, red cell folate and serum B₁₂ and negatively correlated with plasma homocysteine.
2. Colonic tissue folate was significantly higher in non-smokers than in smokers.
3. Colonic tissue folate was marginally and positively correlated with age.
4. Colonic tissue folate levels did not differ between genders, ethnic groups, and drinkers and non-drinkers.
5. Colonic tissue folate was higher in the folic acid supplement user group than in the non-user group but no differences were observed in colonic tissue folate between nutritional supplement users and non-users.
6. Multiple linear regression revealed a positive association between colonic tissue folate and serum folate and a negative non-significant association with smoking.

Analysis of the subjects' blood samples showed that none of the subjects were serum or red cell folate deficient. Data from FFQs completed by only 60 subjects (representing ~18% of the cohort) indicated that the population consumed adequate levels of dietary folate. Upon colonoscopy, none of the subjects showed any sign of neoplasia or other colonic pathologies, despite having originally presented with symptoms of problems with the large bowel.

Analysis of colonic tissue folate and biomarkers in this neoplasia-free cohort revealed significant positive associations between systemic and colonic folate status and regression analysis confirmed the positive association between colonic folate levels and serum folate. Interestingly, the strong positive association with serum folate and colonic tissue folate remained the same even after adjustment in the second regression model. These results were consistent with findings of Kim *et al.* (1998) who reported that concentrations of colonic mucosal folate were strongly correlated with folate biomarkers in blood and, in particular, serum homocysteine was the most sensitive indicator of colonic tissue folate in their study (Kim *et al.*, 1998). Notably, the Kim *et al* (1998) study used normal recto-sigmoid mucosa, but tissues were sampled from patients with excised colorectal polyps which could affect the biochemical composition of anatomically related normal tissue. In contrast, in the current study colonic mucosa from neoplasia-free subjects was assayed.

Serum folate rather than homocysteine was most closely correlated with colonic tissue folate, however the patients in the Kim study had all had polypectomies.

Associations between folate in the colonic mucosa and serum of healthy volunteers could be useful in assessing CRC risk but this would need validation in larger studies. Colonic neoplasia originates in the mucosa, and folate metabolism is a critical factor for this cancer. In a key review, Brockton (2006) suggested that the beneficial relationship between folate in the colon and reduced CRC risk might be due to the fact that folate facilitates DNA stabilisation in colonic tissue (Brockton, 2006). Thus, accurate and fast determination of folate levels by a simple biochemical blood-based test could be an invaluable tool to estimate CRC risk when combined with other tests, genetic associations and other patient-related risk factors. Such combined tests could facilitate the biological understanding of disease pathogenesis.

Another key aim of this study was to examine the relationship between the MTHFR C667T genotype and folate status in normal mucosa. In the current study, colonic tissue folate did not differ by the MTHFR C667T genotype in the colon of a neoplasia-free cohort with adequate folate status.

Further examination into the demographic and lifestyle characteristics of the 336 subjects revealed that folate levels in colonic tissue were higher in non-smokers than in smokers. Cigarette smoke is a known source of free radicals and a major contributor of oxidative stress (Burke and Fitzgerald, 2003). It has been long been established that smoking is associated with lowered level of serum folate and red cell folate and that the association remains after adjustment for dietary intake (Walmsley et al., 1999, Piyathilake et al., 1994, Mannino et al., 2003). Vardavas *et al.* (2008) reported that smokers were likely to have different lifestyles including drinking more alcohol, and different dietary patterns compared to non-smokers, with lower consumption of fruit, vegetables and fibre but similar amounts of meat products. These dietary habits were associated with lower levels of serum folate, vitamin C and antioxidants but had little effect on serum iron or vitamin B₁₂ (reflecting meat consumption) (Vardavas et al., 2008). The Vardavas *et al* report was based on healthy populations and provides useful insight for the interpretation of our findings, suggesting depletion of colonic tissue folate in smokers.

The association between dietary variations in smokers and non-smokers reported by Vardavas *et al.* could not be explored further in this study due to the small number of diet-related FFQs returned (60/336; ~18%). Additionally, the low number of completed FFQs prevented the accurate assessment of possible relationships between total dietary folate intake (or other micronutrients). Nevertheless, in a comparison of folic acid supplementation users and non-users, a significant increase in colonic tissue folate was observed ($P=0.03$). Diet and lifestyle differences described by Vardavas *et al.* do not provide an explanation for the fact that lower folate has been reported in those exposed to second hand smoke (Ortega *et al.*, 2004, Ortega *et al.*, 2010), suggesting that smoking reduces the absorption of folate or affects folate metabolism and other one-carbon components of folate metabolism (Walmsley *et al.*, 1999), especially as smoking was also shown to deplete vitamin B₆ and B₁₂ in parallel with tetrahydrofolate and 5, 10-methylenetetrahydrofolate whilst pteroylmonoglutamate (folic acid) formyltetrahydrofolate and 5,10-methylenetetrahydrofolate accumulated in the tissue.

This study was the first of its kind to investigate folate coenzyme distribution in tissue, but samples were taken from the buccal mucosa which is directly exposed to the smoke, thus comparison with the colon is difficult (Gabriel *et al.*, 2006). In rats, inhibition of the methionine synthase vitamin B₁₂ complex in response to nitrous oxide (a particulate in cigarette smoke) caused reduction in tetrahydrofolate (Horne *et al.*, 1989).

In a nationally representative US sample, of all of the multiple demographic and lifestyle variables, age and smoking came out as the strongest correlates of biomarkers including folate. Biomarker concentrations increased with age and decreased with cigarette smoking (Pfeiffer *et al.*, 2013) and this is continuous with the findings reported in this chapter.

In summary, this study demonstrates that serum folate predicts folate levels in the colonic mucosa in a population free from colonic neoplasia (and not deficient in folate or vitamin B₁₂) but the *MTHFR* C667T genotype does not influence colonic folate levels in this population. Systemic markers of folate status showed varying associations with demographic, lifestyle and folate intake and there were significant differences in the level of folate biomarkers between the population groups in the cohort for these variables (i.e. gender, ethnicity, smokers and non-smokers, and users and non-users of folate

supplementation), after adjustment with regression analysis these associations were not observed. Only serum folate was associated with folate status in the colonic tissue.

6 Effects of short-term folate supplementation on DNA methylation and systemic folate biomarkers in the colorectal mucosa of subjects without colorectal neoplasia: A Randomised Controlled Trial.

6.1 Introduction

Folate plays a critical role in 1-carbon metabolism during DNA synthesis and methylation, and defective DNA processing could lead to carcinogenesis. Impaired DNA methylation in colorectal cancer could be bimodal, with hypomethylation of genomic DNA and concomitant hypermethylation of DNA in promoter regions of tumour suppressor genes. Findings on the role of folate supplementation in colorectal cancer is conflicting. Folic acid supplementation has been reported by some to reverse genomic DNA hypomethylation in individuals with adenoma (Cravo et al., 1994, Pufulete et al., 2005a) while others have shown folic acid to have no effect in adenoma patients (Figueiredo et al., 2009a), nor in healthy volunteers (Fenech et al., 1998, Basten et al., 2006). A summary of previous randomised controlled studies investigating the effects of folate supplementation on DNA methylation are described in **Table 1.5 (Chapter 1, Section 1.5.4.3)**. In Chapter 3 of this thesis, DNA methylation and folates in the blood (from dietary intake and supplementation) were investigated for associations in neoplasia-free subjects. In order to investigate a causal relationship in this cohort, an intervention trial with folic acid supplementation was carried out.

