# *In Vitro* and *In Vivo* approaches to the examination of folate-related nutritional genetics in health and disease

Thesis submitted as partial fulfilment of the requirements for the degree of
in the School of Environmental and Life Sciences, University of Newcastle

By

Jeong-Hwa Choi
BSc
MSc
PhD (Food Science)

Supervisors:
Dr Zoe R. Yates
Associate Professor Mark D. Lucock
Associate Professor Martin Veysey

August 2013
School of Environmental and Life Sciences
University of Newcastle
Australia

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# ACKNOWLEDGMENTS

**This thesis is dedicated to my late dad who encouraged me to pursue my dream. I love you.**

I would like to express my deep appreciation and gratitude to the following people for helping me complete this research/thesis.

First and foremost, I am deeply grateful to Dr Zoe Yates for her support and kindness. Her generosity and patience has truly encouraged me during my PhD studies. She has both challenged and enriched my academic career with her immense understanding of cell culture. I am truly blessed to have worked with her as my principal supervisor.

Also, I would particularly like to thank Associate Professor Mark Lucock, for tirelessly guiding me through every step of this research/ thesis, by thoroughly looking over my writing and statistical analysis. He has been a major source of knowledge, and without his advice and criticism this thesis would not have been completed. I admire his scientific and analytical mind and professional approach to research.

I would like to express my gratitude to both Dr Zoe Yates and Associate Professor Mark Lucock for devoting much time to reading my work over and over again. Indeed, without their guidance, I would not have been able to complete this thesis. I wish to thank them both for their advice, knowledge and, most of all, patience.

I would particularly like to thank Associate Professor Martin Veysey for financially supporting me through my studies. He has provided me with the opportunity to achieve my dreams. I thank him for seeing the potential in me and my academic career.

I would like to thank Dr Teresa Bates for her editorial assistance in the preparation of this thesis.

I would also like to thank my friends: Jacquie Rutland, Jannellies Van Berkel, Elies Van Berkel, Dr Sathira Hirun, Selin Tan, Vincent Candrawinata, and Jessica Huh for their moral support and encouragement. Without them I would be lost.

Finally, I would also like to acknowledge the support given to me by my fellow postgraduates: Charlotte Martin, Lyndell Boyd, Dr Sarah Tang, Dr Xiaowei Ng and Kelly McDowell. I am truly grateful for their generous contributions to this research/ thesis.

I would like to offer my special thanks to my wonderful family for their unconditional love, support and encouragement. In particular, the patience and understanding shown by my mum during the completion of this thesis is greatly appreciated. I know, at times, my temper is particularly trying. I would also like to thank my sister for having faith in me and my abilities as it is what kept me going and allowed me to overcome many obstacles.

Most importantly, I would like to thank God for being my strength and guide in completing this thesis. Without Him I would not have had the wisdom or the physical ability to do so. He has blessed me with opportunities and taken me on journeys that have truly enriched my life.

## ACKNOWLEDGMENTS OF COLLABORATION

*I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers, or carried out in other institutions. I have included as part of the thesis a statement clearly outlining the extent of collaboration, with whom and under what auspices.*

**CHAPTER 2: B-VITAMIN NUTRITIONAL GENETICS IN THE OCCURRENCE OF ADENOMATOUS POLYP AS AN ANTECEDENT OF COLORECTAL CANCER; CLINICAL *IN VIVO* APPROACH**

Funding source: Associate professor Martin Veysey trust fund, Northern Sydney Central Coast Health

Chief investigator: Associate Professor Martin Veysey

Co-Investigators: Associate Professor Mark Lucock, Dr Paul Roach

Associate investigators and students: Dr Zoe Yates, Dr Xiaowei Ng, Dr Virginia Skinner (Interviewing subjects and collecting blood samples), Charlotte Martin (food frequency questionnaire analysis), Ron Wai (genetic variant analysis), Dr Sarah Tang (genetic variant analysis), Jeremy Koh (genetic variant analysis), Lyndell Boyd (thiol and genetic analysis)

**CHAPTER 3: POTENTIAL EFFECT OF FOLYL VITAMERS, ANTIOXIDANTS AND ITS PHOTO-OXIDATIVE DERIVATIVES ON COLON CANCER CELL BIOLOGY; *IN VITRO* APPROACH**

Funding source: Ramaciotti Foundation

Chief investigator: Dr Zoe Yates

Funding source: Northern Sydney Central Coast Health, University of Newcastle

Co-Investigators: Associate Professor Mark Lucock, Associate Professor Martin Veysey, Dr Zoe Yates

## ACKNOWLEDGMENTS OF AUTHORSHIP

*I hereby certify that the work embodied in this thesis contains a published paper/s/scholarly work of which I am a joint author. I have included as part of the thesis a written statement, endorsed by my supervisor, attesting to my contribution to the joint publication/s/scholarly work.*

**Original research article**

1) Lucock M., Ng X., Boyd L., Skinner V., Wai R., Tang S., Naylor C., Yates Z., **Choi JH**., Roach P., Veysey M. TAS2R38 bitter taste genetics, dietary vitamin C, and both natural and synthetic dietary folic acid predict folate status, a key micronutrient in the pathoaetiology of adenomatous polyps. *Food Funct. 2012, 2(8):457-65.*

2) Lucock M., Yates Z., Boyd L., Naylor C., **Choi JH**., Ng X., Skinner V., Wai R., Kho J., Tang S., Roach P., Veysey M. Vitamin C related nutrient-nutrient and nutrient-gene interactions that modify folate status. *Eur J Nutr. 2013, Mar;52(2):569-82*

3) Lucock M., Yates Z., Naylor C., **Choi JH**., Boyd L., Tang S., Ng X., Dufficy L., Naumovski N., Blades B., Traves C., Lewis P., Sturm J., Roach P., Veysey M. (2012) Hydrogen sulphide related thiol metabolism and genetics in relation to hypertension in an elderly population. *Genes Nutr. 2013, 8(2):221-9.*

I endorse the above statement by Jeong-Hwa Choi:

Dr Zoe R Yates

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# ABSTRACT

Folic acid has been the focus of extensive research since it plays an essential role in the human body as a major coenzyme in one-carbon metabolism. This metabolic role facilitates both methionine and DNA synthesis. It has been suggested that increased folate intake, and hence blood status, provides benefits in maintaining health.

However, a growing body of literature now reports differing opinions with respect to the level of folic acid intake, the type of vitamer and pathoaetiological outcomes. Part of this controversy lies in the study of cancer aetiology, particularly in the context of adenomatous polyp (AP) occurrence. AP is an antecedent of colorectal cancer. As part of the present study an *in vivo* model was therefore developed in order to re-examine the role of folate in carcinogenesis, taking into account both genetic variants and environmental factors. To obtain further evidence, the biological characteristics of folate vitamers in promoting cancer cell proliferation were examined using an *in vitro* model.

202 individuals were recruited and placed into two groups depending upon whether they had been diagnosed with AP or were control subjects. The blood folate, thiol levels, dietary folate intake, including synthetic and natural forms, and intake of nutrients related to one-carbon metabolism (pyridoxine, riboflavin, cobalamin, niacin, methionine and vitamin C) of each subject were evaluated. Sixteen major genetic variants in folate metabolism were also determined as potential risk factors in AP aetiology: MTHFR C677T, A1208C, G1793A, SHMT C1420T, TS 1496del6, TSER 2R3R, TS 3RG>C, GCPII C1561T, CBS 844*ins*66, DHFR 19bp del , RCF G80A, CTH G1364T, IVS 10-430 C>T, BHMT G595A, MS A2756G and MSR A66G.

Statistical analysis has provided evidence to suggest that AP aetiology depends upon a range of interactions between genes and nutrition. The following factors were found to be associated with an increased risk of developing AP: RBC folate level (*p* for females[1] = 0.0021 and males[2] = 0.004), nutritional intake (*p* for methylfolate intake below the median[2] = 0.0189 and cobalamin intake above median[2] = 0.0402) and genetic variants (*p* for CBS 844*ins*68[1] = 0.035, TS 1496del6[1] = 0.048, MSR A66G[1] = 0.025 and MTHFR C677T- CBS 844*ins*66[1] = 0.0403). Although no direct correlation was found between PteGlu intake and the occurrence of AP, genetic variants predicted a differential AP risk depending on total dietary folate intake level (in subjects with total dietary folate intake above the RDI: *p* for MTHFR A1298C[1]= 0.026, MTHFR C677T-A1298C-G1793A[3]=0.016, TS 1496del6[2] = 0.024, DHFR 19bp del[2] = 0.032, and, in subjects


[1] The variable predicts increased risk for AP, [2] signifies decreased risk, [3] limited detail due to lability of statistical software

with total dietary folate intake below the RDI: MTHFR A1298C[2]= 0.011, RFC G80A-GCPII C1561T[3]= 0.033). Additionally, vitamin C was also associated with occurrence of AP when examined by RCF G80A and GCPII C1561T genotype (*p* for RFC G80A AA[1] = 0.0473, GCP II C1561T CT[2]= 0.0276 and RFC G80A-GCPII C1561T AA/CC[1] = 0.0026).

An *in vitro* model utilizing both colon (Caco-2) and breast cancer (MCF 7) cell lines was developed. The cells were cultured for 8 days. There was found to be a differential growth response depending upon which folyl vitamer was used as a source of one-carbon units, its concentration and the presence/absence of a folate protecting antioxidant.

At a relatively low concentration (≤ 1µg/mL) of PteGlu no significant difference in Caco-2 cell growth was observed, but the cells showed a differential growth pattern at higher concentrations (50µg/mL and 500µg/mL). Fully reduced, labile 5-$CH_3H_4$PteGlu with no added antioxidant inhibited Caco-2 cell growth in a dose-dependent manner, but this inhibition was altered by the presence of an antioxidant. The addition of 10µg/mL of GSH resulted in a similar, but enhanced, inhibition of Caco-2 cell growth to that of 5-$CH_3H_4$PteGlu in the absence of any additional antioxidant. By contrast, 5-$CH_3H_4$PteGlu in the presence of ascorbic acid (10µg/mL) exhibited a differential change depending on time of incubation and vitamin concentration. Partially oxidised 5-$CH_3H_2$PteGlu increased cell growth in both cell lines, providing evidence that 5-$CH_3H_2$PteGlu could potentially enter the one-carbon metabolic pool in this *in vitro* model. The photo-oxidative degradation products of 5-$CH_3H_2$PteGlu did not show a significant effect on growth in Caco-2 cells.

Findings from the current study provide new information on the physico-chemical characteristics of folate vitamers and their role in cell proliferation. The interactions between these vitamers and nutrients related to methyl group metabolism and genetic variants collectively illuminate the multifactorial nature of AP aetiology. In terms of gene-gene, gene-nutrient and nutrient-nutrient interactions, this study has generated valuable new information about the molecular mechanisms that play a role in AP and their response to the nutritional environment.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 5,10-$CH_2H_4$PteGlu | 5,10-methylentetrahydrofolate |
| 10-$CHOH_4$PteGlu | 10-formyltetrahydrofolate |
| AICAR | 5-amino-4-imidazole carboxamide ribonucleotide |
| 5-FU | 5-fluorouracil |
| 5-$CH_3H_2$PteGlu | 5-methyldihydrofolate |
| 5-$CH_3H_4$PteGlu | 5-methyltetrahydrofolate |
| 6-FP | 6-fomylpterine |
| SBD-F | 7-fluorobenzo-2-oxa-1,3-diazole?4-sulphonate |
| AP | Adenomatous polyp |
| AMDA | Asymmetric dimethylarginine |
| BHMT | Betaine-homocysteine methyltransferase |
| BSA | Bovine serum albumin |
| CVD | Cardiovascular disease |
| CoI | Cob(I)alamin |
| CoII | Cob(II)alamin |
| CRC | Colorectal cancer |
| CI | Confidence interval |
| CAD | Coronary artery disease |
| CIMP | CpG island methylator phenotype |
| CTH | Cystathionine gamma-lyase |
| CBS | Cystathionine β-synthase |
| CTH | Cystathionine γ-lyase |
| Cys | Cysteine |
| Cys-Gly | Cysteinyl-glycine |
| CpG | Cytosine-guanine dinucleotide |
| DMSO | Demethyl sulfoxide |
| dNTP | deoxyribonucleotide triphosphate |
| dTMP | deoxythymidine monophosphate |
| dTTP | deoxythymidine triphosphate |
| dUMP | deoxyuridine monophosphate |
| dUTP | deoxyuridine triphosphate |
| DHFR | Dihydrofolate reductase |

| | |
|---|---|
| DMG | Dimethylglycine |
| DTT | Dithiothreitol |
| ECACC | European Collection of Cell Cultures |
| EtBr | Ethidium bromide |
| EDTA | Ethylenediaminetetraacetic acid |
| EC-SOD | Extracellular superoxide dismutase |
| FAD | Flavin adenine dinucleotide |
| FBS | Foetal bovine serum |
| FOLR | Folate receptor |
| FPGS | Folylpolyglutamate synthase |
| FFM | Folate free medium |
| FFQ | Food frequency questionnaires |
| FSANZ | Food Standards Australia New Zealand |
| FIGLU | Formiminoglutamic acid |
| GCPII | Glutamate carboxypeptidase II |
| GSH | Glutathione |
| GAR | Glycinamide ribonucleotide, |
| GNMT | Glycine N-methyl transferase |
| HPLC | High performance liquid chromatography |
| Hcy | Homocysteine |
| HCl | Hydrochloric acid |
| $H_2S$ | Hydrogen sulphide |
| IQR | Interquartile range |
| IVF | *In-vitro* fertilization |
| LD | Linkage disequilibrium |
| LOD | Logarithm of odds |
| MTHFS | Methenyltetrahydrofolate synthetase |
| MS | Methionine synthase |
| MSR | Methionine synthase reductase |
| MTX | Methotrexate |
| $CH_3$-CoIII | Methyl-Cob(III)alamin |
| MTHFR | Methyltetrahydrofolate |
| MSI | Microsatellite instability |
| MEME | Minimum essential medium eagle |

| | |
|---|---|
| Maf | Minor allele frequencies |
| NHANES | National Health Study and the Nutrition Examination Survey |
| NK cells | Natural killer cells |
| NTDs | Neural tube defects |
| NADP | Nicotinamide adenine dinucleotide phosphate |
| NS | Not significant |
| OR | Odds ratio |
| p-ABG | p-aminobezoylglutamate |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PCA | Pteridine-6-carboxylic acid |
| PteGlu | Pteroylmonoglutamate |
| PWV | Pulse-wave velocity |
| PLP | Pyridoxal 5`- phosphate |
| RT-PCR | Real-time polymerase chain reaction |
| RDI | Recommended Daily Intake |
| RBC | Red blood cell |
| RFC | Reduced folate carrier |
| RFLP | Restriction-enzyme fragment length polymorphism |
| RPMI 1640 | Roswell Park Memorial Institute 1640 |
| SAH | S-adenosylhomocysteine |
| SAM | S-adenosylmethionine |
| SHMT | Serine hydroxymethyltransferase |
| SNP | Single nucleotide polymorphism |
| SE | Slope estimate |
| NaOH | Sodium hydroxide |
| SD | Standard deviation |
| $H_4$PteGlu | Tetrahydrofolate |
| MTT | Thiazolyl blue tetrazolium bromide |
| TS | Thymidylate synthase |
| TCEP | Tris (2-carboyethyl) phosphine |
| TAE | Tris-acetate-EDTA |
| TBE | Tris-borate-EDTA |
| UL | Upper intake level |

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# I. LITERATURE REVIEW

Folic acid, a water soluble B vitamin, is the generic term for both natural folyl polyglutamate derivatives in food, and synthetic folic acid (pterolymonoglutamic acid). Folate plays an essential role in the human body as a major coenzyme in one-carbon metabolism, including purine, pyrimidine and methionine biosynthesis, the inter-conversion of serine and glycine, and histidine catabolism. A growing body of literature informs us that these critical roles in cellular homeostasis influence the risk factors for disease. Therefore, having an appropriate level of folate intake and an adequate blood level is now thought to be an important nutritional consideration.

Although the health benefits of folate have been studied intensively over the last couple of decades, researchers still report differing opinions with respect to recommended intake levels, types of vitamers and interactions with other pathoaetiological factors. In light of such controversy, the present study was developed in order to establish a better understanding of the role of folate in carcinogenesis, taking into account both genetic variations and the characteristics of folate vitamers and using both *in vivo* and *in vitro* models.

### 1.1 History of folate

Although it was not yet given this name, folate was first discovered by Lucy Wills in 1931 who discovered that extracts from yeast and liver were effective against tropical macrocytic anaemia in pregnant women [1]. In 1941, following further studies, Mitchell and his colleagues isolated folate from spinach and characterized it as N-(4-(((2-amino-4-hydroxy-6-pteridinyl) methyl) amino) benzoyl) glutamic acid, an active growth factor [2]. It was named after the Latin word "*folium*" meaning leaf. Folate is sometimes referred to as vitamin $B_9$, vitamin $B_C$ or folacin. The term folic acid (PGA or PteGlu) is also used to describe the synthetic form of the vitamin.

Folate is abundant in green leafy vegetables as its name indicates, while some fruits such as kiwi fruit, oranges and lemons are also rich in folate. Kidney and liver are good animal sources of the vitamin, and more moderate amounts are present in potatoes and dairy products. Some bacteria which colonise the large intestine synthesize a small amount of folate.

Folate has been implicated in many health issues including complications of pregnancy and birth defects [3]. It is well known that low blood folate nutritional status is involved in neural tube defects (NTDs). An insufficient folate intake is also a risk factor for megaloblastic anaemia, the deficiency syndrome for the vitamin. Folate is considered to be an independent risk factor

for hyperhomocysteinemia and cardiovascular disease (CVD). It has been confirmed that folate supplements and mandatory folic acid fortification improve blood folate status and reduce both risk factors for disease and deficiency symptoms.

As a result of the human genome project, a large amount of information that is functionally relevant to gene polymorphisms has been discovered. One such polymorphism is the C677T methyltetrahydrofolate (MTHFR, EC 1.5.1.20) variant which was first reported in 1995 [4]. Since that time research examining genetic variation in folate metabolism genes has led to a better understanding of the relationship between folate nutritional status and disease risk. A major role for folate in the development and progression of cancer has been suggested, and many researchers are working to verify and establish more detailed mechanisms in disease processes. Some of the latest issues that have arisen with respect to folate such as mandatory folate fortification of foods and its potential adverse effects, and photolysis of folate also need further investigation.

### 1.2 Chemical structures of folate and its vitamers

Folate consists of three distinctive structures: a pteridine ring which has a 2-ring structure that mammals cannot synthesize [5], para-aminobenzoic acid and glutamic acid (see Figure 1.1). Folate is the generic term used to describe a large number of chemically similar compounds which differ by

i) the oxidation status of the pteridine ring,

ii) the number of glutamate residues joined together via a series of gamma glutamyl bonds and

iii) the one-carbon substitution at N5 and N10 (see Figure 1.1).

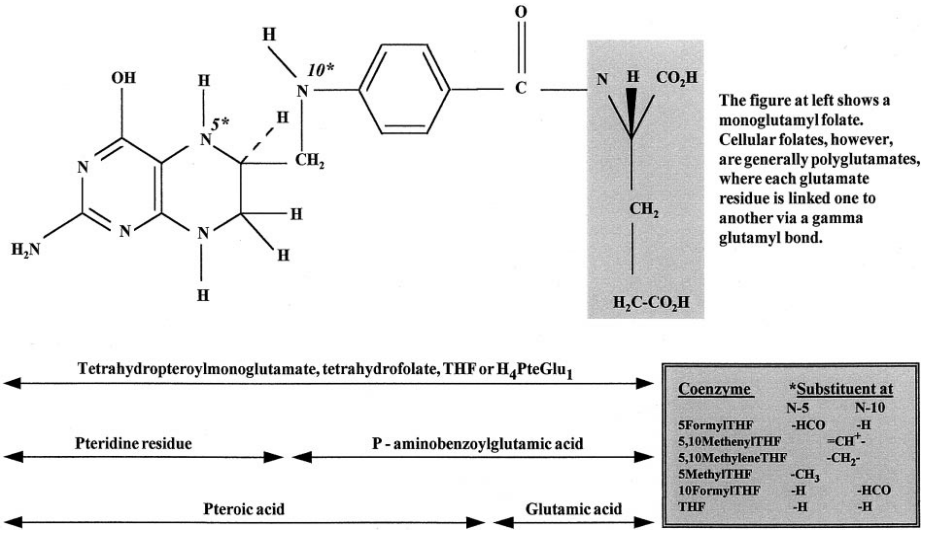



Figure 1.1 The chemical structure of tetrahydrofolate and its various derivatives [6]

The natural form of folate found in food generally has five to eight glutamic acid residues, whilst PteGlu, the form used for supplements and fortification of food, has only one glutamate residue and is fully oxidized.

Folate exists in various vitamer forms in food and blood, each exhibiting a different level of stability (see Table 1.1). The stability of folate is affected by various environmental conditions such as pH, $O_2$, metal ion concentration, temperature and UV radiation. As a result of these oxidative stresses, the pteridine rings of folate derivatives are oxidized and lose the C9-N10 bond readily. This breakage leads to a loss of their activity as vitamins, and yields degradation residues such as p-aminobezoylglutamate (p-ABG).

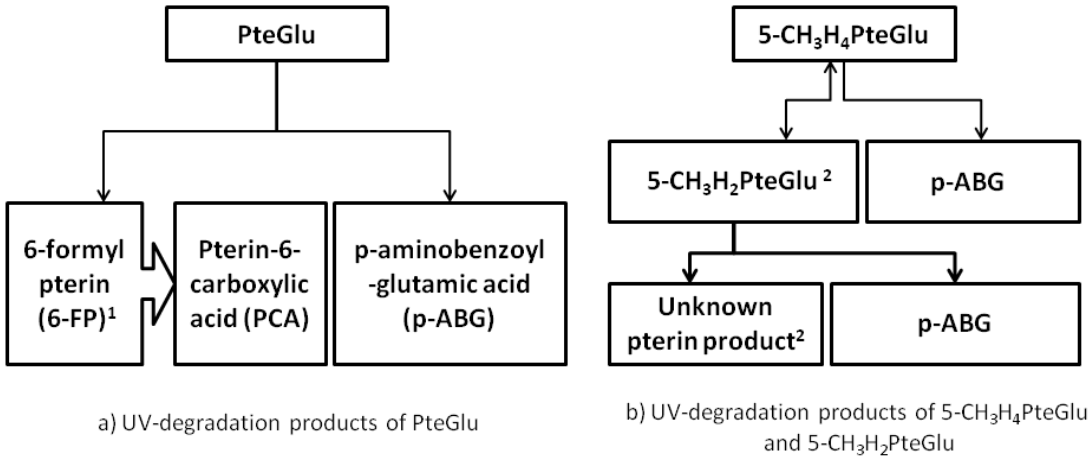
5-methyltetrahydrofolate (5-$CH_3H_4$PteGlu), the predominant form of folate found in food, is easily oxidized to 5-methyldihydrofolate (5-$CH_3H_2$PteGlu) [7]. This reaction means that up to 50% of total food folate exists as 5-$CH_3H_2$PteGlu [8]. 5-$CH_3H_4$PteGlu presents very limited stability. It is particularly affected by factors including the concentration of metal ions and increased pH. 5-$CH_3H_4$PteGlu is relatively stable between pH 3.5 and pH 7, but exhibits instant oxidative degradation at pH 9 [9].

Table 1.1 Various types of folate vitamers, adapted from [10].

| Synthetic form | |
|---|---|
| PteGlu | Very stable under majority of conditions<br>Mainly consumed from supplements and fortified food.<br>High dose may exceed metabolic pool |
| **Natural occurring forms** | |
| $H_2$PteGlu | Unstable – minor food folate |
| $H_4$PteGlu | General natural folate- unstable; may undergo degradation in GI trace |
| 5-$CH_3H_4$PteGlu | Predominant vitamer form in food |
| 5-$CHOH_4$PteGlu | General natural folate and pharmaceutical agent |
| 10-$CHOH_4$PteGlu | General natural folate |
| 5,10-$CHH_4$PteGlu | General natural folate; also formed in acidic equilibrium with 5-$CHOH_4$PteGlu and 10-$CHOH_4$PteGlu |
| 5,10-$CH_2H_4$PteGlu | Readily altered when heated to yield $H_4$PteGlu |
| 10-$CHOH_2$PteGlu | Oxidation derivative of 10-$CHOH_4$PteGlu |
| 10-CHOPteGlu | Oxidation derivative of 10-$CHOH_2$PteGlu |

Antioxidants such as ascorbic acid, dithiothreitol (DTT) and glutathione (GSH) may protect against the decay of 5-$CH_3H_4$PteGlu at different pH values [9]. Under mildly acidic conditions, 5-$CH_3H_4$PteGlu is fairly stable, but 5-$CH_3H_2$PteGlu degrades quickly, resulting in the irreversible loss of the vitamin via C9-N10 bond cleavage [8]. However, 5-$CH_3H_2$PteGlu is relatively stable in a neutral environment. Ascorbic acid reduces 5-$CH_3H_2$PteGlu back to acid stable 5-$CH_3H_4$PteGlu under acidic conditions, and this may be critical for the bioavailability of dietary folate in the human gastrointestinal system [8].

It is known that photo-degradation is responsible for a 30% loss of folate in human blood [11]. Although PteGlu exhibits strong stability compared to other folate vitamers, it degrades into p-ABG, and other pteridine moieties such as pteridine-6-carboxylic acid (PCA) and 6-fomylpterine (6-FP), particularly following UV irradiation. *In vitro* research also confirms that UV exposure converts 5-$CH_3H_4$PteGlu into 5-$CH_3H_2$PteGlu, with eventual C9–N10 bond scissions, forming p-ABG and a benign pteridine residue (see Figure 1.2) [12].




a) UV-degradation products of PteGlu

b) UV-degradation products of 5-$CH_3H_4$PteGlu and 5-$CH_3H_2$PteGlu

Figure 1.2 Photo-oxidative degradation products of PteGlu and methylfolate at neutral pH and absence of antioxidant [8, 11, 12](1: 6-FP is extremely unstable to light, readily oxidized to PCA, 2: degradation and salvage between two methylfolate vitamers and production of this benign pterin compound are influenced by pH and presence/absence of antioxidants (i.e. ascorbic acid))

## 1.3 Folate bioavailability and metabolism

### 1.3.1 Folate bioavailability

Folate bioavailability is a major concern when formulating public health policies. Unlike PteGlu, the estimated bioavailability of dietary folate varies greatly, ranging from 10 to 98% [13]. The

concept of dietary folate equivalence was introduced to accurately estimate dietary folate bioavailability, and is generally defined as the sum of the quantity of natural folate and 1.7 times the quantity of synthetic folic acid present in the diet [14].

The variation of folate bioavailability depends on many factors including

i) intestinal deconjugation of polyglutamyl folate,
ii) the food matrix,
iii) the stability of folate coenzymes during digestion,
iv) the pH of the jejunem and
v) age and variation in dietary folate intake [13].

Dietary folate exists as polyglutamates, mainly 5-$CH_3H_4PteGlu_n$ and 10-formyltetrahydrofolate (10-$CHOH_4PteGlu_n$)**.** It is believed that there is no difference between the bioavailability of polyglutamates and monoglutamates [14]. Unlike monoglutamates, polyglutamate forms need to be hydrolysed by folate deconjugase (glutamate carboxypeptidase II (GCPII) EC 3.4.17.21) into monoglutamates for effective absorption. There are two types of folate deconjugase: a brush border exopeptidase which is Zn ion dependent [15] and works optimally at pH 6.7 to 7.0, and a non-metal ionic dependent intracellular endopeptidase of mainly lysosomal origin which works at an optimum pH of 4.5 [16]. These brush-border enzymes have been reported to be influenced by pH [17], certain drugs, alcohol, and genetic variations [18]. Synthetic folic acid is absorbed easily into intestinal cells as it does not require deconjugation.

Bound folate in food matrices occasionally cannot be released completely, potentially influencing folate bioavailability. Additionally, heating and washing during cooking and preparation processes may considerably reduce the amount of folate ingested [13].

### 1.3.2 Absorption, transport and cellular metabolism

The intestinal absorption of folate is a multistep process which includes crossing of the brush border membrane, a temporary retention and/or metabolism within the enterocyte, and crossing of the basolateral membrane [19].

#### 1.3.2.1 Absorption

Folate is absorbed throughout the duodenum and upper jejunum. Polyglutamate forms of folate are hydrolysed to monoglutamate by folate deconjugase. The transport of this folylmonoglutamate across the brush-border membrane is achieved by two parallel processes of folate transportation which are responsible for intestinal lumen-cellular uptake [19].

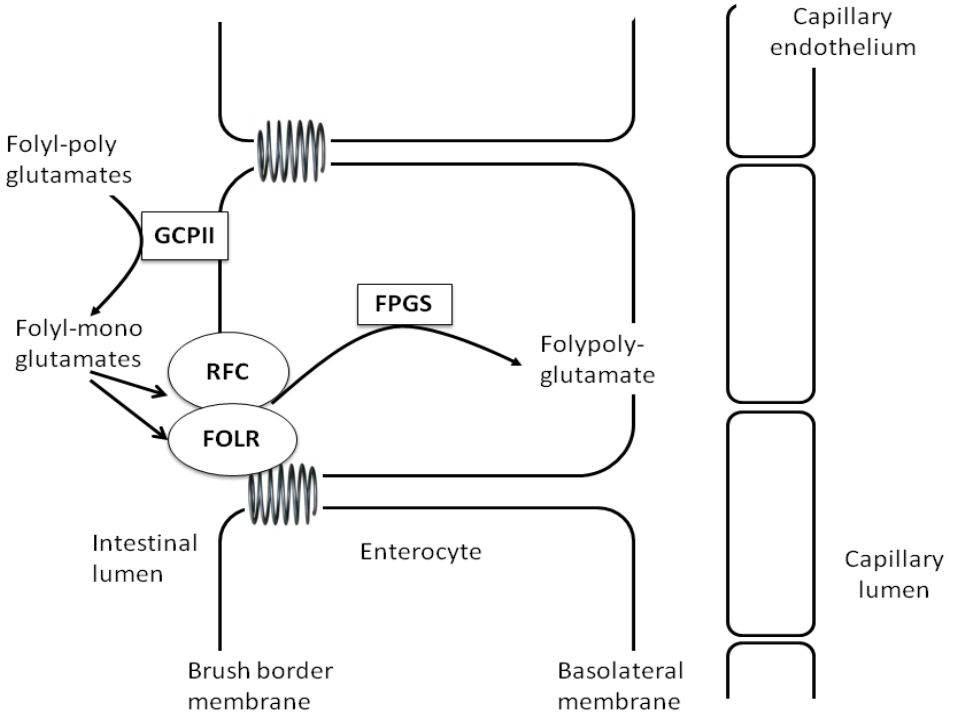



Figure 1.3 Intestinal absorption of folate, adapted from [20] (GCPII: glutamate carboxypeptidase II , RFC-reduced folate carrier: FOLR- folate receptor, FPGS- folylpolyglutamate synthase)

At physiological folate concentrations (<10μM), a saturable process involving a pH dependent carrier mediated mechanism occurs. This involves the folate receptor (FOLR) or reduced folate carrier (RFC) as the dominant mechanism. RFC is abundant in the small intestine. Its action is highly pH-dependent and optimal at pH 6 which is very similar to the pH of the proximal small intestine [21-25]. By contrast, at a high concentration of luminal folate (>10μM), the primary transport of folate is achieved by a non-saturable ion-mediated transport procedure and simple diffusion [24]. A similar system to the one described above has been reported in the human colon in which the folate synthesized by the bacteria living in the large intestine is directly absorbed [26, 27].

#### 1.3.2.2 Transport and cellular metabolism

Before entering the portal blood circulation, folylmonoglutamates absorbed through the intestinal membrane are converted into 5-$CH_3H_4$PteGlu within the enterocyte [28]. 5-$CH_3H_4$PteGlu is the most abundant form of folate in human plasma where 30-40% is bound loosely to plasma proteins such as albumin, α2 macroglobulin and transferrin. Plasma also contains a less abundant high-affinity folate binding protein which increases during folate

deficiency, pregnancy and disease. 5-$CH_3H_4PteGlu_n$ is transported to the peripheral tissues where it is converted to tetrahydrofolate ($H_4PteGlu$) by vitamin $B_{12}$ dependent methionine synthase (MS EC 2.1.1.13). This conversion of intracellular 5-$CH_3H_4PteGlu$ into the more biologically useful form, $H_4PteGlu$, is an important reaction for the cellular accumulation of folate and subsequent nucleotide biosynthesis [6].

$H_4PteGlu$ is converted to a polyglutamyl form by folypolyglutamate synthase (FPGS EC 6.3.2.17) and is the preferred substrate for the FPGS enzyme. Due to the anionic charge of the glutamate chain, this polyglutamylated form of folate (predominantly hexaglutamyl- $H_4PteGlu$) cannot cross the cellular membrane and is retained within the cell [29]. These folypolyglutamate forms of folate are then hydrolysed to monoglutamate by GCPII for their release.

### 1.3.3 Folate homeostasis and excretion

The liver stores approximately 50% of total body folate and plays a central role in maintaining folate status in the form of tetra-, penta-, hexa- and heptaglutamates of 5-$CH_3H_4PteGlu_n$ and 10-$CHOH_4PteGlu_n$. The majority of 5-$CH_3H_4PteGlu$ is released into the blood and is taken up by the liver. However, some 5-$CH_3H_4PteGlu$ is recirculated and absorbed in the intestine by means of the enterohepatic cycle in bile. The liver cells do not respond to short term dietary folate deficiency by releasing their folate stock. Rather, folate pools within the cells and the enterohepatic cycle is mobilized. Folate released by the liver cells via the enterohepatic cycle is distributed to the tissues that have the greatest requirement, for example, actively proliferating cells. Decreased tissue uptake induces reduced cellular folylpolyglutamate synthesis and increased folylpolyglutamate hydrolysis to folylmonoglutamates [30].

Folate which is not bound to protein in plasma is filtered by the glomerulus and reabsorbed in the proximal renal tubules. The renal reabsorption appears to mainly be a nonspecific process although a folate binding protein-mediated process at the renal brush-border membrane and basolateral membrane has been demonstrated [31].

The major pathways for folate excretion are via urine and bile. Although a very small amount of intact folate (≤ 1% of the total body folate store per day) is excreted into urine, most folate is excreted as the catabolized form [32]. The rate of folate loss by faecal excretion is difficult to determine due to the bacterial synthesis of folate in the colon. The first step of folate catabolism is cleavage of folypolyglutamates at the C9-N10 bond, a process catalyzed by

carboxypeptidase (EC 3.4.17.11). This is followed by acetylation to yield the final excretory metabolites of para-acetamidobenzoylglutamate and p-ABG [28].

## 1.4 Biochemical functions of folate

As previously mentioned, folate is a major source of one-carbon units in human metabolism. Its main function is to transfer these one-carbon units from donor molecules into the biosynthetic pathways necessary for the synthesis of purine, thymidylate and methionine. Folate coenzymes also mediate the inter-conversion of serine to glycine and play a major role in histidine catabolism.

### 1.4.1 Conversion of serine and glycine

The 3-carbon of serine is the major source of one carbon units in $H_4$PteGlu conversion [33]. The conversion of serine to glycine is a reversible reaction catalyzed by serine hydroxymethyltransferase (SHMT, EC 2.1.2.1) which depends on vitamin $B_6$ (pyridoxal 5`-phosphate (PLP)) as a cofactor. This reaction is very significant in that $H_4$PteGlu is converted to 5,10-methylentetrahydrofolate (5,10-$CH_2H_4$PteGlu) as a secondary reaction. 5,10-$CH_2H_4$PteGlu is an important folate vitamer as it is required for several biosynthetic pathways critical to life processes such as homocysteine (Hcy) remethylation and the synthesis of thymine, adenine and guanine.

### 1.4.2 Purine and pyrimidine synthesis

During pyrimidine synthesis the transfer of a one-carbon unit from 5,10-$CH_2H_4$PteGlu methylates deoxyuridine monophosphate (dUMP) forming deoxythymidine monophosphate (dTMP) which is the indirect precursor of thymine. This reaction is catalyzed by thymidylate synthase (TS EC 2.1.1.45) which has maximized expression during the S phase of the cell cycle and limits the replication rate of DNA.

Another function of folate is purine biosynthesis in which glycinamide ribonucleotide (GAR) reacts with 10-$CHOH_4$PteGlu to produce formylglycinamide ribonucleotide. This reaction is catalyzed by GAR transformylase (EC 2.1.2.2) and gives rise to the eighth carbon atom of the final purine ring. 10-$CHOH_4$PteGlu also donates a carbon unit to 5-amino-4-imidazole carboxamide ribonucleotide (AICAR). The one-carbon unit from 10-$CHOH_4$PteGlu eventually becomes the carbon atom in position 2 of a purine ring. This reaction forms formyl- AICAR and is catalyzed by AICAR transformylase (EC 2.1.2.3).

### 1.4.3 Catabolism of histidine

The formimino group of formiminoglutamic acid (FIGLU) catabolised from histidine is transferred to $H_4$PteGlu by glutamate-formimino transferase (EC 2.1.2.5). The ammonia group is then liberated from FIGLU by formiminotetrahydrofolate cyclodeaminase (EC 4.3.1.4), producing 5,10-$CHH_4$PteGlu. FIGLU is excreted in urine. An increase of urinary FIGLU is a marker for inadequate body folate status and therefore a histidine load test can be used for the diagnosis of folate deficiency.

### 1.4.4 Homocysteine remethylation and transsulphuration

Hcy, a sulphur containing amino acid which does not exist in food naturally, was discovered by Vigneaud in 1932 [34]. It is produced as an intermediate product during the conversion of methionine to cysteine. Depending on the dietary intake of methionine and choline, between approximately 50 and 80% of the synthesized Hcy is remethylated [33].

Hcy metabolism is composed of two parts: methionine biosynthesis by remethylation and the transsulphuration processes regulated by S-adenosylmethionine (SAM) (see Figure 1.4) [35]. During the initial step of Hcy remethylation, 5,10-$CH_2H_4$PteGlu is irreversibly reduced to 5-$CH_3H_4$PteGlu by the flavoprotein MTHFR. 5-$CH_3H_4$PteGlu then donates a methyl group to Hcy. Both of these reactions are of great significance, not only for maintaining an adequate intracellular concentration of methionine, but also for regulating the level of Hcy. High Hcy concentrations (>13 µmol/L) have been reported to be neuro-, vasculo- and embryo-toxic [6, 36, 37].

The remethylation of Hcy to methionine is catalyzed by MS which is vitamin $B_{12}$ (cobalamin) dependent. The methyl group liberated from 5-$CH_3H_4$PteGlu is transferred by MS to cobalamin and then shifted to Hcy. In addition, an auxiliary enzyme methionine synthase reductase (MSR EC 2.1.1.135) is involved in this methylation reaction to sustain proper methylated status. Methionine is adenylated to form SAM, the principal methyl donor for a variety of acceptors in many methylation reactions. It is then converted into S-adenosylhomocysteine (SAH). SAH is reversibly hydrolyzed back to Hcy and adenosine to begin a new remethylation cycle. SAM allosterically regulates the utilization of Hcy for *de novo* synthesis of methionine, i.e. it inhibits MTHFR, leading to a decrease in the concentration of 5-$CH_3H_4$PteGlu and the subsequent restriction of MS activity.

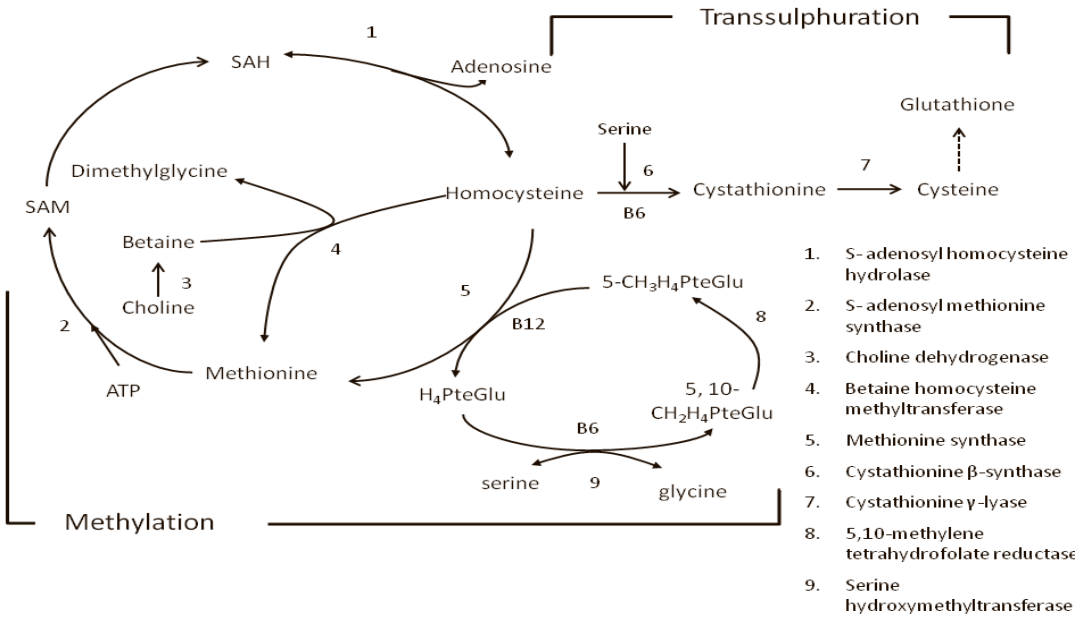



Figure 1.4 Pathway for homocysteine remethylation and transsulphuration

During the transsulphuration process, Hcy is condensed with serine to form cystathionine, a reaction catalyzed by the vitamin $B_6$ dependent enzyme cystathionine β-synthase (CBS EC 4.2.1.22). Cystathionine is subsequently hydrolysed by cystathionine γ-lyase (CTH, EC 4.4.1.1) to cysteine and α-ketobutyrate. As for Hcy remethylation, the transsulphuration pathway is regulated by SAM. An increased cellular concentration of SAM leads to the activation of CBS, diverting Hcy through the transsulphuration pathway to cysteine. On the other hand, a low concentration of SAM induces the activation of 5-$CH_3H_4$PteGlu synthesis, but inhibits cystathionine synthesis [38]. Excess 5-$CH_3H_4$PteGlu also inhibits glycine N-methyl transferase (GNMT) which controls SAM concentration in the liver. GNMT methylates the nonessential amino acid glycine and catalyzes the transformation from sarcosine into SAH. Sarcosine is transferred by sarcosine dehydrogenase into the mitochondria and reconverted into glycine via 5-$CH_3H_4$PteGlu.