Hypothesis

1. Increasing folate levels with short-term dietary supplementation influences DNA methylation in the colonic mucosa of subjects without colorectal adenoma or cancer.
2. The *MTHFR* C677T polymorphism influences the response of DNA methylation to folate supplementation.

Aims

3. To determine whether short-term folate supplementation causes changes in systemic and colonic folate status which modify DNA methylation.
4. To determine whether the response differs according to the *MTHFR* C677T

genotype.

Objectives

1. To conduct a randomised, double blind, randomised controlled trial (RCT) of folic acid supplementation or placebo in a subset of participants from the cross-sectional study presented in **Chapter 3 (Study 1)**.
2. To compare the change from baseline in DNA methylation levels between subjects taking folic acid and those taking placebo.
3. To examine whether DNA methylation changes differ according to the MTHFR C677T genotype.

6.2 Methods

6.2.1 Subject Recruitment

The present study was conducted in a subgroup of subjects originally recruited into **Study 1 (Chapter 3)**, recruitment methods are described fully in **Chapter 2**. Briefly, of the 336 subjects in **Study 1**, all of those with the CC and TT genotype were approached during their follow-up appointment and invited to take part in a randomised, controlled, double-blind trial. Subjects who agreed to participate signed a consent form and were assigned to either folic acid or placebo for a period of 12 weeks. Throughout the study, subjects were telephoned every two weeks to monitor whether they were taking their tablets and if they were willing to continue in the trial. At the end of the intervention period, subjects were invited back to the hospital and asked to return their remaining tablets so that they could be counted for compliance. Repeat fasting blood samples and colorectal biopsies were collected, this time using sigmoidoscopy. Systemic and colonic folate levels, and DNA methylation in the colonic mucosa, were measured as described previously (**see Chapter 2, Sections 2.4**).

Originally, the aim was to measure uracil content as part of the intervention trial but it was not possible due to insufficient samples remaining.

6.2.2 Statistical Analysis

Sample size for the RCT was calculated from a previous folate intervention study performed in this laboratory which investigated genomic DNA methylation in patients with colorectal adenoma as an endpoint. In this study, the aim was to recruit 23 subjects

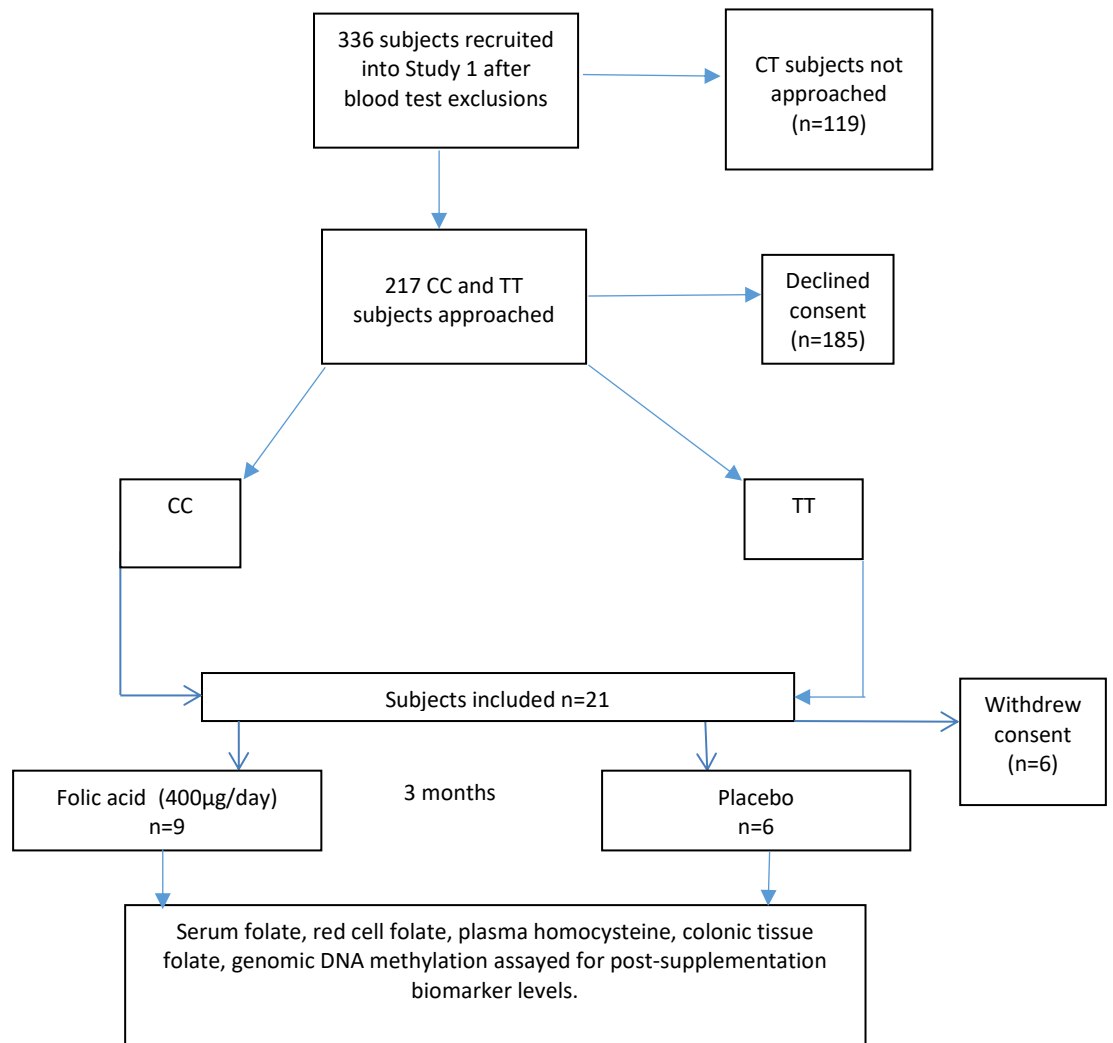
into each arm to receive either 400µg/d of folic acid or placebo pills for 3 months. These numbers were calculated in order to detect a 0.8SD (136Bq/µg/DNA) change in DNA methylation following folate supplementation, at $P < 0.05$ (80% power) (Pufulete et al., 2005a). In the current study the aim was to recruit a total of 60 subjects to allow for potential exclusions and to be able to compare equal numbers in each arm in relation to the *MTHFR* C677T polymorphism.

6.3 Results

6.3.1 Subject Recruitment

Details of numbers in the flowchart below are described in full in **Section 6.4**.

Figure 6.1 Flow diagram: Study 2 - Recruitment of subjects who were initially recruited into Study 1



6.3. Subject characteristics

A total of 15 subjects completed the 12-week study. Of these, six were randomised to placebo of which two subjects had the CC genotype and four subjects had the TT genotype. Nine subjects were randomised to folic acid of which six subjects had the CC genotype and three had the TT genotype. Subject characteristics are shown by treatment arm in **Table 6.1**.

Table 6.1 Subject characteristics by treatment group at baseline

		Placebo (n=6)	Folic acid (n=9)
Age (years)		50 (41-59)	71 (59-71)
Gender	Male	3	2
	Female	3	7
BMI (kg/m ²)		27.2 (21.9-30.2)	22.5 (20.9-29.8)
MTHFR genotype	CC	2	6
	TT	4	3
Smoking	Non-smoker	4	7
	smoker	2	2
Drinking	Non-drinker	3	3
	Drinker	4	5
Dietary folate intake (mg/d)		272 (179-350)	387 (216-426)

Data are median (IQR) for age, BMI, dietary folate intake. Gender, the MTHFR C677T genotype, smoking and drinking are all displayed as frequencies

Biomarkers of folate status, serum vitamin B₁₂ and genomic DNA methylation pre- and post-intervention are shown by the placebo and folic acid arms in **Table 6.2**. Change from baseline values in the folic acid and placebo groups were compared using the Mann-

Whitney test to determine whether folic acid supplementation affected biomarker profile.

Genomic DNA methylation measured in the colonic mucosa did decrease as a result of folic acid supplementation but this was not significant. Change in serum folate from baseline was greater in the folic acid group ($P=0.05$) than the placebo group. There were no significant differences in red cell folate, colonic tissue, plasma homocysteine, and serum vitamin B₁₂.

Table 6.2 Change from baseline in biomarkers of folate status, serum B₁₂ and DNA methylation after supplementation

	Placebo			Folic acid			P
	Baseline	Post RCT	Change	Baseline	Post RCT	Change	
Serum Folate (nmol/l)	20.6 (17.1-28.7)	15.9 (11.4-21.0)	-6.9 (-8.60- -2.58)	23.3 (16.9-30.4)	34.3 (26.4-52.0)	9.90 (0-27.0)	0.05
Red cell folate (nmol/l)	924 (830-992)	866 (760-1009)	11.3 (-119-36.2)	902 (679-994)	1026 (979-1200)	239 (0-328)	0.11
Colonic tissue folate (nmol/g tissue)	0.83 (0.44-1.37)	0.47 (0.30-1.44)	-0.11 (-1.18-0.25)	0.71 (0.47-1.07)	1.13 (0.97-1.96)	0.88 (0.20-1.3)	0.07
Serum B ₁₂ (pmol/l)	566 (463-748)	545 (379-695)	-83.5 (-249-83.5)	485 (380-598)	398 (332-433)	-70.0 (-217-0)	0.9
Plasma homocysteine (μmol/l)	17.5 (12.5-29.4)	16.40 (14.9-23.5)	-2.35 (-10.7-7.45)	13.4 (9.03-17.6)	14.9 (1.35-20.8)	0 (-13.0-10.6)	0.73
DNA Methylation (% methylcytosine)	4.10 (4.00-4.38)	4.40 (4.05-4.5)	0.20 (4.05-4.5)	4.20 (3.5-4.75)	3.90 (3.50-4.70)	-0.10 (3.50-4.70)	0.61

Data are median (IQR) for baseline, post-intervention and change from baseline.

Change from baseline (post-supplementation) between folic acid and placebo were tested using the Mann-Whitney U test.

An attempt was made to examine the effects of supplementation by genotype on the biomarkers. **Table 6.2** was reproduced with each column split into CC and TT genotypes. Some of the groups were too small (n=2) to produce interquartile ranges and the information was very difficult to assimilate. Additionally groups were too small for change from baseline to be tested statistically. **Table 6.3** is included in **Appendix 5** for information.

6.4 Discussion

Given the role of folate in DNA methylation and the fact that impaired DNA methylation is an intermediary marker of cancer (Okugawa et al., 2015), the aims of the current study were to a) determine whether genomic DNA methylation in healthy colonic tissue was modified by short-term folic acid supplementation in subjects with adequate folate status; b) clarify the effects of folic acid supplementation on folate levels in the blood and in the colonic mucosa; and c) understand the influence of the *MTHFR* C677T genotype. Serum folate was the only variable that was significantly increased as a result of folate supplementation. Red cell folate, plasma homocysteine, serum vitamin B₁₂ and genomic DNA methylation in the colon did not change following supplementation with folic acid.

This study was powered to recruit 60 subjects in total and the aim was to recruit 30 subjects into the placebo group and 30 subjects in the folic acid group. Subjects were recruited from the same cohort of 336 subjects participating in **Study 1** (Chapter 3). Unfortunately, a number of logistical challenges affected recruitment of 60 subjects.

All CC and TT subjects (identified by genotyping 336 subjects from **Study 1**) were asked to participate in this trial (**Study 2**) which involved daily intake of either folate or placebo for 12 weeks and a request to return to the hospital for repeat sample collection. Biopsies were to be taken by sigmoidoscopy, an invasive procedure which can involve pain, stress and has some clinical risks. Thus, it is not surprising that many were reluctant to participate. 217 of the 336 subjects were approached but only 30 agreed to participate. Three developed pathologies that violated the inclusion criteria and six could no longer be contacted prior to commencing the study. A total of 21 subjects entered the study and received a supply of either folic acid or placebo. These subjects were followed throughout the study by phone to check if they were still participating and had remembered to take their pills. Maintaining compliance and retention in **Study 2** was also challenging. Two

subjects lost their tablets, two subjects admitted to taking them sporadically with breaks of up to a week, and three subjects started treatment but it became impossible to contact them after the study commenced. Only subjects who had missed one day and had remembered to take 2 tablets the next day could remain in the study for further analysis. Eventually, only 15 subjects (placebo n=6 and folic acid n=9) completed the study and attended the follow-up for collection of repeat blood and colonic tissue samples. Thus, this study is severely underpowered and caution should be taken with the interpretation of results. Recruitment and retention may have been improved by putting more time and resources into preparation prior to study start-up and in obtaining more prior agreement from the hospital clinical team. The study is presented here as a pilot study to guide future work. In addition, limited availability of biopsy DNA samples did not allow examination of uracil misincorporation as samples were prioritised for analysis of DNA methylation.

As previously discussed, folate deficiency has been suggested as one of the possible factors contributing to the development of CRC and tumour progression. DNA hypomethylation is often followed by hypermethylation of the promoter region of tumour suppressor genes and this can also affect tumour progression (Duthie et al., 2004). Folate supplementation was shown to reverse global DNA hypomethylation in disease-free rectal mucosa by the PI of this study. Supplementation with 400µg/d for 10 weeks resulted in increased serum and red cell folate and a concurrent decrease in genomic DNA methylation by 31% in leucocytes and 25% in the colonic mucosa of adenoma patients (Pufulete et al., 2005a). Meanwhile folic acid has also been shown to cause an increase in DNA methylation on the promoter of specific genes (van den Donk et al., 2007a).

Older rat and mouse studies suggest that folate insufficiency could predispose the normal epithelium to neoplasia while folate intake could suppress tumorigenesis. More specifically, physiological doses (2-400µg) of folic acid supplementation during the early stages of colorectal neoplasia were found to reduce tumour growth, whereas pharmacological doses after the onset of carcinogenesis were found to promote tumour growth (Kim et al., 1996b, Song et al., 2000b). This paradoxical effect has been termed the 'dual modulatory effect' of folate supplementation (Kim, 2007b). An investigation into the effects of folic acid supplementation in rats during the early stages of colorectal cancer (aberrant crypt foci, ACF) found that tumour multiplicity was positively correlated with dietary folic acid dosage indicating that folic acid supplementation may promote the

progression of ACF to colorectal tumours (Lindzon et al., 2009). In mice with no precancerous lesions, Lin et al. (2011) found that folic acid supplementation was more effective in reducing CRC risk compared to those with adenocarcinomas (Lin et al., 2011).