The cellular SAH concentration controls the tissue-specific bioregulatory function, increases CBS activity and constrains the Hcy methyltransferase and MTHFR activities. The conversion from SAM to SAH is irreversible. SAH has higher affinity for methyltransferase than SAM. Thus condensed cellular SAH suppresses methyltransferase activity [39]. These reactions are involved in controlling the cellular SAM concentration, and maintaining the ratio of SAM to SAH. Hence they assist the metabolic balance between the remethylation and transsulphuration pathways, as well as the utilisation of *de novo* synthesized methyl groups and dietary methionine.

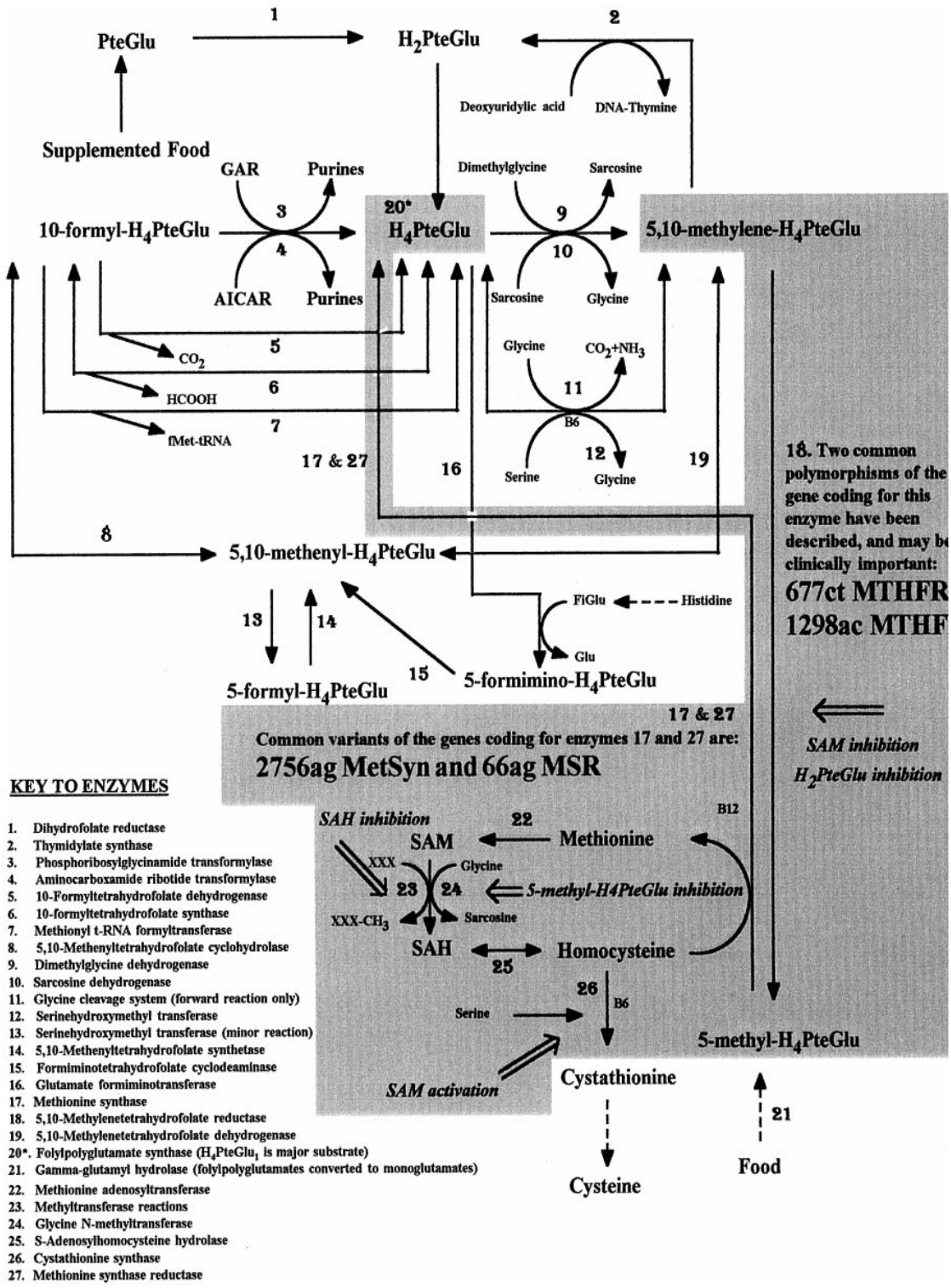



Figure 1.5 Folate-dependent one-carbon metabolism, highlighting the homocysteine remethylation cycle and common single-nucleotide polymorphisms [6]

## 1.5 Folate in health and disease

In recent decades folate has been highlighted as having significant health benefits. The Medical Research Council Vitamin Study (1991) in which randomized volunteers took folate supplements at 4mg/day confirmed that folate plays a major role in preventing congenital malformations such as anencephaly, spina bifida and encephalocele [40]. Other studies have demonstrated numerous health benefits of folate including reduced risks of pregnancy complications, cognitive disorders, Alzheimer's disease, several cancers as well as lowering Hcy levels [41-44]. Around 27 genes have been identified that encode the proteins involved in folate-dependant one-carbon metabolism (see Table 1.5) [45]. An increased risk of developing these disorders can occur due to aberrant folate and other relevant B-vitamin status which arises because of specific genetic mutations, particularly single nucleotide polymorphisms (SNPs). These mutations may affect the activity or expression of folate metabolism genes and hence alter the normal cellular and blood distribution of folate vitamers. Folate status and risk of disease may also be influenced by other nutritional and environmental factors such as diet, use of supplements, alcohol intake, smoking, ethnicity and seasonal changes.

### 1.5.1 Roles, characteristics and polymorphisms of genes in folate metabolism

#### 1.5.1.1 Methylenetetrahydrofolate reductase

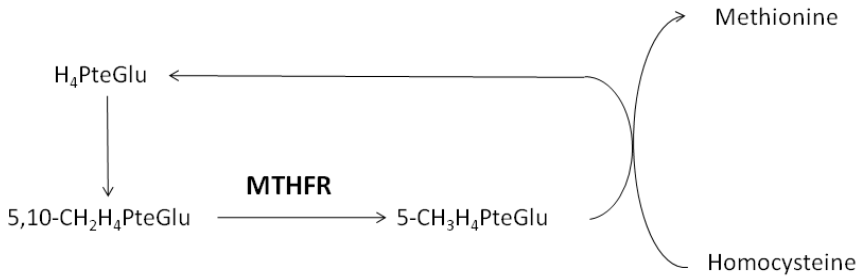



Figure 1.6 Simple reaction diagram for methylenetetrahydrofolate reductase (MTHFR)

MTHFR is a cytoplasmic enzyme whose gene is located on chromosome 1p36.3. It is considered to be a key enzyme because of its importance in folate metabolism. As previously mentioned, MTHFR catalyzes the reduction of 5,10-$CH_2H_4$PteGlu to 5-$CH_3H_4$PteGlu (Figure 1.6), supplying one-carbon units for the methylation of Hcy to methionine [46]. This reaction produces the precursor of SAM which is the methyl group donor in more than 100 reactions. The SAM/SAH ratio regulates MTHFR activity.

There are many polymorphisms in the MTHFR gene, two of them having been described as commonly occurring polymorphisms: C677T and A1298C. Frosst et al. [4] identified a 677C→T mutation in the MTHFR gene (rs1801133) which results in a nucleotide substitution of cytosine to thymine: an amino acid change of alanine to valine at position 222. The C677T transition occurs within the predicted catalytic domain of the MTHFR enzyme. Guenther et al. [46] used a thermolabile variant MTHFR model expressed in E. coli to show that the C677T mutation results in the exposure of binding sites for the flavin adenine dinucleotide (FAD) cofactor which otherwise would be embedded in a barrel-like structure. Such exposure results in a weakened enzyme and FAD complex and subsequent loss of MTHFR activity.

The frequency of alleles and C677T genotypes differs with ethnic groups and geography. Research with Caucasian populations has shown that the prevalence of the variant homozygous genotype (TT) is between 10.3 % and 18.2%. However, in a South African study researchers found that only 1.8% of people had the homozygous recessive genotype, whilst in an Asian study around 50% of the population were found to be heterozygotes (CT) and 14% were homozygous recessive for the C677T MTHFR variation [47].

Those carrying the variant MTHFR C677T allele have been found to exhibit altered blood folate and Hcy levels in addition to a lower proportion of 5-$CH_3H_4$PteGlu and a higher proportion of formylated folate than wild types (CC) [48]. Since 5-$CH_3H_4$PteGlu is a source of methyl groups in the remethylation of Hcy to methionine, it has been reported that homozygous recessive individuals with low-normal plasma folate levels have increased Hcy concentration, whereas TT individuals with high plasma folate levels have virtually normal Hcy levels [49, 50].

There is significant literature highlighting the occurrence and critical impact of the C677T MTHFR variation, particularly its association with CVD [4, 51-53] and cancers such as colorectal cancer (CRC) [54-56] and breast cancer [57-60]. It is widely believed that this genetic variant plays a role relevant to the function of folate methylation and DNA synthesis. However, studies considering these functional consequences in cancers do not always show significant results. This genomic variation also influences the cellular compartmentalisation of folate. In general, it is accepted that the C677T MTHFR variation is associated with an increased risk of oesophageal cancer [61], gastric cancer [62-64], endometrial cancer [65, 66], ovarian cancer [60, 67] and breast cancer [59, 68]. By contrast, the presence of this variation reduces the risk of developing leukaemia and CRC when other factors such as folate intake, smoking and alcohol consumption are taken into account [69]. MTHFR variants interact with folate status and can reduce the risk of cancer if individuals are replete in folate [70].

Weisberg et al. [71] identified the common A1298C polymorphism (rs1801131) of the MTHFR gene which results in a base change of adenine 1298 to cytosine: a glutamine 429 to alanine (E429A) substitution located in the presumed regulatory domain. Although the evidence suggests that this MTHFR polymorphism is not an individual risk factor for disease and therefore not a critical problem, when examined in combination with the C677T mutation individuals have increased levels of plasma Hcy and lower blood folate status [72, 73]. Similarly, Van der Put et al. [74] examined the effects of the combined mutations at the two polymorphic sites (C677T and A1298C) in NTD patients and found that the heterozygote/heterozygote combination (677CT/1298AC) resulted in lower MTHFR activity than heterozygosity of either of the MTHFR mutations individually, resulting in significantly increased Hcy and decreased plasma folate levels. This may account for folate-related NTDs.

Another variation of the MTHFR gene is the G1793A polymorphism (rs2274976). This is located on exon 11 and generates a base change of guanine 1793 to adenine: an amino acid change at position 594 converts an arginine into a glutamine (R594Q) [75]. To date there are few studies that show an association between this variation and incidence of disease. However, in 2013, Lucock et al. reported that this genetic variation had an influence on blood folate status [76]. Another study reported that it increased the risk of sporadic CRC [77]. Additionally, when combined with the C677T variation, the 677CT/1793GA genotype is associated with increased risk for prostate cancer [78] and ulcerative colitis [79].

#### 1.5.1.2 Methionine synthase

MS catalyzes the final step of methionine biosynthesis. This gene is located on chromosome 1q43 and catalyzes the transportation of a methyl group from 5-$CH_3H_4$PteGlu to Hcy, generating $H_4$PteGlu and methionine which can be used for protein synthesis or SAM production (see Figure 1.7). MS requires the coenzyme MSR and the oxidation of cobalamin which

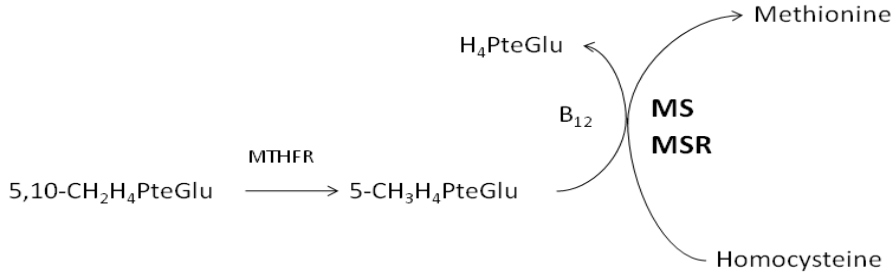



Figure 1.7 Simple reaction diagram for methionine synthase (MS) and methionine synthase reductase (MSR)

exists in three different forms (Cob(I)alamin: CoI, Cob(II)alamin: CoII and methyl-Cob(III)alamin: $CH_3$-CoIII). The inhibition of MS induced by inadequate consumption of cobalamin or exposure to nitric oxide leads to impaired enzyme activity and causes the redistribution of cellular folate derivatives and an aberrant total intracellular folate status [80].

Several SNPs have been identified in MS [81]. The A2756G (rs1805087) mutation in the open reading frame of this gene is the most common: an aspartic acid residue is replaced by glycine at codon 919. This mutation is considered to have an effect on folate metabolism. In some Asian countries such as Japan, China and Korea, the prevalence of the recessive genotype (GG) is only 2-3%. Similarly, in many European countries, 3% of the population are homozygote recessive [47]. However, population groups of African and African origin showed a range of prevalence of 1-12% for the recessive genotype [82].

Some studies have associated the A2756G MS polymorphism with elevated Hcy concentration and an increased risk of multiple myeloma, thromboembolic events, NTDs and cleft lips and palates [83-87]. However, a number of studies have found that this mutation has no effect on human health [88-90].

#### 1.5.1.3 Methionine synthase reductase

The MSR gene is located on chromosome 5p15.2-15.3. The product of this gene is a novel member of the ferredoxin-nicotinamide adenine dinucleotide phosphate (NADP) + reductase family of electron transferases; it is approximately 34 kb in length, is composed of 15 exons containing 698 amino acids and has a molecular mass of 77,700 [91, 92].

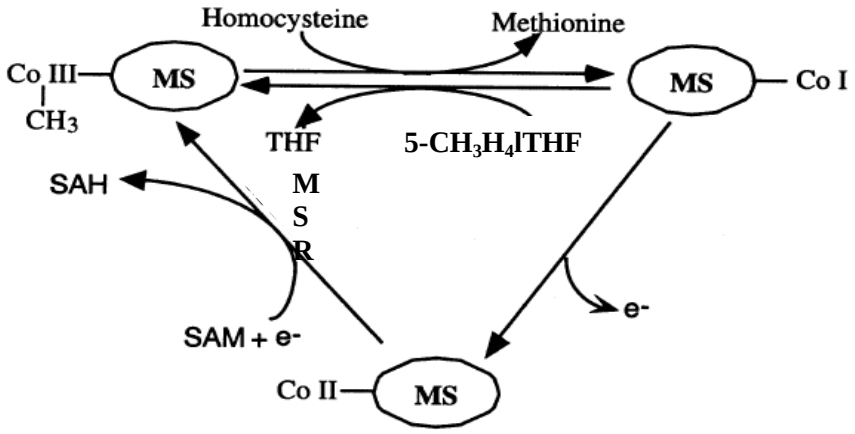



Figure 1.8 The roles of methionine synthase reductase (MSR) in homocysteine metabolism (MS: methionine synthase) [93]

Cobalamin acts as an intermediate methyl carrier between 5-$CH_3H_4$PteGlu and Hcy during the MS dependent Hcy remethylation pathway. MSR is essential for retaining adequate levels of activated cobalamin which is the enzyme bound form of MS. Cobalamin circulates as a cofactor as shown in Figure 1.8. CoI is a strong reducing agent which can be oxidized to generate an inactive form of the enzyme CoII. The methylation reaction of CoII to $CH_3$-CoIII using SAM as a methyl donor [94] is catalyzed by MSR. Hence, MSR plays a significant role in sustaining activated cobalamin [93].

A common variant in MSR, A66G (rs1801394), leads to an amino acid substitution of isoleucine to methionine at codon 22 of exon 2. The HapMap project results (2005) showed that the frequency of the G allele in healthy controls was 44.8% in European countries. The frequency of the G allele was found to be decreased in Chinese (25%), Japanese (30.2%) and Sub-Saharan African groups (23.3%) [93]. An association of this SNP with increased levels of Hcy [93], increased risk of NTDs [95], Down syndrome [96] and congenital heart defects mostly associated with maternal hyperhomocysteinemia and low blood cobalamin status [97] has been reported.

#### 1.5.1.4 Cystathionine β-synthase

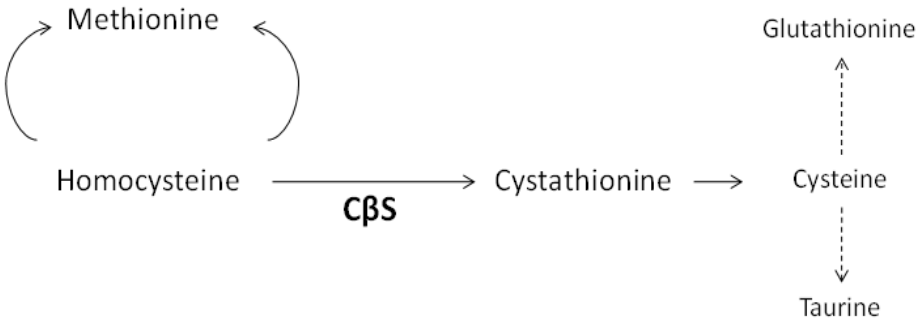



Figure 1.9 Simple reaction diagram for cystathionine β-synthase (CBS)

CBS is a key enzyme in Hcy metabolism residing within the transsulphuration pathway (see Figure 1.9). The methionine remethylation pathway produces approximately half the amount of Hcy we require. The transsulphuration pathway, where CBS is located, produces the other half. Beyond this point in the transsulphuration pathway, Hcy cannot serve as a provider for methionine biosynthesis [6].

The human CBS gene is located on chromosome 21q22.3, is approximately 25 kbp long and is a homotetramer of a 63-kDa polypeptide. CBS metabolizes Hcy into cystathionine and requires vitamin $B_6$ (pyridoxine) as an essential cofactor and heme as a nonessential cofactor, although

the function of heme is unknown [98]. However, the heme group is combined with cysteine 52 and histidine 65 at the N-terminal which is involved in the correct folding of CBS. Mutations of these moieties therefore give rise to the oxidation of heme and reduce CBS activity [99]. As mentioned previously, CBS is allosterically regulated by SAM but inactivated by MTHFR. SAM is a V-type allosteric effecter which activates CBS two to threefold (the V*max* of the enzyme is altered by the effecter and is hence called V-type) [100].

An aberrant remethylation route and/or inhibition or saturation of the transsulphuration pathway results in a change in Hcy concentration. In addition, an increase in Hcy can be the consequence of genetic variations within the enzymes MTHFR and CBS. Such genetic variations and the corresponding response in plasma Hcy level are modulated by the individual's health and nutritional status. In May, 2013 approximately 164 mutations had been identified, most of which are single point mutations (this information is updated online at http://cbs.lf1.cuni.cz/index.php). These mutations occur in all areas of the CBS gene including, for example, dimmer interface junctions, the heme-binding site, the active site and the presumed regulatory domain. They affect CBS gene activity as well as folate and Hcy levels. Out of these 164 mutations, 110 have been identified to be missense mutations and 6 to be nonsense mutations.

Recessive homozygosity for CBS variations can be lethal or lead to a severe increase in Hcy. The most prevalent mutations are T833C (I278T) which is pyridoxine-responsive and G919A (G307S) which is pyridoxine-nonresponsive [101]. G307S is considered to be of "Celtic" origin with 71% of Irish homocystinuria patients possessing this SNP [102]. The I278T mutation which is located in the C-terminal domain is panethnic and can be found in approximately 25% of patients with homocystinuria [103].

Another reported variant of CBS is CBS 844*ins*68: the CBS 844*ins*68 allele contains an insertion of 68bp in the coding region of exon 8. It has been proposed that in about 90% of cases this insertion is closely associated with the T833C transition [104]. Homozygosity for this insertion is quite rare: it occurs with the highest frequency (3-4%) in Africans and Brazilians. In Europe, Australia and the United States 13-15% of the population are heterozygous, whilst only 5% of Japanese and Chinese populations have the heterozygous genotype [47]. Many studies have now been published linking the CBS 844*ins*68 variant to increased Hcy levels [105] and an increased risk of developing a number of diseases including conotruncal heart defects [106], schizophrenia [107], idiopathic mental retardation [108] and cancers [104, 109, 110]. However, the associations between this insertion and breast cancer and colorectal adenoma are still

controversial [110-112]. Interestingly, Summers et al. [113] suggested that the CBS 844*ins*68 allele may have a normalizing effect on Hcy concentrations in MTHFR C677T TT homozygotes who would ordinarily have an increased Hcy level compared to MTHFR 677 heterozygotes (CT) and wild types (CC).

#### 1.5.1.5 Glutamate carboxypeptidase

GCPII, Zn-dependant exopeptidase, is involved in dietary folate hydrolysis, a process which cleaves polyglutamate forms of folate into mono glutamate forms. GCPII exists on the brush-border of the intestine and is a type II transmembrane glycoprotein [15]. Folate nutritional status and the presence of disease can alter the activity of GCPII. Mutations in the GCPII gene may affect the intestinal absorption of dietary folate leading to low blood folate levels and, consequently, hyperhomocysteinemia. It has been reported that one particular mutation of this gene, C1561T in which 475 histidine is changed to tyrosine (rs61886492), resulted in a 50% reduction of GCPII activity, leading to lower blood folate concentration and higher Hcy [15, 114]. In addition, this variation has been associated with increased risks of breast and prostate cancer, autism, coronary artery disease (CAD) and miscarriages [115, 116]. However, Afman et al. [117] suggests that this variation increases the deconjugation activity of the enzyme which, in turn, could increase plasma folate and decrease plasma Hcy levels.

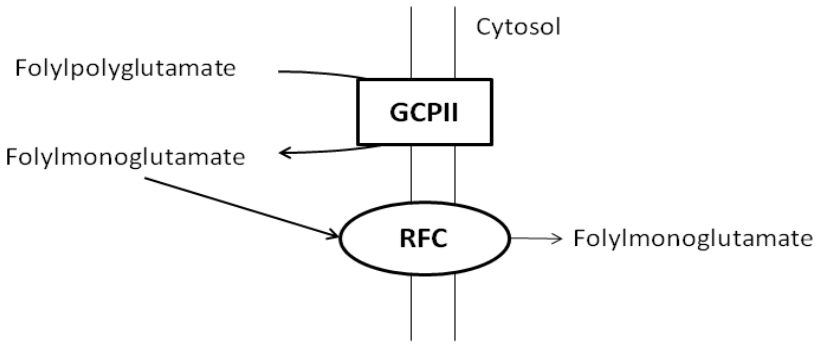



Figure 1.10 Simple reaction diagram for glutamate carboxypeptidase (GCPII) and reduced folate carrier (RFC)

#### 1.5.1.6 Reduced folate carrier

RFC, also known as SLC19A1, is an example of an anion exchanger involved in the cellular uptake of folate. Once dietary folate has been hydrolyzed to its monoglutamate form by GCPII it can be absorbed in the proximal small intestine via an RFC mediated procedure involving the folate receptor (see Figure 1.10). RFC exhibits a comparatively high affinity for reduced folate

($K_m$ 1-10µM). However, it shows an inferior affinity for oxidized folic acid ($K_m$ 200-400µM). Moreover, RFC shows a high capacity as a cellular transporter of 5-$CH_3H_4$PteGlu, but has a lower capacity as a transporter of PteGlu. RFC also facilitates the transport of antifolate drugs such as methotrexate (MTX) [118].

The best studied SNP of this gene is G80A (rs61510559) which involves the substitution of an arginine residue with histidine at position 27. The way in which this variation affects folate and its metabolism is unclear at this time. Several papers suggest that RFC G80A on its own does not influence folate and Hcy levels [119-121]. However, when combined with the MTHFR C677T variation, significant effects on Hcy level have been observed [120, 122]. In addition, it has been reported that this variation may be responsible for the absorption and cellular translocation of dietary folate [121, 123], it may alter the outcome of MTX therapy [124], and an association with blood pressure levels has been seen in an elderly population. Furthermore, G80A appears to be protective against thrombosis via a non Hcy related mechanism [121, 123].

#### 1.5.1.7 Dihydrofolate reductase

DHFR (EC 1.5.1.3) is located on chromosome 5q11.2-q13.2. Its main role is to catalyze the reduction of $H_2$PteGlu to $H_4$PteGlu which is NADPH dependant. However, it is also responsible for the conversion of PteGlu to $H_2$PteGlu. This is the only known way to metabolize synthetic PteGlu from supplements and fortified food, although the enzyme exhibits a very high $K_m$ towards PteGlu [125].

The main metabolite of DHFR, $H_4$PteGlu, is catabolized to 5,10-$CH_2H_4$PteGlu and glycine with serine and $NAD^+$ by SHMT. 5,10-$CH_2H_4$PteGlu donates a one-carbon unit for thymidylate synthesis, a nonreversible methylation reaction involving TS where dUMP is converted into dTMP. This reaction generates $H_2$PteGlu which is then catabolized to $H_4$PteGlu by DHFR again. Therefore DHFR is critical for both the continuous circulation of reduced folate in the body and synthetic PteGlu metabolism.

Many genetic variations of DHFR have been reported. Of these, a 19 base pair insertion/deletion has been the most thoroughly investigated. This DHFR 19del in intron 1 predicted a putative Sp1 transcription factor-binding site. There is some controversy over whether this variation decreases the expression and activity of DHFR according to the genotype [125, 126]. This variation has been shown to have an influence on several disorders, especially spina bifida, independent of folate status [125-127].

There is another reason why DHFR is relevant: antifolate drugs for cancer and rheumatism therapy, including MTX and aminopterin, mainly target DHFR via competitive inhibition [128, 129]. However, the overexpression of DHFR due to aberrant application or genetic variants results in a decrease in the affinity of these antifolates for DHFR, leading to drug resistance for the curative antifolate-based chemotherapy of various human diseases [128]. DHFR also possesses various potential inhibition levels depending on the type of folate vitamers it is exposed to. PteGlu presents maximum competitive substrate inhibition on its activity towards $H_2$PteGlu concentration. DHFR activity also varies between mammalian species [130] with the activity of DHFR in the rat being higher than in humans [131]. In addition, DHFR activity differs approximately 5-fold between individuals [131-134].

#### 1.5.1.8 Thymidylate synthase

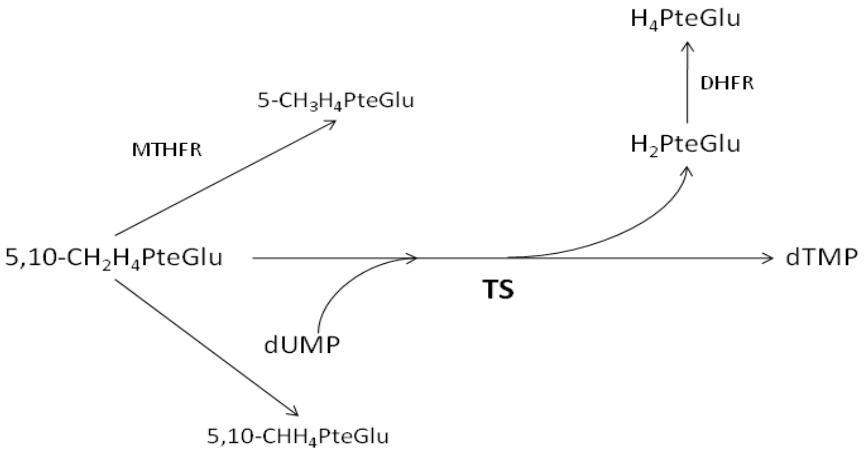



Figure 1.11 Simple reaction diagram for thymidylate synthase (TS)

TS methylates dUMP to dTMP, leading to the essential provision of thymine biosynthesis (deoxythymidine triphosphate (dTTP)), using 5,10-$CH_2H_4$PteGlu as its one-carbon methyl source (see Figure 1.11). This enzyme is therefore of interest in oncology research. Applications include the optimization of anticancer therapy with drugs such as 5-fluorouracil (5-FU) [135, 136].

Some TS genetic variations are known to affect enzyme expression and hence may affect risk factors for human disease. Examples include a variable nucleotide tandem repeat of either two or three 28-bp repeats located in the 5' UTR region (TSER 2R3R). Within this TSER variation exists another SNP, a G>C at the 12th nucleotide in the second repeat of its 3R allele (TSER 3RG>C). One other TS variation involves a 6bp (TTAAAG) deletion/insertion (0bp/6bp) at 1494 in the 3`UTR (TS 1494del6) and has been associated with diseases via affected TS expression [137]. Evidence suggests that these variations are particularly associated with cancer risk,

perhaps via anticancer drug mechanisms, and include CRC [137, 138], renal cancer [139], non-Hodgkin's lymphoma [140], adult acute lymphocytic leukemia [141], esophageal squamous cell carcinoma and clinical outcomes of chemotherapy [142].

#### 1.5.1.9 Serine hydroxymethyltransferase

As mentioned previously, SHMT catalyzes the reversible conversion of serine and $H_4$PteGlu to glycine and 5,10-$CH_2H_4$PteGlu (see Figure 1.12). This process provides one-carbon units for the synthesis of pyrimidine and purine using vitamin $B_6$ as a cofactor [143]. There are two types of SHMT which are encoded by different genes in the human cell. One exists in the cytosol and is localized on chromosome 17p11.2. This form is involved in folate-metabolic pathways. The other isoform exists in mitochondria and is located on chromosome 12q13.2 [143-145]. Pyridoxine (vitamin $B_6$) is firmly bound to a lysine residue in SHMT, has homotetramer structure and has the active site at the interface of two monomers in an obligate homodimeric structure [146]. However, vitamin $B_6$ restriction does not affect rates of Hcy remethylation or synthesis [147].

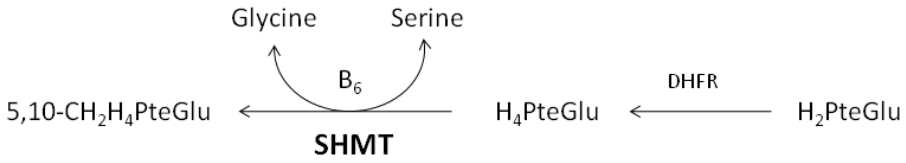



Figure 1.12 Simple reaction diagram for serine hydroxymethyltransferase (SHMT)

The SHMT C1420T polymorphism (rs1979277) results in a leucine to phenylalanine substitution in exon 12. In the United States and Nigeria 11-20% of the population have the homozygous recessive (TT) genotype, whilst Japanese and Chinese populations have not been found to possess this genotype [148]. There are several reports showing that the SHMT C1420T variation is associated with lower risks of developing malignant lymphoma [149], esophogeal squamous cell carcinoma, gastric cardia adenocarcinoma [61], Down syndrome [150] and oxidative stress levels [151]. Additionally, when combined with TSER variations, SHMT C1420 decreases the risk of adult acute lymphocytic leukemia [141] and increases the risk of CVD when combined with the MTHFR C677T variation [152]. However, the influence of this SNP on breast cancer remains controversial [145, 153] and it has been found to have no association with prostate cancer [154]. Some studies suggest that SHMT might play a role in schizophrenia

[155]. This is because the concentrations of serine and glycine, and the activity of SHMT are abnormal in the plasma and brains of schizophrenics. SHMT is the only enzyme which converts serine to the neurotransmitter, glycine in the brain [156].

#### 1.5.1.10 Betaine-homocysteine S-methyltransferase

The predominant pathway for methionine-Hcy conversion in the liver is mediated by BHMT and remethylates about 50% of Hcy [157]. It has been suggested that mutated BHMT may cause a structural change to the Hcy-BHMT binding site. Betaine supplementation can reduce the Hcy concentration, acting as a methyl donor for direct remethylation of Hcy to methionine [157].

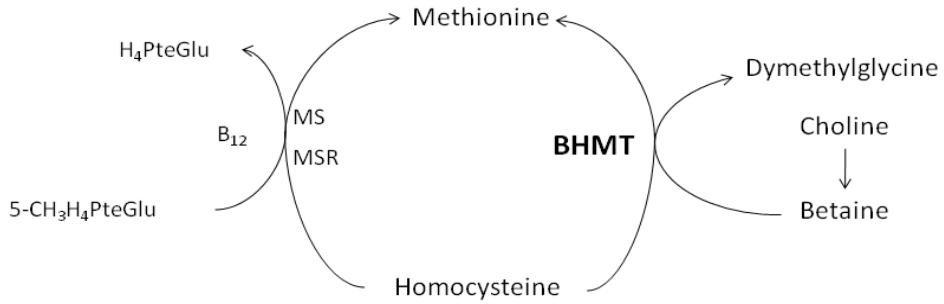



Figure 1.13 Simple reaction diagram for betaine-homocysteine S-methyltransferase (BHMT)

A few genetic variations of BHMT have been studied. It has been shown that G742A (rs 3733890) in exon 6 may modulate the risk for disease. For instance, it may be associated with the risk of Down syndrome [158, 159], non-syndromic cleft lip with/without cleft palate [160], congenital heart defect affected pregnancy [161] and telomere length [162]. BHMT G595A (rs59866108), another nonsense variation which exists in exon 5, was predicted to potentially influence blood folate and Hcy levels, although it presents with a rare minor allele frequency [157].

#### 1.5.1.11 Cystathionine γ-lyase

CTH is situated on chromosome 1p31.1 and is expressed in the liver, kidney, thoracic aorta, portal vein, brain as well as the uterus and placenta [163]. Although CTH does not metabolize folate intermediates directly, it converts cystathione, which is produced from methionine, into cysteine in the transsulfuration pathway [164]. CTH is of interest because it produces hydrogen sulphide ($H_2S$) from Hcy and cysteine (see Figure 1.14). Cysteine has been reported to be a signal molecule, antihypertensive agent and cytoprotectant. $H_2S$ has also been found to be

responsible for the modulation of neuronal transmission and insulin, smooth muscle relaxation and inflammation [163].

To date, few studies have investigated CTH polymorphisms and their influence on disease risk factors. Two mutations of the CTH gene have been identified that are associated with cystathioninuria [165]. The G1364T CTH (S403I, rs1021737) homozygous TT genotype showed significantly higher Hcy levels than the other genotypes [166, 167] and IVS10-430 C > T CTH (rs559062) is also reported to be associated with the occurrence of bladder cancer [168] .

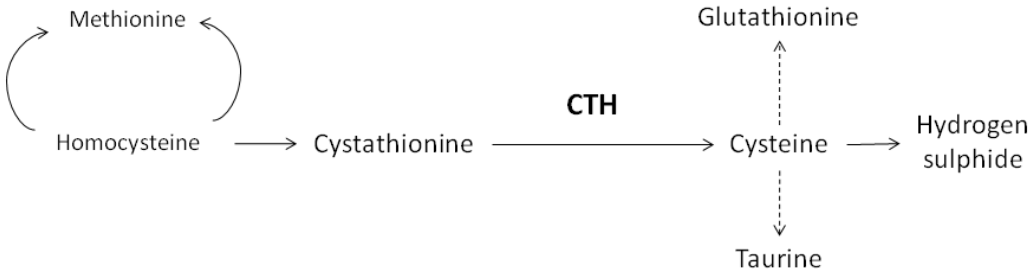



Figure 1.14 Simple reaction diagram for cystathionine γ-lyase (CTH)

Although the existing literature has not shown consistent associations between these polymorphisms and disease, many studies are currently being conducted to verify the influence of genetic variations on folate metabolism and disease risk. The question of whether nature or environmental influences play the predominant role in disease pathoaetiology has been the focus of much controversy. The current literature suggests that the development and progression of disease is a multifactorial process involving the interaction between genetic and environmental factors. The interactions of gene-gene, nutrient-gene and nutrient-nutrient factors, together with a myriad of other factors, are all relevant, resulting in a variety of disease phenotypes. Further research is thus needed to verify the underpinning mechanisms between these factors in order to develop effective pathways for disease prevention and remedy.

### 1.5.2 The role of folate in disease aetiology

#### 1.5.2.1 Evaluating folate nutritional status and deficiency

To evaluate folate nutritional status, plasma folate, red blood cell (RBC) folate and Hcy indices have been used. While haematological deficiency symptoms, for example megaloblastic anemia, manifest in severe deficiency, the former indices are important for estimating folate nutritional status under less extreme, relatively normal conditions.

A plasma folate concentration of under 6.8nM (3.0µg/L) is considered low, but the plasma concentration can respond rapidly to decreased or increased folate consumption. For this reason, it is not appropriate to estimate long-term folate storage in the body using this parameter. RBC folate is a more reliable index for evaluating long-term body folate storage. An erythrocyte folate concentration of under 317nM (140µg/L) is considered low [169].

Megaloblasts are large, irregular cells that have a nucleus. They appear in the blood in folate deficiency as inadequate folate status causes impaired cell division (proper nucleic acid synthesis is a key function of folate). The appearance of these immature cells in the blood is a characteristic of megaloblastic anaemia.

Another useful index for evaluating folate status is the plasma Hcy concentration. As previously mentioned, Hcy is inversely related to folate. A plasma Hcy concentration of ≤13 µmol/L is considered normal. However, the Hcy level is not an absolute index for estimating folate nutrition status as it can be affected by many other factors such as other one-carbon source nutrients, genetic variation, vitamin $B_{12}$ and $B_6$ status and kidney failure [28].

#### 1.5.2.2 The importance of folate in pregnancy complications and birth defects

Folate was originally discovered by Lucy Wills as a 'factor' effective against the anaemia which was common in pregnant women in India. Since this time, folate has been implicated in many issues with respect to pregnancy complications and birth defects. It is now confirmed that folate supplementation improves maternal folate nutritional status [3] and reduces risks of pregnancy complications [170].

##### 1.5.2.2.1 Complications of pregnancy and birth defects

Adequate folate consumption is important, not only for pregnant women, but also for women of child bearing age. Neural tube closure occurs in the early stages of pregnancy (within 28 days) before most women recognize that they are pregnant [171], and over half of pregnancies are unplanned [172]. Pregnant women are particularly sensitive to folate deficiency; their

metabolism, particularly DNA synthesis and one-carbon transport reactions, is enhanced during pregnancy as a result of the growth of the fetus and uteroplacental organs [173]. McPartlin reported that excretion of folate catabolites was increased in late pregnancy [174, 175]. Thus pregnant women who do not take folate supplementation show lower blood folate concentrations [176, 177].

To date, considerable research data has been published, but there is no consistent theory regarding the association between the role of folate, Hcy status and pregnancy complications [173]. However, since Hibbard first suggested a putative association between miscarriage and folate deficiency [178], various kinds of pregnancy complications, for instance placental abruption, preeclampsia, spontaneous abruption and birth defects such as cleft lip, cleft palate, certain heart defects, and limb malformations have been considered to be related to inadequate folate status [3]. Subsequent studies have shown correlations between birth weight, maternal serum folate and erythrocyte folate [170, 179-181]. Others have shown that folic acid supplementation results in an increase in birth weight and a significant reduction in the number of low birth weight infants and preterm deliveries [181]. Folic acid supplementation has also been shown to reduce the risks of placental abruption [182] and preeclampsia [183]. It has been demonstrated that the Hcy concentration of women who have experienced placental abruption is increased when compared to that of normal controls. Furthermore, there is evidence to suggest that Hcy may contribute to preeclampsia [184, 185], but no consensus theory with respect to Hcy and pregnancy complications has yet been reached [173].

It is also possible that genetic variation might be involved in pregnancy complications. Although a small number of reports have found no association between the MTHFR genotype and pregnancy complications [186, 187], many others have reported that MTHFR C677T on its own, or in combination with the A1298C MTHFR polymorphism, seems to act as a risk factor for placental abruption. This effect may be mediated by Hcy [188-191]. In addition, the association of MTHFR C677T with pregnancy complications has been repeatedly confirmed in Dutch and French women who had unexplained early pregnancy loss [189, 192] and placental vasculopathy (placental abruption or placental infarction with fetal growth restriction) [193]. The MTHFR C677T variation has similarly been shown to influence low birth weight in a UK cohort [194].