Human studies have also produced variable results with regards to folic acid supplementation both in patients with CRC or history of colorectal cancer and in healthy individuals. An intervention study in patients following resection of colorectal adenomas found that a high dose of folic acid supplementation (n=49; 5 mg/day 0.02) for 3 years significantly reduced recurrence of adenomas compared to a placebo group (n=45). It was also shown that the clinical response was improved in patients below 70 years of age (Jaszewski et al., 2008).

In healthy volunteers, Basten *et al.* (2004) reported that short-term folic acid supplementation (12 weeks; 1.2 mg/day) could significantly increase red cell folate and total lymphocyte folate but tissue folate (buccal mucosa, not the colon) was unchanged. In a subsequent study in lymphocytes the same group showed no difference in global DNA methylation in healthy volunteers following supplementation in the folic acid group compared to placebo (Basten et al., 2006). They also suggested that DNA methylation was less sensitive to changes in folate status than other markers such as uracil misincorporation and reported that folic acid supplementation had significantly lowered plasma homocysteine levels. In another study, young Australian adults (aged 18-32) with adequate folate status (red cell folate) were given cereal supplemented with 700µg folic acid and 7mg vitamin B₁₂ or identically matched cereal without supplementation. No effect on DNA methylation in lymphocytes was observed in the supplementation group but plasma homocysteine was decreased by 37% (Fenech et al., 1998). Two larger studies of healthy volunteers examined DNA methylation using the LC-ES MS/MS method in uncoagulated blood. One measured DNA methylation changes in coagulated and uncoagulated blood samples from women of reproductive age (n=195) after supplementation with 4mg/day for six months (Crider et al., 2011). The other measured DNA methylation changes in leucocytes of healthy volunteers (n=216) after daily supplementation of 800 µg/day for three years (Jung et al., 2011). Neither showed any effect of supplementation on DNA methylation apart from a minor effect seen in the coagulated samples in the Crider study.

In the few subjects who took part in the current study, an increase in serum folate was confirmed after 12 weeks of folic acid supplementation. A marginal decrease in genomic DNA methylation was found from baseline to post intervention in the folic acid group but this was not significant. As highlighted before, this cohort was underpowered but the results are in agreement with van den Donk *et al.* (2007a) results observed in patients with a history of adenoma. More importantly (for comparison with the current study) in healthy volunteers Fenech *et al.* (1998), Basten *et al.* (2006), Jung *et al.* (2011) and Crider *et al.* (2011) (the latter two both used LC-ESI-MS/MS employed in the current study) showed that DNA methylation was not significantly altered by short-term folic acid supplementation.

In summary, the results from this pilot study show that short-term folic acid supplementation at physiological doses may increase serum folate status but this had no significant influence on colonic tissue folate or genomic DNA methylation in the colonic mucosa of healthy folate-replete subjects. Furthermore, a response was not seen according to the *MTHFR* C667T genotype but numbers were too small to perform meaningful analysis on the effects of folic acid and placebo according to the *MTHFR* genotype. The results presented in this chapter are based on a very small cohort and not statistically powered to allow for robust conclusions.

Future studies would need to involve larger cohorts of healthy volunteers and a carefully selected folic acid dosage. Folate and placebo supplementation would need to be tightly monitored for subject compliance. In addition, extensive and detailed data on subject lifestyle variables – especially diet, smoking and alcohol intake – must be collected in order to fully investigate confounders and ensure meaningful results. In order to recruit truly healthy volunteers, recruitment should involve pathology-free subjects with no history of bowel issues and no history of diagnostic colonoscopy. Obviously such a study design would be challenging because persuading subjects to volunteer for procedures with a risk of perforation would not be straightforward and would most likely require some form of financial incentive.

7 Discussion and Future Work

7.1 Results Summary

The results of this thesis were in contrast to the original hypothesis that the *MTHFR* C677T genotype and systemic folate biomarkers and colonic tissue folate influence genomic DNA methylation and uracil misincorporation in DNA in healthy, folate-replete subjects.

The initial objectives in this study were: a) to determine the influence of the *MTHFR* C667T polymorphism on genomic DNA methylation and uracil misincorporation in DNA (precursors of colorectal neoplasia) in the colonic mucosa; b) to explore these associations in respect of demographic, lifestyle and folate intake (dietary and supplemental) characteristics of this population; c) to establish that systemic folate status could reliably predict colonic tissue folate status in healthy folate-replete subjects; and d) to assess the effect of folate supplementation on these relationships.

The main findings in this thesis are summarised below:

1. DNA methylation in the colon was inversely associated, albeit non-significantly, with red cell folate (i.e. DNA hypomethylation was associated with increased red cell folate).
2. Uracil misincorporation into DNA in the colonic mucosa was positively associated with serum folate (significantly) and red cell folate (non-significantly). It was also higher in the colonic mucosa of nutritional supplement users (n=60) when compared with that of non-users.
3. DNA methylation, uracil misincorporation, colonic tissue folate and systemic markers of folate status and serum vitamin B₁₂ did not differ by the *MTHFR* C677T genotype.
4. Colonic tissue folate significantly correlated with serum, red cell folate and serum B₁₂ and negatively correlated with plasma homocysteine, it was higher in non-smokers than in smokers, and higher in the folic acid supplement user group than in the non-user group.
5. Genomic DNA methylation in the colon did not change as a result of daily folic acid supplementation for three months when compared with placebo (n=15). Serum folate was the only variable that was significantly increased after 3 months of supplementation.

At the time this clinical trial was underway, it was the largest (n=336) investigation of its kind to examine these relationships in a healthy, folate-replete population. The

distribution of *MTHFR* C667T alleles (55% CC, 35% CT and 10% TT) was in Hardy-Weinberg equilibrium ($\chi^2=3.80$) and also in agreement with the reported ethnic distribution of this genotype. Importantly, folate biomarkers were measured systemically and also in the colon, and DNA methylation and uracil misincorporation were measured in colonic tissue, the site of interest, enabling a comprehensive profile of subjects with regards to these markers.

Unexpectedly, red cell folate was negatively correlated with genomic DNA methylation, and uracil misincorporation was positively correlated with serum and red cell folate and was higher in supplement-users. These results are in contrast to the general consensus that folate deficiency is associated with DNA hypomethylation and increased uracil misincorporation into DNA (Choi and Mason, 2000, Linhart et al., 2009). This data suggests that high folate status might increase CRC risk in healthy, folate replete subjects. To the best of my knowledge this is in line with only one other human study which found that promoter methylation and uracil misincorporation increased as a result of folate supplementation in subjects with a history of adenoma (van den Donk et al., 2007a).

Common dietary habits in older populations (high bread and breakfast cereal consumption) (Smith et al., 2008), the number of subjects who were taking multivitamins and folic acid supplements and the fact that Bovril and Marmite (60-120 μ g of folate per serving) were the only drinks which could be consumed in the two days prior to colonoscopy may have all contributed to high levels of circulating folate in these subjects. Ideally, it would have been good to have Marmite and Bovril as an exclusion criteria but this would have been an extra pressure to place on subjects who were unable to consume anything other than these drinks and clear liquids in the run up to the procedure. Folic acid is known to be 100% bioavailable (compared with 50% bioavailability of dietary folates) and it is not clear whether higher concentrations of serum folate may have had a transient effect on DNA methylation and uracil misincorporation. Average serum folate levels were at the upper limits of normal. 40 subjects had levels which were above the upper limit of normal in the assay and only 2 out of 336 subjects had values indicative of deficiency (<6.3nmol/l). Nevertheless, no subject was defined as red cell folate deficient (<337nmol/l). Folate supplementation in animal models has been shown to be protective in healthy animals; but, importantly, at very high doses or in the early stages of neoplasia it has been shown to promote carcinogenesis (Song et al., 2000a, Bashir et al., 2004)

The *MTHFR* C677T genotype had no significant influence on genomic DNA methylation in this population and this was consistent with multiple previous studies (Narayanan et al., 2004). Genomic DNA methylation has been shown to be strongly influenced by the *MTHFR* C667T genotype in disease-free folate-deficient subjects (Friso et al., 2002b) suggesting that the polymorphism may influence DNA methylation only when folate levels are low. Of note, another primary endpoint of this work which was led by another student in the laboratory demonstrated that gene-specific DNA methylation was also unaffected by the *MTHFR* C667T genotype in the same study population (Hanks et al., 2013).