#### 1.5.2.2.2 Neural tube defects

NTDs are one of the best known effects of folate deficiency and can be grouped into two categories: closed and open. Anencephaly and spina bifida, the most frequent types of NTDs, belong to the former category. Worldwide, approximately 300,000 children are born annually with NTDs exhibiting variable levels of severity [195]. Although some rare cases have genetic, geographical or socioeconomic causes, it is generally accepted that insufficient maternal folate status is the decisive risk factor for NTDs [196].

The work of Smithells and colleagues in the early 1980s commenced research into the role of folate in preventing NTDs [197]. Three major studies followed, confirming the role of folic acid supplementation in the prevention of NTDs, even for mothers who had previously had an affected newborn.

One of these studies was conducted by the British Vitamin Study Research Group of the Medical Research Council and involved seven European countries from which 1817 volunteers, including a number who had previously had pregnancies affected by NTDs, were recruited [40]. Women received 4 mg of folic acid daily from the time they planned their pregnancy until 12 weeks into it. After their delivery, the NTD recurrence rate of the group which received folic acid supplementation was 1%, but the recurrence rate of those in the non-folic acid group was 3.5% [40].

Another important study in Hungary illustrated that periconceptional folic acid supplementation was effective in reducing the prevalence of NTDs by over 90% [16, 198]. The third study was carried out in two regions of China: one region with a high rate and the other with a low rate of NTDs [199]. This study also showed a significantly reduced incidence of NTDs in both regions after folic acid supplementation with reductions of 41% in the 'low rate' region and 80% in the 'high rate' region, respectively.

The precise mechanism by which folate supplementation reduces the prevalence of NTDs is not yet clear although a couple of theories have been advanced [3, 16]. However, we do know that the risk of NTDs is inversely correlated with early pregnancy erythrocyte folate levels. Daly et al. (1995) [200] reported that in women who had an erythrocyte folate concentration over 400ng/mL, the risk of NTDs was about 88% lower when compared to women in a group with lower erythrocyte folate levels. In 1993, Kirke et al. [201] ascertained that not only folate, but also vitamin $B_{12}$ is related to risk of NTDs. Rosenquist et al. [202] used an avian embryo model to explain the mechanism of folate prevention of NTDs by direct treatment with Hcy and folic

acid. They concluded that maternal hyperhomocysteinaemia gives rise to dysmorphogenesis through altered neurulation related gene expression which is based on a growth factor like effect. Lucock et al. [203] gave another possible mechanism under which folate supplemation protects against NTDs, postulating that by reducing endotoxic Hcy or inhibiting MTHFR (as does $H_2$PteGlu), one-carbon units might be diverted toward DNA thymine. Genetic variants are also considered a determinant of NTD risk. Shields et al. [205] reported that the homozygous mutant of MTHFR C677T is related to NTD risk.

Christensen et al. proposed that the gene-nutrient interaction, i.e. MTHFR polymorphism and low folate status, is associated with a greater risk for NTDs than either variable on its own [88]. To confirm this proposal they assessed genotype and folate status in patients with spina bifida, mothers of spina bifida patients, children without NTDs (controls) and mothers of the controls. In 20% of spina bifida patients and 18% of their mothers homozygosity for the MTHFR polymorphism was found, compared to 11% of the control children and 11% of the control mothers. This may indicate that the mutant genotype confers an increased risk for NTDs. The risk was further increased if both the mother and the child had this genotype. RBC folate levels were lower in cases and case mothers, when compared to their respective controls. The combination of homozygous recessive MTHFR genotype and RBC folate in the lowest quartile conferred an odds ratio (OR) for being an NTDs case of 13.43 and an OR for having a child with an NTDs of 3.28. However, in a similar study, Lucock et al. [204] found no difference in MTHFR C677T mutation frequency between the control and NTDs groups. They also examined folate cellular distribution, finding an increased incidence of 5,10-$CH_2H_4$PteGlu particularly in mothers who gave birth to babies with NTDs.

The associations of most of the well-known polymorphisms in folate metabolism genes with NTDs have been investigated. Some significant results have been obtained. A66G MSR was found to be related to NTDs risk when combined with low vitamin $B_{12}$ status [95, 205]. MTHFR A1298C in combination with C677T, Catechol-O-methyl transferase G472A [206], DHFR 19-deletion [127] and GCP II C1561T [207] also showed an association with NTD incidence.

#### 1.5.2.3 Cardiovascular disease

In 1969, McCully first alluded to the significance of high blood Hcy in vascular pathology [208]. Since this time, high concentrations of blood Hcy have been considered an independent risk factor in promoting the development of vascular disease. A large number of retrospective studies provide evidence of this. According to Boushey's meta-analysis, the OR for CAD in males due to a 5μM Hcy increment is 1.6 (95% confidence interval (CI): 1.4 - 1.7) and in

females is 1.8 (95% CI: 1.3 - 1.9) [209]. Based on these findings, 10% of the population's CAD risk might be considered to be due to high concentrations of blood Hcy. Additionally, research suggests that an increase in plasma Hcy of 5µM is associated with an approximately 1.5-fold increase in the risk of CAD[210]. Recent results do not consistently confirm that Hcy is a risk factor for CVD, but indicate that it may, instead, be a simple risk marker for CVD [211]. However, two updated meta-analyses reported by Ueland et al. [212] and Humphrey et al. [213] continue to confirm the relationship between increased Hcy and risk of CVD.

#### 1.5.2.3.1 Vascular toxicity of homocysteine

Several mechanisms for the vascular toxicity of Hcy have been proposed [214]. A modest increase of Hcy concentration is related to endothelial dysfunction [215]. The first proposed mechanism could possibly contribute to Hcy-induced endothelial dysfunction via an elevated synthesis of asymmetric dimethylarginine (AMDA), an endogenous nitric oxide synthase inhibitor (nitric oxide induces vasodilatation) [216]. AMDA is suggested to be a significant mediator of Hcy-induced dysfunction. Small amounts of AMDA can be managed by endothelial cells, and methionine or Hcy up-regulates this reaction. The production of AMDA by endothelial cells is controlled by SAM dependent methyltransferases and up-regulated by native or oxidized low density lipoprotein. This means that Hcy can theoretically impair endothelial function [214].

A second vascular toxicity theory suggests that Hcy may have a pro-oxidant effect which leads to oxidative damage to endothelial cells [217, 218]. However, work in 2000 of Wilcken et al. [219] suggested that extracellular superoxide dismutase (EC-SOD) and Hcy could represent a positive relationship between an antioxidant response to Hcy-induced oxidative damage, indicating that the human body may have a protective mechanism for cardiovascular risk.

Another proposal is that Hcy might incite the release of inflammatory mediators. According to Hofmann et al. [220], Hcy induces enhanced vascular inflammation, atherogenesis and vulnerability within established atherosclerotic plaques. The authors also observed that the increase in oxidation of low density lipoprotein in the presence of increased Hcy supports this view. They advocated the concept of a “two-hit model,” as Hcy modulates vascular lesion development and sequence, likely by aggravating the risk associated with traditional factors such as hyperlipidemia. Recently, further evidence in support of this model was published which suggested that accumulated Hcy could be related to chronic immune activation in CAD patients [221, 222].

Finally, Hcy is a vigorous procoagulant that enhances the accumulation of fibrin and thrombosis in vein walls. Hcy generates the connective tissue changes of atherosclerotic plaques causing fibrosis, calcification, proteoglycan deposition and damage to elastic tissue layers, thus indicating that arterial stiffness is an independent determinant of CVD[223-225]. Pulse-wave velocity (PWV) is an index for stiffness of the arterial wall and Hcy might be related to PWV in patients with renal failure and hypertension [223, 226]. Hyperhomocysteinemia has been shown to affect smooth-muscle cells [227]. This effect can be ameliorated by folate administration [228].

Aside from the above theories, there is a report that Hcy may also affect the vascular endothelial cell cycle at the G1-S junction by the reduction of carboxyl methylation, membrane association, and activity of $p21^{ras}$-a major G1 regulator [229]. Hcy is related to renal function and hyperhomocysteinemia may reflect the presence of early nephrosclerosis [212].

#### 1.5.2.3.2 Folate, homocysteine and vascular disease

Folate status is one of the major factors to have an effect on blood Hcy status [230, 231]. Therefore, folate status possibly affects vascular lesions through its interaction with Hcy. Recently an alternative theory was postulated, suggesting that a low blood folate status might be an independent risk factor for CVD [232, 233]. Two representative studies demonstrated the relationship between folate levels and CVD risk. The Framingham Heart Study demonstrated that folate consumption and plasma folate concentration were inversely related to extracranial carotid stenosis. The lowest folate quartile (<2.51 μg/L) showed an OR of 1.9 for stenosis [234]. Another large case-control study, the COMAC European multi–cancer case control study demonstrated that the male case group had low plasma folate levels when compared to the control group. In addition, the concentration of erythrocyte folate under the $10^{th}$ centile was associated with increased risk of vascular disease [235]. In addition, vitamins $B_2$, $B_6$ and $B_{12}$, which are known to be related to folate metabolism, also have an effect on CVD, most likely through the Hcy transsulphuration pathway [236].

#### 1.5.2.3.3 The influence of genetic variation in vascular disease

Genetic elements, especially polymorphisms in folate metabolism genes, are major determinants in CVD aetiology. Deficiency of enzyme activity such as in CBS leads to a notable increase in Hcy and can bring about homocysteinemia [208]. In addition, MTHFR C677T was first reported to be a genetic risk factor for vascular disease [4]. Recently, it has been shown that the modest risk increase induced by the TT genotype is mostly generated by elevated Hcy and low plasma folate concentrations [51], yet not all studies consistently support the idea

that the recessive mutation genotype (TT) increases risk for CVD [237]. SHMT in combination with MTHFR C677T [238] and GCPII C1561T [239] has also been found to be associated with CVD risk. Interestingly, the MS A2756G polymorphism is reported to confer a protective effect against thromboembolism [86] and vascular disease by increasing the activity of MS and lowering Hcy concentration [240].

#### 1.5.2.4 Cancers

Folate has been considered to be a significant nutrient associated with cancer aetiology since the 1950s. Based on many folate-carcinogenesis studies [241-245], various epidemiological data suggest that folate is protective against cancer [246]. However, they do not show consistent relationships between carcinogenesis and folate nutrition.

Table 1.2 Candidate mechanisms for folate related carcinogenesis

| DNA methylation | DNA synthesis |
|---|---|
| -Aberrant DNA methylation | |
| -Alteration of gene expression | - Misincorporation of uridylate in place of DNA thymidilate |
| - Impaired DNA stability | |
| -Secondary deficiency of choline | |

# Adapted from Folate status Effects on Carcinogenesis [247]

##### 1.5.2.4.1 Epigenetic factors in carcinogenesis

Putative carcinogenesis mechanisms are likely to be influenced by various factors such as species, type of tumour, timing, dose and carcinogen [248, 249]. As folate is a major one-carbon source, it is considered to be a very critical nutrient for cancer development and progression. A number of proposed mechanisms for folate-carcinogenesis are listed in Table 1.2 [249]. Researchers infer that diminished folate status can cause aberration in major biosynthesis pathways involving folate. These include altered SAM, purine and pyrimidine synthesis. Folate deficiency possibly suppresses the progression of pre-existing neoplasms. By contrast, folate deficiency in normal tissues appears to predispose them to neoplastic transformation, with folate supplementation suppressing the development of tumours [250].

###### 1.5.2.4.1.1 Altered DNA methylation

DNA methylation is the addition of a methyl group at the carbon 5' position of cytosine within the cytosine-guanine (CpG) dinucleotide. DNA methylation occurs at CpG dinucleotides and

some of the CpA and CpT dinucleotides within the genome [251]. Methylation in the 5´ promoter regions or opening exons of genes which are CpG-dinucleotide rich regions (or "CpG islands") is considered to be significant [252, 253] since DNA methylation is a fundamental pathway for the epigenetic modulation of gene expression and evaluation of genomic integrity in mammalian cells. Elevated methylation in the promoter region of CpG islands generates reduced expression in general [253]. Hence, evaluation of the genomic methylation status is important in any study relating to cell growth control, tissue-specific differentiation and carcinogenesis [70].

A decline of genomic methylation is easily observed in early tumorigenesis and is consistent with the development of many kinds of cancer such as those of the colon, stomach, uterine, cervix, prostate, thyroid and breast [254]. Global DNA methylation status was observed to respond to folate intake [255-258] and folate intake seems to be very critical in the prevention of chromosome instability and DNA hypomethylation [257].

Site-specific aberrant methylation can occur in the absence of global DNA methylation changes [259]. It has been confirmed that some kinds of proto-oncogenes such as *p53*, *MYC*, *FOS* and *HRAS* are hypomethylated and mRNA concentration is elevated in folate deficiency. This suggests that hypomethylation of proto-oncogenes could generate the conversion of proto-oncogenes into oncogenes and play a significant role in hepatocellular cancer, cervical cancer, prostate tumours, B-cell chronic lymphocytic leukemia and gastric carcinogenesis [260-263].

Hypermethylation of CpG islands is also involved in the mechanism of carcinogenesis. Gene-specific hypermethylations are observed in many tumours. Methylated *RARβ, RASSF1A, CDNK2A* and *APC* were observed in lung cancer [264]. Oestrogen receptor α, the progesterone receptor, *BRCA1*, *GSTP1* and *TIMP-3* are generally methylated in breast cancer [265]. In haematological diseases, tumour-suppressor genes- $p15^{INK4B}$ were investigated and a higher prevalence of hypermethylation was found in high-risk myelodysplastic syndromes [266]. However, hypermethylation of the CpG islands can be prevented by mechanisms involving replication timing, local chromatin structures, active transcription and demethylation of DNA methyltransferase [267].

Recently, a CpG island methylator phenotype (CIMP) has been established to classify cancers and their aetiology in terms of 'epigenetic instability' [268]. CRC is classified into three groups by level of promoter methylation, frequency of microsatellite instability (MSI), location of tumour and mutations of significant genes in carcinogenesis such as *BRAF, KRAS* and *p*53 [269].

Genomic variations such as the MTHFR C677T polymorphism also have an influence on the methylation status of genes. It is reported that DNA from individuals with the 677TT genotype is hypomethylated and therefore has a higher capacity to accept the methyl group compared to DNA from 677CC individuals [270].

Other influences on methylation status include a group of methyltransferases and the other dietary methyl donors (lipotropes) and cofactors such as methionine, choline and vitamin $B_{12}$ [253].

#### 1.5.2.4.1.2 Disruption of DNA synthesis and stability

Folate is a critical substance for DNA synthesis and repair. Aberrant DNA synthesis due to abnormal folate nutrition status is another major factor in carcinogenesis. In folate deficiency the methylation of dUMP to dTMP is blocked. This methylation reaction is responsible for the provision of thymine. Insufficient methylation of dUMP to dTMP also interferes with the balance of DNA precursors to induce an accumulation of surplus deoxyuridine triphosphate (dUTP) in the nucleotide pool [271]. The results of such an accumulation include uracil miscorporation into DNA instead of thymine, chromosomal breaks in the phosphodiester backbone of DNA and the disruption of DNA repair.

DNA from cultured human myeloid cells grown in folate depleted media exhibits high uracil and reduced thymine concentrations [272]. In bone marrow cells from megaloblastic anaemia patients uracil is substituted for approximately 30% of the thymidine [273]. The human body has a mechanism for expunging DNA-incorporated uracil via glycosylase enzymes. This excision process, if continued, can lead to genomic DNA instabilities which include chromosomal translocation and deletion [253, 274].

MSI is another form of DNA instability which may be involved in carcinogenesis. Microsatellites are short tandem repeat sequences in DNA. MSI is reflected in the length change of these tandem repeats and is often associated with defects in DNA mismatch repair genes in many types of cancer including CRC and breast cancer [275, 276].

All cells have repair mechanisms for DNA strands that have been damaged, not only due to folate deficiency, but also by the actions of endogenous or exogenous reactive oxygen species (ROS). Folate deficiency decreases the deoxyribonucleotide triphosphate (dNTP) pool, and folate deficiency arising from insufficient consumption of folate is often accompanied by a deficiency of other antioxidant nutrients such as vitamin C, vitamin E and carotenoids. Therefore, it is possible that folate deficiency in conjunction with insufficient intake of other

antioxidants may bring about a limitation in DNA repair and impaired resilience to radical damage [277]. In a folate deficient rat model, the rate of DNA excision repair was decreased in folate deficient colonic mucosal cells when compared to normal mucosal cells [278]. There is also evidence that folate deficiency affects the DNA mismatch repair system in ulcerative colitis patients [279]. The above mentioned DNA/chromosomal breaks could be ameliorated by folate repletion [280].

Elevated genomic integrity can be achieved as a result of the increased availability of the MTHFR substrate 5,10-$CH_2H_4$PteGlu for DNA synthesis [253]. MTHFR variation shows a cancer modulating effect due to altered 5,10-$CH_2H_4$PteGlu. An elevation in 5,10-$CH_2H_4$PteGlu would lead to increased nucleotide synthesis [274]. The distribution of folyl vitamers is different for each MTHFR C677T genotype. According to Bagley [274], MTHFR TT individuals showed formylated forms of $H_4$PteGlu in their erythrocytes, but CC individuals only had 5-$CH_3H_4$PteGlu. This result possibly explains the protective effect of MTHFR TT genotype via an altered folyl vitamer partitioning within the cell.

#### 1.5.2.4.2 Colorectal cancer

According to the World Health Organization about 608,000 people died of CRC in 2008 worldwide, making CRC the second most common cancer in adults globally [281]. Mortality from this condition is approximately 50%. The prevalence of CRC is highest in North America, Australia/New Zealand, Western Europe, and Japan [55, 282].

CRC is caused by accumulated genetic and epigenetic factors transforming normal glandular epithelium into invasive carcinoma. The pathway for this transformation could be a classic adenoma→carcinoma sequence model (see Figure 1.15). Approximately 5-10% of adenomas turn into carcinomas, with the major histological type (90-95%) of CRC being adenocarcinomas [283-285].

Amassed data indicates that there are multiple molecular pathways which contribute to CRC development and progression. For instance, MSI and DNA methylation are related to serrated polyps, whilst inactivation of tumour suppressor gene and chromosomal instability are associated with tubular adenomas [285]. Oncogenes or tumour suppresser genes such as *MLH1*, *MGMT* and *APC* are likely to play a critical role in CRC aetiology. For example *APC,* a tumour suppressor gene, is known as the gatekeeper gene for colorectal epithelium cells because approximately 85% of CRC cases show the loss of function of *APC* in the initial stage and it also appears in aberrant crypt foci. When *APC* is silenced by aberrant methylation and

mutation, it activates expression of oncogenes such as *C-MYC* and *CYCLIN D1* via cascade reactions [283, 285].

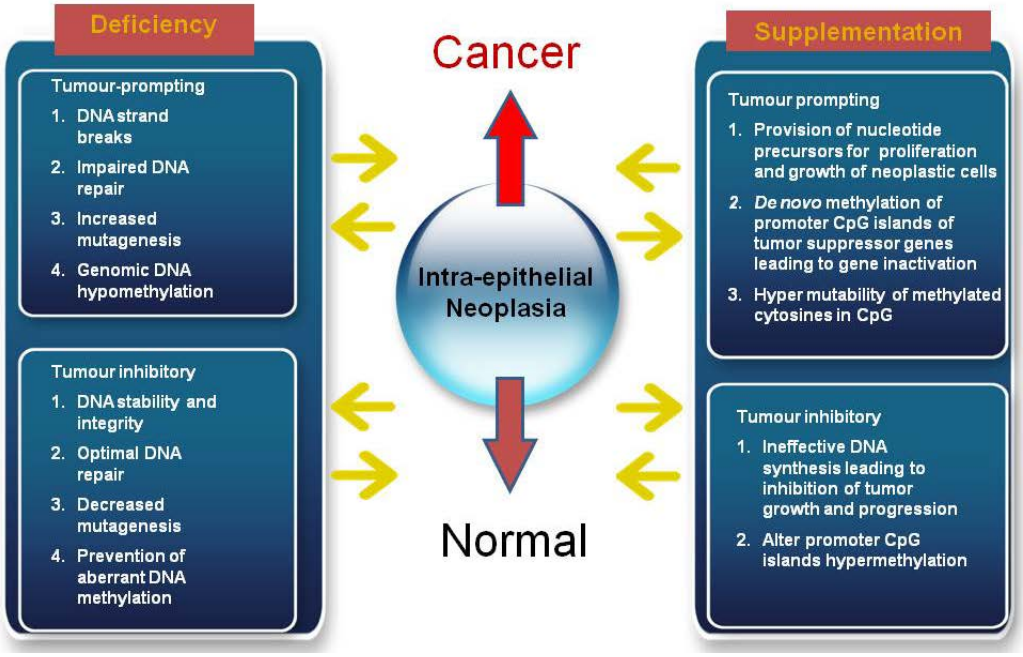



Figure 1.15 Putative mechanisms for folate related carcinogenesis indicating that folate may have opposing biological effects [134, 286]

CRC is the best studied disease for which folate is considered to be a major aetiological factor [250]. However, the results of studies are not consistent and depend on factors such as gender and the site (colon or rectum) of its origin. Several large studies (the Netherlands Cohort Study on Diet and Cancer and the First National Health Study and the Nutrition Examination Survey (NHANES) and its epidemiologic follow-up study) indicated that folate is clearly related to CRC with high folate intake reducing CRC risk by about 40% when compared to low folate intake [250, 287-289]. The Nurses' Health Study conducted in the United States showed that there was a 75% reduction in CRC risk in women using multivitamin supplements containing 400µg of PteGlu [290]. According to Sanjoaquin [243], meta-analysis showed that folate intake and CRC risk are inversely related, with dietary folate consumption, in particular, looking more significant than synthetic folic acid supplements (OR= 0.75, 95% CI: 0.64-0.89). In addition, Jaszewski et al. [291] published results showing that a high dose of folic acid could reduce CRC risk and high serum and erythrocyte folate might have a protective effect against CRC [292, 293].

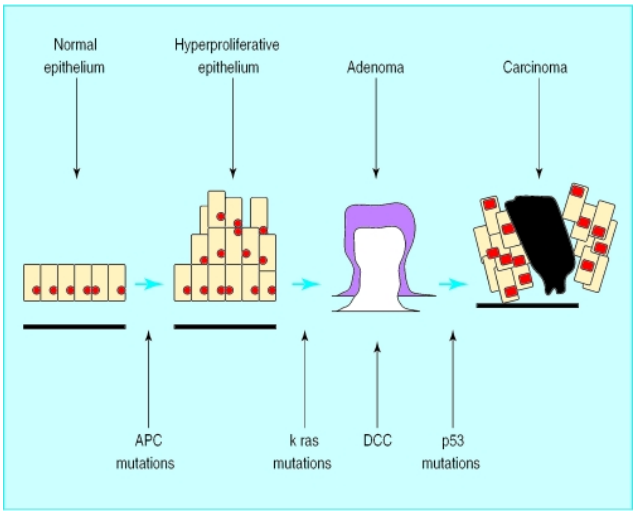



Figure 1.16 Brief scheme of adenoma to carcinoma sequence in CRC [294].

However, a small number of published studies suggest that high folate status or folate supplementation might have a harmful effect. A Swedish study published in 2006 showed that low folate levels might have a protective effect against CRC, with high plasma folate concentrations significantly increasing CRC risk (OR= 3.87, 95% CI: 1.52-9.87) [54]. In addition, in one massive randomized cohort study (the Aspirin/Folate Polyp Prevention Study, 2007[295]) 516 participants were administered 1mg of folic acid on a daily basis, whilst 505 controls were given a placebo. An eight year follow-up survey showed that the folic acid supplementation group tended to develop more colonic lesions than the control group. However, this finding was not statistically significant.

MTHFR C677T variation is also considered to be one of the important factors involved in colorectal carcinogenesis. There are many studies examining the question of whether this variation reduces CRC risk. Several conflicting research findings have been reported. However, Ulvik [56] and Van Guelpen [54] reported that TT genotype may reduce CRC risk and, according to recent meta-analysis, which includes 29 studies and 14 pooled analyses, MTHFR C677T variation decreases CRC risk. The OR for CRC for people with the CT genotype was not statistically significant, but the OR for the TT genotype was 0.83 (95% CI: 0.77-0.90). The finding of an inverse association between MTHFR 677 TT genotype and CRC was statistically significant in Asian and Caucasian populations in the United States, but was not statistically significant in European populations [55].

As previously mentioned, MSI may also be involved in CRC carcinogenesis. It is estimated that MSIs are associated with approximately 10 to 15% of sporadic colorectal carcinomas, particularly proximal colon cancer [296, 297]. These results indicate that the MTHFR 677TT genotype effect on MSI-colon cancer [298, 299], and the relationship between MTHFR genotypes and MSI is influenced by folate status [296, 300]. It is also estimated that 35% of CRC arises via aberrant DNA methylation [301]. Global hypomethylation appears in the early steps of the polyp → cancer progression [285]. It has also been specifically observed within neoplasm tissue. Schernhammer et al. found that for participants in their study with colon cancer, low dietary folate intake was significantly associated with low tumoral LINE-1 methylation levels [304]. In addition, Pufulete et al. found that, when compared to controls, subjects in their study population with colon cancer exhibited lower blood folate status and DNA hypomethylation in their colonic mucosa [111].

#### 1.5.2.4.3 Breast cancer

Breast cancer is the most prevalent type of cancer among women worldwide [302]. The most common histologic types of invasive breast cancer are ductal and lobular carcinomas, accounting for approximately 75% and 15% of all breast cancer cases, respectively [303].

Breast cancer, like many other cancers including CRC, is a manifestation of genetic and epigenetic changes. The promoter hypermethylation of genes such as *P16INK4α, BRCA1, BRCA2, ERα, RARβ2, APC*, and *RASSF1A* has been observed in the early stages of breast cancer and is usually included in the models which evaluate individual breast cancer risk [276]. A variety of significant factors such as genetics, hormones (especially oestrogens), inheritance of mutations, ionising radiation and dietary factors (notably vitamin A, E, C, carotenoids and folate) are associated with this epigenetic level of change and risk of breast cancer [304].

Much research has examined the association between breast cancer and folate consumption, but some studies have not shown significant results [249, 305, 306]. However, in a Uruguayan study, total dietary folate as well as vegetable, fibre, vitamin C and E intakes were inversely associated with breast cancer risk ($4^{th}$ quartile OR for total vegetable intake = 0.41, 95% CI: 0.26-0.65) [307], and an Italian study reported that high folate intake reduced breast cancer risk (OR for $1^{st}$ quartile folate intake group compared to lower quartiles was 0.45 (95% CI: 0.27-0.74)) [308].

Several other studies such as the Nurses' Health Study and Canadian National Breast Screening Study have suggested that breast cancer risk reduction by folate is particularly

relevant in women consuming alcohol. Zang et al. [41] explained that the risk of breast cancer was associated with alcohol intake in women whose total folate intake was less than 300μg/day (multivariate RR, 1.32; 95% CI: 1.15-1.50). Similarly, the effectiveness of folate in reducing breast cancer risk in women who consumed alcohol was reported by Rohan et al [309]. The protective effect was shown to be stronger in postmenopausal women whose alcohol intake was over 14g per day (OR=0.28, 95% CI: 0.14–0.55). Since alcohol is considered to be an antagonist of folate [310], alcohol consumption could increase the requirement for folate intake. One meta-analysis published in 2007 showed a non-significant association of breast cancer risk with folate intake and blood folate concentration overall. However, the meta-analysis did find a statistically significant association of breast cancer risk with folate intake and blood folate concentration among those who consumed moderate or high amounts of alcohol (OR = 0.51, 95% CI = 0.41 - 0.63) [311]. These results suggest that folate supplementation could reduce the risk of breast cancer and maintain adequate circulating levels of folate where alcohol consumption is elevated [312].

However, recent studies conducted in France and Sweden have suggested that the protective effect of folate may be distinct from alcohol intake [313, 314]. Additionally, there is an interesting report that increased dietary folate intake or plasma folate concentration is positively associated with breast cancer risk [315, 316]. In addition, high Hcy and cysteine concentrations have been shown to place women at greater risk for developing breast cancer when their folate levels are low [317]. Whether other one-carbon sources such as the nutrients betaine and choline are related to breast cancer risk is still controversial [318, 319].

The MTHFR C677T polymorphism has been intensively studied to verify any association with breast cancer in terms of gene-nutrient interactions. Some pooled and meta-analyses reported no significant associations between this variation and breast cancer risk, but many individual studies reported significant findings [320]. Suzuki et al. [57] suggested that the MTHFR 677 TT genotype increased risk of postmenopausal breast cancer (OR = 1.83, 95% CI: 1.08-3.11) and this association was stronger in women with a lower consumption of dietary folate compared with those with the 677 CC genotype and adequate folate consumption (OR = 2.80, 95% CI: 1.11-7.07). Maruti et al. [58] observed an approximately 60% increase in risk for MTHFR 677 TT individuals. The latest meta-analysis report [59] shows that the increase in risk for individuals with the MTHFR 677 TT genotype was significant, but was a low-penetration risk factor for developing breast cancer (TT vs. CC: OR = 1.11, 95% CI: 1.01-1.23).

Johanning et al. [324] found that breast cancer tissues had globally hypomethylated DNA and a decreased vitamin $B_{12}$ level, but that folate concentrations in breast cancer tissues were increased. Another report suggests that DNA methylation profiles for breast cancer vary depending on the size of the tumour as well as alcohol and folate intake [321]. The differences in methylation pattern according to MTHFR C677T genotypes have not been reported yet [322], but in an *in vivo* breast cancer model it was confirmed that the C677T mutation could produce genomic DNA methylation alteration. At high or moderate folate status the MTHFR C677T mutation was significantly associated with decreased genomic DNA methylation, but did not respond when folate status was low [323]. The DNA damage status of breast cancer patients' mononuclear cells is significantly higher when compared to benign breast disease controls [324].

#### 1.5.2.5 Other folate related disorders

##### 1.5.2.5.1 Down syndrome

Down syndrome is a consequence of trisomy occurring at human chromosome 21. 95% of Down syndrome patients have a third copy of this chromosome. In the remaining 5% there is a partial copy or mosaicism of chromosome 21 [325].

Trisomy of chromosome 21 alters folate and Hcy metabolism as at least 7 folate metabolism genes are located on chromosome 21. Triple copies of these genes possibly elevate folate requirements, leading to a functional folate deficiency in the developing Down syndrome fetus [326, 327].

In 1999 it was first reported that aberrant folate and Hcy metabolism due to variation in a folate metabolism gene might be involved in an increased risk of having a baby with Down syndrome. Mothers who have the MTHFR C677T polymorphism show a higher risk of having a Down syndrome infant when compared with mothers who do not carry the MTHFR 677T allele (OR= 2.6, 95% CI: 1.2-5.8). Indeed, Hcy concentration was found to be significantly higher in MTHFR CT+TT group mothers with Down syndrome children [328]. This association has been examined in many countries with inconsistent findings. Studies in North America, India, China and Egypt showed that the MTHFR C677T polymorphism increased the risk of Down syndrome [328-331]. In Brazil, Japan, Italy, and Northern India [332-335] the association was found to be not statistically significant. This conflicting evidence may reflect the frequency of this polymorphism in these areas and/or the interaction of this genotype with environmental factors such as diet [327].

A further risk factor for Down syndrome that is related to folate metabolism is Hcy concentration. A few studies have shown that mothers of Down syndrome children have a higher Hcy concentration than controls [328, 333, 336]. Another hypothesis is that altered folate metabolism might affect meiosis I. If this phenomenon was initiated during the foetal development of the mother, then it is possible that the maternal grandmother's environment during her pregnancy is of importance in the determination of Down syndrome risk [327].

#### 1.5.2.5.2 Dementia

Folate deficiency is known to be associated with neuropsychiatric disorders, for example, depression, cognitive decline, vascular dementia, schizophrenia, bipolar disorder and Alzheimer's disease (which accounts for 70% of dementia cases) [337]. About 80% of psycho-geriatric patients are assumed to be affected by folate insufficiency [28]. It is postulated that adequate folate and cobalamin consumption moderates cognitive brain functioning through effects on cerebrovascular health and that Hcy is associated with poor memory and cognitive performance as well as dementia [338]. Previously published studies showed that Hcy concentration and cognitive function were inversely associated [339-341]. Seshadri et al. [341] found that a 5µM increment in the plasma Hcy concentration elevated the risk of Alzheimer's disease by 40%.

To date, several theories have been postulated to explain the association between high Hcy levels and Alzheimer's disease. As dementia is worse in the presence of a brain infarction [346], it is possible that hyperhomocysteinaemia could be one factor in the development of Alzheimer's disease via its induction of vascular changes. In cell culture models Hcy has a neurotoxic effect by activating N-methyl-D-aspartate receptors [342]. Another hypothesis which could explain this association is that neuronal death by folate deficiency and high Hcy levels promotes accumulation of DNA damage and sensitizes neurons to amyloid beta-peptide toxicity [343, 344].

The accumulated literature has implicated folate in the development and progression of multiple kinds of degenerative diseases and congenital disorders. However, due to the complexity and diversity of disease aetiology, it is not yet possible to explain the precise mechanisms behind the action of folate in all disease aetiologies. Experimental evidence shows that increased folate intake could give us many benefits in maintaining health. In particular, the administration of folic acid supplements has resulted in a significant reduction in the occurrence of NTDs, strengthening the advocacy for, and the implementation of, mandatory folic acid fortification in a large number of countries. However, some controversial and

unexpected issues have been reported with mandatory folic acid fortification. Section 1.6 is devoted to a description of these issues.

## 1.6 Folic acid metabolism, fortification and contemporary Issues

In the late 1990s the food industry began to offer folic acid fortified food to the health conscious consumer. In addition, since 1998, mandatory folic acid fortification has been implemented in the United States. To date, about 60 countries have introduced mandatory folic acid fortification as a population health measure [345]. The level of fortification differs between countries, but in all cases it is designed to reduce the prevalence of NTD pregnancy. It appeared that this folic acid supplementation was successful because population folate status was enhanced [346] and there were dramatic reductions in NTD rates in Canada and the United States [347-350]. However, emerging evidence suggests that folic acid supplementation might be responsible for the development and progression of undiagnosed malignancies and other adverse phenomena [286, 351].

### 1.6.1 Folate fortification and policies in selected countries

Table 1.3 presents data on the folic acid fortification levels and the rate of NTD reduction in four countries including the United States, Canada, Australia and Chile. Cereals and flour for baking bread are the main vehicles for fortification because bread and cereal are commonly consumed by the target population: women of child bearing age (16–44 years) [352]. Mandatory folate fortification was implemented in Australia in September, 2009. As a result, no post-fortification data is available as yet.

Table 1.3 Recommendations for folic acid intake, folic acid fortification policies and reduction of NTDs in four countries

| Country | Folate RDI | Folic acid fortification policy | Implemented year | NTD reduction |
|---|---|---|---|---|
| United States | 400μg/day<br>600μg/day- pregnancy<br>500μg/day- lactation | 140μg folic acid/100g grain product [353] | 1998 | 19% [347]<br>31% [348] |
| Canada | United States requirements | 150μg folic acid/100g flour & 200μg folic acid/100g pasta [354] | 1998 | 46% [350] |
| Chile | Ambiguous | 220μg folic acid/100g flour [355] | 2000 | 51% [356] |
| Australia | 400μg/day<br>600μg/day - pregnancy<br>500μg/day - lactation | 135μg folic acid/100g flour [357] | 2009 | N/A |

# Adapted from Lawrence et al. 2009 [352]

However, in Australia, voluntary folic acid fortification of food has been allowed since 1995, with the prevalence of NTDs being reduced by approximately 30%, except in Aboriginal populations [358, 359]. Australia and New Zealand have a common committee (Food Standards Australia New Zealand) which is responsible for fortifying food with vitamins and minerals including folate. Australia decided to administer mandatory fortification of flour (excluding flour in organic bread) from September, 2009. New Zealand deferred the implementation of mandatory folic acid fortification for 3 years [360] and decided to keep fortification voluntary in August, 2012 (imposing a maximum level of 250µg folic acid/100g flour) [361].

### 1.6.2 Improved blood folate level

In the United States the folate status of the population has been monitored since mandatory folic acid fortification was first introduced. From NHANES data it can be seen that, comparing pre-fortification (1988–1994) and post-fortification (1999–2010) periods, serum and RBC folate concentrations have increased dramatically (see Table 1.4) [362].

Table 1.4 Mean concentrations of serum and RBC folate for two periods in the United States (±SD)

| | 1988–1994 (pre-fortification) | 1999-2010 (post-fortification) |
|---|---|---|
| Serum folate (nM) | 16.7 (0.5) | 41.0 (0.3) |
| RBC folate (nM) | 747 (10) | 1120 (7) |

# Adapted from Pfeiffer et al. 2012 [362], from NHANES data, pre-and post-fortification, participants ≥ 4 years old

### 1.6.3 PteGlu absorption and dihydrofolate reductase

As explained above, PteGlu is not a natural form of folate and it requires additional metabolic steps before it can enter the circulating plasma folate pool as 5-$CH_3H_4$PteGlu. PteGlu is absorbed in the proximal jejunum through a saturable, carrier-mediated, pH and energy dependent transport mechanism. Excess PteGlu above normal physiological concentrations (400µg) is inactively transported [363] and daily administration of 260-280µg of PteGlu may give rise to the appearance of unmetabolized PteGlu in the plasma [364].

In order to enter folate metabolism, PteGlu needs to be reduced first to $H_2$PteGlu and then to the active form, $H_4$PteGlu. DHFR converts synthetic PteGlu to $H_2$PteGlu and on to $H_4$PteGlu which is the methyl group shuttle required for the *de novo* synthesis of purine and

thymidylate. The activity of DHFR in the human mucosa is significantly less than in other mammals and shows an inferior capacity to reduce synthetic folic acid. Therefore, the habitual intake of a moderately high dose of folic acid (mainly from folic acid supplements or fortified food) could result in the chronic appearance of unmetabolized folic acid in the circulatory blood system (see Figure 1.17) [365].

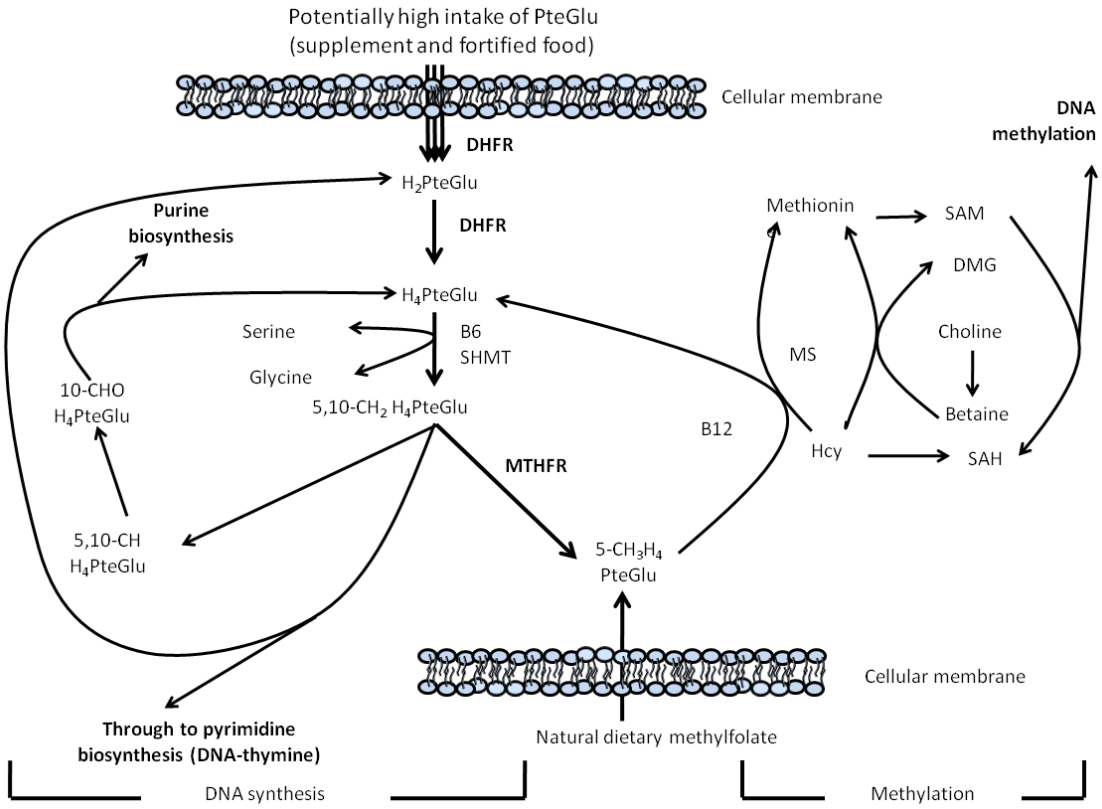



Figure 1.17 Folate metabolism for high folic acid intake (DHFR: dihydrofolate reductase, SHMT:Serine hycroxymethyltransferase, MTHFR: methyltetrahydrofolate reductase, MS: methionine synthase, DMG: dimethylglycine)

### 1.6.4 Adverse effects of excess folic acid and current controversial issues

Since the initiation of mandatory folic acid fortification in several countries, the main goal of reducing the prevalence of NTDs has been achieved. However, since all people are now exposed to plentiful folate, unexpected adverse phenomena are likely to arise. In addition, since folic acid fortification has commenced, more circulating unmetabolized folic acid has been observed as a component of the total blood folate value. How this newly changed folly vitamer distribution influences human folate metabolism is not yet known, but it needs to be understood because of possible long term effects.