Analyses of the demographic and lifestyle characteristics of this cohort also revealed that these had little influence on genomic DNA methylation in the colonic mucosa. These relationships were confirmed by multiple linear regression after adjusting for *MTHFR* C677T genotype, age, gender, BMI, smoking, drinking, use of folic acid and systemic folate biomarkers (serum folate, red cell folate, plasma homocysteine and serum vitamin B₁₂). In addition, the under-powered (n=15) short-term folic acid intervention with physiological doses (400 µg/day) increased serum folate but had no significant effect on genomic DNA methylation or on colonic tissue folate in the colonic mucosa in healthy, folate replete subjects.

Despite the close relationship between folate status, the *MTHFR* C667T genotype and CRC risk, information specific to the colonic epithelia of neoplasia-free individuals with adequate folate status is scarce. This is partly due to the lack of effective, non-invasive methods to evaluate folate levels in the colonic mucosa of subjects without any history of bowel complaints and who are not undergoing surgery as part of their care. At the time of this study, much of the data on these relationships had been acquired from patients with colorectal neoplasms which are subject to the confounding effects of cancer (van den Donk et al., 2007b). Conversely studies in subjects free from colorectal adenoma or cancer have generally focused on systemic folate biomarkers. The key strength of this study include the fact that subjects were free from any obvious colorectal pathology, and this reduced the potential confounding effects that neoplasia may have exerted on tissue folate, DNA methylation and uracil misincorporation in the colon. It should be highlighted that although these subjects were classified as 'normal' healthy volunteers, they were undergoing colonoscopy because of specific bowel symptoms including bloating, bleeding, and pain. Thus it is possible that they might have had microscopic lesions or

gastrointestinal inflammation that were difficult to detect but could have contributed to changes in DNA methylation and uracil misincorporation (both biomarkers of damage in the colon).

Other possible limitations include the assay which was employed in the hospital for routine measurement of folate. The traditional method of measuring red cell folate is the microbiological assay. Immune competitive binding assay used at King's College Hospital (for the current study) is generally thought to be appropriate for assaying plasma and serum folate but falsely high results have been produced for red cell folate analysis due to a build of metabolites. The MBA measures total folate by growth of the organism whereas in the binding assay, folate in the human sample competes with a labelled folate standard and an unlabelled folate standard and binding is dependent on binding affinities with the protein. Different folates have different binding abilities. Folic acid tends to bind more than 5-methyl-THF and formyl THF. Additionally, monoglutamate standards and folylpolyglutamate standards bind with different affinities and polyglutamates known to show falsely high folate levels if conversion of red cell folate from the polyglutamate to the monoglutamate derivative is incomplete. These different binding affinities of the folates are not problematic if 5-methyl-THF is the main one-carbon present. However, when other one-carbon forms are present, for example in red blood cells of methylenetetrahydrofolate reductase 677C/T subjects, results have been shown to be less reliable (Shanes, 2011). In general, serum and red cell folate levels measured by these kits are considerably lower than the concentrations measured using the MBA. With the advent of folate fortification programmes, serum samples have contained higher concentrations than the upper limit that these assays are able to measure, and dilution is required in compensation for this and, on dilution, matrix effects can produce a mixture of results.

It is important to note that in the current population, median serum and red cell folate are both unusually high for the whole sample, and there are no subjects with folate deficiencies representative of the UK population.

Median serum folate was 25.5nmol/L (IQR 17.1-38.0), whereas in the national median level (reported in the NDNS Rolling programme between 2008-12) mean serum total folate concentration was 18.1nmol/L and 21.4nmol/L for 19-64-year-old men and women respectively and mean serum total folate concentration was 22.7nmol/L and, 27.6 nmol/L

for over 65 year-old men and women respectively. The proportion of adults in the whole population with a serum total folate concentration below the WHO threshold (10nmol/L), an indication of biochemical folate deficiency, was between 8.5-15.5%. Red cell folate was 965 nmol/L (IQR 765-1139) compared with a national mean red blood cell folate concentration of 621nmol/L and 652nmol/L for 19-64-year-old men and women respectively and mean red blood cell folate concentration was 729nmol/L and 787nmol/L for over 65 year-old men and women respectively. The proportion of adults in the whole population with a mean red blood cell folate concentration below the WHO threshold (340nmol/L), an indication of biochemical folate deficiency, was between 6.8-10.8%.

The Rolling NDNS programme used LC-MS/MS to measure serum folate and the MBA to measure red cell folate concentration and therefore it is not known whether the overall results in this population are higher due to a problem with the competitive binding assays for both serum and red cell folate (as described above) or, as mentioned previously, whether another external dietary factor may have caused this.

As described previously, the lack of association between the MTHFR TT genotype and DNA methylation is in agreement with other studies in healthy subjects where DNA methylation was measured in lymphocyte DNA and similarly to our population, these subjects had adequate or high folate status whereas in studies with subjects possessing poor folate status, the TT genotype influences genomic DNA methylation in lymphocytes and TT individuals have lower levels of genomic DNA methylation

An association between the *MTHFR* C677CT genotype and DNA methylation in the colon of neoplasia free subjects may only be observed when folate status is low. In the Friso study a difference in genomic DNA methylation (measured in lymphocytes) between CC and TT subjects was apparent only when serum folate was below the low. In the current study median serum folate was 25 nmol/L, approximately twice that in the Friso study (Friso et al, 2002a).

The lack of association between the MTHFR TT genotype and DNA methylation in this study could also have been due to the fact that differences in serum and red blood cell folate between genotypes were not seen.

In addition to the primary endpoints, the multiple testing between subgroups such as smokers/non-smokers, drinkers/non drinkers, those who did/did not take daily folic acid supplementation (in Chapters 3, 4 and) should be considered with caution because these were not prespecified endpoints and the P value was not adjusted accordingly to compensate for this, the disadvantage of this is sort of multiple statistical testing is that Type I errors can occur and false positive results can arise.

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7.2 Future work

Future experiments would need to be designed around the unanswered questions generated in this thesis. Large studies need to be designed to test the effect of folate status and supplementation in truly disease-free volunteers with no history of bowel symptoms (recruited directly for the study) on DNA methylation and uracil misincorporation. Given the sufficient folate status of subjects living in developed

societies, it would be challenging to test a folate-deficient cohort over time (asking subjects to go through depletion would be controversial) but subjects could be stratified carefully in terms of different dietary folate levels. The challenge of low compliance of FFQ completion would also need to be overcome.

If the only option was to do the same experiment again, and to recruit subjects at clinically indicated colonoscopy, data collection would need to be far more extensive, for example, on past medical history, family history of IBD and CRC, smoking and drinking habits and current dietary habits to assess confounders. Additionally, follow-up would need to occur at subsequent points in the future (i.e. at 6, 12 and 18 months) to determine whether the result of undetected pathology was reliable at the time or whether something underlying developed into inflammation (IBD, Crohn's and Coeliac disease) or even neoplasia in the bowel. If the cohort was large enough, subjects could be split by phenotype for comparison of influence on DNA methylation and uracil misincorporation.

To improve on the RCT design, numbers would need to be greatly increased for statistical power but this would be difficult without remuneration because of the risks associated with colonoscopies and sigmoidoscopies. In addition to the FFQ, details of folic acid supplementation and diet and lifestyle habits would need to be recorded during supplementation so that adjustment could be adjusted for confounders in the post-supplementation analysis.

Though less surprising than the results in **Chapter 3** and **Chapter 4**, this study confirmed that smoking caused a reduction in folate levels in the colonic mucosa. This is the first report to examine this relationship in the colorectal tissue in disease-free volunteers. It would be useful to develop this and investigate the mechanism for depletion (whether folate is depleted by smoking particulates or folate is low as a result of diet and lifestyle

habits) and also to assess for relationships with other biomarkers of neoplasia in the colon. A future experiment might include more details on smoking, drinking and dietary habits (past and present) including consideration of important cofactors including serum vitamin B₁₂ using a prospective design and careful monitoring of diet over time to examine folate change in relation to these biomarkers of damage in the colon.

In addition to folate and B vitamins, other factors such as medications, alcohol, choline and methionine may play an important role in folate metabolism (Giovannucci, 2001). Although no effect was seen in this study, alcohol is a folate antagonist that reduces folate absorption and may influence folate metabolism. Folate and methionine deficient diets combined with high alcohol consumption are termed 'methyl poor' and have generally been associated with CRC risk (Hubner and Houlston, 2009).

Collectively, the unexpected evidence of an association between genomic DNA methylation (non-significant) and uracil misincorporation, and systemic folate levels and folate supplementation suggest that current folate levels in the population may cause temporary non-favourable epigenetic changes. Important questions remain as to whether the effect seen in this cohort is robust, long-term and if it might be associated with future neoplastic changes. It is not known what the optimum level of folate (dietary and supplemental) is to avoid aberrations in markers such as genomic DNA methylation and uracil misincorporation and early pathological changes in CRC.

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9 **Appendices**
 Appendix 1
 INFORMED CONSENT FORM (ICF)

Patient Identification Number:

Title of Project:

The relationship between diet and genetic makeup in determining folic acid levels in the body and chemical changes in genes

Effects of folic acid supplements on bowel tissue folic acid levels and chemical changes in genes

Name of Researcher:

Please initial box

I confirm that I have read and understood the information sheet dated.....

☐

(version) for the above study. I have had the opportunity to consider the information,
ask questions and have had these answered satisfactorily.

☐

I understand that my participation is voluntary and that I am free to withdraw at any time,
without giving any reason, without my medical care or legal rights being affected.

☐

I understand that relevant sections of any of my medical notes and data are collected during the study,
may be looked at during the study, may be looked at by responsible individuals from the department of
Nutrition & Dietetics, King's College London, from regulatory authorities or from the NHS Trust, where it is
relevant to my taking part in this research. I give permission for these individuals to access my records.

☐

I agree to my GP being informed of my participation in the study.

☐

I agree to take part in the above study.

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Name of Patient	Date	Signature

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Name of person taking consent (if different from researcher)	Date	Signature

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Researcher	Date	Signature

When completed, 1 for patient; 1 for researcher site file, 1 (original to be kept in medical notes).

Appendix 2

GP Letter

Dear Dr. X

Re: The effect of folic acid supplementation on blood and tissue folate levels and markers of DNA damage in the colon

I am writing to ask whether you have any objections to Mr *name*, D.O.B ***/**/*** of *address* participating in the above study, which will look at the relationship between folic acid and colorectal cancer.

Epidemiological evidence has shown an inverse association between folate status and colorectal cancer risk. Folate supplements have been shown to reduce the incidence of neoplasia in patients with ulcerative colitis and polyp recurrence in patients with adenomas. Furthermore, a common mutation in a gene encoding a folate metabolising enzyme (5,10-methylenetetrahydrofolate reductase, MTHFR C677T) also influences disease risk. Folate plays a key role in DNA methylation and DNA synthesis, which are required to maintain the integrity of the genome. Aberrant DNA methylation and DNA damage are common phenomena of many cancers and have been observed in the early stages of colorectal carcinogenesis in humans.

Folate supplementation in pharmacological and physiological doses has been shown to reverse aberrant DNA methylation in normal-appearing colonic mucosa of patients with adenoma and carcinoma. However, it is not known whether folate supplementation has a similar effect in people without colorectal neoplasia. Also, it is not known whether increasing folate status has a positive effect on markers of DNA damage associated with abnormal DNA synthesis. We propose to give patients with no colorectal abnormalities a 400µg/d folate supplement or a placebo for a period of 3 months. We will look at the effect this has on folate status (serum, red cell and bowel tissue folate levels) and markers of DNA damage (global and gene-specific DNA methylation and uracil misincorporation) in the colon.

Please find enclosed a copy of the patient information sheet, detailing the procedure.

The exclusion criteria for this study include:

- Patients with tumours/polyps at colonoscopy
- Patients with previous history of colorectal cancer, a strong family history of colorectal cancer or adenomatous polyposis coli
- Patients with inflammatory bowel disease
- Current or past history of gluten-sensitive enteropathy
- Pregnancy, epilepsy, alcoholism, pernicious anaemia and the use of anti-folate (*ie* methotrexate) medication

I would be grateful if you could spare a few minutes to complete and return the section below in the reply paid envelope, or fax it to on 0207 848 4185. Thank you for your help. Please do not hesitate to contact me if you require further information.

Yours sincerely

Re: The effect of folic acid supplementation on blood and tissue folate levels and markers of DNA damage in the colon

Please tick the appropriate box

- ☐ I am in agreement that Mr/Mrs *name*, D.O.B ***/**/***, of *address*, taking part in the above study.
- ☐ I am not in agreement that Mr/Mrs *name*, D.O.B ***/**/***, of *address*, taking part in the above study.

Signature -----

Name -----

Date -----

Appendix 3
Health Questionnaire

Health Screening Questionnaire
King's College London

FOLGEN Study

Folate, genetic polymorphisms and markers of DNA damage in the colon.

REC number no: 06/Q0703/29

Thank you for agreeing to help us with our study. Please answer all the questions as fully as you can. All information will be held in the strictest confidence.

Subject number:

Date:

Name and Occupation:	DOB/Age:
Sex:	Telephone No.
Address:	Email:

Height:	Weight:
Ethnicity:	Have you had anything to eat or drink in the last 48 hours?

Did you eat Bovril/Marmite in last 48 hours If so, approximately how many teaspoons?	Have you eaten Bovril/Marmite in the last week? (how many days/how many teaspoons per day?)
---	--

Do you smoke now? ☐ Don't smoke regularly ☐ Occasionally ☐ Yes,	If not, have you ever smoked? ☐ Yes ☐ No
How old were you when you started smoking?	How old were you when you stopped smoking?
How much do you/did you smoke per day?	