### 1.6.4.1 Changed cellular folate distribution by folic acid fortification

To date, most of the limited amount of available data comparing changed blood folyl vitamer distribution as a consequence of fortification has come from the United States.

A study by Kelly et al. [364] indicated that no one had PteGlu in their fasting blood before the PteGlu intervention. Kalmbach and colleagues [366] examined the concentration of blood PteGlu before and after folic acid fortification within the Framingham Offspring Cohort (see Table 1.5). The results showed that, prior to PteGlu fortification, the proportion of subjects with detectable PteGlu stood at 55% in non-B vitamin supplement users, but after PteGlu fortification this increased to 74.4%. Even in B vitamin supplement users the ratio for subjects with detectable PteGlu increased from 72.5% to 80.7%. It was hypothesized that this high ratio of subjects with detectable PteGlu from the pre-fortification period was due to voluntary folic acid fortified foods.

Table 1.5 5-$CH_3H_4$PteGlu and circulating unmetabolized folic acid status from the Framingham Offspring Study pre- and post-fortification (median, 95% CI)

| | Non B vitamin supplement users | | B vitamin supplement users | |
|---|---|---|---|---|
| | Pre | Post | Pre | Post |
| PteGlu intake (μg/d) | 32.4 (27.9, 37.0)[a] | 241.4 (224.1,259.2)[b] | 399.4 (378.2, 421.2)[c] | 601.4 (568.6, 635.1)[d] |
| Total plasma folate (nM) | 19.5 (18.8, 20.4)[a] | 37.2 (35.3, 39.4)[b] | 30.8 (29.2, 32.9)[c] | 40.6 (37.8, 43.5)[b] |
| Total 5-$CH_3H_4$PteGlu (nM) | 19 (18.3, 19.9)[a] | 36.3 (34.2, 38.3)[b] | 30.1 (28.5, 31.9)[c] | 39.2 (36.5, 41.9)[b] |
| Circulating PteGlu (nM) | 0.25 (0,15.18)[a] | 0.5 (0–24.11)[b] | 0.54 (0–19.78)[b] | 0.68 (0–33.94)[c] |
| Subjects with detectable PteGlu (%) | 55 (51.1, 58.9)[a] | 74.7 (69.5,79.9)[b] | 72.5 (66.9,78.1)[b] | 80.7 (74.2,87.2)[b] |

# Adapted from Kalmbach et al.[366], PteGlu intake square root transformed and values were adjusted for total energy. Values in a row with common superscript letters are significantly different, $p<0.05$

In Troen's study [367] unmetabolized PteGlu was detected in 78% of subjects. Unmetabolized circulating PteGlu may promote aberrant DNA synthesis (see Figure 1.16) and affect the methylation process. It is also possible that it is an antimetabolite for gene expression products in folate metabolism. This may be one of the causes for the reported adverse effects of excess PteGlu intake, but little clear research evidence exists as yet [131].

#### 1.6.4.2 Masking cobalamin deficiency and declined cognition

Vitamin $B_{12}$ is an important coenzyme of MS and is related to folate metabolism via methionine biosynthesis. Therefore, since vitamin $B_{12}$ is closely connected with folate nutrition, insufficient cobalamin levels may generate megaloblastic anaemia as occurs in folate deficiency alone. The excessive intake of PteGlu can mask vitamin $B_{12}$ deficiency and interrupt the diagnosis of its symptoms, such as megaloblastic anaemia and some well-recognized neurologic consequences (pernicious anaemia). This vitamin $B_{12}$ masking could possibly delay the diagnosis of pernicious anaemia until demyelination becomes irreversible [368].

Declined cognition has also been reported as an adverse effect of excess folate, while serious deficiency of cobalamin can cause neurologic abnormalities [369]. 10 to 15% of the elderly population (over 60 years old) are not taking sufficient vitamin $B_{12}$ [370]. Morris et al. [371] suggested that a high intake of folate may be associated with faster cognitive decline in the elderly. When compared to a group with high folate intake, a group with high total vitamin $B_{12}$ intake showed slower cognitive decline. As a result, in the United States, the Food and Nutrition Board of the Institute of Medicine established a tolerable upper intake level for folate (UL) of <1000µg per day [368].

#### 1.6.4.3 Antifolate medication (methotrexate)

MTX has been widely used as an antifolate metabolite and was the first drug that was curative for patients with solid tumours. It is also the most commonly used anti-inflammatory drug for rheumatoid arthritis. MTX inhibits DHFR which reduces PteGlu to its biologically active form, depleting the intracellular activated folate form and perturbing cellular metabolism [372]. MTX can be converted into a polyglutamate just like natural intracellular folates by FPGS which converts folylmonoglutamates into folylpolyglutamates [373]. Although PteGlu supplementation, in concert with MTX therapy, has been recommended to reduce the toxicity of MTX on blood cells, gastrointestinal tract and liver [374], one concern with respect to PteGlu fortification is how elevated blood folate levels and the presence of unmetabolized PteGlu interfere with folate antagonists.

Arabelovic and his colleagues [375] computed MTX doses per patient per year, comparing the overall mean MTX doses before and after 1997 (when mandatory folic acid fortification was instituted) for thirty-six rheumatoid arthritis subjects in the United States. Although the study involved a small number of subjects and reported only preliminary data, it determined that mean annual MTX dose was statistically higher after folic acid fortification [376].

#### 1.6.4.4 Reduction in cytotoxicity of natural killer cells

As explained above, Troen et al. [367] reported that unmetabolized PteGlu was found in the circulation of 78% of high folate intake subjects after folic acid fortification was implemented, and that this unmetabolized PteGlu in plasma is associated with decreased natural killer cell cytotoxicity. The association with PteGlu was also independent of circulating 5-$CH_3H_4$PteGlu and total folate. As there has been no follow-up research related to this reduced cytotoxicity, further studies are clearly needed.

#### 1.6.4.5 Elevated maternal folate may influence fat mass and insulin resistance of offspring

In a six year follow-up study of pregnant Indian women, higher maternal erythrocyte folate levels predicted higher offspring adiposity and higher homeostatic model assessment of insulin resistance. Furthermore, high insulin resistance was observed in the offspring born to mothers with a combination of high folate and low vitamin $B_{12}$ levels. It is hypothesised that folate trapped as 5-$CH_3H_4$PteGlu (by vitamin $B_{12}$ deficiency) and increased methylmalonyl-CoA could confer elevated lipogenesis [377]. There is another interesting report with regard to high PteGlu and breast milk. According to Houghton, pregnant women who were administrated a PteGlu supplement during their pregnancy showed the presence of unmetabolized PteGlu in their milk and low milk folate binding protein synthesis [378].

#### 1.6.4.6 Increased twin births

Twin or multiple pregnancies are a risk factor for maternal and infant morbidity and mortality [379]. Folic acid fortification has been reported to be associated with an increase in twin births. A randomized-cohort study of Hungarian women showed around a 40% increase of twin births in women who took multivitamin supplements, including PteGlu, compared with women who took just trace elements [380]. In addition, some Swedish [381, 382] and Chilean [383] studies report an increased incidence of twin births after folic acid fortification was implemented. This increasing incidence of twin births has also been observed in the United States [384-387].

Recently it has been suggested that folate fortification might increase the success rate of in-vitro fertilization (IVF). Haggarty and colleagues [388] reported that high plasma and erythrocyte folate contribute to increased IVF success. However, it is a complicated issue because multiple births, as mentioned above, are risky and might lead to a “fear of folate”. Hence, the implementation of folate fortification should be carefully assessed with further research evaluating the role of current nutritional status, diet, food distribution, and genetic background [389, 390] .

#### 1.6.4.7 Increase of colorectal cancer risk

In Chile, mandatory folic acid fortification has been implemented for wheat flour since January 2000. Hirsch et al. [391] examined the rates of hospital discharges of patients with colon cancer in Chile. Interestingly, CRC was increased by 162% in the 45–64 year group and by 192% in the 65–79 year group. Increased CRC incidences after folic acid fortification were also reported by Mason et al. in Canada and the United States where folic acid fortification commenced earlier [392]. As explained above, in the Aspirin/Folate Polyp Prevention Study, participants in the group treated with folic acid were administered 1000µg of PteGlu per day for 5 years. This administration did not decrease the risk of adenoma incidence in the large intestine, and it is possible that the folic acid supplementation may have contributed to the recurrence of colorectal adenomas [295]. A Swedish study found that low folate levels possibly have a protective effect against CRC. However, there is some clear evidence that folate is associated with a low incidence of CRC [243, 250, 287-289, 291-293]. These contrasting results could form the basis for an hypothesis that only natural dietary folate has protective effects with respect to CRC risk, whereas PteGlu from supplements and fortification may elevate the disease risk [393].

#### 1.6.4.8 Photosensitization of PteGlu

Scientists have known about the break-down of PteGlu through photolysis since the 1940s [394]. When exposed to UV radiation, PteGlu breaks down into the products p-ABG and 6-FP, the latter of which eventually oxidizes to form PCA. The photosensitivity of PteGlu suggests that there may be an association between *in vivo* PteGlu photolysis rates and skin pigmentation since dark skin protects body folate status from photolysis by UV radiation [11, 395].

Interesting evidence has been put forward suggesting that altered folate cellular distribution may play a role in carcinogenesis, possibly by affecting DNA stability. Degradation products of PteGlu, 6-FP and PCA could be potential mutagens and/or carcinogens. PCA has been shown to produce sequence-specific DNA lesions [396]. This DNA cleavage could be a cause of diseases including cancer. Indeed, folic acid fortification in high doses could be limited by the saturation of DHFR. Over supplementation may result in more unmetabolized circulating PteGlu in the blood which likely accelerates the DNA break-down process. In addition, the activity of DHFR varies between individuals [131]. It has also been proposed that 6-FP is likely to be associated with cancer development and progression. Halpern et al. [397] discovered that only cancer cell

lines secreted 6-FP into their media and, additionally, that 6-FP only existed in the urine of patients with malignant disease [398, 399].

Despite such conjecture, folic acid fortification has achieved its main goal: the reduction of NTDs along with other health benefits such as decreased mortality rates after stroke [400]. In the context of public health benefits, folic acid fortification should be evaluated with caution. New perspectives and approaches, especially at a molecular level, with respect to folate research in relation to dietary intervention are required.

## 1.7 Folate research involving an *in vitro* model

To date, a large number of studies have been conducted which attempt to explain how folate functions in the human body. Although these studies have provided some answers, many important questions remain unanswered. An important and new question is 'what influence does excess folic acid have on cellular processes?' This question leads to further questions such as 'what is the possibility for other folate vitamers to replace synthetic PteGlu?', and 'what might their relative efficiency, stability and economic advantages be?'.

Most previous studies focus on the interaction of folate and anti-cancer medicines in the context of inhibiting neoplastic growth using transformed cancer cell lines. There is not much evidence to explain the effects of long-term exposure to excess synthetic PteGlu and the various natural folyl vitamers. These questions are arguably most relevant to non-cancer and cancer cell growth kinetics where the dietary and therapeutic use of folate has its primary focus.

### 1.7.1 *In vitro* research - cell line model

Mammalian cell culture is an experimental method which has rapidly grown in use over the last couple of decades as an *in vitro* technique [401, 402]. In many fields of the life sciences, cell culture has become a basic and critical technology [403]. In higher organisms, such as *in vivo* models, a high degree of interrelationship and interaction occurs between the myriad of intercellular and intracellular mechanisms. On the other hand, cell line models provide a simplified paradigm for experimental modification, allowing the researcher to minimize the interference of other factors without ethical constraints. Table 1.6, which is adapted from Freshney [408], highlights the advantages and limitations of cell culture models.

Table 1.6 The main advantages and shortcomings of cell culture models

| **Advantages** | |
|---|---|
| Complete control of environment | Ensures the reproducibility of results |
| Economic model | Much less expensive than working on live animals |
| Avoids ethical issues | No informed consent required; researcher may be freely make modifications |
| Cryopreservation | Long term conservation and indefinite use of cell lines |
| **Limitations** | |
| Simplicity | Over simplicity implies difficulties in extrapolating |
| Limited representativeness | Cell lines cannot cover all of the tumour features |
| Stability/instability | Dedifferentiation and divergence induced from long-term cell culture process result the loss of special characteristics |

Cell cultures can be categorized into primary and secondary culture models, defined by the cell types to which the cell lines belong. They are also sub-classified by their life span, cell growth patterns and origin. Various types of cell lines have been established commercially or non-commercially, providing researchers with a selection depending on their experimental requirements.

### 1.7.2 Latest research relating to folate and colon and breast cancer in *in vitro* models

As previously described, folate plays a significant role in colon and breast cancer. Therefore many studies have been carried out using *in vitro* models, particularly with regards to colon cancer. According to the Pubmed data base, the first paper involving folic acid and a colon cancer cell line was published in 1981 by Houghton [404]. This study investigated the responsiveness to 5-FU and its derivatives in xenografts of human colorectal adenocarcinomas in mice. Much research has subsequently been done to understand the pharmacological effects of chemotherapy drugs such as MTX, 5-FU, gefitinib and erlotinib and their influence on folate metabolism [405-408].

The biochemical roles of folate in basic cellular processes were discovered over 30 years ago. Further attention has been focused on these roles in the latest folate-depletion studies. Not much information is available to ascertain the precise long-term exposure effects to different levels of extracellular folic acid and other folyl vitamers. However, this information is definitely necessary for an evaluation of the effects of chronic or sub-chronic exposure to increased systemic blood folate levels by dietary (discretionary or mandatory) fortification [409].

Folate depletion and gene expression have previously been reported. Novakovic and colleagues [410] studied the effects of folate deficiency on gene, apoptosis and cancer pathways in colon cancer cells and proved that folate deficiency affects the expression of key genes that are involved in cell cycle control, DNA repair, apoptosis and angiogenesis. Lemos [411] studied the expression of a specific gene (ABCG2) as the result of folate deprivation. Further research on folate deficiency and gene expression was carried out by Crott [412] who ascertained that moderate folate depletion controls the expression of selected cancer risk genes involved in the cell cycle, intracellular signalling and folate uptake in human colonic epithelial cell lines.

However, not many studies showing the action of folate and its vitamers on cells have been published. Jaszewski and colleagues [413, 414] reported that folic acid inhibits EGFR-mediated

proliferation in human colon cancer cell lines, but they only experimented with a fixed concentration of PteGlu and no other folyl vitamer forms or concentrations.

Akoglu et al. [420] carried out a study in which the hyperproliferation status of colon cancer cells which were treated by Hcy was reversed by PteGlu and 5-$CH_3H_4$PteGlu. However, the characteristics of folyl vitamers with regard to their stability and interactions with antioxidants were not considered, and the cell proliferation was only measured over a period 24 hours. The cells were treated with a high concentration of Hcy (1μg/mL and 10μg/mL) prior to the folyl vitamer treatment, and the concentrations of folyl vitamers used were relatively high (above the levels that can be obtained from dietary means). Thus the model used was not a representative *in vitro* model in which to evaluate the influence of folyl vitamers in terms of folic acid fortification.

Pellis et al. [409] and McCabe et al. [415] analyzed the effects of high PteGlu on human HT29 colon cancer cells and oral cancerous cell lines, confirming increased cell turnover, lower cell differentiation and dose dependant cell growth. However, they only examined the synthetic PteGlu form of folate. Recently Charles et al. [416] reported that the supra-physiological concentration of PteGlu from cell culture medium induces an aberrant DNA methylation pattern, although they also only considered the effect of PteGlu.

When compared to colon cancer, very little has been done to investigate the effects of folate vitamers on breast cancer cells. Most existing studies, like those with colon cancer cells, have focused on chemotherapeutic aspects such as anticancer medicine and cancer diagnosis [417-420].

One research group in the United States has investigated the influences of lipotropes on breast cancer via an *in vitro* cell line. Their results suggest that lipotropes can inhibit the growth of the breast cancer cells [421]. However, the research was carried out using a number of lipotropes including folate and in the presence of many other methylation related nutrients. Therefore it is not sufficient to verify whether there is a clear influence of folate and/or each nutrient in cancer progression. Additionally, there was no preliminary preparation of the cell lines prior to the main experiment: a potentially uncharacterized environment for the cells which, together with the base line effects from the culture medium, may have influenced the outcome of the experiment. Other research investigating breast cancer cell proliferation and folate has mainly focused on changed intracellular distribution of folyl vitamers for 24 hours [422].

The presence of unmetabolized PteGlu resulting from mandatory folate fortification may be an issue of relevance given the potential for *in vivo* photolysis of PteGlu. The photo-oxidative products 6-FP and PCA induce DNA cleavage [423, 424]. Hirakawa et al. (2003) reported that PCA caused DNA cleavage at consecutive G residues especially through electron transfer and this may be a risk factor for skin cancer [424]. This was confirmed by Offer et al. (2007) who also interestingly found that this catalyzed process could be protected by 5-$CH_3H_4$PteGlu [423]. It has been reported that this photo-degradation is accelerated by riboflavin [425] and that accumulated PCA exists in the epidermis of vitiligo patients [426]. 6-FP is likely to be associated with several health issues such as carcinomas [397], hypoxia [427] and loss of cochlea function [428]. Although research has been carried out to investigate this with *in vitro* models, the results are conflicting. 6-FP induced apoptosis in HL-60 cell lines [429, 430] and suppressed the proliferation and production of cytokines of T-cells [431], but did not affect the growth of pancreatic cancer cells [432].

To date, the studies performed have not been sufficient to ascertain information about the physicochemical effects of folyl vitamers on cell proliferation, particularly with respect to their concentrations, disease specific characteristics and oxidative degradation derivatives. In order to obtain a deeper understanding of the long-term effects of various folate vitamers on cancer cell proliferation, further studies need to carefully consider the chemical characteristics of vitamers. Therefore the present study aimed to address these gaps in the research.

## 1.8 Aims of research

The studies described in this thesis were designed to better address the question of how folate nutrition interacts with genes and other nutrients to modify the risk for the development of early neoplastic change, and to investigate the role of folate vitamers in cancer cell physiology via two different approaches: studying adenomatous polyp (AP) as a predictor for colorectal cancer development and progression *in vivo*, and studying cancer cell lines as an *in vitro* model.

Clinical approach: AP study

1) To assess how methylation related nutrient intake including folate (5-$CH_3H_4$PteGlu and PteGlu) riboflavin, niacin, pyridoxine, vitamin C (natural and synthetic), blood folate and thiol status are associated with the occurrence of AP.
2) To investigate how the various SNPs in folate metabolism, nutritional intake and thiol levels interact and determine disease risks in AP aetiology: MTHFR C677T, A1298C, G1793A, SHMT C1420T, TS 1496del6, TSER 2R3R, 3R G>C, GCPII C1561T, CBS 844*ins*66, DHFR 19bp deletion, RCF G80A, CTH G1364T, CTH IVS 10 430 C>T, BHMT G595A, MS A2759G and MSR A66G.
3) To study specific haplotypes involving genetic variations in MTHFR and CTH to see whether they are associated with increased susceptibility for AP.

*In vitro* model: colon cancer and breast cancer cell lines

1) To evaluate the influence of PteGlu concentration on cancer cell line proliferation.
2) To study whether folate vitamers (5-$CH_3H_4$PteGlu, 5-$CH_3H_2$PteGlu, 5-$CHOH_4$PteGlu) have differing effects on cancer cell growth.
3) To verify the interaction of methylfolate vitamers with antioxidants (vitamin C, GSH, DTT) and the potential influence this has on cell proliferation.
4) To determine whether photo-oxidation derivatives of 5-CH3H2PteGlu and PteGlu (p-ABG, PCA, 6-FP) affect cell proliferation via genotoxicity.

# II. B-VITAMIN NUTRITIONAL GENETICS IN THE OCCURRENCE OF ADENOMATOUS POLYP AS AN ANTECEDENT OF COLORECTAL CANCER: CLINICAL *IN VIVO* APPROACH

## 2.1 Introduction

The central dogma of molecular biology suggested by Francis Crick is often rephrased as the following [433]: "DNA makes RNA, RNA makes proteins and proteins make us." Genetic variations may be one of the most important elements in rendering this assertion a reality. Simply put, variations in a DNA sequence may result in altered RNA expression, protein levels or structure. Therefore, genetic variation has been extensively studied as a significant risk factor in disease phenotype occurrence, either alone or in combination with other biochemical and milieu components such as nutrition, xenobiotics (medicine, alcohol) and pathogens.

CRC is the second most prevalent cancer in Australia, being responsible for 9.3% of cancer related deaths in Australia in 2010 [434]. Each year approximately 12,600 new cases are diagnosed and 4,700 patients die of CRC. It has been suggested that 5% of Australians may develop CRC during their life time, with the risk surging dramatically after the age of 50 [435]. As described above, AP is considered to be a common precursor of CRC [436]. Therefore, identifying critical factors in AP occurrence is an important step in understanding the aetiology of CRC.

As folate has been reported to be a significant nutrient in CRC development and progression, genetic variations in folate metabolism genes should be examined to see what contribution they make to CRC risk. This research project analysed 16 common genetic variations in folate metabolism: MTHFR C677T, A1208C, G1793A, SHMT C1420T, TS 1496del6, TSER 2R3R, TS 3RG>C, GCPII C1561T, CBS 844*ins*66, DHFR 19bp del , RCF G80A, CTH G1364T, IVS 10-430 C>T, BHMT G595A, MS A2756G and MSR A66G. The genetic data was examined in combination with blood folate, vitamin $B_{12}$, and thiol levels, along with dietary nutrient intake with respect to the micronutrients important in DNA methylation and, hence, in modifying risk for the occurrence of AP.

## 2.2 Study design

This blind, case-controlled study was performed as a component part of the larger 'Bitter taste phenotype, dietary pattern and nutritional genetics in the pathaetiology of colonic adenomatous study' (adenomatous polyp (AP) study) carried out by the Nutrition, Food and Health Research Group of the School of Environmental and Life Science, University of Newcastle. Sample collection, genotype assays of MTHFR C677T, MTHFR A1208C, GCP II C1561T, RFC G80A, SHMT C1420T, TSER 2R3R, TS 1496del6, DHFR 19bp del, MS A2756G, MSR

A66G, thiols and FFQ analysis were partially carried out by Honours and Ph.D students as part of their projects.[1]

### 2.2.1 Ethics approval

Ethics approval for this research was given by the Human Research Ethics Committee (HREC) of the University of Newcastle prior to the commencement of the research (H-429-0407).

### 2.2.2 Recruitment of Subjects

202 participants (86 male and 116 female) were recruited from a gastroenterology clinic at Gosford hospital (NSW) between May 2007 and September 2009. Participants were aged between 40 and 89 years (median age 64 years). Sporadic AP is considered a disease of ageing with the prevalence increasing after 40 years of age [435]. People do not generally present the signs and symptoms of the occurrence of AP under 40 years and the case of colorectal neoplasm is rare [435, 437, 438]. Additionally, inclusion of subjects <40 years may have led to a biased cohort due to the very specific reason such as strong familial inheritance [438, 439], which is not associated with folate nutrition, or resulted in a younger control group compared to AP cases. Colonoscopy was conducted as a screening protocol for colonic pathology. 38 participants were found to have an AP during their colonoscopy (cases). Controls were defined as participants who had no evidence of an AP during colonoscopy.

All subjects participated voluntarily and provided informed consent prior to entering the study. An individual interviewer-administrated FFQ was conducted on the day of examination, and all participants were mentally capable of completing the FFQ. Subjects receiving high-dose vitamin $B_{12,}$ antifolate chemotherapy, who had previous bowel surgery and/or CRC, or who were less than 40 years old were excluded from this study. Information regarding each participant's age, medical and surgical history, colonoscopy history, polyp status and medication were recorded. Each subject and sample was given a study and lab number, respectively, to ensure anonymity and confidentiality.

### 2.2.3 Sample collection

Blood was collected from participants by venipuncture to analyse folate levels, thiol levels and genetic variation. In order to perform the analysis of genetic variations and RBC folate, 2 and 4mL of whole blood, respectively, was collected in an EDTA (Ethylenediaminetetraacetic acid) Vacuette. For plasma samples, 8mL of whole blood was collected in a lithium heparin Vacuette. For serum samples, two x 4mL of whole blood was collected in a Vacuette containing

[1] See the acknowledgement on page iv

a serum separator and a clot activator. All tubes, apart from the one for genetic analysis, were centrifuged at 3000xg for 15 minutes within half an hour after venipuncture. These samples were transferred to the lab on ice and were protected from light exposure. Samples were aliquoted into 2mL cryo tubes. Whole blood samples were stored at -20°C, while plasma and serum samples were stored at -80°C until analysed (see Figure 2.1).

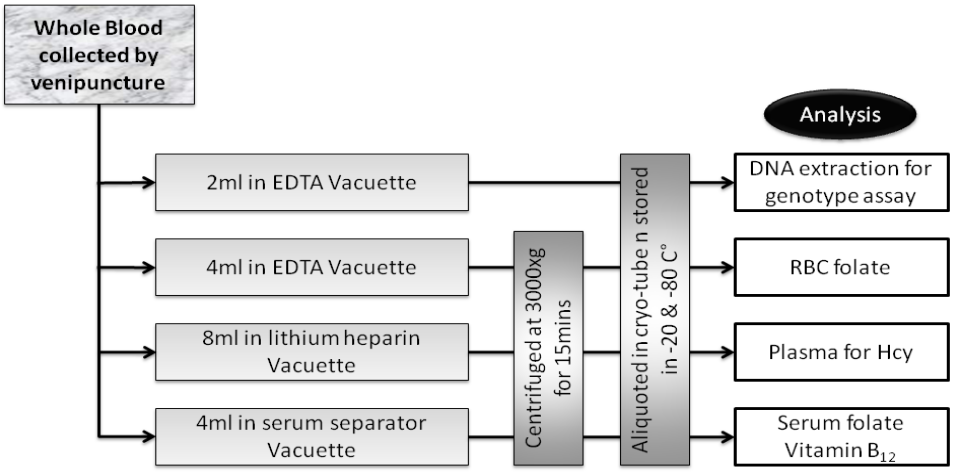



Figure 2.1 The procedure for blood sample collection

## 2.3 Materials and methods

Briefly, genetic variation was assessed using restriction-enzyme fragment length polymorphism (RFLP) analysis. Blood folate and $B_{12}$ status were analysed using a chemiluminescence assay. Thiol analysis was performed using HPLC with fluorescence detection and methylation-related nutrient intake analysis was performed using data from FFQ.

### 2.3.1 Genotype determination

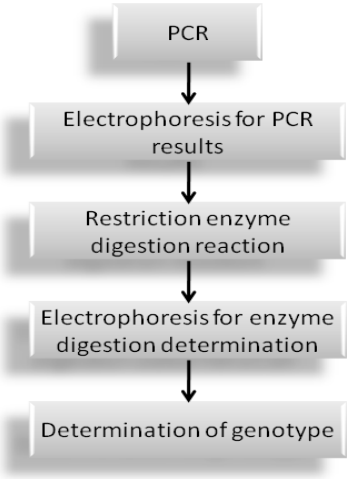



Figure 2.2 The procedure for genotype determination using RFLP

#### 2.3.1.1 Chemicals and equipment

A DNA blood mini kit (Cat. No. 51106, Qiagen, Chadstone, VIC) was used for DNA extraction from whole blood. Molecular biology grade ethanol for DNA extraction was purchased from Sigma (E7023, Castle Hill, NSW). For the polymerase chain reaction (PCR), Promega GoTaq® Green Master Mix was used (M7123). All primers were synthesized by Invitrogen (Mulgrave, VIC). 100bp and 25bp DNA markers were supplied by Promega (G2101) and Bioline (BIO-33029, both from Alexandria, NSW). Demethyl sulfoxide (DMSO, D8418) was purchased from Sigma. Restriction enzymes used for each determination of genotype and bovine serum albumin (BSA) were purchased from Promega and NEB (Arundel, QLD). Ultrapure agarose was supplied by Invitrogen (Cat. No. 16500500), Nusieve GTG agarose (Cat. No. 50084) was obtained from Cambrex Bio Science, Australia. Tris-borate-EDTA (TBE, 93290) buffer used for electrophoresis and ethidium bromide (E1510, EtBr, 10mg/mL) were purchased from Sigma. Micro- and PCR tubes were supplied by SSI (Melbourne, VIC), and micro-tips were purchased from Greiner (Melbourne, VIC). For PCR, a Bio-Rad thermocycler (Sydney, NSW) was used. Electrophoresis tanks and a power supply were purchased from Bio-rad. Agarose gels were visualized using a gel doc system (Image Quant 300) and the software used was iQuant Capture (GE Healthcare, Sydney, NSW).

#### 2.3.1.2 Genomic DNA extraction

Genomic DNA (gDNA) was extracted from whole blood using a commercial kit, following the manufacturer`s instructions. Briefly, frozen whole blood samples were defrosted at room temperature. A 200µL whole blood sample was treated with 20µL protease and incubated at 56°C for 10mins. 100% ethanol was added to precipitate molecular gDNA which was then filtered using a QIAamp spin column. The column, which retained the gDNA, was washed with buffers AW1 and AW2 in that order. gDNA was eluted with AE buffer and then samples were stored at -20°C until analysed.

#### 2.3.1.3 Restriction fragment length polymorphism analysis of genotype

Various methodologies have been developed to determine genotype including RFLP, pyrosequencing, denaturing high performance liquid chromatography (dHPLC), real-time polymerase chain reaction (RT-PCR) and SNPlex. RFLP, based on PCR, is a common and simple genotype analysis method which generates accurate results. RFLP is comprised of two major steps: PCR and restriction enzyme digestion, followed by electrophoresis.

Table 2.1 All primer sequences for genotype detection

| Genetic variation | Sense | Antisense | Reference |
|---|---|---|---|
| MTHFR C677T | TGAAGGAGAAGGTGTCTGCGGGA | AGGACGGTGCGGTGAGAGTG | [4] |
| MTHFR A1208C | CTTTGGGGAGCTGAAGGACTACTAC | CACTTTGTGACCATTCCGGTTTG | [74] |
| MTHFR G1793A | CTCTGTGTGTGTGTGCATGTGTGCG | GGGACAGGAGTGGCTCCAACGCAGG | [75] |
| SHMT C1420T | AGAGTTCAAGGAGAGACTGGCAG | GTCAACAGTTCCCTTTGGAGC | [440] |
| TS 1492del6 | CAAATCTGAGGGAGCTGAGT | CAGATAAGTGGCAGTA | [441] |
| TSER 2R3R, 3R G>C* | GTGGCTCCTGCGTTTCCCCC | GGCTCCGAGCCGGCCACAGGCATGGCGCGG | [141] [299] |
| GCPII C1561T | CATTCTGGTAGGAATTTAGCA | AAACACCACCTATGTTTAACA | [239] |
| CBS 844*ins*66 | CTGGCCTTGAGCCCTGAA | GGCCGGGCTCTGGACTC | [442] |
| DHFR 19bp deletion | CCACGGTCGGGGTACCTGGG (sense primer1)<br>ACGGTCGGGGTGGCCGACTC (sense primer2) | AAAAGGGGAATCCAGTCGG | [125] |
| RCF G80A | AGTGTCACCTTCGTCCC | TCCCGCGTGAAGTTCTTG | [443] |
| CTH G1364T | AGGGAGCTCAGTCAAAGTGC | CACCTCCTTCAGAGGCAAAC | CTH SNPs designed using Primer3 program, restriction enzyme was selected using NEB cutter |
| CTH IVS 10-430C>T | AGGCCTCTATGCCAGGAGTT | TGGAGGCATGTTTCTGTCTC | |
| BHMT G595A | AGTACTCTAACCTTAACTGATTCCAG | GGCACTAGTGTGTTCTTTTAAG | [444] |
| MS A2756G | GGTGTGTTCCCAGCTGTTAGATG | GACACTGAAGACCTCTGATTTGAAC | [445] |
| MSR A66G | GCAAAGGCCATCGCAGAAGACAT | GTGAAGATCTGCAGAAAATCCATGTA | [95] |

* Identical PCR product was used for TSER 2R-3R and 3R G>C genotype determination

Table 2.2 PCR conditions and product size of each genetic variation

| Genetic variation | Stage 1 | Stage 2 (35 cycles) | | | Stage 3 | PCR product size (bp) |
|---|---|---|---|---|---|---|
| | Initial denaturation | Denaturation | Elongation | Extension | Final extension | |
| MTHFR C677T | 95℃ 1min | 95℃ 1min | 56℃ 1min | 72℃ 1min | 72℃ 7mins | 198 |
| MTHFR A1208C | 95℃ 5mins, 55℃ 2mins, 72℃ 2mins | 95℃ 75sec s | 55℃ 75seC s | 72℃ 90sec s | 72℃ 6mins | 163 |
| MTHFR G1793A | 94℃ 2mins | 94℃ 1min | 66℃ 1min | 71℃ 2mins | 72℃ 10mins | 310 |
| SHMT C1420T | 95℃ 2mins | 95℃ 1min | 56℃ 1min | 72℃ 1min | 72℃ 7mins | 217 |
| TS 1492del6 | 94℃ 2mins | 94℃ 30sec s | 58℃ 45seC s | 72℃ 45sec s | 72℃ 5mins | 158 |
| TSER 2R3R[1], 3R G>C [2] | 94℃ 2mins | 94℃ 30sec s | 63℃ 30seC s | 72℃ 30sec s | 72℃ 5mins | 243, 215 |
| GCPII C1561T | 94℃ 3mins | 94℃ 30sec | 56℃ 1min | 72℃ 1 min | 72℃ 7mins | 244 |
| CBS 844*ins*66 [1] | 95℃ 3mins | 95℃ 1min | 64℃ 1min | 72℃ 2mins | 72℃ 7mins | 252, 184 |
| DHFR 19bp deletion [1] | 94℃ 4mins | 94℃ 55sec s | 62℃ 55sec s | 72℃ 55sec s | 72℃ 12mins | 113, 92 |
| RCF G80A | 94℃ 2mins | 94℃ 30sec s | 58℃ 30secs | 72℃ 45sec s | 72℃ 7mins | 230 |
| CTH G1364T,IVS 10-430C>T [3] | 95℃ 3mins | 95℃ 30sec s | 60℃ 30sec s | 72℃ 30sec s | 72℃ 7mins | 401, 498 |
| BHMT G595A | 92℃ 2mins | 92℃ 30sec s | 47℃ 30sec s | 72℃ 30sec s | 72℃ 7mins | 235 |
| MS A2756G | 92℃ 2mins | 95℃ 30sec s | 60℃ 30sec s | 72℃ 30sec s | 72℃ 7mins | 265 |
| MSR A66G | 92℃ 2mins | 95℃ 60sec s | 56℃ 1min | 72℃ 1 min | 72℃ 7mins | 66 |

[1] These variations did not require enzyme digestion. Each allele presents different sized of PCR products, [2] Two genetic variations in TS were determined from same PCR product, [3] Same PCR conditions used for two CTH SNPs

#### 2.3.1.3.1 Polymerase chain reaction

PCR is a very efficient tool which is used in modern molecular biology to amplify a particular sequence of any given gene within template DNA. Oligonucleotide primers designed to be complementary to the end sections of a target sequence are mixed with template DNA as well as other components such as Taq DNA polymerase (thermostable DNA polymerase; elongates the DNA strands), dNTPs (a mixture of all four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP)), $MgCl_2$ (stabilizing double strands and cofactor for Taq polymerase) and an appropriate buffer. Repeated denaturation, annealing and extension steps at different temperatures intensify the primary signal on the template DNA in the sample mixture. The procedure eventually produces a large amount of DNA containing the desired target sequence at a theoretical abundance of $2^n$, where n is the number of PCR cycles conducted [446]. Various critical factors such as annealing temperature, GC content of template or primers, and the concentration of $MgCl_2$ may influence the outcome of PCR. For this reason, commercial PCR master mix was used to optimize the experimental conditions for this study.

The GoTaq® Green Master Mix used contains Taq polymerase, 2X Green GoTaq® Reaction Buffer (pH 8.5), 400µM of each dNTP and 3mM $MgCl_2$. 13-60ng of gDNA was used for each PCR sample. DMSO was used for TSER 2R3R and TS 3R G>C analysis, and DHFR 19bp del genotype was determined by a nested PCR method which uses two allele specific sense primers and one antisense primer. Table 2.1 shows the sequence of oligonucleotides used for genetic variation analysis. Table 2.2 provides details of the PCR conditions for each of the genetic variants analysed.

#### 2.3.1.3.2 Restriction enzyme digestion reaction

Amplified PCR products were digested using restriction enzymes. Restriction enzymes recognize short specific base sequences, leading to a cut at a precise point on the DNA strands. For instance, the restriction enzyme *Eco*RI which is used for CTH G1364T analysis, recognises the sequence 5`-GATTC-3` and digests it into two fragment at between the G and A nucleotides. Various kinds of bacteria produce different types of restriction enzymes which provide diverse cutting sites [447].

Restriction enzyme digestion was carried out to determine the following genotypes; MTHFR C677T, A1208C, G1793A, SHMT C1420T, TS 3`UTR, TS 3R G>C, GCPII C1561T, RCF G80A, CTH G1364T, CTH IVS 10-430C>T, BHMT G595A, MS A2756G and MSR A66G. TSER 2R3R, CBS 844*ins*66 and DHFR 19bp deletion did not need enzyme digestion because each allele produced a different sized PCR product, and therefore, their genotypes could be identified

from the PCR product directly. See Table 2.3 and Figure 2.3 for details of the restriction enzymes used and genotype fragment pattern generated.

Each reaction mixture for enzyme digestion consisted of the PCR product, five to eight units of restriction enzyme, appropriate 10X reaction buffer suggested by the supplier, and water. BSA was added where appropriate. Total reaction volume was 10µl.

#### 2.3.1.3.3 Electrophoresis

Electrophoresis is a practical biotechnology technique used to separate macromolecules such as DNA, RNA and protein. DNA molecules present an overall negative charge due to their phosphate back bone and therefore migrate well through an agarose or polyacrylamide gel under an electric field. While the DNA migrates from cathode to anode, the molecules are separated depending on their physical properties such as charge and molecular weight [448]. To increase resolution of small fragments, ultrapure agarose is mixed with Nusieve GTG agarose. To maintain an even distribution of the electric field, and to get the best resolution, the appropriate type of buffer is required. TAE (tris-acetate-EDTA) and TBE buffers are commonly used for DNA oligonucleotide separation. TAE buffer is a better choice for separating larger molecules and DNA gel elution, while TBE has a better buffering capacity and is suitable for smaller molecules.

In this study, electrophoresis was performed to separate and visualize the PCR amplicons and restriction enzyme digestion products. This was done using agarose gel and EtBr in a TBE buffer. The specific electrophoresis conditions such as running voltage, time and gel percentage were adjusted, depending on the size of the product fragments (see Table 2.3).

#### 2.3.1.3.4 Genotype banding pattern

As described above, to determine the genotype for CBS 844*ins*66, TSER 2R3R and DHFR 19bp deletion variants, restriction enzyme digestion was not required. For CBS 844*ins*66 variation, the allele without the insertion presented as a single 184bp band, whereas the allele with an insertion produced a 252bp fragment.

The DHFR PCR product that retains the 19bp deletion variation was generated by three oligonucleotides that were designed to be sequence specific. DNA samples from individuals having no deletion allele generated an 113bp sized amplicon, while those from individuals having a single deletion showed 113 and 92bp bands. A single 92bp band was present in individuals where both alleles have a 19bp sized deletion.

Two primers designed to target the TSER 2R3R sequence were used to amplify a 243bp PCR amplicon to allow determination of the genotype. The 2R allele produced a 215bp oligonucleotide band and the 3R allele generated a 243bp oligonucleotide band. Therefore, homozygous individuals showed a 243 bp band, heterozygous individuals showed 243 and 215 bp bands and homozygous recessive individuals exhibited a 215bp fragment.

The primer set designed for TSER 2R3R was also used for TS 3R G>C. Samples that had the 3R allele were selected and were digested with the restriction enzyme *Hae*III. Five different potential genotype patterns were generated from this reaction (2R/3Rc. 2R/3Rg. 3Rc/3Rc, 3Rc/3Rg and 3Rg/3Rg). The 2R allele exhibited 13, 44, 45, 47 and 65bp fragments, 3Rc showed 13, 33, 34, 37 and 94bp fragments, and the 3Rg allele produced 13, 28, 44, 45, 47 and 65bp bands.

The restriction enzyme *Dra*I cut the 158 bp TS 1492del6 PCR amplicon into 70 and 88bp fragments within the homozygous insertion genotype. Individuals with heterozygous insertion exhibited 158, 88 and 70bp fragments. The samples from individuals without this insertion remained uncut.

The PCR product of MTHFR C677T was digested by the restriction enzyme *Hinf*I. This reaction yielded a 198bp band for wildtype individuals (CC), 198, 175 and 23bp fragments for the heterozygotes (CT), and 175 and 23bp bands for homozygote recessives (TT).