Do you suffer from any allergies?	Do you suffer from any medical problems?
Are you taking/have you taken any of the following medication?	If so, how much, how often and for how many months in the last year?
Co-trimoxazole (Sulfamethoxazole & trimethoprim)	
Carbamazepine	
Cycloserine	
Erythromycin	
Fansidar® (sulfadoxine & pyrimethamine)	
5-Fluorouracil (chemotherapy)	
Iron salts	
Lamotrigine	
Lometrexol	
Oral contraceptives	
Methotrexate (rheumatoid arthritis)	
Malarone® (atovaquone & proguanil)	
Nitric oxide	
Oxcarbazepine	
Parenteral nutrition	
Pemetrexed	
Pentamidine	
Phenobarbital	
Phenytoin	

Primidone	
Pyrimethamine	
Raltitrexed	
Rifampicin	
Sulfadoxine	
Sulfadiazine	
Sulfasalazine	
Triamterene	
Trimethoprim	
Trimetrexate	
Thymitaq	

Have you taken any of the following in the last six months?	If so, how much, how often and for how many months in the last year?
Vitamin C	
Vitamin E	
Folic Acid	
Multivitamins	
Multivitamins and minerals	
Calcium	
Cod liver oil	
Evening primrose oil	
Garlic pearls	
Others (please specify)	

Do you have a family history of:	If so, please give details:
Colorectal cancer	
Irritable bowel syndrome (IBD)	
Coeliac disease	
Epilepsy	
Do you exercise regularly at all?	If so, what do you do, how much and how often?

ALCOHOL INTAKE	FREQUENCY OF CONSUMPTION								AMOUNT PER DAY ON DAYS CONSUMED											
	Never eaten	Once per month or less	Once per fortnight	Number of days per week																
				1	2	3	4	5	6	7										
SHANDY AND CIDER											Type • Pints $\frac{1}{4}$ $\frac{1}{2}$ 1 2 • ☐ ☐ ☐ ☐ • ☐ ☐ ☐ ☐ • ☐ ☐ ☐ ☐									
BEERS AND LAGERS											Type • Pints $\frac{1}{4}$ $\frac{1}{2}$ 1 2 • ☐ ☐ ☐ ☐ • ☐ ☐ ☐ ☐ • ☐ ☐ ☐ ☐ Bottles 1 2 3 4 • ☐ ☐ ☐ ☐ • ☐ ☐ ☐ ☐ Cans 1 2 3 4 • ☐ ☐ ☐ ☐ • ☐ ☐ ☐ ☐									
WINE (white, red or rose)											Glasses 1 2 3 4 • ☐ ☐ ☐ ☐ OR How many days does it take you to finish a bottle?									
MARTINI, VERMOUTH, SHERRY, PORT, etc											Glasses 1 2 3 4 • ☐ ☐ ☐ ☐									
SPIRITS (vodka, whisky, gin, etc)											Pub measures 1 2 3 4 • ☐ ☐ ☐ ☐ • ☐ ☐ ☐ ☐									

Appendix 4

Food Frequency Questionnaire

Food Frequency Questionnaire
King's College London

FOLGEN Study

Folate, genetic polymorphisms and markers of DNA damage in the colon.

REC number no: 06/Q0703/29

Thank you for agreeing to help us with our study. Please answer all the questions as fully as you can. All information will be held in the strictest confidence.

Subject name:

Subject number:

Date:

How to complete the FOOD QUESTIONNAIRE

A	B	C	D	E							F
FOOD EATEN	FREQUENCY OF CONSUMPTION							AMOUNT PER DAY ON DAYS EATEN			
	Never eaten	Once per month or less	Once per fortnight	Number of days per week				Describe amounts using the measures in PHOTO 1 and PHOTO 2			
				1	2	3	4	5	6	7	
BREAKFAST CEREALS (Cornflakes, Branflakes, All Bran, muesli etc)			✓			✓					Type of cereal Number of bowls [R in PHOTO 1] Include brand names <u>Cornflakes (Kellogg)</u> 1R <u>Luxury muesli (Tesco)</u> 1/2R
BREAD <i>Toast</i>									✓		Type of slices Number [Thick ☒ Medium ☐ Thin ☐ <u>White</u> <u>Mighty White</u> <u>Brown/wholemeal</u> 2 <u>Granary</u> <u>BeMax/VitBe</u> <u>Other</u>
PLAIN RICE											Type and size of Number spoonfuls [PHOTO 2] <u>White</u> 2E <u>Brown</u>
FRUIT JUICE (orange juice, tomato juice, lemonade)										✓	Type Volume [PHOTO 1] <u>Orange juice</u> 1H

Column A: Lists the foods/food groups of interest.

Columns B to E: Tick one box to show how often you eat the food.

Column F: Write down the exact names of the foods you eat and, where indicated, describe the amounts using the household measures in **PHOTO 1** and **PHOTO 2**. Alternatively, write down the weight (g or ounces) or volume (ml or fluid ounces) of food items if you know them.

Please describe your eating habits over the **PAST YEAR** by filling in the questionnaire on the following pages

FOOD EATEN	FREQUENCY OF CONSUMPTION								AMOUNT PER DAY ON DAYS EATEN		
	Never eaten	Once per month or less	Once per fortnight	Number of days per week							Describe amounts using the measures in PHOTO 1 and PHOTO 2
				1	2	3	4	5	6	7	
BREAKFAST CEREALS (Cornflakes, Branflakes, All Bran, muesli etc)											Type of cereal bowls [R in PHOTO 1] Include brand names
BREAD <i>Toast</i>											Type Number of slices [Thick ☐ Medium ☐ Thin ☐ <i>White</i> <i>Mighty White</i> <i>Brown/wholemeal</i> <i>Granary</i> <i>BeMax/VitBe</i> <i>Other</i>
<i>Sliced bread (ie in sandwiches)</i>											Type Number of slices [Thick ☐ Medium ☐ Thin ☐ <i>White</i> <i>Mighty White</i> <i>Brown/wholemeal</i> <i>Granary</i> <i>BeMax/VitBe</i> <i>Other</i>
<i>French bread, rolls</i>											Type Number of rolls/pieces <i>White</i> <i>Brown/wholemeal</i>
CAKES (fruitcake, sponge cake, gâteau, ...)											Type Number of slices

chocolate, ginger etc)												
TEA BREADS (scone, malt loaf etc)												Type Number of items/slices
												Type Number of items

FOOD EATEN	FREQUENCY OF CONSUMPTION										AMOUNT PER DAY ON DAYS EATEN	
	Never eaten	Once per month or less	Once per fortnight	Number of days per week							Describe amounts using the measures in PHOTO 1 and PHOTO 2	
				1	2	3	4	5	6	7		
PUDDINGS <i>Rice pudding/ other milk puddings</i>											Type size of [PHOTO 2]	Number and spoonfuls
<i>Cheesecake</i>											Type slices	Number of
<i>Bread and butter pudding</i>											size of [PHOTO 2]	Number and spoonfuls
<i>Fruit crumble/ pie/tart</i>											Type slices or [PHOTO 2]	Number of spoonfuls
<i>Sponge puddings</i>											Type slices	Number of
BISCUITS											Type biscuits	Number of
MILK AND MILK PRODUCTS <i>Plain milk to drink/in coffee or tea/on cereals etc</i>											Type [PHOTO 1] [whole ☐ s-skimmed ☐ skimmed ☐ UHT ☐	Volume