MTHFR A1298C genotype analysis was carried out using the restriction enzyme *Mbo*II. It produced 56, 31, 30, 28 and 18bp fragments for the wildtype (AA), 84, 56, 31, 30, 28 and 18bp bands for the heterozygous genotype (AC), and 84, 31, 30 and 18bp DNA fragments for the homozygous recessive genotype (CC).

The MTHFR G1793A PCR primer set produced a 310bp product. This amplicon was digested by restriction enzyme *Bsr*BI. Wildtype GG samples yielded 233 and 77bp fragments, while heterozygous samples yielded 310, 233 and 77 bp bands. Since homozygous recessive genotypes do not possess a restriction enzyme digestion site, only the undigested 310bp single band remained.

When the RFC G80A 230bp PCR product was digested with the enzyme *Cfo*I it yielded 125, 68 and 37bp fragments for the wildtype (GG), 162, 125, 68 and 37bp bands for the heterozygote (GA), and 162 and 68bp fragments for homozygote recessive individuals (AA).

A 244bp PCR amplicon was produced to determine GCPII C1561T genotype. Incubation with the restriction enzyme *Acc*I generated 141 and 103bp fragments for the TT genotype whilst the CC genotype remained uncut, leaving a 244bp band. The heterozygous genotype was represented by 244, 141 and 103bp bands.

The 215bp PCR amplification product of SHMT C1420T contains a recognition sequence for the restriction enzyme *Ear*I. Wildtype CC yielded 126 and 89bp bands, the heterozygous genotype presented 89, 126 and 215bp fragments, but the homozygous recessive T allele abolished the restriction site. As a result, the recessive TT genotype exhibited a single 215bp band which remained uncut.

The primer set used for CTH G1364T amplified a 401bp PCR product which has a specific recognition sequence for *Eco*RI. The heterozygous genotype GT generated three fragments: 401, 265 and 136 bp. The TT homozygous recessive genotype yielded two fragments: 265 and 136bp, whilst the GG wildtype remained as a single 401bp band.

The CTH IVS 10-430C>T variation creates a restriction site for the enzyme *Mbo* I. The heterozygous genotype (CT) yielded 498, 388 and 110 bands, and the CC genotype produced 388 and 110 fragments. However, the TT wildtype remained uncut, leaving one 488bp band.

In examining the BHMT G595A genotype, primers were designed to amplify a 235bp PCR product. The restriction enzyme *Nci* digested wildtype GG DNA into two fragments: 143 and 92bp. Heterozygous GA genotype DNA generated three fragments: 235, 143 and 92bp.

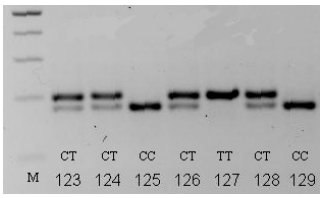
The MS A2756G 265bp PCR product was amplified by a dual set of primers. Wildtype samples with this genetic variation are not digested by the enzyme *Hae*III. However, heterozygous and recessive genotypes present 265, 180 and 22bp digested fragments. Homozygous recessive genotypes present 180 and 85bp digested fragments.

In order to genotype the MSR A66G variation, the primer set amplified a 66bp PCR product which has a specific recognition sequence for the restriction enzyme *Nde*I. The AA wildtype generated 44 and 22 bp DNA fragments, AG heterozygous DNA yielded 66, 44 and 22 bp bands, and GG homozygous recessive DNA remained uncut, leaving a 66bp band.

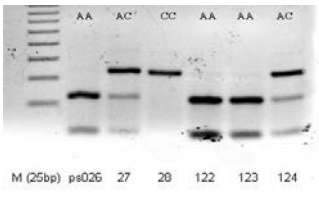
Table 2.3 Summary of the restriction enzyme digestion reactions and electrophoresis

| Genetic variation | PCR product size (bp) | Restriction enzyme | Enzyme units | Electrophoresis gel (%) | Genotype banding pattern (bp) | | |
|---|---|---|---|---|---|---|---|
| | | | | | Wild | Heterozygous | Recessive homozygous |
| MTHFR C677T | 198 | *Hinf*I | 5 | 2 | 198 | 198, 175, 23 | 175, 23 |
| MTHFR A1208C | 163 | *Mbo*II | 5 | 4 | 56, 31, 30, 28 | 84, 56, 31, 30, 28, 18 | 84,31,30,18 |
| MTHFR G1793A | 310 | *Bsr*BI | 5 | 2 | 310 | 310, 233, 77 | 233, 77 |
| SHMT C1420T | 217 | *Ear*I | 4 | 2.5 | 126, 89 | 215, 126, 89 | 215 |
| TS 1492del6 | 158 | *Dra*I | 5 | 3 | 88,70 | 152, 88, 70 | 152 |
| GCPII C1561T | 244 | *Acc*I | 5 | 2 | 244 | 141,103 | 103 |
| RFC G80A | 230 | *Cfo*I | 5 | 3 | 125, 68, 37 | 162, 125, 68, 37 | 162, 68 |
| CTH G1364T | 401 | *Eco*RI | 8 | 2.5 | 401 | 401, 265, 136 | 265, 136 |
| CTH IVS 10-430 C>T | 498 | *Mbo*I | 4 | 2 | 388, 110 | 498, 388, 110 | 498 |
| BHMT G595A | 235 | *Nci* | 8 | 2.5 | 143, 92 | 235, 143, 92 | 235 |
| MS A2756G | 265 | *Hae*III | 5 | 2.5 | 265 | 265,180,85 | 180,85 |
| MSR A66G | 66 | *Nde*I | 5 | 4 | 44,22 | 66,44,22 | 66 |
| Genetic variation | PCR product size (bp) | Restriction enzyme | Enzyme units | Electrophoresis gel (%) | No insertion or deletion | Single insertion or deletion | Double insertion or deletion |
| CBS 844*ins*66 | 252, 184 | N/A | N/A | 2 | 184 | 252, 184 | 252 |
| DHFR 19bp del | 113, 92 | N/A | N/A | 4 | 92 | 113, 92 | 113 |
| TSER 2R3R | 243,215 | N/A | N/A | 3 | 215 | 243, 215 | 243 |
| TS 3R G>C | 215 | *Hae*III | 5 | 4 | 2R/3Rg-65,47,45,44,28,13; 2R/3Rc-94, 65, 47, 45, 44,13; 3Rg/3Rg-65, 47, 45, 44,28, 13; 3Rg/3Rc-94, 65, 47, 45, 44, 28, 13; 3Rc/3Rc- 94, 47, 45, 44, 13 | | |

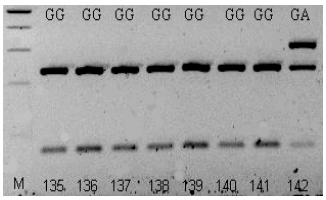
# All enzyme digestion reactions were carried out at 37°C, 2µg of EtBr was used for each 100mL of agarose gel solution



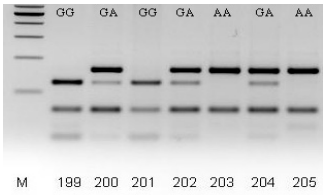

a) MTHFR C677T (CC: WT, CT: Het, TT: Rec)



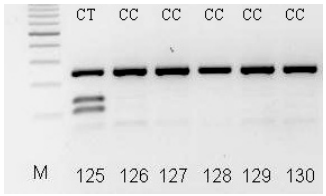

b) MTHFR A1298C (AA: WT, AC: Het, CC: Rec)




c) MTHFR G1793A (GG: WT, GA: Het)

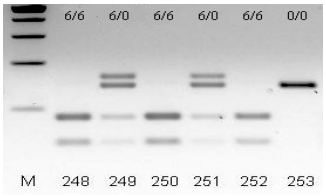



d) RFC G80A (GG: WT, GA: Het, AA: Rec)

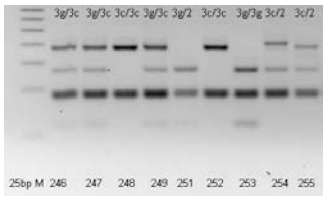



e) GCPII C1561T (CC: WT, CT: Het)

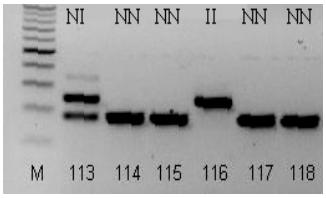

f) TSER 2R3R (3R/3R: WT, 3R/2R: Het, 2R/2R: Rec)



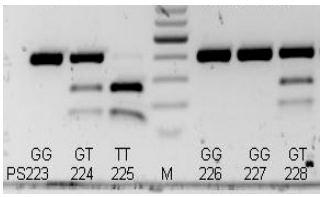

g) TS 1496del6 (6/6: WT, 6/0: Het, 0/0: Rec)



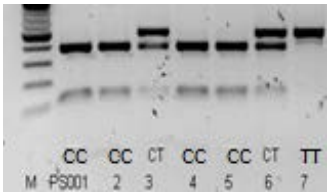

h) TSER 3RG>C



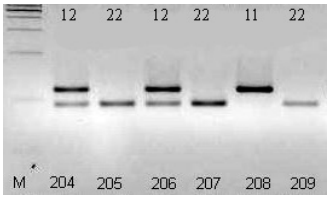

i) CBS 844*ins*68 (NN: WT, NI Het, II: Rec)



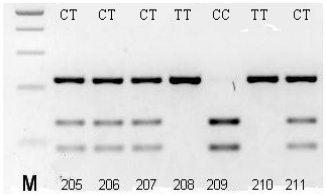

j) CTH G1364T (GG: WT, GT: Het, TT: Rec)



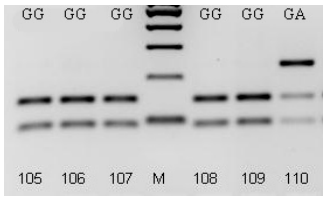

k) CTH IVS 10-430 C>T (CC: WT, CT: Het, TT: Rec)



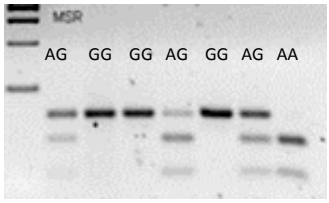

l) DHFR 19bp del (11: WT, 12: Het, 22: Rec)



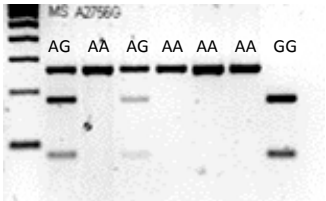

m) SHMT C1420T (CC: WT, CT: Het, TT: Rec)




n)BHMT G595A (GG: WT, GA: Het)




o) MSR A66G (AA: WT, AG: Het, GG: Rec)




p) MS A2756G (AA: WT, AG: Het, GG: Rec)

Figure 2.3 RFLP banding pattern of each genotype

(M- marker, number refers to lab ID of sample, WT- wild type, Het- heterozygous genotype, Rec- homozygous recessive genotype )

### 2.3.2 Blood folate and vitamin $B_{12}$ determination

Blood samples were transported on ice to the Institute of Clinical Pathology and Medical Research at Westmead Hospital in Sydney for folate and vitamin $B_{12}$ assays. All folate and vitamin $B_{12}$ measurements were carried out using an automated Access Immunoassay System (Beckman Coulter, Sydney NSW) based on paramagnetic particle and capture antibody chemiluminoscence detection technology. For serum folate and RBC folate analysis a competitive-binding receptor assay was used, whilst vitamin $B_{12}$ status was determined via a competitive-binding immunoenzymatic assay using proprietary kits from Abbott Diagnostics.

Serum samples and whole blood samples for RBC were processed to release folate from their endogenous binding proteins. These were treated with a lysing agent containing ascorbic acid and folate-free human serum albumin. After neutralisation of the reaction mixture, rat antifolate binding protein, folate binding protein, folate-alkaline phosphate conjugase, and goat anti-mouse capture antibody combined with paramagnetic particles were added to the reaction vials (see Figure 2.4).

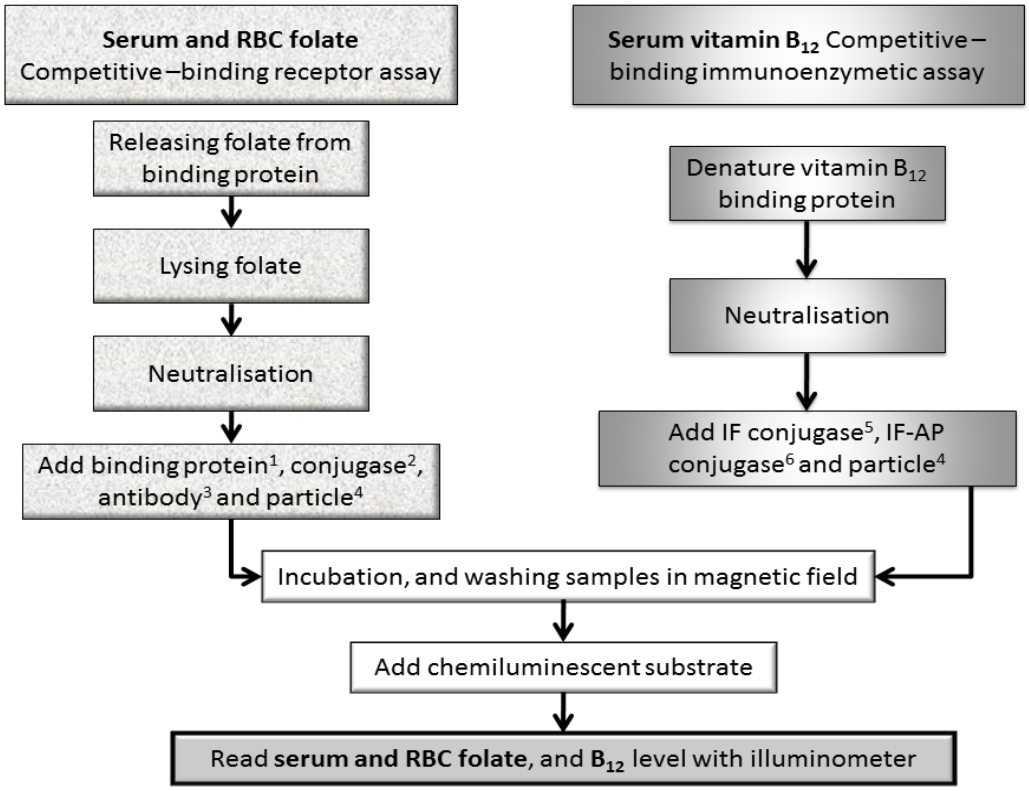



Figure 2.4 Simplified procedure for determining serum and RBC folate, and vitamin $B_{12}$ level (1: rat antifolate binding protein and folate binding protein, 2: folate-alkaline phosphate, 3: goat anti mouse capture antibody, 4: paramagnetic particle, 5: intrinsic factor (IF) conjugase, 6: IF-alkaline phosphatase conjugase)

As all vitamers of $B_{12}$ must be converted to the cyanocobalamin form, alkaline potassium cyanide and DTT were added to the serum sample to denature vitamin $B_{12}$ binding protein in preparation for the serum vitamin $B_{12}$ analysis. After neutralisation, to bind vitamin $B_{12}$ in the sample with intrinsic factor (IF), IF-alkaline phosphatase, conjugase and paramagnetic particles coated with goat antimouse IgG were added to the samples.

After incubation, to remove materials not binding to the solid phase, samples were washed in a magnetic field, and then a chemiluminescent substrate (Lumi-Phos 530) was added to each sample. These samples were processed with an illuminometer. Folate and vitamin $B_{12}$ levels were determined by the amount of light emitted from each reaction and analyte in the samples.

Normal distribution ranges for these analyses are 5-21nM for serum folate, 370-1050nM for RBC folate and 125-780pM for serum vitamin $B_{12}$.

### 2.3.3 Thiol determination

The determination of plasma thiols including cysteine (Cys), Hcy, cysteinyl-glycine (Cys-Gly) and GSH was performed using HPLC with fluorescence detection. The thiol assay used for this study was modified from the methods of Araki and Sako [449], Dudman [450] and Krijt [451].

#### 2.3.3.1 Chemicals and Equipment

The following chemicals were used for sample preparation: 1M sodium hydroxide, 4M potassium hydroxide, 0.5M potassium borate/5mM disodium EDTA (pH10.5), 0.1M potassium hydroxide/2mM disodium EDTA (pH 9.5), 0.6M perchloric acid/1mM EDTA solution, 15% (w/v) tris (2-carboyethyl) phosphine (TCEP). SBD-F 1mg/mL in 0.1M potassium tetraborate/2mM disodium EDTA (pH 9.5) was used as a fluorescent labelling agent. 10mM of cysteine and cystinyl-glycine, 1mM of glutathione and 5mM of homocysteine in deionised water were prepared as standard solutions and 10mM of N-(Mercaptopropionyl)-glycine was used as an internal standard. All liquid solvents were filtered with a 0.45µm cellulose filter from Millipore (North Ryde, NSW). All chemicals were purchased from Sigma.

A Varian ProStar System TM (Varian, Clyton, VIC) with two pumps (ProStar 210) and a temperature control tray set at 8°C was used to determine plasma thiol concentration. To separate thiol compounds, a Phenomenex sphereclone$^{TM}$ 5µ ODS (2) (250 X 4.60mm 5µM

spherical bonded silica) column was used. Guard column - Security Guard™ (C18 4.0 x 3.0mm, Phenomenex, Pennants Hills, NSW) was employed to protect the analytical column. Fluorescence intensities were measured using a Varian ProStar 363 fluorescence detector. The excitation and emission wavelengths were 385nm and 515nm, respectively. The chromatogram was analysed using Star Chromatography Workstation TM software (version 6.0).

A gradient employing two different mobile phases was used for this assay. The gradient involved: (A) 4% methanol in 0.1M sodium acetate pH 4 and (B) 15% methanol in 0.1M sodium acetate pH 4.

Mobile phase A was run for the first 3.6 minutes. The HPLC system was switched to mobile phase B between 3.6 and 7.2 minutes and then the system was switched back to mobile phase A for 10 minutes to re-equilibrate the column on solvent A prior to next sample injection.

**2.3.3.2 Plasma thiol derivatisation**

Plasma samples including study specimens, 4 standards and 2 pooled plasmas for quality control were defrosted at room temperature for 30 minutes. Plasma samples were centrifuged at 14,000rpm for 15 minutes to remove any fibrin clots.

60µL of the supernatant and 20µL of the standard samples were aliquoted to new labelled tubes, and then 8µL of the reducing agent TCEP was added to the samples.

After 30 minutes of incubation at room temperature, 50µL of 0.6M perchloric acid/1mM EDTA solution was added to the samples. This solution denatures the plasma proteins to release thiols. The samples were left for 5 minutes at room temperature and then centrifuged for 10 minutes at 14,000rpm to remove the protein residue. 40 µL of the clear supernatant from each sample was transferred to new tubes, and 80µL of 0.5M potassium borate/5mM disodium EDTA (pH10.5) was added. 40µL of SBD-F solution was added as a last step, and then then samples were incubated at 60°C in a water bath for 1 hour, protected from light. All samples were then moved onto ice immediately to cool down, after which they were vortexed and centrifuged for 5 minutes. The samples were then transferred to brown HPLC vials and 20µL of each sample was injected onto the HPLC using the auto-injector. Figure 2.5 presents a simplified schematic for the technique for determining plasma thiol levels.

To obtain accurate results, all samples were derivatised in duplicate and injected onto the HPLC column twice. Using the pooled plasma samples, pure thiols and internal standards, the elution time of each thiol peak was confirmed.

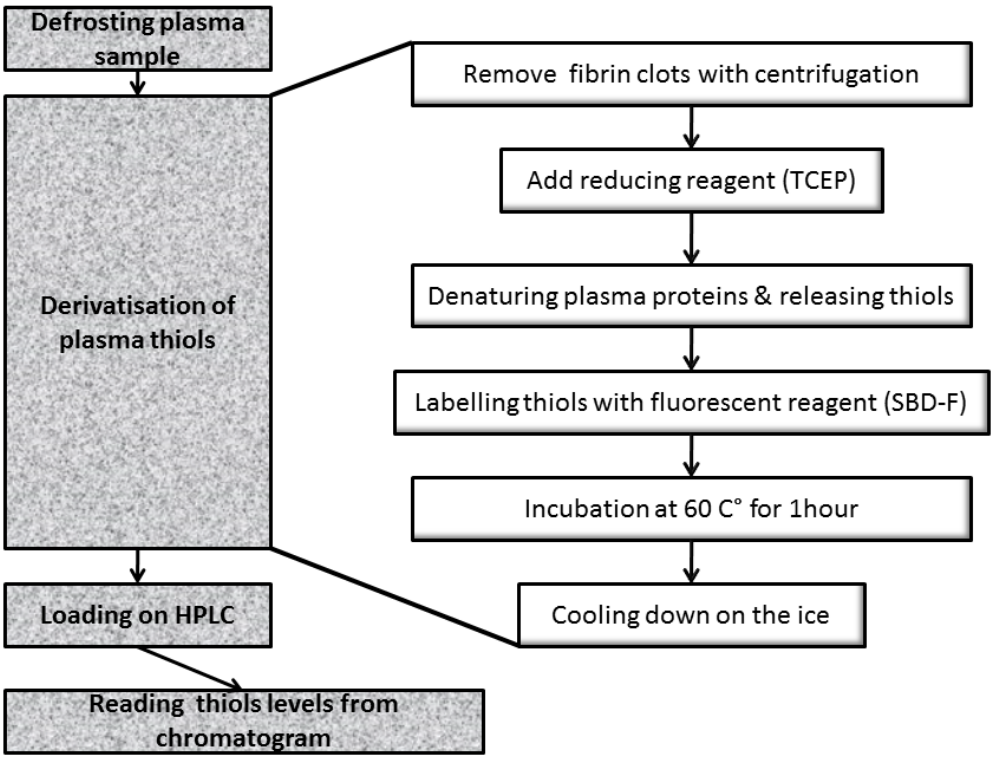



Figure 2.5 Simplified procedure for determining plasma thiol level

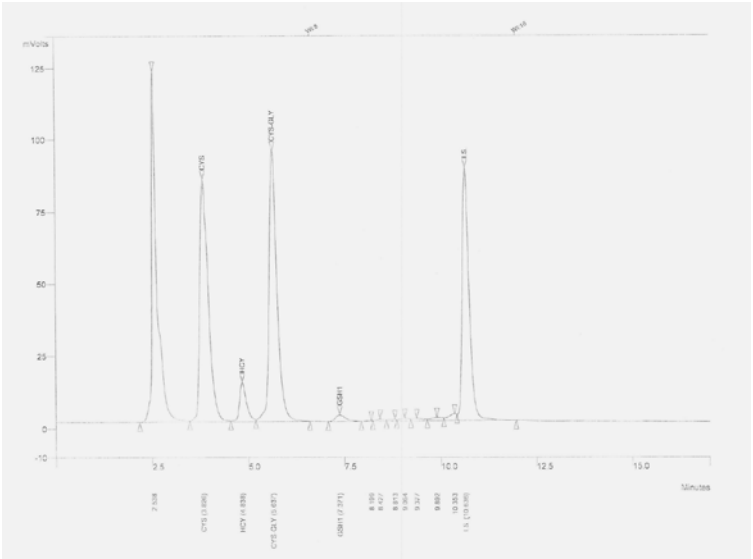


Figure 2.6 Chromatogram of SBD-F derivatised plasma thiols (I.S-internal standard:N-(Mercaptopropionyl)-glycine)

### 2.3.4 Nutrition intake assessment

A number of methods have been developed to assess nutritional intake including weighed food records, estimated food records, 24-hour recall and multiple pass recall [452, 453]. FFQ, one of these commonly used methods, has been designed to collect accurate information from comparatively large sample sizes (more than 100 individuals) [452, 453]. The FFQ used for this study was adapted from the NSW Health Survey Nutrition Questionnaires [454], the Short Fat Survey [455] and the CSIRO Diet and Health Booklet [456].

#### 2.3.4.1 Food Frequency Questionnaire

The FFQ consisted of a list of food items (see Table 2.4) and multiple options relating to the frequency of consumption of each food such as daily, weekly, monthly, yearly or never. Participants were asked to specify the quantity of food consumed for each food group. To help in the estimation of food quantity, average serving sizes and a couple of photographs were supplied for some specific common foods. Participants were asked to list all food items not listed in the FFQ, but which they consumed regularly at the end of the questionnaire. The consumption of vitamin and mineral supplements was also surveyed.

Nutrient intake analysis from the FFQ was performed using Foodworks ™ 2.10.146 (Xyris Software, Brisbane, QLD). This database package included AusFoods (brands), AusNut (base foods) and the New Zealand – Vitamin and Mineral Supplements 1999, and therefore covered the majority of food and supplements consumed by Australians.

To assist people who could not recall precisely how often and what types of food items they consumed, the interviewer asked further questions about the type of food, the quantity of cooked food consumed at one time and the times when they consumed these foods. This commonly occurred in relation to vegetables. Take-away meals such as Chinese and Thai food were included in the list as common foods.

Due to variations in availability, certain assumptions were used in the estimation of seasonal food intake. For instance, the amount, type and frequency of stone fruit intake was surveyed. This amount was then divided by three (corresponding the average period that stone fruits are in season e.g. a third of the year). Similarly, certain foods consumed only in a particular season were recorded for four months of the year, unless otherwise stated. For better accuracy, participants were also asked to name the brands of food that they generally ate.

### 2.3.4.2 Estimation of dietary nutrients important for methyl group metabolism

For a complete understanding of the impact of nutrients on one-carbon metabolism, the intake of not only folate, but also of other nutrients involved in methyl group generation was estimated. This included niacin, riboflavin, pyridoxine, methionine, cobalamin and vitamin C.

Table 2.4 Food groups in food frequency questionnaire

| Food Groups | Foods |
|---|---|
| Dairy Products | Milk |
| | Cream |
| | Cheese |
| | Yoghurt |
| Fats and Oils | Spreads – margarine and butter |
| | Oils |
| Meat and Seafood | Red meats – beef, lamb, pork, sausages, offal |
| | Poultry |
| | Fish |
| | Other seafood |
| Eggs | Eggs |
| Cereals and Grain Foods | Bread |
| | Crisp bread biscuits |
| | Breakfast cereals |
| | Pasta |
| | Rice |
| | Noodles |
| Fruits, Vegetables and Herbs | Fruit – raw, cooked, canned, dried |
| | Vegetables – raw, cooked, frozen, canned |
| | Fruit or vegetable juices |
| | Herbs – fresh or dried |
| Nuts and Legumes | Nuts, nut spreads or nut sauces |
| | Legumes – fresh, canned , cooked, dried, frozen |
| Snack Foods | Fast Foods e.g. McDonalds, Chinese, KFC |
| | Desserts and sweets e.g. lollies, chocolate, ice creams, cakes, biscuits |
| | Hot chips, pies, hot dogs etc. |
| | Sauces e.g. gravy, cheese sauce |
| | Soft drinks |
| Alcohol | Beer and Wine |
| | Spirits |
| | Liqueurs |
| | Premixed drinks |
| Vitamin and Mineral Supplements | Multivitamins |
| | Specific vitamin supplements |
| | Herbal supplements |
| | Fish oil products |
| | Protein drinks |
| Miscellaneous | Spreads e.g. vegemite, honey, jam |
| | Sugar (in cereal, tea, coffee in cooking) |
| | Tea and coffee |
| | Herbal drinks |

Total folate intake was determined based on the sum of dietary intake values from Foodworks ™ and additional vitamin supplements. Synthetic PteGlu intake was estimated from any known food sources such as fortified powder products, juice and cereal along with vitamin supplements, whilst intake of $CH_3H_4$PteGlu, the natural form of folate, was calculated only from the natural forms of food such as vegetables, fruits, grains and dairy products. Due to the high temperature and pressure used in the manufacturing process for cereal, cereal was considered to contain only PteGlu; no methylfolate remains in cereal products. All blood samples and FFQs were collected prior to the commencement of mandatory folate fortification of bread flours (19th September, 2009 in Australia). Dietary intake of riboflavin, pyridoxine and methionine were also estimated using Foodworks ™. Due to niacin biosynthesis from tryptophan, endogenous and estimated synthesized niacin from tryptophan was determined separately. Similarly, vitamin C was estimated separately according to its source: natural endogenous vitamin C and the artificial form from vitamin supplements and fortified food. The intake of cobalamin was also considered.

#### 2.3.4.3 Estimation of dietary cobalamin

Cobalamin is a critical nutrient and must be considered in relation to folate due to its important role as a cofactor in the re-methylation and, hence, transsulphuration of Hcy. However, Foodworks ™ software (version 2.10.146) does not include cobalamin information and there is no available database to help determine the intake of this vitamin from Australian foods and supplements. Therefore, to assess cobalamin intake, the United States Department of Agriculture National Nutrient Database for Standard References was used.

### 2.3.5 Haplotype analysis

In order to examine any association between the combined alleles of MTHFR and CTH polymorphisms and the occurrence of AP, haplotype analysis was conducted using Haploview software version 4.1 (http://www.broad.mit.edu/mpg/haploview/). The D` value for linkage disequilibrium (LD) between the three polymorphisms in MTHFR and the two in CTH were also estimated using Haploview software [457].

### 2.3.6 Statistical analysis

All statistical analysis was performed using the JMP program for Windows (version 9.0; SAS Institute Inc., Cary, NC, USA). Chi-square analysis was used to determine the Hardy-Weinberg equilibrium. All continuous variables such as blood folate, thiols, $B_{12}$ and dietary nutrition intake levels were log10 transformed prior to performing a t-test. This t-test was used to assess the differences in mean values for each variable between phenotypes. All genetic and non-genetic variables were analysed using simple and multiple logistic regression analysis to establish a prediction model for AP susceptibility.

To verify the interaction of nutrition intakes and genetic variations, variables were grouped according to their biochemical characteristics. Stepwise regression was performed in a mixed direction with significant probability (0.250) for a parameter to be considered as a forward step and entered into the models or considered as a backward step and removed from the model. Mallow's Cp criterion was used for selecting the model where Cp first approaches p variables. Standard least squares analysis was carried out to establish a prediction model for biochemical data with related genetic or non-genetic variables. All statistical analysis was performed using a confidence level of 95%.

As this thesis covers a large volume of data/results, to help readability and comprehension only significant results with corresponding *p*-values are tabulated. All other results are either not presented or marked "not significant (NS)".

## 2.4 Results

In order to develop a better understanding of folate-related AP aetiology, various types of data were collected and analysed using appropriate statistical methods. In order to provide a logical approach, collected data was classified according to its characteristics, and analysed either separately or combined as appropriate. All data was separated into two groups: genetic and non-genetic variables. Non-genetic parameters were then further subdivided into two groups: population and biochemical variables, and dietary intake data.

The following variables lie in the population and biochemical variable group category: age, gender, serum and RBC folate, Hcy, Cys, Cys-Gly and GSH level. Nutritional intake data includes dietary intake of folate (synthetic and natural) as well as other nutrients related to methyl group metabolism such as pyridoxine, cobalamin, methionine, niacin, riboflavin and vitamin C. Sixteen individual genetic variations and eight compound genotypes were classified within the genetic data group.

Statistical analysis was carried out to identify the significant variables predicting the occurrence of AP. The main results are presented in this section. Details of the analysis methods used and their outcomes are presented in the following order.

1) The descriptive data for 'population and biochemical indices', and 'nutritional intake' in study subjects was tabulated, and their nutritional intake and blood folate status were evaluated. Additionally, dietary data was analysed in various ways to ascertain any interesting characteristics and associations between variables. Using a t-test, the possibility of the existence of any difference in the non-genetic data according to clinical phenotype was evaluated.

2) Population and biochemical data was analysed to examine whether these variables were associated with the occurrence of AP using simple nominal and stepwise regression models. The question of whether the interaction between these variables influenced AP occurrence was also analysed.

3) Nutritional intake data was analysed to examine whether it was associated with risk for AP. For a more in-depth analysis, dietary data was subdivided according to whether it was a synthetic or natural form and by intake level (above/below median or RDI). The association between dietary intake and an AP event was also analysed.

4) The prevalence of genotypes for 16 individual and 8 combined folate genetic variants was presented. Using a chi-square test, this genetic data was analysed to see whether any association exists between nutrient related polymorphisms and the occurrence of AP. For a more in-depth analysis, genotype distribution data was examined using simple nominal and stepwise regression models, taking into account gender and total folate intake level.

5) The genetic and nutritional intake variables were grouped according to their biochemical/ metabolic locus, and, using simple nominal regression analysis, the interaction between genetic variants, nutritional intake and any possible association with risk for AP was investigated.

6) To assess the most important parameters in predicting the occurrence of AP (taking into account all genetic and non-genetic variables), stepwise regression analysis was performed.

7) In order to support significant findings in the biochemical data associated with the occurrence of AP, and investigate the critical variables, standard least square analysis was performed to examine components predicting RBC folate and thiol levels.

8) Finally, the results of a case-control association test involving MTHFR and CTH haplotypes are presented for the current study population. HapMap data is also presented as a tool for reference.

### 2.4.1 The evaluation of nutritional intake status and biochemical indices in the study population

Table 2.5 presents descriptive data for all study subjects. No statistical difference was found between control and AP subject groups for any variables. However, Cys, Hcy, total methylfolate and niacin intake in male subjects were significantly higher than in female subjects (see Table 2.6). Subjects with a total folate intake above the RDI had significantly higher blood folate concentrations and other methyl group metabolism-related nutritional intake levels (total dietary folate, methylfolate, PteGlu, pyridoxine, cobalamin, methionine, niacin, niacin intake equivalence, riboflavin, total vitamin C and natural vitamin C intake) than the group of subjects whose folate intake was below the RDI for folate (see Table 2.6).

Table 2.5 Descriptive non-genetic data for all subjects

A. Population and biochemical indices

| Parameters | All subjects | | |
|---|---|---|---|
| | Mean ± SD | Median | IQR |
| Subjects (female, male) | 202 (116, 86) | N/A | N/A |
| Age | 63.17 ± 10.99 | 64 | 56-72 |
| Serum folate (nM) | 21.65 ± 12.02 | 30 | 12.0-30.0 |
| Serum folate excluding outliers (nM)* | 18.93 ± 9.48 | 17 | 11.0-25.0 |
| RBC folate (nM) | 983.4 ± 520.4 | 868.5 | 583.6-1180 |
| RBC folate excluding outliers (nM)* | 928.4 ± 440.2 | 848 | 579.0-1151 |
| Cys (μM) | 264.2 ± 39.94 | 261.74 | 237.6-288.5 |
| Hcy (μM) | 9.96 ± 2.85 | 9.64 | 7.61-11.80 |
| Gly-Cys (μM) | 23.8 ± 5.0 | 23.23 | 20.41-26.84 |
| GSH (μM) | 13.91 ± 5.68 | 12.04 | 9.8-17.45 |
| Serum $B_{12}$ (pM) | 280.1 ± 148.5 | 251 | 194.5-322.0 |

* Serum and RBC folate level excluding ≥ 45nM and ≥ 2500nM, respectively. Refer to the definition in section 2.4.2.1

B. Nutritional intake

| Nutritional intake | Total | | |
|---|---|---|---|
| | Mean ± SD | Median | IQR |
| Total dietary folate intake (μg/day) | 454.5 ± 239.4 | 398.8 | 314.9-565.7 |
| Total dietary methylfolate intake (μg/day) | 324.7 ± 124.5 | 310.3 | 239.9-385.6 |
| Total dietary PteGlu intake (μg/day) | 128.8 ± 199.1 | 79.92 | 12.99-148.2 |
| Total dietary pyridoxine intake (μg/day) | 5.96 ± 8.72 | 2.75 | 2.119-5.33 |
| Total dietary cobalamin intake (μg/day) | 19.91 ± 72.29 | 10.2 | 5.6-15.7 |
| Total dietary methionine intake (g/day) | 2.28 ± 1.13 | 2.01 | 1.76-2.46 |
| Total dietary niacin intake equivalence (mg/day) | 45.71 ± 14.98 | 27.31 | 36.61-52.53 |
| Total dietary niacin intake (mg/day) | 32.87 ±20.1 | 27.31 | 21.56-37.26 |
| Total dietary riboflavin intake (mg/day) | 4.86 ± 5.81 | 2.52 | 1.871-4.168 |
| Total dietary vitamin C intake (mg/day) | 250.7 ± 224.1 | 208.9 | 135.8-282.4 |
| Total dietary natural vitamin C intake (mg/day) | 200.5 ± 132.2 | 178.3 | 120.3-264.5 |

Table 2.6 Comparison of indices by clinical phenotype, gender and total folate intake levels (*p* value)

| Parameters | Clinical phenotype | Gender | Folate intake * level by RDI |
|---|---|---|---|
| Adenomatous polyp | N/A | NS | NS |
| Gender | NS | N/A | NS |
| Age | NS | NS | NS |
| Serum folate (nM) | NS | NS | 0.0033 |
| RBC folate (nM) | NS | NS | 0.0033 |
| Cys (μM) | NS | 0.0354 | NS |
| Hcy (μM) | NS | 0.0004 | NS |
| Gly-Cys (μM) | NS | NS | NS |
| GSH (μM) | NS | NS | NS |
| Serum $B_{12}$ (pM) | NS | NS | NS |
| Total dietary folate intake (μg/day) | NS | NS | <0.0001 |
| Total dietary methylfolate intake (μg/day) | NS | 0.0353 | <0.0001 |
| Total dietary PteGlu intake (μg/day) | NS | NS | <0.0001 |
| Total dietary pyridoxine intake (μg/day) | NS | NS | <0.0001 |
| Total dietary cobalamin intake (μg/day) | NS | NS | <0.0001 |
| Total dietary methionine intake (g/day) | NS | NS | <0.0001 |
| Total dietary niacin (mg/day) | NS | NS | <0.0001 |
| Total dietary niacin intake equivalence (mg/day) | NS | 0.0042 | <0.0001 |
| Total dietary riboflavin intake (mg/day) | NS | NS | <0.0001 |
| Total dietary vitamin C intake (mg/day) | NS | NS | <0.0001 |
| Total natural vitamin C intake (mg/day) | NS | NS | <0.0001 |

# See appendix 1 for the values * RDI –recommended dietary intake, 400 μg/day of folate for study subjects, significance presents the difference at <0.05 in each variable between two group of subjects with total dietary folate intake above RDI and subjects with total dietary folate intake below RDI level.

Table 2.7 Evaluation of blood folate, serum $B_{12}$ and Hcy level in the study subjects

| | Reference range | Subjects with blood folate-related indices level out of normal range (%) | | | Reference |
|---|---|---|---|---|---|
| | | Total | Control | AP | |
| Serum folate (nM) | < 6.8 nM | 10 (5.0) | 8 (4.9) | 2 (5.3) | NHANES Study [458] |
| RBC folate (nM) | < 317 nM | 6 (3.0) | 4 (2.5) | 2 (5.3) | NHANES Study [458] |
| Hcy (μM) | ≥13 μM | 22 (12.9) | 17 (12.3) | 5 (16.1) | Mayo clinic medical laboratories [459] |
| Serum $B_{12}$(pM) | < 111 pM | 3 (1.5) | 2 (1.2) | 1 (2.6) | Mayo clinic medical laboratories original value was < 150ng/L [460] |

Table 2.7 exhibits the evaluation of blood folate, $B_{12}$ and Hcy levels in the study population. 5% and 3% of subjects had low serum and RBC folate, respectively. However their blood folate deficiency was only borderline. Approximately 13% of subjects had a high Hcy level, and three subjects showed a vitamin $B_{12}$ deficiency. There was no statistical difference in the distribution of abnormal levels by clinical phenotype.

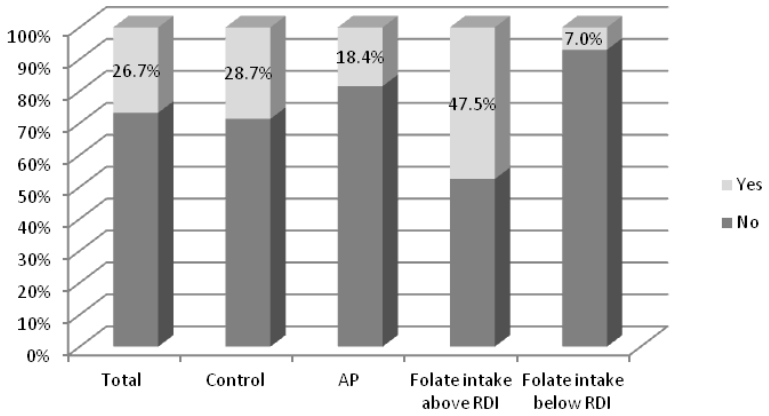



Figure 2.7 Percentages of subjects consuming multivitamin supplements

Table 2.8 Analysis of nutritional intake compared to RDI

| | RDI | | Subjects with nutritional intake below RDI (%) | | |
|---|---|---|---|---|---|
| | Female | Male | Total | Control | AP |
| Folate (μg/day) | 400.0 | 400.0 | 100 (50.3) | 82 (50.9) | 18 (47.4) |
| Pyridoxine (μg/day) | 1.3 (1.5)[1] | 1.3 (1.7)[1] | 11 (5.5) | 8 (4.9) | 3 (7.9) |
| Cobalamin (μg/day) | 2.4 | 2.4 | 1 (0.5) | 1 (0.6) | 0 |
| Niacin (mg/day) | 14.0 | 16.0 | 0 | 0 | 0 |
| Riboflavin (mg/day) | 1.1 (1.3)[2] | 1.3 (1.6)[2] | 13 (6.5) | 10 (6.1) | 3 (7.9) |
| Vitamin C (mg/day) | 45.0 | 45.0 | 4 (2.0) | 2 (1.2) | 2 (5.3) |

[1] Numbers in the bracket means RDI for over 50 years old, [2] numbers in the bracket means RDI for over 70 years old

The level of dietary vitamin intake and the use of multivitamin supplements were investigated (see Figure 2.6 and Table 2.8). 28.7% of controls and 18.4% of AP subjects were taking a multivitamin supplement, yet there was no statistically significant difference in the number of multivitamin users between each group (see Figure 2.6). Folate intake in approximately 50% of all subjects did not meet the RDI level (see Table 2.8). 47.5 % of subjects with a folate intake level above the RDI were consuming multivitamin supplements. However, surprisingly, 7% of subjects who were taking multivitamin supplements still had a total folate intake below the RDI (see Figure 2.6). Ten subjects had a total dietary folate above the upper limit (UL, as

defined in chapter 1.6.4.2). 5.5% and 6.5% of subjects had pyridoxine and riboflavin intakes lower than the RDI level, respectively. All subjects had an adequate intake of dietary niacin. There was no statistical difference in the distribution of multivitamin supplement use and under-intake of vitamins between controls and AP cases.

Table 2.9 explains whether the dietary intake of folate is related to the intake of other nutrients important for methyl group metabolism using stepwise regression analysis. The intake of pyridoxine and niacin was significantly associated with total dietary folate intake in all subject groups ($p$ = 0.0008 and <0.0001, respectively). Total dietary folate intake was increased with natural vitamin C intake, but this association was neither observed in the control group, nor in subjects who took dietary folate above the RDI level. In the group of subjects with folate intake below the RDI there was a significant association between the intake of all dietary nutrients important for methyl group metabolism, apart from animal source nutrients such as methionine and cobalamin, and total folate intake.

Table 2.9 Nutrient intake important for methyl group metabolism associated with total dietary folate intake by stepwise regression analysis

| | | Nutrient | $p$ | Slope estimate | $r^2$ |
|---|---|---|---|---|---|
| All subjects | | Pyridoxine | 0.0008 | 8.3759 | 0.4633 |
| | | Niacin equivalence | <0.0001 | 6.3091 | |
| | | Riboflavin | 0.0408 | 7.5160 | |
| | | Natural vitamin C | 0.0131 | 0.3242 | |
| Clinical phenotype | Control | Pyridoxine | 0.0027 | 8.6000 | 0.4293 |
| | | Niacin equivalence | <0.0001 | 6.2630 | |
| | Adenomatous polyp | Pyridoxine | <0.0001 | 17.421 | 0.8576 |
| | | Niacin | 0.0014 | 5.6269 | |
| | | Natural vitamin C | <0.0001 | 0.6050 | |
| Folate intake | Above RDI | Pyridoxine | <0.0001 | 9.1890 | 0.2493 |
| | | Niacin equivalence | 0.0043 | 3.9026 | |
| | Below RDI | Pyridoxine | 0.0023 | 11.508 | 0.6185 |
| | | Niacin | 0.0204 | -2.5671 | |
| | | Niacin equivalence | <0.0001 | 4.6412 | |
| | | Riboflavin | 0.0203 | 6.4702 | |
| | | Natural vitamin C | <0.0001 | 0.5125 | |

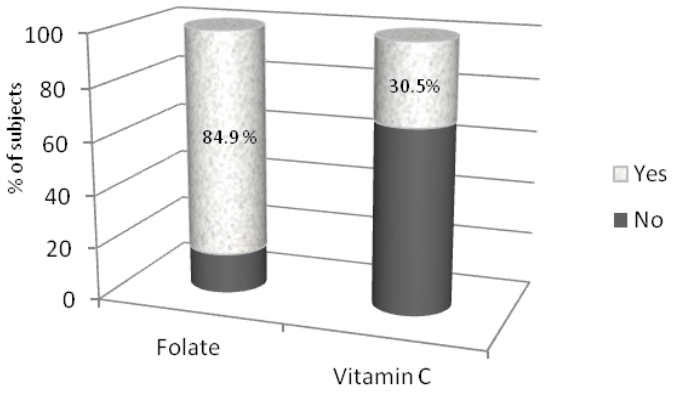



Figure 2.8 Percentages of subjects who consumed the synthetic form of folate and vitamin C as part of their total dietary intake

It was found that 84.9% and 30.5% of subjects were consuming the synthetic form of folate or vitamin C, respectively, via one or both of fortified food and vitamin supplements (see Figure 2.7). The ratios of the synthetic form of folate and vitamin C intake are presented in Table 2.10.

Table 2.10 Composition (%) of synthetic folate and vitamin C-related to total intake

| | Total % (SD) | Clinical phenotype | | Total dietary folate intake | |
|---|---|---|---|---|---|
| | | Control | AP | Above RDI | Below RDI |
| Folate | 22.54 (20.07) | 22.72 (20.33) | 21.82 (19.15) | 29.93 (22.23)* | 15.24 (14.41) |
| Vitamin C | 10.94 (20.97) | 11.21 (21.14) | 9.78 (20.46) | 16.35 (23.79)* | 5.41 (6.03) |

*statistical difference at $p<0.05$, mean % of synthetic form in each total dietary vitamin intake (SD)

Table 2.10 exhibits the mean percentage intake of the synthetic vitamer (PteGlu) within the total dietary folate intake and the percentage of supplemental vitamin C intake. In the control and AP groups, respectively 22.72% and 21.82% of the total dietary folate intake was the synthetic PteGlu form. However, the distribution did not differ significantly between the two groups. This result, in conjunction with the results from Table 2.6 and Figure 2.5 (no difference in PteGlu intake and multivitamin use between the case and control groups), suggest that, in the current study population, consuming PteGlu may not be associated with the occurrence of AP. When the study populations were grouped according to their total folate intake level using the RDI (above or below RDI), subjects with a total dietary folate intake above the RDI showed

a significantly higher percentage intake of PteGlu (29.93%) than subjects with a total dietary folate intake level below the RDI (15.24%, $p$ <0.05). Along with the results from Table 2.6 (subjects with total folate intake above the RDI level consumed significantly higher level of total dietary folate, PteGlu and methylfolate, all $p$<0.0001), this clearly supports the view that PteGlu intake contributes to the increased total population dietary folate level.

Interestingly, analysis showed that synthetic PteGlu intake may be related to synthetic vitamin C intake. Subjects with total dietary folate intake above the RDI had a significantly higher level of synthetic vitamin C intake (16.35%) than subjects with a total dietary folate intake below the RDI (5.41%, $p$<0.05). This increased synthetic vitamin C intake in subjects taking high levels of folate may suggest that both synthetic vitamers have a similar source such as a multivitamin supplement, fortified cereal and health foods. Vitamin C is known to be a critical coenzyme in folate metabolism [76, 461]. Therefore increased intake of vitamin C and folate may generate a potentially important interaction in folate metabolism. This will be discussed further in chapter 3 which describes a cell culture model of folate metabolism.

In summary, approximately half of the study population did not reach the RDI for folate. However, their blood folate status was relatively normal. A high level of total dietary folate intake was mainly due to consumption of PteGlu: 85% of subjects were taking PteGlu prior to the commencement of mandatory folic acid fortification. As yet there is no clear evidence that either synthetic or natural folate is associated with the occurrence of AP.

### 2.4.2 The effect of non-genetic variables on the occurrence of adenomatous polyps

In order to explore the potential mechanisms between multiple variables and the occurrence of AP in depth, variables were analysed by genetic and non-genetic parameters. Non-genetic variables are considered in two subgroups. The first is population and biochemical data such as blood folate, serum $B_{12}$ and thiol concentrations along with gender and age, and the second is nutritional intake data. This section will first examine the population and biochemical variables associated with AP occurrence. Subsequently the relationships between nutritional intakes and AP occurrence will be considered. The investigation of data concerning genetic variables and their interactions with nutritional intake as a risk factor for AP is presented in section 2.4.3.

#### 2.4.2.1 The effects of biochemical variables on the occurrence of adenomatous polyps

Table 2.11 lists the variables included in the statistical analysis for this section. Some subjects had serum and RBC folate levels out of the reference range and are referred to as 'outliers' (≥ 2500nM for RBC and ≥ 45nM for serum folate). For these subjects, it is not clear whether this value was an accurate concentration or due to the calibration range limitations of the assays. For these reasons, serum and RBC folate levels were statistically analysed inclusive and exclusive of these values (outliers).

Table 2.11 Non-genetic biochemical parameters for statistical analysis

| Classification | | Indices |
|---|---|---|
| Population and biochemical data | | Gender |
| | | Age |
| | Blood folate and $B_{12}$ level | Serum folate (nM) |
| | | Serum folate excluding outliers (nM) |
| | | RBC folate (nM) |
| | | RBC folate excluding outliers (nM) |
| | | Serum $B_{12}$ (pM) |
| | Blood thiol levels | Cys (μM) |
| | | Hcy (μM) |
| | | Cys-Gly (μM) |
| | | GSH (μM) |

When the study population was considered as a whole, no variables associated with AP occurrence were found. However, when gender was taken into account, RBC folate (including and excluding outliers) was associated with the development of AP (see Table 2.12). Interestingly, RBC folate level showed a contrasting effect on AP occurrence by gender. In females the risk of an AP increased with RBC folate concentration ($p$ = 0.0021, $r^2$ = 0.0986, slope estimate (SE) = 0.0014). However, in males, RBC decreased the risk for AP ($p$ = 0.0040, $r^2$ = 0.0869, SE = -0.0019). It was found that the increment of 100nM in RBC folate concentration increases the risk of AP 1.15 times in females, but the same RBC folate increment decreases AP risk 0.83 times in males (see Table 2.13). The relationship between RBC folate level and AP risk was confirmed using stepwise regression analysis (see Table 2.14). No variables were found to be associated with AP risk when total dietary folate intake level was taken into account.

Table 2.12 Parameters that predict the occurrence of adenomatous polyps with respect to population and biochemical data within all subjects, by gender and according to total dietary folate intake level using simple nominal regression analysis

| Group | | Variables | *p* | $r^2$ | Slope estimate |
|---|---|---|---|---|---|
| All subjects | | None | - | - | - |
| Gender | Female | RBC folate | 0.0021 | 0.0986 | 0.0014 |
| | | RBC folate excluding outliers | 0.0007 | 0.1253 | 0.0018 |
| | Male | RBC folate | 0.0040 | 0.0869 | -0.0019 |
| | | RBC folate excluding outliers | 0.0097 | 0.0712 | -0.0019 |
| Total dietary folate intake | Above RDI | None | - | - | - |
| | Below RDI | None | - | - | - |

Table 2.13 The risk for the occurrence of AP according to an increment of 100nM of RBC folate concentration

| | Female | | | Male | | |
|---|---|---|---|---|---|---|
| | OR | 95% CI | *p* | OR | 95% CI | *p* |
| RBC folate | 1.15 | 1.05-1.26 | 0.0021 | 0.83 | 0.70-0.95 | 0.004 |
| RBC folate excluding outliers | 1.20 | 1.08-1.35 | 0.0007 | 0.83 | 0.70-0.96 | 0.0097 |

Table 2.14 Population and biochemical variables that predict the occurrence of an adenomatous polyp within all subjects, by gender and according to total dietary folate intake level using stepwise regression analysis

| Group | | Indices | *p* | Slope estimate | $r^2$ |
|---|---|---|---|---|---|
| All subjects | | None | - | - | - |
| Gender | Female | RBC folate | 0.0021 | 0.00136 | 0.0986 |
| | Male | RBC folate | 0.0018 | -0.00357 | 0.1428 |
| Total dietary folate intake | Over RDI | None | - | - | - |
| | Below RDI | None | - | - | - |

The data analysis for the interaction between each biochemical variable and the occurrence of AP is presented in Table 2.15. Age and Hcy concentrations interact with serum and RBC folate level to predict AP occurrence. Cys was found to predict AP prevalence in concert with RBC folate and serum $B_{12,}$ respectively. Interestingly, all of these interactions showed a reduced risk for an AP event. Increased age or Hcy concentration in combination with blood folate level decreased the risk for AP (Age*RBC folate; $p$ = 0.0143, $r^2$= 0.0434, SE = -0.0001, Age*serum folate; $p$ =0.0135, $r^2$=0.0438, SE = -0.0040, Hcy*RBC folate; $p$ = 0.0203, $r^2$= 0.0345, SE = -0.0003, Hcy*serum folate; $p$ = 0.0110, $r^2$ = 0.0436, SE = -0.0170). In addition, an elevated Cys level in combination with RBC folate ($p$ = 0.0342, $r^2$ = 0.0282, SE = -0.0003) and serum $B_{12}$ ($p$ = 0.0151, $r^2$= 0.0376, SE = -0.0001) lowered the risk for AP.

Table 2.15 The interaction between population and biochemical variables in predicting the occurrence of adenomatous polyps analysed using nominal logistic regression analysis

A. Significant interactions between variables predicts the occurrence of adenomatous polyps ($p$-value)

| | Age | Serum $B_{12}$ | Cys | Hcy | Cys-Gly | GSH |
|---|---|---|---|---|---|---|
| Age | | NS | NS | NS | NS | NS |
| Gender | NS | NS | NS | NS | NS | NS |
| Serum folate | 0.0135 | NS | NS | 0.0110 | NS | NS |
| Serum folate excluding outliers | 0.0076 | NS | NS | NS | NS | NS |
| RBC folate | 0.0143 | NS | 0.0342 | 0.0203 | NS | NS |
| RBC folate excluding outliers | 0.0008 | NS | NS | 0.0055 | NS | NS |
| Serum $B_{12}$ | | | 0.0151 | NS | NS | NS |
| Cys | | | | NS | NS | NS |
| Hcy | | | | | NS | NS |
| Cys-Gly | | | | | | NS |
| GSH | | | | | | |

B. Slope estimate and $r^2$ value for the significant interaction of multiple components in predicting adenomatous polyps

| Interaction | $p$ | $r^2$ | Slope estimate |
|---|---|---|---|
| Serum folate*Age | 0.0135 | 0.0438 | -0.0040 |
| Serum folate excluding outliers*Age | 0.0076 | 0.0586 | -0.0060 |
| RBC folate*Age | 0.0143 | 0.0434 | -0.0001 |
| RBC folate excluding outlier*Age | 0.0008 | 0.0727 | -0.0001 |
| RBC folate*Cys | 0.0342 | 0.0282 | -0.0003 |
| Serum $B_{12}$*Cys | 0.0151 | 0.0376 | -0.0001 |
| Serum folate*Hcy | 0.0110 | 0.0436 | -0.0170 |
| RBC folate*Hcy | 0.0203 | 0.0345 | -0.0003 |
| RBC folate excluding outliers*Hcy | 0.0055 | 0.0511 | -0.0010 |

Age is a critical risk factor for colorectal neoplasia. The risk of CRC increases with age [462, 463]. However, in the current study, the age of the subjects showed no association with the risk for AP on its own, whereas, it decreased the risk for AP when combined with serum and RBC folate level. The reasons for these contrasting results may include the following: the study subjects were relatively young (mean age 63.2 and 65.7 years for the whole study and AP subjects, respectively and only 10 subjects were aged above 80 years) and the sample size was small. In addition, the decreased risk for AP found with the interaction between blood folate and age could be explained as follows: although RBC folate concentration did not show an association with AP events in all study subjects, it is possible that it is having a protective effect as observed in previous research [111]. Therefore, increased blood folate levels could have a dominant effect on the occurrence of AP over age. In line with this, the increased RBC folate and serum $B_{12}$ levels possibly have a dominant effect over Hcy and Cys levels. Therefore, the interaction between RBC folate and Hcy, and serum $B_{12}$ and Cys also decreased the occurrence of AP.

#### 2.4.2.2 Nutritional intake and the occurrence of adenomatous polyps

In this section, the dietary intake data was analysed with the aim of answering the question: "Does nutritional intake predict an AP event?" Nutrients which are utilised for methyl group metabolism such as folate, including its synthetic and natural forms, pyridoxine, niacin, riboflavin, and methionine were included in this statistical model. Vitamin C intake was also considered. It is common knowledge that vitamin C plays an important role as an antioxidant. Indeed, there is evidence suggesting that vitamin C has a potential beneficial role in maintaining folate in the reduced states and, hence, in influencing the stability of folate [76, 464].

Nutritional intake was analysed, not only in terms of total dietary intake, but also subdivided according to 1) whether it was synthetic/supplemental or natural, and 2) intake level (above/below median and/or RDI) (see Table 2.16). For instance, pyridoxine, cobalamin, methionine and riboflavin were analysed according to total intakes and whether they were above/below the median value (≥ median and <median). This aimed to provide data with regards to different levels of nutrient intake (low and high) and allow an examination of their potential associations with the occurrence of AP.

Total dietary folate intake data was divided by RDI level instead of median value as previous analysis carried out on nutritional intake suggested that a significant number of subjects were not reaching the RDI for total dietary folate (see Table 2.8). By contrast, most subjects in this

study population had an intake of other nutrients such as pyridoxine, cobalamin, niacin, riboflavin and vitamin C intake which met the RDI (see Table 2.8). For this reason, the median value was used to differentiate the nutritional intake level of these nutrients. Analysis of niacin intake was performed using two measures: total niacin intake and niacin intake equivalence. When considering vitamin C intake, the analysis was performed for both the natural form of vitamin C intake from food and for total dietary vitamin C intake including both synthetic and natural forms.

Table 2.16 List of indices used for statistical analysis to investigate significant nutritional intake associated with adenomatous polyp risk

| Classification | | | Indices |
|---|---|---|---|
| Nutrition intake | Dietary folate intake | Total intake (both natural and synthetic form) | Total dietary folate intake (μg/day) |
| | | | *Total dietary folate intake above / below RDI (μg/day)* |
| | | Natural form intake | Total methylfolate intake (μg/day) |
| | | | *Total methylfolate intake above / below median (μg/day)* |
| | | Synthetic form intake | Total PteGlu intake (μg/day) |
| | | | *Total PteGlu intake above / below median (μg/day)* |
| | Pyridoxine intake | | Total pyridoxine intake (μg/day) |
| | | | *Total pyridoxine intake above / below median (μg/day)* |
| | Cobalamin intake | | Total cobalamin intake (μg/day) |
| | | | *Total cobalamin intake above / below median (μg/day)* |
| | Methionine intake | | Total methionine intake (g/day) |
| | | | *Total methionine intake above / below median (g/day)* |
| | Riboflavin intake | | Total riboflavin intake (mg/day) |
| | | | *Total riboflavin intake above / below median (mg/day)* |
| | Niacin intake | | Total niacin intake (mg/day) |
| | | | Total niacin equivalent intake (mg/day) |
| | Vitamin C intake | | Total vitamin C intake (mg/day) |
| | | | Total natural vitamin C intake (mg/day) |

# See the median value from Table 2.5, median subject was included in above group, and variables in italic font (dietary intake subdivide by median or RDI value) were excluded for stepwise regression analysis

These variables for niacin and vitamin C intake provide additional information with respect to dietary intake patterns and their varied physico-chemical roles (see section 2.3.4.2). Due to the requirement for mathematical stability in establishing a statistical prediction model only the total dietary nutritional intake data was used to perform the stepwise regression analysis.

Table 2.17 Nutritional intake associated with the occurrence of an adenomatous polyp using simple nominal regression analysis

A. Significant nutritional intake predicts the risk of adenomatous polyps in entire population

| Total nutritional intake | *p* | $r^2$ | Slope estimate |
|---|---|---|---|
| Methylfolate intake below median | 0.0189 | 0.0612 | -0.0115 |
| Cobalamin intake above median | 0.0402 | 0.0407 | -0.0398 |

B. Significant nutritional intake predicts the occurrence of adenomatous polyps by gender

| | Gender | | | | | |
|---|---|---|---|---|---|---|
| | Female | | | Male | | |
| Total nutritional intake | *p* | $r^2$ | Slope estimate | *p* | $r^2$ | Slope estimate |
| Methylfolate intake below median | | NS | | 0.0172 | 0.1332 | -0.0195 |
| Pyridoxine intake above median | 0.0386 | 0.082 | -0.1214 | | NS | |
| Methionine intake | 0.0037 | 0.0873 | 0.6497 | | NS | |
| Methionine intake above median | 0.0034 | 0.166 | 0.9859 | | NS | |

C. Significant nutritional intake predicts the occurrence of adenomatous polyps taking into account total dietary folate intake

| | Folate intake | | | | | |
|---|---|---|---|---|---|---|
| | Above RDI | | | Below RDI | | |
| Total nutritional intake | *p* | $r^2$ | Slope estimate | *p* | $r^2$ | Slope estimate |
| Methylfolate intake below median | | NS | | 0.0206 | 0.0754 | -0.013 |
| Cobalamin intake | 0.0194 | 0.0549 | -0.051 | | NS | |
| Cobalamin intake above the median | 0.0060 | 0.1129 | -0.122 | | NS | |
| Methionine intake | | NS | | 0.0476 | 0.0416 | 0.3875 |
| Methionine intake above the median | | NS | | 0.0131 | 0.1737 | 0.7408 |
| Natural vitamin C intake | | NS | | 0.0194 | 0.058 | -0.0090 |

Table 2.17 presents significant relationships between nutritional intake and the occurrence of AP. In the entire population subjects who had a methylfolate intake below the median were less likely to have AP ($p$ = 0.0189, $r^2$ = 20.0612, SE = -0.0115). The higher intake of cobalamin (≥ median) also decreased the risk for an AP ($p$ = 0.0402, $r^2$ = 0.0407, SE = -0.0398). Low methylfolate intake (<median) in male subjects also had a protective effect against AP ($p$ = 0.0172, $r^2$ = 0.1332, SE = -0.0195). In females, a high intake of pyridoxine (> median) decreased the occurrence of AP ($p$ = 0.0386, $r^2$ =0.082, SE = -0.1214). However, a high intake of methionine significantly increased the risk of AP in female subjects (total methionine intake: $p$ = 0.0037, $r^2$ = 0.0873, SE = 0.6497, total methionine intake above the median: $p$ = 0.0034, $r^2$ = 0.166, SE = 0.9859). When analysing total folate intake level, subjects taking total folate above the RDI and in combination with a high level of cobalamin were found to be less likely to have AP ($p$ = 0.0060, $r^2$ = 0.1129, SE = -0.122). This potential protective effect of cobalamin intake was not retained within the subject group consuming a total dietary folate below the RDI level. However, low methylfolate intake reduced AP occurrence within this group ($p$ = 0.0206, $r^2$ = 0.0754, SE = -0.013). Intake of natural vitamin C also had a protective effect against AP in subjects taking below the RDI for folate ($p$ = 0.0194, $r^2$ = 0.058, SE = -0.009). Meanwhile, a high intake of methionine was shown to increase the risk of AP (methionine; $p$ = 0.0476, $r^2$ = 0.0416, SE = 0.3875, methionine intake above the median; $p$ = 0.0131, $r^2$ = 0.1737, SE = 0.7408).

Table 2.18 Nutritional intake predicts the occurrence of an adenomatous polyp by stepwise regression analysis

| Group | | Total dietary nutrition intake | $p$ | Slope estimate | $r^2$ |
|---|---|---|---|---|---|
| All subjects | | None | - | - | - |
| Gender | Female | Methionine | 0.0037 | 0.6497 | 0.0873 |
| | Male | None | - | - | - |
| Folate intake | Above RDI | Cobalamin | 0.0127 | -0.0562 | 0.0638 |
| | Below RDI | Niacin | 0.0311 | -0.0943 | 0.1659 |
| | | Natural vitamin C | 0.0298 | -0.0107 | |
| | | Pyridoxine | 0.0199 | 0.3941 | |
| | | Methionine | 0.0202 | 0.6130 | |

Nutrient intake levels which significantly predicted the occurrence of AP by stepwise regression analysis are presented in Table 2.18. Results suggest that there is no dominant effect of nutritional intake for an AP event when all subjects are considered. Total methionine intake increased the risk for AP occurrence in females ($p$ = 0.0037, $r^2$ = 0.0873, SE = 0.6497).

Cobalamin was the only nutrient associated with the risk of AP in subjects taking folate above the RDI level ($p$ = 0.0127, $r^2$ = 0.0638, SE = -0.0562) as predicted by simple regression analysis. Total dietary niacin ($p$ = 0.0311, SE = -0.0943) and natural vitamin C ($p$ = 0.0298, SE = -0.0107) intake decreased AP risk. However, methionine ($p$ = 0.0202, SE = 0.6130) and pyridoxine ($p$ = 0.0199, SE = 0.3941) increased risk for AP when folate intake was below the RDI level.

Table 2.19 demonstrates the interaction between nutrient intakes and any trend in predicting AP. In the group of subjects consuming total dietary folate below the RDI level, the interaction between total folate and pyridoxine ($p$ = 0.0065, $r^2$ = 0.1010, SE = -0.0059), and riboflavin ($p$ = 0.0403, $r^2$ = 0.0514, SE = -0.0074) intake was shown to reduce AP risk. The interaction between PteGlu and methionine ($p$ = 0.012, $r^2$ = 0.0471, SE = -0.0046) was also found to be associated with an AP event. Total dietary pyridoxine intake showed a protective effect when combined with various nutrients. Cobalamin ($p$ = 0.0013, $r^2$ = 0.0681, SE = -0.0221), methionine ($p$ = 0.0315, $r^2$ = 0.0433, SE = -0.086), niacin ($p$ = 0.011, $r^2$ = 0.0382, SE = -0.010), riboflavin ($p$ =0.0415, $r^2$ = 0.0274, SE = -0.0216) and vitamin C ($p$ = 0.0491, $r^2$ = 0.0376, SE = -0.0003) all exhibited an interaction with pyridoxine, decreasing the risk of AP in each case.

In summary, nutritional intakes showed interesting associations in relation to the occurrence of AP either in the entire group or according to gender and folate intake levels. When all subjects were considered, low intake of methylfolate and high intake of cobalamin decreased the risk for AP. In females, a high intake of methionine increased the risk. The effect of nutritional intake on the risk of AP was also modulated by the level of total dietary folate intake. In the high folate intake group, cobalamin decreased the occurrence of AP, although in the low folate intake group, methylfolate and natural vitamin C intake decreased the risk, while high intake of methionine significantly increased the occurrence of AP. Additionally, total dietary pyridoxine intake reduced the risk of AP when interacting with various other nutrients such as folate, cobalamin, methionine, niacin and riboflavin.

Table 2.19 The significant nutrition-nutrition interaction in predicting the occurrence of adenomatous polyp analysed using logistic regression analysis

A. The significant interaction between nutritional intakes associated with AP events (*p*-value)

| Total dietary nutrition intake | Total dietary folate | Folate intake over RDI | Folate intake under RDI | Methyl folate | PteGlu | Pyridoxine | Cobalamin | Methionine | Niacin | Niacin equivalence | Riboflavin |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Pyridoxine | NS | NS | 0.0065 | NS | NS | | | | | | |
| Cobalamin | NS | NS | NS | NS | NS | 0.0013 | | | | | |
| Methionine | NS | NS | NS | NS | 0.012 | 0.0315 | NS | | | | |
| Niacin | NS | NS | NS | NS | NS | 0.0111 | NS | NS | | | |
| Niacin equivalence | NS | NS | NS | NS | NS | NS | NS | NS | NS | | |
| Riboflavin | NS | NS | 0.0403 | NS | NS | 0.0415 | NS | NS | NS | NS | |
| Vitamin C | NS | NS | NS | NS | NS | NS | 0.0491 | NS | NS | NS | NS |
| Natural vitamin C | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS |

B. Slope estimate and $r^2$ value for the interaction between nutritional intake modifying AP risk

| Total dietary intake | *P* | Slope estimate | $r^2$ |
|---|---|---|---|
| Pyridoxine* Total dietary folate intake below RDI | 0.0065 | -0.0059 | 0.1010 |
| Riboflavin* Total dietary folate intake below RDI | 0.0403 | -0.0074 | 0.0514 |
| PteGlu*Methionine | 0.0120 | -0.0046 | 0.0471 |
| Pyridoxine*Cobalamin | 0.0013 | -0.0221 | 0.0681 |
| Pyridoxine*Methionine | 0.0315 | -0.086 | 0.0433 |
| Pyridoxine*Niacin | 0.0111 | -0.010 | 0.0382 |
| Pyridoxine*Riboflavin | 0.0415 | -0.0216 | 0.0274 |
| Cobalamin*vitamin C | 0.0491 | -0.0003 | 0.0376 |

### 2.4.3 The effects of genetic variations on the occurrence of adenomatous polyps

Sixteen individual and eight combined genetic variants were analysed to examine whether there are any associations between them and AP risk. The effects of genetic variation on the occurrence of AP was also analysed in combination with nutritional intake data. To achieve these aims a chi-square test, simple and stepwise regression analysis was used.

#### 2.4.3.1 Genotype distribution for folate-related genes

The observed prevalence of genotypes and minor allele frequencies (Maf) for fifteen genetic variations are summarised in Table 2.20. As TS 3RG>C possesses seven genotypes it is tabulated separately in Table 2.21.

Significant differences between each clinical phenotype were found in the genotype distributions and Maf for TS 1496del6, CBS 844*ins*68 and MSR A66G. However, no statistical difference between the genotype distributions and Maf of each clinical phenotype was found for MTHFR C677T, A1298C, G1793A, SHMT C1420T, TSER 2R3R, GCPII C1561T, DHFR 19bp del, RCF G80A, CTH G1364T, CTH IVS 10-430C>T, BHMT G595A and MS A2756G.

The frequency of the variant MTHFR C677T T allele in the control and AP case groups was 0.31 and 0.36, respectively. This did not equate to a significant difference in the prevalence of this allele between these two groups. The carriage of the T allele was 53.7% and 55.3% in control and case groups, respectively, and this mutant allele did not increase the risk of an AP event. The Maf of MTHFR A1298C was 0.29, which was presented equally in both clinical phenotypic groups. The prevalence of carriage for the mutant C allele was 50% and 47.37% in each of the case and control groups, respectively. The Maf of the MTHFR G1793A variant was rare: only 0.06 and 0.03 in control and case subjects, respectively. No homozygous recessive genotype was found in either the case or the control subjects. This genetic variation was not associated with risk for AP.

The frequency of the RFC G80A A allele was 0.43 for controls and 0.42 for subjects with AP. This genetic variation was also not associated with AP occurrence.

The GCPII C1561T TT genotype did not exist in either the case or the control group, and Maf was only 0.04 and 0.09 in the control and case groups, respectively. This result is consistent

with findings from other genetic studies from South Australia [465] and the Framingham Offspring cohort in the United States [466]. Although the homozygous recessive genotype was found in these two studies, it was only present in one out of 164 subjects in the South Australian study, and five out of 1913 subjects in the Framingham Offspring study. Although the GCP II C1561T variation gave a high OR for risk of AP, this finding was not significant (OR: 2.67, CI: 0.91-7.63, $p$ = 0.064). For the TSER 2R3R variation, Maf was 0.47 and 0.39 in the control and AP groups, respectively. No significant difference in the prevalence of this variation was found between the two groups.

The presence of the TS 1496del6 deletion allele was scored as 0.30 and 0.42 in the control and case groups, respectively. This difference in deletion allele prevalence between case and control groups predicts an increase in the likely occurrence of AP (OR: 1.73, CI: 1.00-2.98, $p$ = 0.048), but this was not observed in individuals who carried at least one copy of the TS 1496del6 deletion allele (i.e. carriage).

Table 2.21 shows the pattern of TSER 3R>C genotype prevalence, although this genetic variation had no effect on the susceptibility for an AP event.

The following prevalence of CBS 844*ins*68 genotypes was observed: in control subjects 88% were wildtype, 12% were heterozygous and 1% were homozygous recessive. Among the AP subjects 71% were wildtype, 29% were heterozygous and no homozygous recessive individuals were observed. Only one individual was homozygous recessive in the control group. This low prevalence of the homozygous recessive genotype corresponds to the findings of other reports [106, 113]. The Maf of this genetic variation in the control and case groups was 0.06 and 0.14, respectively, predicting an increased risk for an AP event (OR: 2.47 95% CI: 1.06-5.71, $p$ = 0.035). It was also observed that carriage of the insertion allele strongly predicted the risk for an AP event (see Table 2.22, OR: 2.93, 95% CI: 1.16-7.35, $p$ = 0.0197).

Two genetic variations in CTH (G1364T and IVS 10-430C>T) were found to have no effect on the occurrence of AP. The Maf of CTH G1394T was 0.31 and 0.25 in control and case subjects, respectively. However, the lower Maf in the AP group was not associated with a decreased risk of an AP event. No difference was observed in the Maf values for CTH IVS 10-430 C>T between control and AP subjects: 0.27 for the control group and 0.28 for the case group.

Table 2.20 Distribution of genotype and minor allele frequency for genetic variants of folate metabolism, and there associated risk for the occurrence of adenomatous polyp

| | Control | | | | | | | Adenomatous polyp | | | | | | | OR (95%CI, *p*) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | WT | | Het | | Rec | | Maf | WT | | Het | | Rec | | Maf | |
| | N | (%) | N | (%) | N | (%) | | N | (%) | N | (%) | N | (%) | | |
| MTHFR C677T | 76 | (46) | 74 | (45) | 14 | (8) | 0.31 | 17 | (45) | 15 | (39) | 6 | (16) | 0.36 | 1.22 (0.70-2.13, 0.542) |
| MTHFR A1298C | 82 | (50) | 70 | (43) | 12 | (7) | 0.29 | 20 | (53) | 14 | (37) | 4 | (11) | 0.29 | 1.01 (0.56-1.82, 0.928) |
| MTHFR G1793A | 148 | (90) | 14 | (9) | 2 | (1) | 0.06 | 36 | (95) | 2 | (5) | N/A | | 0.03 | 0.47 (0.07-2.15, 0.390) |
| RCF G80A | 57 | (35) | 73 | (45) | 34 | (20) | 0.43 | 14 | (37) | 16 | (42) | 8 | (21) | 0.42 | 0.96 (0.56-1.65, 0.991) |
| GCPII C1561T | 152 | (93) | 12 | (7) | N/A | | 0.04 | 31 | (82) | 7 | (18) | N/A | | 0.09 | 2.67 (0.91-7.63, 0.064) |
| TSER 2R3R | 46 | (28) | 80 | (49) | 37 | (23) | 0.47 | 11 | (29) | 22 | (58) | 4 | (11) | 0.39 | 0.76 (0.44-1.31, 0.360) |
| **TS 1496del6** | **79** | **(48)** | **73** | **(45)** | **12** | **(7)** | **0.30** | **13** | **(34)** | **18** | **(47)** | **7** | **(18)** | **0.42** | **1.73 (1.00-2.98, 0.048) *** |
| **CBS 844ins68** | **144** | **(88)** | **19** | **(12)** | **1** | **(1)** | **0.06** | **27** | **(71)** | **11** | **(29)** | **N/A** | | **0.14** | **2.47 (1.06-5.71, 0.035)*** |
| CTH G1364T | 72 | (44) | 82 | (50) | 10 | (6) | 0.31 | 20 | (53) | 17 | (45) | 1 | (3) | 0.25 | 0.74 (0.40-1.35, 0.365) |
| CTH IVS10-430C>T | 90 | (55) | 61 | (37) | 13 | (8) | 0.27 | 21 | (55) | 13 | (34) | 4 | (11) | 0.28 | 1.06 (0.58-1.91, 0.958) |
| DHFR 19bp del | 53 | (32) | 79 | (48) | 32 | (20) | 0.44 | 18 | (47) | 14 | (37) | 6 | (16) | 0.34 | 0.67 (0.39-1.17, 0.172) |
| SHMT C1420T | 85 | (52) | 64 | (39) | 15 | (9) | 0.29 | 18 | (47) | 15 | (39) | 5 | (13) | 0.33 | 1.22 (0.69-2.15, 0.555) |
| BHMT G595A | 160 | (98) | 4 | (2) | N/A | | 0.01 | 38 | (100) | N/A | | N/A | | 0.00 | 0.00 (0.00-6.68, 1.000) |
| MS A2756G | 109 | (66) | 52 | (32) | 3 | (2) | 0.18 | 28 | (74) | 8 | (21) | 2 | (5) | 0.16 | 0.87 (0.42-1.79, 0.822) |
| **MSR A66G** | **39** | **(24)** | **79** | **(48)** | **46** | **(28)** | **0.52** | **5** | **(13)** | **15** | **(39)** | **18** | **(47)** | **0.33** | **1.87 (1.07-3.28, 0.025) *** |

* Significant at $p<0.05$, WT: wildtype, Het: heterozygous genotype, Rec: homozygous recessive genotype, Maf: Minor allele frequency, one control and case subjects had aberrant TSER 2R3R genotype and were excluded from analysis (multiple repeats more than 3R)

Table 2.21 Genotype distribution for the TSER 3RG>C polymorphism

| TSER 3RG>C genotype | Control (%) | | Adenomatous polyp (%) | |
|---|---|---|---|---|
| 3RC/2R | 47 | (29) | 12 | (33) |
| 3RG/2R | 33 | (20) | 9 | (25) |
| 3RG/3RC | 21 | (13) | 4 | (11) |
| 3RC/3RC | 20 | (12) | 4 | (11) |
| 3RG/3RG | 5 | (3) | 3 | (8) |
| 2R/2R | 37 | (23) | 4 | (11) |
| X | 1 | (1) | 2 | (5) |
| Total | 164 | (100) | 38 | (100) |

# X denotes three samples possessed multiple repeats more than 3R or altered TSER 3RG>C genotype. These samples were excluded from statistical analysis.

Table 2.22 Analysis of allele carriage for genetic variations in the occurrence of adenomatous polyps

| | Control (n) | | AP (n) | | OR | 95% C.I | *P** |
|---|---|---|---|---|---|---|---|
| | WT | Het + Rec | WT | Het + Rec | | | |
| MTHFR C677T | 76 (46.34) | 88 (53.66) | 17 (44.74) | 21 (55.26) | 1.07 | (0.5-2.3) | NS |
| MTHFR A1298C | 82 (50) | 82 (50) | 20 (52.63) | 18 (47.37) | 0.90 | (0.42-1.93) | NS |
| MTHFR G1793A | 148 (90.24) | 16 (9.76) | 36 (94.74) | 2 (5.26) | 0.51 | (0.08-2.5) | NS |
| RFC G80A | 57 (34.76) | 107 (65.24) | 14 (36.84) | 24 (63.16) | 0.91 | (0.41-2.03) | NS |
| GCPII C1561T | 152 (92.68) | 12 (7.32) | 31 (81.58) | 7 (18.42) | 2.86 | (0.93-8.65) | NS |
| TSER 2R3R | 46 (28.22) | 117 (71.78) | 11 (29.73) | 26 (70.27) | 0.93 | (0.4-2.19) | NS |
| TS 1496del6 | 79 (48.17) | 85 (51.83) | 13 (34.21) | 25 (65.79) | 1.79 | (0.81-3.99) | NS |
| **CBS 844ins68** | **144 (87.8)** | **20 (12.2)** | **27 (71.05)** | **11 (28.95)** | **2.93** | **(1.16-7.35)** | **0.0197*** |
| CTH G1364T | 72 (43.9) | 92 (56.1) | 20 (52.63) | 18 (47.37) | 0.70 | (0.33-1.51) | NS |
| CTH IVS 10-430C>T | 90 (54.88) | 74 (45.12) | 21 (55.26) | 17 (44.74) | 0.98 | (0.46-2.12) | NS |
| DHFR 19bp del | 53 (32.32) | 111 (67.68) | 18 (47.37) | 20 (52.63) | 0.53 | (0.24-1.15) | NS |
| SHMT C1420T | 85 (51.83) | 79 (48.17) | 18 (47.37) | 20 (52.63) | 1.20 | (0.56-2.57) | NS |
| BHMT G595A | 160 (97.56) | 4 (2.44) | 38 (100) | N/A | 0 | (0-6.78) | NS |
| MS A2756G | 109 (66.46) | 55 (33.54) | 28 (73.68) | 10 (26.32) | 0.71 | (0.3-1.66) | NS |
| MSR A66G | 39 (23.78) | 125 (76.22) | 5 (13.16) | 33 (86.84) | 2.06 | (0.7-6.47) | NS |

* Significant at $p<0.05$

Analysis of the data produced from the DHFR 19bp del and SHMT C1420T assays showed that these two genetic variations were not associated with the risk for AP. Although the Maf of DHFR 19bp del was 0.44 for control subjects and 0.34 for AP subjects, this difference was not statistically significant, suggesting that this genetic variant does not play a role in AP development.

Subjects possessing the mutant allele of BHMT G595A were rare in the study population. Only four heterozygous subjects were observed in the control group, while no mutant allele was found in the AP group. Therefore, in this cohort, this rare mutant allele did not have a significant effect on the occurrence of an AP event.

The G allele has been considered to be the MSR A66G variant allele in other studies. However, the prevalence of this allele was dominant in this study. The frequency of the G allele was 0.52 for controls and 0.33 for the case group. This phenomenon has already been observed in previous studies [112, 316, 467]. The analysis of the MSR A66G genetic variation provided evidence for an association between the G allele and risk for AP. Prevalence of AA, AG and GG genotypes among the control subjects was 24%, 48% and 28%, respectively, whilst in AP subjects it was 13%, 39% and 47%, respectively. The frequency of the variant G allele in the control and case groups generated an OR of 1.87 (95% CI: 1.07-3.28, $p$ = 0.025). However, this increased risk with respect to the mutant G allele was not retained in individuals with G allele carriage (see Table 2.22). MS A2756G genetic variation was not associated with risk for an AP event.