<i>Spaghetti/ tagliatelle etc</i> <i>Macaroni cheese/tinned spaghetti/other pasta</i> <i>Lasagne</i>												
												Type of dish Number and size of spoonfuls [PHOTO 2]
												Type of dish Number and size of spoonfuls [PHOTO 2]
												Type Amount [Small ☐ Medium ☐ Large ☐
QUICHE AND SAVOURY FLANS												Type Number of slices
MEAT/ CHICKEN <i>Roast or boiled</i> <i>Steaks</i> <i>Beef burger</i> <i>Minced beef dishes (chilli con carne, shepherd's pie etc)</i> <i>Meat stew or casserole</i>												Type Number of pieces/slices
												Number & size [Small ☐ Medium ☐ Large ☐
												Number & size [Small ☐ Medium ☐ Large ☐
												Type Number and size of spoonfuls [PHOTO 2]
												Number and size of spoonfuls [PHOTO 2]

FOOD EATEN	FREQUENCY OF CONSUMPTION				AMOUNT PER DAY ON DAYS EATEN
	Never eaten	Once per month	Once per	Number of days per week	

		or less	fort- night								Describe amounts using the measures in PHOTO 1 and PHOTO 2
				1	2	3	4	5	6	7	
LIVER AND KIDNEY											Type Number of slices
<i>Steak and kidney pie</i>											Number of slices
POTATOES <i>Boiled</i>											Number of pieces
<i>Chips</i>											Amount on plate [$\frac{1}{4}$ ☐ $\frac{1}{2}$ ☐ $\frac{3}{4}$ ☐ Whole ☐
<i>Jacket potato</i>											Number [Small ☐ Medium ☐ Large ☐
<i>Mashed potato</i>											Number and size of spoonfuls [PHOTO 2]
<i>Roast potato</i>											Number of pieces
ROOT VEGETABLES <i>Carrots</i>											Number of carrots [Small ☐ Medium ☐ Large ☐ [Raw ☐ Cooked ☐ Both ☐
<i>Beetroot</i>											Number of slices
<i>Parsnips</i>											Number of pieces
BROCCOLI, CAULIFLOWER											Number of florets

<i>Broccoli</i>															
															Number of florets
<i>Cauliflower</i>															
GREEN LEAFY VEGETABLES <i>Cabbage</i>															Number and size of spoonfuls [PHOTO 2] [Raw ☐ Cooked ☐ Both ☐
	<i>Brussels sprouts</i>														Number of sprouts
FOOD EATEN	FREQUENCY OF CONSUMPTION													AMOUNT PER DAY ON DAYS EATEN	
	Never eaten	Once per month or less	Once per fortnight	Number of days per week							Describe amounts using the measures in PHOTO 1 and PHOTO 2				
				1	2	3	4	5	6	7					
<i>Spinach, spring greens, kale, watercress, mustard and cress</i>															Number and size of spoonfuls [PHOTO 2] [Raw ☐ Cooked ☐ Both ☐
PEAS															Number and size of spoonfuls [PHOTO 2]
GREEN BEANS (runner beans, French beans, mange tout, sugar snaps etc)															Number and size of spoonfuls [PHOTO 2] [Raw ☐ Cooked ☐ Both ☐
SALAD VEGETABLES <i>Lettuce</i>															Number and size of spoonfuls [PHOTO 2]
	<i>Cucumber</i>														Number of slices
	<i>Tomato</i>														Number of tomatoes [Small ☐ Medium ☐ Large ☐

<i>Satsuma, clementine, mandarin, tangerine</i>											[Small ☐ Medium ☐ Large ☐
											Type Number
BANANAS											Number & size [Small ☐ Medium ☐ Large ☐
BERRIES (raspberries, strawberries, blackberries etc)											Type Number and size of spoonfuls [PHOTO 2]
DRIED FRUIT (dates, sultanas, fruit mix)											Type Number and size of spoonfuls [PHOTO 2]
NUTS AND SEEDS (sunflower, sesame etc)											and size of Number spoonfuls [PHOTO 2] Peanut butter Nuts/seeds
CRISPS AND SAVOURY SNACKS											Type Number of packets
LIVER PATE											Number and size of spoonfuls [PHOTO 2]
EGGS (boiled, poached, fried, scrambled etc)											Number of eggs
SOUP											Type Number of bowls [R in PHOTO 1]
FOOD EATEN	FREQUENCY OF CONSUMPTION										AMOUNT PER DAY ON DAYS EATEN

	Never eaten	Once per month or less	Once per fortnight	Number of days per week							Describe amounts using the measures in PHOTO 1 and PHOTO 2
				1	2	3	4	5	6	7	
FRUIT JUICE (orange juice, tomato juice, lemonade)											Type Volume [PHOTO 1]
SQUASH (orange, Ribena etc)											 Volume [PHOTO 1]
BOVRIL AND MARMITE											Type Number & size of spoonfuls [PHOTO 2]
OXO CUBES											Number
MILKY DRINKS (Complan, Horlicks, Ovaltine, Build-up)											Type Volume [PHOTO 1]
TEA											 Volume [PHOTO 1]

Thank you for completing the questionnaire.

Appendix 5

Table 6.3a Biomarkers –RCT - change from baseline split by MTHFR C677 CC and TT genotype – placebo and folic acid subjects

	Placebo arm					
	Baseline		Post RCT		Change from baseline	
	CC (n=2)	TT (n=4)	CC (n=2)	TT (n=4)	CC (n=2)	TT (n=4)
Serum Folate (nmol/l)	27.8	19 (16.0-25.1)	20.9	15.9 (10.5-17.9)	-6.9	-5.9 (-8.6- -1.33)
Red cell folate (nmol/l)	1007	906.5 (740.8-950)	923	867 (783-957)	-83.6	23.7 (-72.3-58.8)
Colonic tissue folate (nmol/g tissue)	0.79	0.95 (0.45-2.2)	1.18	0.37 (0.21-1.08)	0.4	0.68 (-1.19- 0.08)
Serum B ₁₂ (pmol/l)	776	533 (462-643)	570	510 (376-692)	-205	-19 (-188-146.3)
Plasma homocysteine (mmol/l)	11.4	22.6 (17.3-33.4)	20.7	16.4 (15.05- 21.2)	9.3	-6.3 (-12.2-2.18)
DNA Methylation (% methylcytosine)	n/a	4.25 (4.05-4.53)	4.4	4.3 (4.0-4.6)	0.4	0.05 (-0.30-0.25)

Folic acid arm						
	Baseline		Post RCT		Change	
	CC (n=6)	TT (n=3)	CC (n=6)	TT (n=3)	CC (n=6)	TT (n=3)
Serum Folate (nmol/l)	23.4 (17.6-29.4)	19.9	40.5 (24.0-52.5)	19.0	12.9 (-1.7-31.1)	0
Red cell folate (nmol/l)	830 (601-1089)	979	1097 (979-1254)	979	253 (166-352)	n/a
Colonic tissue folate (nmol/g tissue)	0.67 (0.52-0.87)	1.07	1.06 (1.0-2.11)	0.57	0.63 (0.20-1.39)	0.09
Serum B ₁₂ (pmol/l)	511 (381-609)	485	408 (359-465)	284	-50 (-184-1.75)	-230
Plasma homocysteine (mmol/l)	13.9 (9.61-17.3)	12	15.2 (1.14-21.1)	n/a	0.75 (-15.3- 11.5)	n/a
DNA Methylation (% methylcytosine)	3.95 (3.38-4.68)	4.3	3.9 (3.30-4.75)	4.4	-0.4 (-1-1.38)	0.2

Data are median (IQR) for biomarkers at baseline, post-intervention and change from baseline

Median only is shown where n<4 and group too small for quartile range to be shown

n/a - where no output produced in SPSS