Table 2.23 List of combined genotypes classified according to their role in folate metabolism for statistical analysis

| Description | | Combined genotype |
|---|---|---|
| Genetic variations in MTHFR | MTHFR C677T | MTHFR C677T-A1298C-G1793A |
| | MTHFR A1298C | |
| | MTHFR G1793A | |
| Genetic variations in folate absorption metabolism | RFC G80A | RFC G80A -GCPII C1561T |
| | GCP II C1561T | |
| Genetic variations in TS | TSER 2R3R | TSER 2R3R-TS 1496del6 |
| | TS 1496del6 | |
| | TSER 3RG>C | |
| Genetic variations in transsulphuration pathway | CBS 844*ins*68 | MTHFR C677T-CBS 844*ins*68 |
| | CTH G1364T | CBS 844*ins*68-CTH G1364T-IVS10-430C>T |
| | CTH IVS 10-430 C>T | CTH G1364T- IVS 10-430 C>T |
| Genetic variations in serine and $H_4$PteGlu metabolism | DHFR 19bp del | SHMT C1420T-DHFR 19bp del |
| | SHMT C1420T | |
| Genetic variations in cobalamin-related MS/MSR metabolism | MS A2756G | MS A2756G-MSR A66G |
| | MSR A66G | |

Genetic variants were categorized into six subgroups, taking into account their roles in metabolism as appropriate (see Table 2.23). Table 2.24 (a-h) provides data for eight combinations of genotypes and their distribution in both the control and AP groups. Each

combined genotype was listed in order of highest to lowest prevalence within the control group. In order to examine whether each combined genotype has an effect on AP occurrence, a chi-square test was performed. Among the eight combinations considered, only the MTHFR C677T-CBS 844*ins*68 variation was associated with the occurrence of AP (see Table 2.24-d, chi-square value: 13.180, $p$ =0.0403). The CBS 844*ins*68 variation (see Table 2.24-g) was also analysed within other combinations such as CTH G1364T and IVS 10-430 C>T, however, no association was observed with the combined cassette of CBS-CTH genetic variations. Further analysis and results, including OR, and the results from logistic regression analysis are described in section 2.4.3.2.

In summary, using simple allele frequency data, it was found that the TS 1496del6, CBS 844*ins*68, MSR A66G and MTHFR C677T-CBS 844*ins*68 genotypes were associated with the occurrence of AP.

Table 2.24 Distribution of combined genotypes according to their biochemical characteristics in folate metabolism (table continued on next page)

a) MTHFR C677T-A1298C-G1793A

| Genotype | Control (%) | | AP (%) | |
|---|---|---|---|---|
| CT/AA/GG | 42 | (26) | 8 | (21) |
| CC/AC/GG | 33 | (20) | 7 | (18) |
| CC/AA/GG | 26 | (16) | 6 | (16) |
| CT/AC/GG | 26 | (16) | 5 | (13) |
| TT/AA/GG | 13 | (8) | 6 | (16) |
| CC/CC/GG | 7 | (4) | 4 | (11) |
| CT/AC/AG | 5 | (3) | 2 | (5) |
| CC/AC/AG | 6 | (4) | N/A | |
| CC/CC/AA | 2 | (1) | N/A | |
| CC/CC/AG | 2 | (1) | N/A | |
| TT/CC/GG | 1 | (1) | N/A | |
| CT/AA/AG | 1 | (1) | N/A | |
| Total | 164 | (100) | 38 | (100) |

b) RFC G80A- GCPII C1561T variations

| Genotype | Control (%) | | AP (%) | |
|---|---|---|---|---|
| GA/CC | 70 | (43) | 15 | (40) |
| GG/CC | 53 | (32) | 10 | (26) |
| AA/CC | 29 | (18) | 6 | (16) |
| AA/CT | 5 | (3) | 2 | (5) |
| GG/CT | 4 | (2) | 4 | (11) |
| GA/CT | 3 | (2) | 1 | (3) |
| Total | 164 | (100) | 38 | (100) |

c) TSER 2R3R-TS 1496del6

| | Control (%) | | AP (%) | |
|---|---|---|---|---|
| 3R/2R6bp/6bp | 39 | (23.8) | 9 | (23.7) |
| 3R/2R6bp/0bp | 37 | (22.6) | 11 | (28.9) |
| 2R/2R6bp/6bp | 27 | (16.5) | 3 | (7.9) |
| 3R/3R6bp/0bp | 25 | (15.2) | 6 | (15.8) |
| 3R/3R6bp/6bp | 13 | (7.9) | 1 | (2.6) |
| 2R/2R6bp/0bp | 10 | (6.1) | 1 | (2.6) |
| 3R/3R0bp/0bp | 8 | (4.9) | 4 | (10.5) |
| 3R/2R0bp/0bp | 4 | (2.4) | 2 | (5.3) |
| X | 1 | (0.6) | 1 | (2.6) |
| Total | 164 | (100) | 38 | (100) |

# One control and one case sample possessing aberrant TSER 2R3R genotypes were excluded from statistical analysis

e) CTH G1364T- IVS 10-430 C>T

| | Control (%) | | AP (%) | |
|---|---|---|---|---|
| GT/CC | 52 | (31) | 10 | (26) |
| GG/CT | 31 | (19) | 6 | (16) |
| GG/CC | 29 | (18) | 10 | (26) |
| GT/CT | 29 | (18) | 7 | (18) |
| GG/TT | 11 | (7) | 4 | (11) |
| TT/CC | 9 | (5) | 1 | (3) |
| GT/TT | 2 | (1) | N/A | |
| TT/CT | 1 | (1) | N/A | |
| Total | 164 | (100) | 38 | (100) |

g) CBS 844ins68- CTH G1364T- IVS 10-430 C>T

| | Control (%) | | AP (%) | |
|---|---|---|---|---|
| NN/GT/CC | 46 | (28) | 7 | (18.4) |
| NN/GG/CT | 28 | 17.1) | 5 | (13.2) |
| NN/GG/CC | 25 | 15.2) | 6 | (15.8) |
| NN/GT/CT | 24 | 14.6) | 5 | (13.2) |
| NN/GG/TT | 9 | (5.5) | 3 | (7.9) |
| NN/TT/CC | 9 | (5.5) | 1 | (2.6) |
| NI/GT/CT | 5 | (3) | 2 | (5.3) |
| NI/GT/CC | 5 | (3) | 3 | (7.9) |
| NI/GG/CC | 4 | (2.4) | 4 | (10.5) |
| NI/GG/CT | 3 | (1.8) | 1 | (2.6) |
| NI/GG/TT | 2 | (1.2) | 1 | (2.6) |
| NN/GT/TT | 2 | (1.2) | N/A | |
| NN/TT/CT | 1 | (0.6) | N/A | |
| NI/GT/CC | 1 | (0.6) | N/A | |
| Total | 164 | (100) | 38 | (100) |

d) MTHFR C677T-CBS 844ins68 variations

| Genotype | Control | | AP (%)* | |
|---|---|---|---|---|
| CC/NN | 67 | (41) | 12 | (32) |
| CT/NN | 64 | (39) | 13 | (34) |
| TT/NN | 13 | (8) | 2 | (5) |
| CT/NI | 10 | (6) | 2 | (5) |
| CC/NI | 8 | (5) | 5 | (13) |
| TT/NI | 1 | (1) | 4 | (11) |
| CC/II | 1 | (1) | N/A | |
| Total | 164 | (100) | 38 | (100) |

* Statistical difference from the control group, $p$ = 0.0403

f) DHFR 19bp del -SHMT C1420T

| | Control (%) | | AP (%) | |
|---|---|---|---|---|
| 11CC | 25 | (15.2) | 8 | (21.1) |
| 11CT | 20 | (12.2) | 8 | (21.1) |
| 11TT | 8 | (4.9) | 2 | (5.3) |
| 12CC | 45 | (27.4) | 6 | (15.8) |
| 12CT | 31 | (18.9) | 5 | (13.2) |
| 12TT | 3 | (1.8) | 3 | (7.9) |
| 22CC | 15 | (9.1) | 4 | (10.5) |
| 22CT | 13 | (7.9) | 2 | (5.3) |
| 22TT | 4 | (2.4) | N/A | |
| Total | 164 | (100) | 38 | (100) |

h) MS A2756G-MS A66G

| | Control (%) | | AP (%) | |
|---|---|---|---|---|
| AA/AG | 51 | (31.1) | 11 | (28.9) |
| AA/GG | 33 | (20.1) | 14 | (36.8) |
| AG/AG | 27 | (16.5) | 4 | (10.5) |
| AA/AA | 25 | (15.2) | 3 | (7.9) |
| AG/AA | 13 | (7.9) | 1 | (2.6) |
| AG/GG | 12 | (7.3) | 3 | (7.9) |
| GG/AA | 1 | (0.6) | 1 | (2.6) |
| GG/GG | 1 | (0.6) | 1 | (2.6) |
| GG/AG | 1 | (0.6) | N/A | |
| Total | 164 | (100) | 38 | (100) |

### 2.4.3.2 The effect of genetic variations on the occurrence of an adenomatous polyp analysed using logistic regression analysis

Logistic regression provides a more in-depth analysis, enabling the establishment of models which predict the occurrence of AP in response to multiple independent variables. Only significant results from the prediction model for an AP event and genetic variants using simple logistic and stepwise regression analysis are presented in this section. Compound genotypes were excluded from stepwise regression analysis due to potential interference between repetitive genotypes. In this section we also present statistical analysis which generated interesting results when gender and total folate intake levels were taken into account.

Table 2.25 Genetic variation in folate metabolism associated with the occurrence of adenomatous polyps

| | All subjects | | Gender | | | | Total dietary folate intake | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Female | | Male | | Over RDI | | Below RDI | |
| | *p* | $r^2$ | *P* | $r^2$ | *p* | $r^2$ | *p* | $r^2$ | *p* | $r^2$ |
| MTHFR A1298C | NS | | NS | | NS | | 0.026 | 0.073 | 0.011 | 0.096 |
| MTHFR C677T-A1298C-G1793A | NS | | NS | | NS | | 0.016 | 0.220 | NS | |
| RFC G80A –GCPII C1561T | NS | | NS | | NS | | NS | | 0.033 | 0.127 |
| TS 1496del6 | NS | | NS | | 0.027 | 0.075 | 0.024 | 0.075 | NS | |
| DHFR 19bp del | NS | | NS | | NS | | 0.032 | 0.069 | NS | |
| CBS 844*ins*68 | 0.035 | 0.035 | 0.013 | 0.064 | NS | | NS | | NS | |
| MTHFR C677T-CBS 844ins68 | NS | | 0.003 | 0.187 | NS | | NS | | NS | |

Table 2.26 Risk for the occurrence of adenomatous polyps with respect to folate genotypes

| Group | | | Genotype (N) | OR | 95% CI | *p* |
|---|---|---|---|---|---|---|
| All subjects | | CBS 844ins68 | NN (171) | 1 | - | - |
| | | | NI (30) | 3.088 | 1.29-7.16 | 0.012 |
| | | | II (1) | 0.00000362 | 0.0-31.66 | NS |
| Gender | Male | TS 1496del6 | 6bp/6bp (37) | 1 | - | - |
| | | | 6bp/0bp (39) | 1.55 | 0.49-5.13 | NS |
| | | | 0bp/0bp (10) | 7.75 | 1.73-39.55 | 0.0075 |
| | Female | CBS 844ins68[1] | NN (100) | 1 | - | - |
| | | | NI (16) | 4.86 | 1.42-15.95 | 0.0128 |
| | | MTHFR C677T- CBS 844 ins68[2] | CC/NN (43) | 1 | - | - |
| | | | CC/NI (7) | 7.31 | 1.13-47.70 | 0.0377 |

[1] No II (double insertion) genotype was observed in female subjects. [2] Only the genotype having statistical significance was presented

Table 2.25 presents genotypes that show statistically significant associations with the occurrence of AP. Out of the total of twenty four genotypes, including eight different combinations, seven were associated with an AP event when either the entire population or groups classified by gender and/or folate intake levels were considered: MTHFR A1298C, TS 1496del6, DHFR 19bp del, CBS 844*ins*68, MTHFR C677T-A1298C-G1793A, RFC G80A–GCPII C1561T and MTHFR C677T- CBS 844*ins*68.

In all subjects, CBS 844*ins*68 predicted an AP event ($p$ = 0.0346) as already observed in the previous section (2.4.3.1) using a chi-square test. This association was confirmed using logistic regression analysis. The subjects with a single insertion of CBS 844*ins*68 were approximately three times more likely to have an AP, compared to subjects with no insertion allele (OR: 3.088, 95% CI: 1.29-7.16, $p$ = 0.012, in Table 2.26). The double insertion genotype showed no effect on AP events. This may be due to the low number (one) of subjects in the study group possessing this genotype.

When gender was taken into account, some very interesting associations for the risk of AP were observed. The association between the risk of AP and having the CBS 844*ins*68 insertion allele was increased in female subjects. Heterozygote female subjects had an OR of 4.86 compared to the wildtype (95% CI: 1.42-15.95, p = 0.0128). No homozygous recessive genotype was found in females. The risk of AP conferred by the CBS 844*ins*68 insertion allele was elevated when combined with the MTHFR C677T genotype. Female subjects with MTHFR C677T-CBS 844*ins*68 CC/NI genotype had a 7.31 times higher risk of AP than subjects with the CC/NN genotype (CI: 1.13-47.70, $p$ = 0.0377).

The risk of AP due to the CBS 844*ins*68 insertion allele was not observed in males. However, TS 1496del6 was associated with AP in the male group. The male subjects with the TS1496del6 homozygous recessive genotype had a 7.75 times higher risk for the occurrence of AP compared to the wildtype (CI: 1.73-39.55, $p$ = 0.0075 in Table 2.27).

Logistic regression analysis taking into account total dietary folate intake demonstrated interesting findings. In the group of subjects consuming folate above the RDI level, MTHFR A1298C, DHFR 19bp del, TS 1496del6 and MTHFR C677T-A1298C-G1793A were associated with an AP event (see Table 2.26). The MTHFR A1298C homozygous recessive genotype (CC) was associated with an increased risk of AP (OR: 7.83, 95%: 1.52-42.38, $p$ = 0.0151). The homozygous recessive genotype for TS 1496del6 was also associated with an increased AP risk

in the high folate cohort. The estimated risk for the homozygous recessive genotype was 8.2 times higher than for the wildtype (OR: 8.2, 95% CI: 1.76-41.29, $p$ = 0.0079).

By contrast, individuals with a double deletion in 19bp DHFR had a significantly lower risk of AP when their total folate intake was above the RDI level (OR: 0.11, 95% CI: 0.01-0.65, $p$ = 0.0118). Nominal regression analysis showed that the MTHFR C677T-A1298C-G1793A genotype is associated with AP occurrence. However, the details of a prediction model could not be obtained due to the lability of the statistical software and the small number of subjects.

The subjects having a total dietary folate below the RDI level showed a contrasting effect for MTHFR A1298C when compared to those consuming above the RDI. The heterozygous and homozygous recessive genotypes of MTHFR A1298C showed a declined risk of AP in those with a low folate intake (AC; OR: 0.256, CI: 0.07-0.79, $p$ = 0.0469, CC; OR: 0.00000001, CI: Unstable, $p$ = 0.0265), while the homozygous recessive genotype of A1298C increased AP risk in subjects having total dietary folate above the RDI (OR: 7.83, CI: 1.52-42.38, $p$ = 0.0151). The combined RFC G80A-GCPII C1561T genotype, which is related to the expression products involved in folate absorption, is also associated with the occurrence of AP in these subjects, but the details could not be obtained due to the low numbers and the instability of the statistical software.

Table 2.27 Risk for occurrence of adenomatous polyps with respect to folate genotype evaluated using nominal regression analysis, taking into account total dietary folate intake as appropriate

| | | | Genotype (n) | OR | 95% CI | $p$ |
|---|---|---|---|---|---|---|
| Total dietary folate intake | Over RDI | MTHFR A1298C | AA (53) | 1 | - | - |
| | | | AC (38) | 2.79 | 0.94-9.02 | NS |
| | | | CC (8) | 7.83 | 1.52-42.38 | 0.0151 |
| | | DHFR 19bp del | 11 (33) | 1 | - | - |
| | | | 12 (47) | 0.41 | 0.14-1.16 | NS |
| | | | 22 (19) | 0.11 | 0.01-0.65 | 0.0118 |
| | | TS 1496del6 | 6bp/6bp (46) | 1 | - | - |
| | | | 6bp/0bp (43) | 2.48 | 0.8-8.64 | NS |
| | | | 0bp/0bp (10) | 8.2 | 1.76-41.29 | 0.0079 |
| | | MTHFR C677T-A1298C-G1793A | Unstable model/ lack of power | | | |
| | Below RDI | MTHFR A1298C | AA (49) | 1 | - | - |
| | | | AC (43) | 0.256 | 0.07-0.79 | 0.0469 |
| | | | CC (8) | 0.00000001 | Unstable | 0.0265 |
| | | RFC G80A -GCPII C1561T | Unstable model/ lack of power | | | |

Table 2.28 exhibits the significant findings from the stepwise regression analysis. In the entire study population, TS 1496del6 and MSR A66G variations were found to have a statistical significance in predicting the occurrence of AP ($p$ = 0.0294 and 0.0179 respectively). These genetic variations were also observed to have an association with AP risk in males ($p$ = 0.015 and 0.0487, respectively). However, in female subjects, the only genetic variation examined that showed a significant association with an AP event ($p$ =0.0062) was CBS 844*ins*68.

The stepwise regression analysis taking folate intake levels into account generated some interesting results. It predicted that the MTHFR A1298C ($p$ =0.0084), TS 1496del6 ($p$ =0.0039) and MSR A66G ($p$ =0.0208) genotypes are associated with the risk of AP when folate intake is above the RDI, generating the higher $r^2$ value 0.3187 when compared to other prediction models. In subjects with a total folate intake level below the RDI level, the MTHFR A1298C ($p$ =0.0005), GCPII C1561T ($p$ =0.0252), TSER 2R3R ($p$ = 0.0197) and MS A2756G ($p$ =0.0242) variations predicted the occurrence of AP.

Table 2.28 Analysis of genetic variations that contribute to the occurrence of adenomatous polyps using stepwise regression analysis

| | All subjects ($r^2$ =0.1112) | Gender | | Folate intake level | |
|---|---|---|---|---|---|
| | | Female ($r^2$=0.1089) | Male ($r^2$=0.161) | Above RDI ($r^2$ =0.3187) | Below RDI ($r^2$ =0.2523) |
| MTHFR A1298C | NS | NS | NS | 0.0084 | 0.0005 |
| GCP II C1561T | NS | NS | NS | NS | 0.0252 |
| TSER 2R3R | NS | NS | NS | NS | 0.0197 |
| TS 1496del6 | 0.0294 | NS | 0.015 | 0.0039 | NS |
| CBS 844*ins*68 | NS | 0.0062 | NS | NS | NS |
| MS A2756G | NS | NS | NS | NS | 0.0242 |
| MSR A66G | 0.0179 | NS | 0.0487 | 0.0208 | NS |

In summary, the two statistical analysis methods employed predicted that different kinds of genetic variables are associated with an AP event. However, CBS 844*ins*68, TS 1496del6 and MTHFR A1298C showed a consistent relationship with AP occurrence, independent of the statistical analysis technique used. Additionally, the effect of genetic variation seems likely to be modulated by total dietary folate intake.

#### 2.4.3.3 The interactive effect of folate genetic variants and nutritional intake on the occurrence of adenomatous polyps analysed using simple logistic regression analysis

The results of the previous section confirm that, when considered independently, nutritional intake and genetic variation are clearly associated with the occurrence of an AP. This finding has led to the investigation of the question of whether genetic variations and nutritional intake interact to modulate risk for AP. In this section, the modulatory effect of nutritional intake on risk for AP is examined by genotype using simple logistic regression analysis. In order to provide a more logical approach, all genetic variants and nutritional intake data were categorized and analysed according to their characteristics with respect to metabolism. Table 2.29 explains the details of each of the groups. As described in section 2.4.2.2, nutritional intake was analysed as total dietary intake, or subdivided according to 1) whether it was synthetic/supplemental or natural, and 2) intake level (above/below or median and/or RDI).

The BHMT G595A genotype is not presented here because it showed no statistically significant associations with AP occurrence.

Table 2.29 Grouped genetic variations and their coenzymes in folate related metabolism

| Description and combined genotype | | Nutrients related to enzyme metabolism |
|---|---|---|
| Genetic variations in MTHFR | | |
| MTHFR C677T-A1298C-G1793A | MTHFR C677T | Folate (total, methyl and PteGlu) |
| | MTHFR A1298C | Niacin, Pyridoxine, Riboflavin |
| | MTHFR G1793A | Vitamin C (natural and synthetic) |
| Genetic variations in folate absorption metabolism | | |
| RFC G80A -GCPII C14561T | RFC G80A | Folate (total, methyl and PteGlu) |
| | GCP II C1561T | Vitamin C (natural and synthetic) |
| Genetic variations in TS | | |
| TSER 2R3R-TS 1496del6 | TSER 2R3R | Folate (total, methyl and PteGlu) |
| | TS 1496del6 | |
| | TSER 3RG>C | |
| Genetic variations in transsulphuration pathway | | |
| MTHFR C677T-CBS 844ins68 | CBS 844ins68 | Folate (total, methyl and PteGlu) |
| CTH G1364T- IVS 10-430 C>T | CTH G1364T | Pyridoxine |
| CBS 844ins68-CTH G1364T- IVS10-430C>T | CTH IVS 10-430 C>T | Vitamin C (natural and synthetic) |
| | | Niacin |
| Genetic variations in serine and $H_4$PteGlu metabolism | | |
| SHMT C1420T -DHFR 19bp del | DHFR 19bp del | Folate (total, methyl and PteGlu) |
| | SHMT C1420T | Pyridoxine |
| Genetic variations in methionine and Hcy metabolism | BHMT G595A | Methionine, Cobalamin, Folate (total, methyl and PteGlu) |
| Genetic variations in cobalamin-related MS/MSR metabolism | | |
| MS A2756G-MSR A66G | MS A2756G | Cobalamin |
| | MSR A66G | Riboflavin, Niacin |

Statistical analysis showed that particular genotypes had altered susceptibility for AP according to nutritional intake. For instance, MTHFR A1298C AA, G1793A GG and C677T-A1298C-G1793A CT/AA/GG genotypes decreased the risk for an AP event when methylfolate intake was below the median level (see Table 2.30). In addition to folate intake, other nutrient intakes were associated with the risk for AP in concert with specific genotypes. MTHFR A1298C AC and C677T-A1298C-G1793A CT/AA/GG genotypes were influenced by niacin and pyridoxine intake with respect to the occurrence of AP. It was interesting to note that nutritional intakes were found to exert no influence on the major SNP in MTHFR, C677T.

The genetic variations related to folate absorption, GCPII C1561T and RFC G80A, showed interesting associations with respect to vitamin C and folate intake (see Table 2.31). It is interesting to note that only methylfolate intake showed a decreased AP risk, while both total dietary intake of vitamin C and natural vitamin C intake changed the risk for AP in association with these two SNPs. The homozygous recessive genotype of RFC G80A, AA, showed an increased risk for AP depending on vitamin C intake, and this trend was also observed in the compound genotype with GCPII C1561T. Natural vitamin C intake modulated the risk for AP in GCP II C1561T heterozygous genotype subjects.

The association of variations in the TS gene with the occurrence of AP (taking into account folate and niacin intake) were analysed (see Table 2.32). The results showed a complicated interaction between genotypes and nutritional intake: the TSER 2R3R 3R/2R genotype reduced AP risk depending on nutritional intake, specifically low total folate, low methylfolate and overall total niacin intake. This decreased AP risk with the 3R/2R genotype was also observed when it was combined with the TS 1496del genotype. On the other hand, intake of total niacin equivalence increased the risk for AP in subjects with the TSER 3R/3R and TS 1496del6 0bp/0bp genotypes, respectively.

Nutritional intake was found to significantly modulate the associations between genetic polymorphisms in the transsulphuration pathway and AP occurrence (see Table 2.33). The CTH G1364T GT and CTH G1364T- IVS 10-430 C>T GG/CT variations showed a significant change in the risk for AP depending on riboflavin intake. In subjects with the CTH G1364T GT genotype, an increased intake of riboflavin (>median) increased the risk of AP by 17.64 times. Increased riboflavin intake decreased the risk of AP approximately 0.19 times within CTH G1364T- IVS 10-430 C>T GG/CT subjects. No nutritional modulation of the association between MTHFR C677T-CBS 844ins68 and AP risk was found.

Table 2.30 MTHFR gene variants associated with adenomatous polyp occurrence taking nutritional intake into account

| | Nutrient intake | Intake level | No. of subjects (AP, Control) | Genotype | *p* | $r^2$ | Slope estimate | OR | 95% CI |
|---|---|---|---|---|---|---|---|---|---|
| MTHFR C677T | | | | | NS | | | | |
| MTHFR A1298C | Total folate | Above RDI | 10,27 | AC | 0.0119 | 0.1467 | -0.0092 | 0.991 | (0.981-0.998) |
| | Methylfolate | Below median | 10,43 | AA | 0.0011 | 0.2065 | -0.0261 | 0.974 | (0.953-0.991) |
| | Niacin | All subjects | 4,12 | CC | 0.0161 | 0.3217 | 0.1850 | 1.203 | (1.030-1.603) |
| MTHFR G1793A | Methylfolate | Below median | 15,83 | GG | 0.0169 | 0.0681 | -0.0123 | 0.988 | (0.977-0.998) |
| MTHFR C677T-A1298C-G1793A | Total folate | Below RDI | 5,20 | CT/AA/GG | 0.0148 | 0.2372 | -0.0219 | 0.978 | (0.951-0.996) |
| | Methylfolate | All subjects | 4,17 | CC/CC/GG | 0.0178 | 0.3893 | 0.0153 | 1.015 | (1.002-1.039) |
| | | Below median | 5,25 | CT/AA/GG | 0.0336 | 0.1669 | -0.0234 | 0.977 | (0.946-0.998) |
| | Pyridoxine | below | 5,20 | CT/AA/GG | 0.0288 | 0.1847 | -2.7820 | 0.062 | (0.002-0.767) |

Table 2.31 RFC and GCPII gene variants associated with adenomatous polyp occurrence taking nutritional intake into account

| | Nutrient intake | Intake level | No. of subjects (AP, Control) | Genotype | *p* | $r^2$ | Slope estimate | OR | 95% CI |
|---|---|---|---|---|---|---|---|---|---|
| RFC G80A | Methylfolate | Below median | 9,36 | GG | 0.0301 | 0.1045 | -0.0152 | 0.98 | (0.969-0.999) |
| | Total vitamin C | | 8,34 | AA | 0.0473 | 0.0972 | 0.0078 | 1.01 | (1.001-1.017) |
| | Natural vitamin C | | 14,57 | GG | 0.0269 | 0.0933 | -0.0102 | 0.99 | (0.980-0.998) |
| GCP II C1561T | Natural vitamin C | | 7,12 | CT | 0.0276 | 0.1940 | -0.0133 | 0.987 | (0.970-0.999) |
| RFC G80A-GCPII C1561T | Total vitamin C | | 6,29 | AA/CC | 0.0026 | 0.2853 | 0.0153 | 1.015 | (1.005-1.031) |
| | Natural vitamin C | | 6,29 | AA/CC | 0.0127 | 0.1985 | 0.0140 | 1.014 | (1.003-1.030) |
| | | | 10,53 | GG/CC | 0.0288 | 0.0867 | -0.0103 | 0.99 | (0.978-0.999) |

Table 2.32 TS gene variants associated with adenomatous polyp occurrence taking nutritional intake into account

| | Nutrient intake | Intake level | Genotype | No. of subjects (AP, Control) | *p* | $r^2$ | Slope estimate | OR | 95% CI |
|---|---|---|---|---|---|---|---|---|---|
| TSER 2R3R | Total folate | Below RDI | 3R/2R | 12,40 | 0.0487 | 0.0691 | -0.0099 | 0.990 | (0.980-0.999) |
| | Methylfolate | Below median | 3R/2R | 11,38 | 0.0178 | 0.1076 | -0.0163 | 0.980 | (0.968-0.997) |
| | Total niacin | | 3R/2R | 12,40 | 0.0402 | 0.0398 | -0.0406 | 0.960 | (0.911-0.999) |
| | Niacin equivalence | | 3R/3R | 12,40 | 0.0250 | 0.0898 | 0.0535 | 1.056 | (1.007-1.114) |
| TS 1496del6 | Total folate | Below RDI | 6bp/6bp | 8,36 | 0.0325 | 0.1096 | -0.0114 | 0.989 | (0.977-0.999) |
| | Niacin equivalence | | 0bp/0bp | 7,12 | 0.0172 | 0.2269 | 0.0788 | 1.082 | (1.012-1.199) |
| TSER 3RG>C | Methylfolate | Below median | 3G/2R | 5,19 | 0.0084 | 0.2828 | -0.0396 | 0.961 | (0.905-0.992) |
| TSER 2R3R-TS 1496del6 | Total folate | Above RDI | 3/26/0 | 6,16 | 0.0121 | 0.2442 | -0.0107 | 0.989 | (0.974-0.998) |
| | | Below RDI | 3/26/6 | 6,18 | 0.0008 | 0.4164 | -0.0342 | 0.966 | (0.925-0.989) |
| | Methylfolate | All subjects | 3/30/0 | 4,8 | 0.0201 | 0.3539 | 0.0179 | 1.018 | (1.002-1.042) |

Table 2.33 Genetic variants in the transsulphuration pathway associated with adenomatous polyp occurrence taking nutritional intake into account

| | Nutrient intake | Intake level | Genotype | No. of subjects (AP, Control) | *p* | $r^2$ | Slope estimate | OR | 95% CI |
|---|---|---|---|---|---|---|---|---|---|
| CBS 844*ins*68 | Methylfolate | Below median | NI | 4,12 | 0.0025 | 0.506 | -0.0492 | 0.952 | (0.889-0.986) |
| MTHFR C677T-CBS 844*ins*68 | | | | | None | | | | |
| CTH G1364T | Vitamin C | Total vitamin C | GG | 20,71 | 0.0340 | 0.0469 | -0.0049 | 0.995 | (0.990-0.999) |
| | Riboflavin | Below median | GT | 9,38 | 0.0073 | 0.157 | 2.8700 | 17.64 | (1.982-309.1) |
| CTH IVS 10-430 C>T | | | | | None | | | | |
| CTH G1364T- IVS 10-430 C>T | Methylfolate intake | All subjects | GT/CT | 7,29 | 0.0168 | 0.1611 | 0.0127 | 1.013 | (1.002-1.028) |
| | | Above median | GG/CC | 4,13 | 0.0069 | 0.3936 | 0.0200 | 1.020 | (1.005-1.049) |
| | Riboflavin | All subjects | GG/CT | 6,31 | 0.0110 | 0.1971 | -1.6678 | 0.189 | (0.020-0.818) |
| CBS 844*ins*68-CTH G1364T-IVS 10-430 C>T | Methylfolate | Above median | NN/GG/CC | 3,11 | 0.0039 | 0.5714 | 0.0259 | 1.026 | (1.006-1.073) |

Table 2.34 SNPs in MS and MSR genes associated with adenomatous polyp occurrence taking nutritional intake into account

| | Nutrient intake | Intake level | Genotype | No. of subjects (AP, Control) | *p* | $r^2$ | Slope estimate | OR | 95% CI |
|---|---|---|---|---|---|---|---|---|---|
| MS A2756G | Methylfolate | Below median | AA | 13,60 | 0.0043 | 0.1192 | -0.0174 | 0.983 | (0.960-0.995) |
| MSR A66G | Methylfolate | Below median | GG | 8,22 | 0.029 | 0.1371 | -0.0173 | 0.983 | (0.963-0.998) |
| | Cobalamin | Below median | AA | 4,18 | 0.0042 | 0.3938 | 0.7924 | 2.210 | (1.251-5.617) |
| MS A2756G-MSR A66G | Methylfolate | Below median | AA/AG | 5,30 | 0.0244 | 0.1766 | -0.025 | 0.985 | (0.945-0.997) |
| | | | AA/GG | 7,18 | 0.031 | 0.1569 | -0.0186 | 0.982 | (0.959-0.998) |

Table 2.35 Genetic variants in DHFR and SHMT genes associated with adenomatous polyp occurrence taking folate and pyridoxine intake into account

| | Nutrient intake | Intake level | Genotype | No. of subjects (AP, Control) | *p* | $r^2$ | Slope Estimate | OR | 95% CI |
|---|---|---|---|---|---|---|---|---|---|
| DHFR 19bp del | PteGlu | All subjects | 22 | 6,32 | 0.0230 | 0.1559 | -0.0143 | 0.986 | (0.965-0.999) |
| SHMT C1420T | Methylfolate | Below median | CC | 7,48 | 0.0021 | 0.2257 | -0.0263 | 0.974 | (0.952-0.991) |
| DHFR 19bp del - SHMT C1420T | Total folate | All subjects | 22/CC | 4,15 | 0.0284 | 0.2456 | -0.0137 | 0.986 | (0.962-0.999) |
| | Methylfolate | Below median | 12/CC | 3,27 | 0.0150 | 0.2977 | -0.0276 | 0.973 | (0.937-0.995) |
| | PteGlu | All subjects | 22/CC | 4,15 | 0.0307 | 0.2387 | -0.0202 | 0.980 | (0.947-0.999) |
| | Pyridoxine | Below median | 12/CC | 3,27 | 0.0415 | 0.2855 | -3.2075 | 0.041 | (0.0001-0.896) |

A significant modulation of the association between the MS A2765G and MSR A66G genotypes and AP risk was found, especially for low levels of nutritional intake. A low level of methylfolate intake in subjects with the MS A2765G AA and MSR A66G GG genotypes decreased the risk for AP. This reduced risk was also observed for the compound genotypes MS A2765G-MSR A66G AA/AG and AA/GG (see Table 2.34). However, the MSR A66G AA genotype showed an increased risk for AP when the dietary cobalamin intake was below the median level.

Interestingly, the only genetic variation examined that showed any association with total dietary PteGlu intake in the context of risk for AP was DHFR 19bp del (see Table 2.35). An increase of total dietary PteGlu lowered AP risk in DHFR 19bp del recessive homozygotes. This declined risk was also observed for individuals with the DHFR 19bp del -SHMT C1420T 22/CC compound genotype. Low intake of pyridoxine significantly reduced the risk for AP in DHFR 19bp del -SHMT C1420T 12/CC subjects.

In summary, genetic variations in MTHFR, TS and the transsulphuration pathway related genes showed associations with AP occurrence which depended on the nutritional intake offolate, riboflavin, niacin and pyridoxine. It is of particular interest that the SNPs in GCP and RFC changed the risk for AP when vitamin C intake was considered. Additionally, DHFR 19bp del demonstrated an association with AP which was dependent on PteGlu intake level.

All of these significant observations provide clear evidence that the interaction between nutritional intake and genetic variation modifies the risk for AP. Although the mechanisms involved are complex and we have not obtained a definitive explanation of what is happening at the molecular level, the data analysed here definitely suggests that the risk of AP development and progression is multifactorial, with nutrient-nutrient, nutrient-gene and gene-gene interactions forming a significant part of the AP aetiology.

#### 2.4.3.4 Analysis to examine the significant variables for the occurrence of adenomatous polyps taking into account folate genotype and nutritional intake using stepwise regression analysis

In order to establish the most important variables in predicting the occurrence of AP (taking into account all genetic and non-genetic variables), data was analysed using stepwise regression. Variables were categorised by their role in folate metabolism, as described in the previous section. The outcomes of this analysis are presented in Table 2.36. Out of all of the genetic and nutritional intake variables, divided according to their characteristics in biochemical metabolism, only CBS 844*ins*68 in the transsulphuration pathway showed a significant association with occurrence of AP ($p$ = 0.0346). None of the nutritional intake variables showed a significant association with AP occurrence.

Table 2.36 Significant parameters predicting the occurrence of adenomatous polyp in each group classified by biochemical role in folate metabolism

| Genetic variables & related metabolism | | Nutrients related to enzyme metabolism | $p$ | $r^2$ |
|---|---|---|---|---|
| MTHFR | C677T | folate (total, methyl and PteGlu) | | |
| | A1298C | niacin, riboflavin | NS | |
| | G1793A | pyridoxine, vitamin C (natural and synthetic) | | |
| Folate absorption metabolism | RFC G80A | folate (total, methyl and PteGlu) | NS | |
| | GCP II C1561T | vitamin C (natural and synthetic) | | |
| TS | TSER 2R3R | folate (total, methyl and PteGlu) | | |
| | TS 1496del6 | | NS | |
| | TSER 3RG>C | | | |
| Transsulphuration pathway | ***CBS 844ins68*** | folate (total, methyl and PteGlu) | 0.0346 | 0.0345 |
| | CTH G1364T | vitamin C (natural and synthetic) | | |
| | CTH IVS 10-430 C>T | niacin, pyridoxine | | |
| Serine and $H_4$PteGlu metabolism | DHFR 19bp del | folate (total, methyl and PteGlu) | NS | |
| | SHMT C1420T | pyridoxine | | |
| Methionine and Hcy metabolism | BHMT G595A | methionine, folate (total, methyl and PteGlu), cobalamin | NS | |
| Cobalamin-related MS/MSR metabolism | MS A2756G | folate (total, methyl and PteGlu) | NS | |
| | MSR A66G | riboflavin, niacin, cobalamin | | |

### 2.4.4 Analysis to examine significant variables in predicting biochemical data

In order to support the significant findings from the biochemical data associated with the occurrence of AP, and investigate the critical variables which may predict this biochemical data in more detail, standard least squares analysis was performed to examine the critical variables predicting RBC folate and thiol levels.

Regression models for RBC folate, Cys, Hcy, Cys-Gly and GSH were established for all types of genetic and non-genetic parameters (see Tables 2.16 and 2.24). There was no significant difference between each clinical phenotype, so the data was analysed only at the total study population level. However, as described in section 2.4.2.1, RBC folate level predicted the differential risk of AP by gender. As a result, the data was also analysed by gender. When performing stepwise regression analysis, only total dietary nutritional intake data was included due to the instability of the statistical model. This means that nutritional intakes above and below the median value were not considered.

#### 2.4.4.1 Significant parameters associated with RBC folate level

Table 2.37 The influence of significant non-genetic variables on RBC folate level (including and excluding outliers)

| | All samples | | | Excluding outliers | | |
|---|---|---|---|---|---|---|
| | *P* | $r^2$ | Slope estimate | *P* | $r^2$ | Slope estimate |
| Age | 0.0122 | 0.0313 | 8.38 | 0.0100 | 0.0342 | 7.37 |
| Serum folate | <0.0001 | 0.3036 | 23.84 | <0.0001 | 0.2980 | 20.36 |
| Serum folate excluding outliers | <0.0001 | 0.2271 | 23.73 | <0.0001 | 0.2377 | 21.25 |
| Serum $B_{12}$ | <0.0001 | 0.0800 | 0.99 | | NS | |
| Hcy | 0.0003 | 0.0764 | -49.1 | 0.0002 | 0.0826 | -42.1 |
| Total folate | 0.0002 | 0.0708 | 0.58 | 0.0002 | 0.0701 | 0.49 |
| Total folate below RDI | 0.0427 | 0.0413 | 1.25 | 0.0052 | 0.7867 | 1.52 |
| Methylfolate | | NS | | 0.0100 | 0.0347 | 0.68 |
| PteGlu | 0.0017 | 0.0493 | 0.58 | 0.0041 | 0.0431 | 0.46 |
| PteGlu above median | 0.0075 | 0.0714 | 0.58 | 0.0166 | 0.0602 | 0.44 |
| PteGlu below median | | NS | | 0.0086 | 0.0704 | 1.43 |
| Pyridoxine below median | | NS | | 0.0071 | 0.0676 | 229.4 |
| Cobalamin | 0.0007 | 0.0567 | 1.71 | | NS | |
| Cobalamin above median | 0.0013 | 0.0945 | 1.70 | | NS | |
| Methionine | 0.0424 | 0.0207 | -66.2 | | NS | |
| Riboflavin | 0.0262 | 0.0248 | 14.12 | | NS | |
| Vitamin C | 0.0188 | 0.0277 | 0.39 | | NS | |

Table 2.37 describes the effects of biochemical and nutritional intake on RBC folate level. In all subjects (cases and controls), multiple parameters such as serum folate, total folate and other nutrient intakes were significantly associated with the concentration of RBC folate. As expected, there was an inverse relationship between Hcy levels and RBC folate concentration.

As described above, the "outliers" are samples whose concentrations are outside the reference range for the parameter under consideration (≥ 2500nM for RBC folate). The RBC folate levels of the outliers are extremely high when compared to the RBC folate levels of samples which lie within the reference range (mean of RBC folate level excluding outliers = 928.4nM and including outliers = 983.4nM).

Interestingly, the standard least squares regression analysis predicted that different intakes of different nutrients were associated with RBC folate concentration, depending upon whether outliers were included or excluded. When outliers were excluded, RBC folate level was associated with dietary methylfolate intake ($p = 0.01$). However, dietary methylfolate intake did not predict RBC folate level when outliers were included. Additionally, RBC folate level (all samples, including outliers) was associated with cobalamin, methionine, riboflavin and vitamin C intake ($p = 0.0007$, 0.0424, 0.0262 and 0.0188, respectively). However, these nutritional intakes did not predict RBC folate concentration excluding outliers.

These findings may suggest that the extreme levels of RBC folate found in the outlier samples are not simply due to the calibration range limitations of the assay. In fact, they may reflect the extremely enhanced RBC folate concentrations of these samples which have arisen due to increased consumption of folate, particularly PteGlu (no association was observed between methylfolate intake and RBC folate level, including outliers). In addition, nutritional intake of cobalamin, riboflavin and vitamin C, the critical coenzymes in folate metabolism, may also facilitate PteGlu metabolism, and hence contribute to the increased RBC folate concentration. In terms of mandatory folic acid fortification and its potential adverse effects, this extremely high level of RBC folate, and its association with PteGlu and other methyl group metabolism related nutrients, needs to be monitored.

Table 2.38 lists the biochemical and nutritional intake variables associated with RBC folate concentration following stepwise regression analysis. Two different statistical methods generated contrasting associations between total pyridoxine intake and RBC folate level. The results of simple regression analysis showed that total pyridoxine intake levels below the median increased the RBC folate concentration excluding outliers (see Table 2.37, $p = 0.0071$,

SE = 229.4), but stepwise regression analysis showed that pyridoxine intake was in an inverse relationship with RBC folate level in all subjects ($p$ = 0.0101, ES = -15.903). Additionally, simple regression analysis result did not show any association with niacin intake, but stepwise regression suggests that niacin intake predicts the RBC folate level (see Table 2.38).

Table 2.38 Stepwise regression analysis shows that nutritional intake predicts RBC folate level

| Group | Total dietary nutrition intake | $p$ | Slope estimate | $r^2$ |
|---|---|---|---|---|
| All subjects | Total folate | 0.0007 | 1.3071 | 0.2342 |
| | Pyridoxine | 0.0101 | -15.903 | |
| | Cobalamin | 0.0023 | 1.4649 | |
| | Niacin | 0.0145 | 7.5680 | |
| | Niacin equivalence | <0.0001 | -16.675 | |
| Excluding outliers | Total folate | 0.0013 | 0.5670 | 0.1704 |
| | Methylfolate | 0.0028 | 1.1251 | |
| | Niacin equivalence | <0.0001 | -15.723 | |
| | Niacin | 0.0408 | 5.6742 | |

Significant findings arose from the analysis of RBC folate concentration with respect to genetic variation (see Table 2.39). In the total study population, both statistical analyses predicted that MTHFR C677T was associated with RBC folate levels. Compound genotype MTHFR C677T-A1298C-G1793A, MTHFR C677T-CBS 844*ins*68, and RFC G80A-GCPII C1561T also had an effect on RBC folate levels. MTHFR G1793A variation was associated with RBC folate levels, but no such association existed with RBC folate when outliers were excluded.

Table 2.39 The effect of folate genotypes on RBC folate level

| | Simple regression analysis | | | | Stepwise regression analysis | |
|---|---|---|---|---|---|---|
| | All samples | | Excluding outliers | | All samples | Excluding outliers |
| | $p$ | $r^2$ | $p$ | $r^2$ | ($r^2$ = 0.2196) | ($r^2$ = 0.2544) |
| MTHFR C677T | <0.0001 | 0.0913 | <0.0001 | 0.1193 | <0.0001 | <0.0001 |
| MTHFR G1793A | 0.0228 | 0.0376 | NS | | 0.0023 | NS |
| GCP II C1561T | NS | | NS | | NS | 0.0314 |
| SHMT C1420T | NS | | NS | | 0.0165 | NS |
| MTHFR C677T-A1298C-G1793A | <0.0001 | 0.1882 | <0.0001 | 0.2053 | N/A* | |
| RFC G80A -GCPII C1561T | 0.0238 | 0.0641 | 0.0296 | 0.0637 | | |
| MTHFR C677T-CBS 844ins68 | <0.0001 | 0.1506 | <0.0001 | 0.1631 | | |

* Compound genotypes were excluded for stepwise regression analysis due to stability of statistical model

Table 2.40 presents the non-genetic parameters that have a significant effect on RBC folate levels by gender. In order to focus on the effect that gender has on RBC folate level, data from all subjects (including outliers) was analysed.

Findings from the analysis, taking gender into account, suggested some interesting effects of nutritional intake on AP occurrence. RBC folate concentration in female subjects exhibited a significant relationship with total dietary folate ($p$ = 0.0069), pyridoxine ($p$ = 0.0411), cobalamin ($p$ = 0.0006), and niacin intake ($p$ = 0.0176), but no relationship was observed with PteGlu intake. By contrast, in male subjects, RBC folate levels showed a relationship with total dietary folate ($p$ = 0.0075), PteGlu intake ($p$ = 0.0113) and the level of pyridoxine intake ($p$ = 0.0336). Stepwise regression analysis also showed that in males total dietary folate intake was associated only with RBC folate levels. However, in females, the total intake of cobalamin and niacin equivalence intake were found to be associated with RBC folate concentration in concert with the total dietary folate intake (see Table 2.41). The findings from both statistical analyses suggested that different kinds of nutritional intake were associated with RBC folate levels for the female and male groups. This may result in the contrasting effect of RBC folate levels on the occurrence of AP by gender.

Table 2.40 Significant non-genetic parameters predicting RBC folate levels by gender

| | Female | | | Male | | |
|---|---|---|---|---|---|---|
| | $p$ | $r^2$ | Slope estimate | $p$ | $r^2$ | Slope estimate |
| Serum folate | <0.0001 | 0.3024 | 24.22 | <0.0001 | 0.3066 | 23.33 |
| Serum folate excluding outliers | <0.0001 | 0.2615 | 24.70 | 0.0002 | 0.1709 | 21.96 |
| Serum B12 | 0.0015 | 0.086 | 0.99 | 0.0135 | 0.0704 | 0.98 |
| Hcy | 0.0026 | 0.0924 | -55.17 | 0.0118 | 0.0848 | -53.78 |
| Cys-Gly | 0.0149 | 0.0614 | 24.30 | | NS | |
| Total folate | 0.0069 | 0.0646 | 0.70 | 0.0075 | 0.0830 | 0.51 |
| Total folate below RDI | | NS | | 0.0053 | 0.1875 | 3.02 |
| PteGlu | | NS | | 0.0113 | 0.0749 | 0.55 |
| PteGlu above median | | NS | | 0.0178 | 0.1326 | 0.53 |
| PteGlu below median | | NS | | 0.0076 | 0.1615 | 8.04 |
| Pyridoxine | 0.0411 | 0.0371 | 10.16 | | NS | |
| Pyridoxine below median | | NS | | 0.0336 | 0.0944 | 319.4 |
| Cobalamin | 0.0006 | 0.1002 | 1.79 | | NS | |
| Cobalamin above median | 0.0032 | 0.1399 | 1.76 | | NS | |
| Niacin | 0.0176 | 0.0498 | 6.22 | | NS | |

Table 2.41 Significant nutritional intakes affecting RBC folate levels in females and males demonstrated by stepwise regression analysis

| Group | Total dietary nutrition intake | *p* | Slope estimate | $r^2$ |
|---|---|---|---|---|
| Female | Total folate | 0.0014 | 1.5552 | 0.2564 |
| | Cobalamin | 0.0009 | 1.6549 | |
| | Niacin equivalence | 0.0008 | -17.501 | |
| Male | Total folate | 0.0007 | 0.7476 | 0.1964 |

Each gender showed a different pattern in the relationship between genetic variation and RBC folate levels (see Table 2.42). In females only two genetic variations were associated with RBC folate levels: MTHFR C677T (*p* = 0.0144 and 0.0001 for simple and stepwise regression, respectively) and MTHFR C677T-CBS 844*ins*68 (*p* = 0.0126). However, in males, several other genetic variations were associated with RBC folate levels. Given these results, the differential significant genetic variants in determining RBC folate level in males and females may also generate the contrasting effect of RBC folate level on the occurrence of AP by gender.

Table 2.42 The effect of significant genetic variation in folate metabolism genes on RBC folate levels according to gender

| | Simple regression analysis | | | | Stepwise regression analysis | |
|---|---|---|---|---|---|---|
| | Female | | Male | | Female | Male |
| | *p* | $r^2$ | *p* | $r^2$ | ($r^2$ =0.3104) | ($r^2$ = 0.4666) |
| MTHFR C677T | 0.0144 | 0.0735 | 0.0052 | 0.119 | 0.0001 | 0.0192 |
| MTHFR A1298C | NS | | 0.004 | 0.1247 | NS | 0.0149 |
| MTHFR G1793A | NS | | 0.0091 | 0.107 | NS | NS |
| MTHFR C677T-A1298C-G1793A | NS | | <0.0001 | 0.386 | NS | NS |
| CBS 844ins68 | NS | | 0.0478 | 0.0707 | NS | NS |
| MS A2756G | NS | | 0.008 | 0.1098 | NS | NS |
| TSER 2R3R | NS | | NS | | NS | 0.0009 |
| RFC G80A -GCPII | NS | | 0.028 | 0.1427 | | |
| MTHFR C677T-CBS 844ins68 | 0.0126 | 0.1242 | 0.0017 | 0.2306 | | |
| CBS 844ins68-CTH G1364T- IVS 10-430 C>T | NS | | 0.0013 | 0.3396 | | |
| MS A2756G-MSR A66G | NS | | 0.0157 | 0.1758 | | |

### 2.4.4.2 An examination of parameters associated with plasma homocysteine levels

All blood folate and serum $B_{12}$ concentrations were inversely related to Hcy levels (see Table 2.43). Plasma Cys was positively associated with Hcy levels ($p$<0.0001), yet no relationship was observed between Hcy levels and plasma Cys-Gly and GSH. Standard least squares analysis found that folate intake (total and methyl), pyridoxine, riboflavin and natural vitamin C showed an inverse relationship with Hcy levels ($p$ = 0.0189, 0.0051, 0.0011, 0.0161 and 0.0218, respectively), whereas stepwise regression analysis predicted that total niacin intake increased Hcy levels (p = 0.0431, SE = 0.0331, in Table 2.43). Interestingly, neither analysis showed any association between PteGlu intake and Hcy levels.

Statistical analysis showed that the only genetic variation considered in this study that was associated with Hcy levels was TS 1496del6. None of the other SNPs in the transsulphuration pathway showed any significant relationship with Hcy (see Table 2.44).

Table 2.43 Significant non-genetic variables associated with plasma homocysteine concentrations

| Parameter | Simple regression analysis | | | Stepwise regression analysis ($r^2$ =0.097) | |
|---|---|---|---|---|---|
| | $p$ | $r^2$ | Slope estimate | $p$ | Slope estimate |
| Gender | 0.0008 | 0.0639 | -0.73 | | NS |
| Age | <0.0001 | 0.1291 | 0.1 | | NS |
| Serum folate | 0.0004 | 0.0721 | -0.06 | | NS |
| Serum folate excluding outliers | <0.0001 | 0.1462 | -0.12 | | NS |
| RBC folate | 0.0003 | 0.0764 | -0.001 | | NS |
| RBC folate excluding outliers | 0.0002 | 0.0826 | -0.002 | | NS |
| Serum B12 | 0.0252 | 0.0292 | -0.003 | | NS |
| Cys | <0.0001 | 0.2781 | 0.038 | | NS |
| Total folate | 0.0189 | 0.0325 | -0.002 | | NS |
| Methylfolate | 0.0051 | 0.046 | -0.005 | 0.002 | -0.006 |
| Riboflavin | 0.0161 | 0.0337 | -0.104 | 0.008 | -0.1519 |
| Niacin | | NS | | 0.043 | 0.0331 |
| Natural vitamin C | 0.0218 | 0.0309 | -0.004 | | NS |
| Total folate intake below RDI* | 0.0072 | 0.0818 | -0.01 | | |
| Methylfolate below median* | 0.0432 | 0.4465 | -0.01 | | |
| Pyridoxine below median* | 0.0011 | 0.1119 | -2.05 | | |

* These variable were excluded for stepwise regression analysis model

Table 2.44 Significant genetic variation in folate metabolism predicted plasma homocysteine levels

| | | *P* | $r^2$ |
|---|---|---|---|
| TS 1496del6 | Simple regression analysis | 0.0068 | 0.0573 |
| | Stepwise regression analysis | 0.0153 | 0.141 |

#### 2.4.4.3 An examination of parameters associated with plasma cysteine concentrations

Table 2.45 shows the significant variables which predicted Cys levels. The level of Cys increased with Hcy levels and the age of the subjects. Nutritional intake had little effect on Cys level: only a low intake of pyridoxine was associated with Cys concentrations ($p$ = 0.0197). The stepwise regression model suggested that none of the nutrients considered in this study were associated with Cys levels. However, DHFR 19bp del, TS 1496del6, TSER 3RG>C and CTH IVS 10-430 C>T variation did have an effect on Cys levels (see Table 2.46).

Table 2.45 Significant non-genetic parameters associated with plasma cysteine levels

| Parameter | *p* | $r^2$ | Slope estimate |
|---|---|---|---|
| Age | <0.0001 | 0.2153 | 1.73 |
| Hcy | <0.0001 | 0.2781 | 7.38 |
| Pyridoxine intake below the median | 0.0197 | 0.0589 | -22.0 |

Table 2.46 Significant folate-related genetic variations that predict plasma cysteine levels

| Analysis | Genetic variation | *p* | $r^2$ |
|---|---|---|---|
| Simple regression test | DHFR 19bp del | 0.0425 | 0.0366 |
| Stepwise regression analysis | DHFR 19bp del | 0.0095 | 0.2373 |
| | TS 1496del6 | 0.0345 | |
| | TSER 3RG>C | 0.0173 | |
| | CTH IVS 10-430 C>T | 0.0321 | |

#### 2.4.4.4 An examination of significant parameters associated with cysteinyl-glycine concentrations

Cys-Gly, a breakdown product of GSH which could be used for GSH intracellular re-synthesis [468, 469], showed a strong relationship with GSH ($p$ <0.0001). Total dietary niacin equivalence exhibited an inverse relationship with Cys-Gly levels ($p$ = 0.0101; see Table 2.47).

Table 2.47 Significant non-genetic variables associated with plasma cysteinyl-glycine levels

| Analysis | Parameter | $p$ | Slope estimate | $r^2$ |
|---|---|---|---|---|
| Simple regression | GSH | <0.0001 | 0.30 | 0.1169 |
| Stepwise regression | Total dietary niacin equivalent intake | 0.0101 | -0.1072 | 0.041 |

Analysis of genetic variations having an effect on Cys-Gly levels led to some interesting findings. The compound genetic variations in the transsulphuration pathway such as CTH G1364T- IVS 10-430 C>T ($p$ = 0.0223) and CBS 844ins68-CTH G1364T- IVS 10-430 C>T ($p$ = 0.0063) showed a significant relationship with Cys-Gly levels (see Table 2.48). The DHFR 19bp del variation showed an association, not only with Cys levels, but also with Cys-Gly levels ($p$ = 0.0454). Stepwise regression analysis showed that TSER 3RG>C had a significant effect in the prediction model and showed an association with Cys-Gly levels (see Table 2.48).

Table 2.48 Significant genetic variations associated with plasma cysteinyl-glycine levels

| Analysis | Genetic variation | $p$ | $r^2$ |
|---|---|---|---|
| Simple regression | DHFR 19bp del | 0.0454 | 0.0359 |
| Stepwise regression | CTH G1364T- IVS 10-430 C>T | 0.0223 | 0.102 |
| | CBS 844ins68-CTH G1364T- IVS 10-430 C>T | 0.0063 | 0.1713 |
| | TSER 3RG>C | 0.0343 | 0.164 |

#### 2.4.4.5 Significant parameters associated with glutathione levels

As described above, GSH levels showed a strong and significant relationship with Cys-Gly ($p$ <0.0001). Age of the subjects ($p$ = 0.0422), dietary methionine ($p$ = 0.0042) and niacin equivalence ($p$ = 0.0152) exhibited an inverse relationship with GSH, yet total cobalamin intake increased GSH levels ($p$ = 0.0051 in Table 2.49). Stepwise regression analysis also showed that

total dietary cobalamin and methionine intakes were related to GSH levels ($p$ = 0.0052 and 0.0043, respectively in Table 2.49).

Table 2.49 Significant non-genetic variables associated with plasma glutathione levels

| | Simple regression analysis | | | Stepwise regression analysis ($r^2$ = 0.091) | |
|---|---|---|---|---|---|
| | $p$ | $r^2$ | Slope estimate | $p$ | Slope estimate |
| Age | 0.0422 | 0.024 | -0.08 | NS | |
| Cys-Gly | <0.0001 | 0.1169 | 0.39 | NS | |
| Cobalamin | 0.0051 | 0.0455 | 0.02 | 0.005 | 0.015 |
| Methionine | 0.0042 | 0.0475 | -1.03 | 0.004 | -1.009 |
| Niacin equivalence | 0.0152 | 0.0343 | -0.07 | NS | |
| Cobalamin over median | 0.0022 | 0.1019 | 0.02 | N/A | |
| Cobalamin below median | 0.0439 | 0.0503 | 0.66 | | |

GSH levels are associated not only with genetic variants in the transsulphuration pathway, but also with the genetic variants that are important in folate metabolism. Simple regression analysis showed that the MTHFR G1793A and CTH G1364T- IVS 10-430 C>T variants may be associated with a possible mechanism regulating GSH levels in the total plasma sample group (see Table 2.50). Stepwise regression analysis also predicted that MTHFR G1793A and TSER 2R3R were associated with GSH levels (see Table 2.50).

Table 2.50 Significant genetic variations associated with plasma glutathione levels

| Analysis | Genetic variation | $p$ | $r^2$ |
|---|---|---|---|
| Simple regression analysis | MTHFR G1793A | 0.0441 | 0.0363 |
| | CTH G1364T- IVS 10-430 C>T | 0.0463 | 0.0905 |
| Stepwise regression analysis | MTHFR G1793A | 0.0338 | 0.0964 |
| | TSER 2R3R | 0.0385 | |

### 2.4.5 Haplotype analysis

The term haplotype defines a set of SNPs that exist on the same chromosome and which tend to be inherited together, a fact that is reflected in a clear statistical association [470]. Certain kinds of haplotype may result in altered protein structure, including instability and altered function of enzymes leading to disease susceptibility. In the present study, an examination of haplotypes for three SNPs in the MTHFR gene and two SNPs in the CTH gene was performed using Haploview software to ascertain whether any association existed with AP. In addition, the linkage disequilibrium (LD) information obtained from the analysis was used for further population-genetic analysis via comparison to the HapMap database. Definitions for the terms and the study group used in this section are described in Table 2.51.

Table 2.51 Definitions of terms and study groups for linkage disequilibrium analysis in the present study

| Term | | Definition |
|---|---|---|
| Linkage disequilibrium (LD) information | LD | When genetic variations at the two loci are not independent of one another. LD is calculated using frequencies of each haplotype and can be explained with several coefficients; D, D`, LOD and $r^2$ [457, 471] |
| | D | Coefficient of LD. The difference between observed and expected frequencies of one haplotype is considered the deviation or D [472] |
| | D` | Another coefficient to measure LD. Normalized value of D (the ratio of observed and theoretical maximum allele frequency. D`=1 indicates complete dependency) [473] |
| | LOD | Logarithm (base 10) of odds, another coefficient to measure LD. Positive value means presence of linkage while negative means linkage is less likely. >2 is considered as an evidence for genetic linkage [474, 475] |
| | $r^2$ | Correlation value between a pair of genetic variation loci, 1 signifies complete LD [476] |
| Definition for each group in current study | A | HapMap reference data obtained from Utah residences with Northern and Western Europe ancestry |
| | B | All subjects including control and AP cases |
| | C | Control subjects only |
| | D | AP subjects only |

#### 2.4.5.1 Haplotypes in methylenetetrahydrofolate reductase SNPs

Figures 2.8 and 2.9 present the results from LD analysis of three SNPs in the MTHFR gene. The MTHFR C677T polymorphism was in LD with both A1298C and G1793A in all study groups (Figure 2.9). Although the D` value for the study's case and control subjects was slightly

different from the HapMap data, it showed very strong LD (D`=1, LOD>2). However, AP cases showed intermediate LD (D`=1, LOD<2), generating colour change within the LD block (Figure 2.8-D).

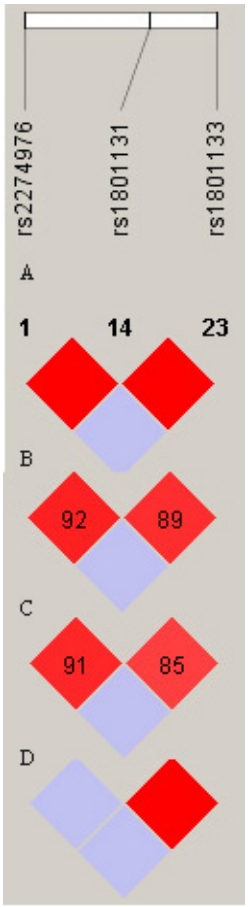



Figure 2.9 Linkage disequilibrium analyses for three SNPs of MTHFR in each population; LD block (Refer to the definition for each group in Table 2.52, D` values of 1.0 are never shown)


Figure 2.10 Linkage disequilibrium test between each SNP of MTHFR in each population: D`, LOD, $r^2$ (Refer to the definitions for each group in Table 2.52)

The results of the case-control association test are presented in Table 2.52. The study population generated six different haplotypes and two of them did not exist in the AP group. However, no haplotype showed a significant association with AP.

Table 2.52 Distribution of the MTHFR Haplotype according to clinical phenotype

| Haplotype | Haplotype frequency | | | | *p* |
|---|---|---|---|---|---|
| | HapMap | All subjects | Control | AP | |
| GAC | 0.389 | 0.412 | 0.414 | 0.41 | NS |
| GAT | 0.242 | 0.305 | 0.307 | 0.30 | NS |
| GCC | 0.311 | 0.230 | 0.218 | 0.27 | NS |
| ACC | 0.058 | 0.041 | 0.047 | 0.03 | NS |
| GCT | N/A | 0.009 | 0.011 | N/A | N/A |
| AAT | N/A | 0.003 | 0.003 | N/A | N/A |

### 2.4.5.2 Haplotypes in cystathionine γ-lyase SNPs

The results of LD analysis for SNPs in CTH are presented in Figure 2.10 and Table 2.53. Both CTH SNPs are in LD. However, the LD for all subjects and for the control group was not as strong as indicated by HapMap. The D` value for AP subjects was stronger than the one indicated by HapMap data, but the LOD value was significantly lower than for other groups, resulting in the change of colour in the LD block (see Table 2.53 and Figure 2.10).

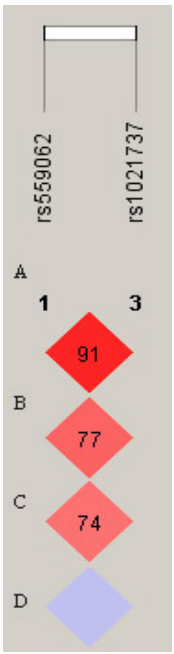



Figure 2.11 Linkage disequilibrium analysis for two SNPs in CTH (G1364T and IVS 10-430 C>T) in each group; LD block (Refer the definition for each group in Table 2.52, D` values of 1.0 are never shown)

Table 2.53 Linkage disequilibrium analysis for two SNPs of CTH in various groups

| | D` | LOD | $r^2$ |
|---|---|---|---|
| HapMap | 0.916 | 5.36 | 0.114 |
| All subjects | 0.778 | 5.03 | 0.094 |
| Control | 0.740 | 3.95 | 0.088 |
| Case | 1.000 | 1.03 | 0.127 |

CTH G1364A and IVS 10-430 C>T generated four different kinds of haplotypes. However, none of these were associated with the susceptibility for AP in this study (Table 2.54).

Table 2.54 Distribution of the CTH Haplotype in various groups

| Haplotype | Haplotype frequency | | | | *p* |
|---|---|---|---|---|---|
| | HapMap | All subjects | Control | AP | |
| CG | 0.470 | 0.451 | 0.446 | 0.483 | NS |
| CT | 0.267 | 0.286 | 0.292 | 0.241 | NS |
| TG | 0.257 | 0.245 | 0.242 | 0.267 | NS |
| TT | 0.006 | 0.018 | 0.020 | 0.009 | NS |

***SUMMARY OF FINDINGS***

A. Assessment of dietary folate intake

a. 50% of the study population did not consume enough dietary folate by comparison to the RDI, but 94% of subjects showed a normal RBC folate level

b. 85% of subjects were taking PteGlu prior to the commencement of mandatory folic acid fortification

c. PteGlu intake contributed to increased total dietary folate intake, and subjects taking high PteGlu also showed a higher synthetic vitamin C intake when compared to subjects who had a folate intake below the RDI

B. Population and biochemical parameters which had an effect on the occurrence of adenomatous polyps

a. There was no significant difference in population and biochemical data by clinical phenotype

b. Increasing RBC folate level increased AP risk in females, but decreased it in males

c. Interactions between certain population and biochemical indices decreased AP risk

- RBC folate ↔ age, Hcy
- Cys↔ RBC folate, serum $B_{12}$

C. Nutritional intake and the occurrence of adenomatous polyps

a. Both low methylfolate and high cobalamin intake decreased risk for AP

b. Gender and total dietary folate intake had an interactive effect with nutritional intakes and AP risk

- Methionine intake increased AP in females while methylfolate intake below the median value decreased AP in males

- In subjects with dietary folate intake above the RDI, cobalamin intake increased AP, but, in subjects with dietary folate below the RDI, low methylfolate and vitamin C decreased AP, whilst pyridoxine and methionine increased AP

c. Some nutrients showed an interaction with each other, resulting in decreased AP risk

- Pyridoxine ↔ low folate, cobalamin, methionine, niacin and riboflavin intake
- Cobalamin ↔ total vitamin C
- PteGlu ↔ methionine intake

D. The effect of genetic variation on the occurrence of an adenomatous polyp

a. CBS 844*ins*68, MSR A66G, TS 1496del6 and MTHFR C677T-CBS 844*ins*68 genotypes were associated with the occurrence of AP.

b. Dietary folate intake showed an interaction with gene variants influencing the occurrence of AP

- MTHFR A1298C, DHFR 19bp del, TS 1492del6, MSR A66G and MTHFR C677T-A1298C-G1793A modulated the risk of AP in subjects with folate intake above the RDI
- MTHFR A1298C, TSER 2R3R, GCPII C1561T, MS A2756G and RFC G80A-GCPII C1561T were associated with AP in subjects with folate intake below the RDI

E. The interaction between genetic variation and nutritional intake on adenomatous polyp occurrence

a. The genetic variations in MTHFR, TS and the transsulphuration pathway gene (CTH) showed an association with AP risk, depending on nutritional intake (folate, riboflavin, niacin and pyridoxine)

b. GCPII C1561T and RFC G80A changed risk for AP when taking vitamin C intake into account. Also DHFR 19bp del showed an association with AP depending on PteGlu intake

F. Significant parameters associated with RBC folate level

a. PteGlu contributed to a high RBC folate level along with cobalamin, riboflavin and total vitamin C intake

b. Nutrients predicted increased RBC folate concentration by gender
   - In female subjects - pyridoxine, cobalamin and niacin intake
   - In male subjects - PteGlu and low level of pyridoxine intake

c. MTHFR C677T, A1298C, G1793A, MTHFR C677T-A1298C-G1793A, MTHFR C677T- CBS 844*ins*68, TSER 2R3R and RFC G80A-GCPII C1561T were associated with RBC folate level

G. Significant parameters associated with plasma thiol level

a. Homocysteine level
   - Blood folate and serum $B_{12}$ exhibited an inverse relationship with Hcy
   - PteGlu intake was not associated with Hcy level. Total dietary folate, pyridoxine, riboflavin and natural vitamin C intake showed an inverse relationship with Hcy level. Total niacin intake increased Hcy level
   - Only TS 1496del6 was associated with Hcy level

b. Cysteine, Cysteinyl-Glycine, Glutathione levels
   - Nutrition had little effect on Cys and Cys-Gly levels
   - Cys-Gly and GSH levels showed a strong positive association with each other
   - DHFR 19bp del, TS 1496del6, TSER 3RG>C and CTH IVS 10-430 C>T was associated with Cys level
   - DHFR 19bp del , TSER 3RG>C, CTH G1364T-IVS 10-430 C>T and CBS 844ins68-CTH G1364T-IVS 10-430 C>T showed a significant relationship with Cys-Gly

- Age, methionine, niacin equivalence and cobalamin intake were associated with GSH level
- MTHFR G1793A, TSER 2R3R and CTH G1364T-IVS 10-430 C>T were associated with GSH level

H. Haplotype analysis for MTHFR and CTH polymorphisms

a. SNPs in these two genes showed six and four haplotypes, respectively, but none were associated with risk for AP

## 2.5 Discussion

A growing body of literature suggests that folate is a critical nutrient associated with the aetiology of multiple diseases. A low dietary folate intake is a significant nutrition-related aetiological factor in congenital disorders, vascular disease and cancer. In order to enhance folate nutritional status, as a preventative measure, approximately sixty countries have introduced mandatory folate fortification since the late 1990s. However, researchers have reported contrasting outcome trends suggesting that the form of folate used as a fortificant may be an aetiological factor in the increase of CRC occurrence. This research project therefore aims to re-investigate the relationship between AP occurrence as an important antecedent of CRC and various nutritional and genetic factors. In particular, the aim is to explore folate intake and metabolism in this context.

- **Analysis for risk factors in AP occurrence**

The Australian National Health and Medical Research Council established an RDI for folate of 400μg/day for adults (excluding pregnant and lactating women). In the present study, only 50% of subjects had a folate intake that reached this RDI level. However, the mean folate intake level (454.5μg/day) of this Australian study population is higher than in other populations such as Europeans (225μg/day), North Americans (352 μg/day and 422 μg/day) and other Australian cohorts (314 μg/day) [477]. However, it is of some concern that 5% of subjects had a dietary folate intake above the UL (1000 μg/day).

Subjects with folate intakes above the RDI showed a higher PteGlu intake, and a higher proportion of PteGlu in their total dietary folate intake (as high as 209μg/day (30%)), when compared to subjects whose folate intake was below the RDI (49.2μg/day, 15% PteGlu). Methylfolate was still a major source of dietary folate intake in both groups (AP and controls). However, it was evident that PteGlu intake was responsible for most of the increased folate intake via consumption of voluntarily fortified foods. Considering that all data was collected prior to the commencement of mandatory folate fortification, it is likely that the proportion of PteGlu in the total dietary folate intake may increase after the implementation of mandatory folic acid fortification, and that a number of people are likely to be overexposed to PteGlu. This trend has already been observed in the United States after the introduction of mandatory fortification in 1998 [44].

The folate absorption and biotransformation process can be saturated by approximately 400μg/day of folate. Doses of PteGlu at or above this level are transported into the blood in a

manner that is directly proportional to the dose taken, without conversion into biologically active 5-$CH_3H_4$PteGlu. This results in the presence of unmetabolized PteGlu in the blood [364, 478]. Unfortunately we know little about the potential of unmetabolized PteGlu as a pathoaetiological factor.

In this study, the comparison of the compositions of dietary folate intake by clinical phenotype did not provide clear evidence with respect to the association between PteGlu intake and AP occurrence. However, there is still sufficient evidence reported in the literature to justify the continued investigation of a possible biochemical role for PteGlu in AP susceptibility.

Findings from this study suggest that nutritional intake clearly predicts risk for AP on its own. A low level of methylfolate intake decreased the occurrence of AP. This may result from the low level of total folate intake, rather than any particular role of methylfolate 'per se' in AP aetiology. The subjects with methylfolate intake below the median value showed relatively low levels of total dietary folate intake (374.49 ± 24.5µg/day) compared to subjects who had methylfolate intakes above the median level (543.5 ± 198.0µg/day).

As described earlier, folate can play a contrasting role in carcinogenesis: it is a 'double edged sword' [286]. The stage of disease development and the intake dose are very critical in folate-related carcinogenic mechanisms [286]. A low level of folate intake may either play a protective or an aggravating role against AP, depending on the stage or cause of potential adenomatous progression. The protective effect of a relatively low level of RBC folate has been previously reported in other studies [54, 479]. It can be postulated that a low level of folate intake may halt the development of adenoma via suppression of oncogene expression, DNA synthesis or apoptosis in neoplasms [480].

By contrast, a decline in the risk for AP associated with total dietary cobalamin intake may be involved in other anti-adenomatous progression mechanisms [481]. For example, cobalamin is a critical coenzyme needed as an intermediate methyl group carrier and is utilised by the MSR gene expression product. Hence, an insufficient intake of cobalamin likely inhibits the MS activity, resulting in reduced methionine biosynthesis and Hcy remethylation. This may therefore imply that increased dietary cobalamin could enhance vitamin $B_{12}$ dependant MS and MSR metabolism, enhancing methyl group turnover, and hence DNA CpG methylation [481].

It has been reported that dietary cobalamin deficiency may increase the risk of CRC via altered DNA methylation [482, 483]. Therefore, enhanced methyl group production may ameliorate

the altered methylation pattern of the promoter region in oncogene or tumour suppressor genes.

Although data analysis generated relatively low $r^2$ values, interaction between nutrients generally showed a decreased risk for AP. Dietary pyridoxine showed an interaction with a variety of other nutrients such as folate, cobalamin, niacin and riboflavin. As pyridoxine is a key coenzyme in a myriad of transamination reactions, including transsulphuration and the serine and glycine conversion pathway, increased pyridoxine intake may have a synergic effect with these nutrients to decrease AP occurrence. Interestingly, methionine intake showed an increased risk for AP on its own. However, when combined with PteGlu intake, the occurrence of AP decreased.

Similar trends with respect to the interaction with biochemical indices were observed between Hcy and age, and blood folate level. Age and Hcy have been found to be critical risk factors for cancer [463, 484, 485], while elevated blood folate level decreases the risk for CRC [292, 293]. This protective effect (increased blood folate and nutritional intake) could countervail the risk from other pathoaetiological factors (age and Hcy). Thus, further examination of potential interactions between risk factors is needed and should be considered when investigating disease aetiology.

Of the biochemical variables considered, only RBC folate was associated with the occurrence of AP, but with contrasting effects with respect to gender. In females, the risk of AP was increased with RBC folate level, yet, in males, the risk was decreased. RBC folate level reflects nutritional intake, but not only of folate. Indeed, other vitamins, folyl coenzymes and genetic factors are also likely to be relevant. In females, pyridoxine, cobalamin and niacin intake were closely related to RBC folate level. However, in the male group, PteGlu and a low intake of pyridoxine were associated with RBC folate concentration. These differences in nutritional intake support the differing findings for RBC folate by gender. The genetic variations in folate metabolism influenced by these nutritional intakes may also help to generate the contrasting trend of RBC folate risk for AP.

In addition to nutrition, genetic variations are a critical pathoaetiological factor for AP. In the present study, three of the investigated individual polymorphisms (CBS 844*ins*68, TS 1494del6 and MSR A66G) were found to be associated with the occurrence of AP in the whole population using chi-square analysis.

Subjects with the CBS 844*ins*68 insertion allele had approximately 2.5 times the risk for AP when compared to subjects with the non-insertion allele. Similarly, this was the only significant variable arising from the stepwise regression analysis, after taking into account all genetic and nutritional intakes. CBS is a central gene in the transsulphuration pathway which metabolises half the systemic Hcy reserve. It has been suggested that the 68 bp insertion in CBS results in altered mRNA and CBS enzyme levels due to an alternative splicing site which eliminates inserted intronic sequences [442]. Therefore, altered CBS enzyme levels could result in increased risk for AP via an alteration in *de novo* methionine biosynthesis. This would also alter SAM levels, leading to an aberrant equilibrium in Hcy/folate metabolism [109].

When this CBS genetic variation is combined with MTHFR C677T, the insertion allele increased the risk for AP approximately 7.3 times in female subjects. Research has suggested that this MTHFR 677TT genetic variation has an association with Hcy and folate levels, and may influence risk for NTD [106, 113, 195]. Both MTHFR and CBS gene expression products are regulated by SAM level. The activity of CBS can be enhanced two to three fold by SAM, while, on the other hand, SAM can decrease MTHFR activity and slow down the turnover rate of the reaction converting 5,10-$CH_2H_4$PteGlu to 5-$CH_3H_4$PteGlu [113]. Although, in the present study, the compound genotype MTHFR C677T- CBS 844*ins*68 did not show any effect on thiol levels, it may have a critical effect in AP aetiology via altered SAM levels. This, however, remains to be proven.

Three genetic variations in TS, TS1494del6, TSER 2R3R and TS3R G>C, were investigated in the current study. TS 1494del6 was observed to be associated with increased AP risk in subjects with double deletion alleles. Pyrimidine is constantly catabolized by the reductive pathway in humans [486]. Therefore, insufficient *de novo* synthesis of pyrimidine possibly decreases the stability of DNA strands (e.g. uracil misincoporation). TS is a critical protein in pyrimidine nucleotide synthesis, a process involving the conversion of dUMP into dTMP which is needed for DNA repair and synthesis. It has been suggested that 3`UTRs could regulate gene expression. Hence, TS 1494del6 deletion variation located in 3`UTRs possibly affects mRNA stability and the translation of TS [487], affecting its expression product. Therefore, insufficient provision for pyrimidine (dTMP) production by decreased TS protein could affect the repair of DNA deficits [488]. This may explain the increased risk of AP observed from the present data.

A SNP in MSR (A66G) showed an association with AP occurrence in this study population. Cobalamin circulates as a cofactor as shown in Figure 1.7. Co I is a strong reducing agent and can be oxidized to generate an inactive form of the enzyme Co II. The methylation reaction of

Co II to $CH_3$-Co III using SAM as a methyl donor is catalysed by MSR. Hence, MSR plays a significant role in sustaining activated cobalamin for both methylation and folate metabolism [93].

Due to the significance of MSR in folate metabolism, the A66G genetic variation has been studied intensively in relation to CRC. The mutant G allele significantly increased AP risk by approximately 1.9 times in the present study, although there was no statistical difference in Hcy levels, dietary intake and serum $B_{12}$ levels between each clinical phenotype.

The increased susceptibility to CRC associated with the MSR variant allele was demonstrated by a meta-analysis including 12 studies [489]. The MSR A66G polymorphism leads to an amino acid change located within the flavin mononucleotide (FMN)-binding domain of the exposed enzyme [92]. This isoleucine to methionine transition, which likely affects the binding site for FMN, could consequently alter MSR reaction status [95]. Along with the significant association between cobalamin intake and AP risk, these findings suggest that cobalamin and its dependant metabolism is a critical factor in folate-related AP aetiology. Therefore, cobalamin and genetic variation in its dependant genes need to be considered alongside folate in the context of disease prevention or remedy.

The findings of the current study show that nutrients and genes exhibit a mutual supportive/interactive relationship. Either as substrates or coenzymes, nutrients could modulate gene expression or its products. Altered gene expression products or enzymes, due to genetic variation, can regulate nutrient metabolism. Such an interaction was clearly observed in the association between genetic variations and AP occurrence where total dietary folate intake level was considered (see Table 2.27).

In subjects whose total dietary folate intake is above the RDI level, the DHFR 19bp del mutation allele decreased AP occurrence. As these subjects showed a higher level of PteGlu intake, it is quite interesting to note that DHFR 19bp del was associated with AP risk in this subject group. DHFR is a decisive enzyme for PteGlu metabolism. It has been verified that DHFR is the only protein that can metabolise PteGlu, thereby allowing it into the folate cycle. However, PteGlu possesses high substrate inhibition for DHFR, leading to a potentially very interesting nutrient-gene interaction centred around this polymorphism. This was also supported by another finding: when PteGlu intake was factored in, the DHFR 19bp del variation showed decreased AP risk in double deletion individuals (see Table 2.27).

By contrast, in the group of subjects with total dietary folate intake levels below the RDI, GCPII C1561T and its compound genotype with RFC G80A, which are critical proteins in methylfolate absorption, showed an association with AP occurrence. In this group, methylfolate was the major source of folate, and these individuals had a lower ratio and amount of PteGlu as part of their total dietary folate intake. Therefore, it is interesting to note that polymorphisms in these genes predict occurrence of AP in this subject group, given that these gene products play a major role in folate absorption and cellular translocation.

A similar altered nutrient-gene interaction involving nutritional intake was also observed with MTHFR. It has been reported that the mutation allele of MTHFR A1298C reduces the activity of MTHFR and decreases the risk of various types of cancer including acute lymphocytic leukaemia, lung and colorectal cancer [71, 73, 490].

In the present study, the MTHFR A1298C variation showed an association with the occurrence of AP in differential ways depending on the total dietary folate intake level. In the group of subjects with a total dietary folate intake below the RDI, heterozygous and homozygous recessive genotypes showed a decreased risk for AP, as previously reported [491, 492]. However, in individuals with folate intake levels above the RDI, the presence of the homozygous recessive genotype showed a decreased risk for AP. This contrasting trend for the association of AP risk and total dietary folate intake with respect to the MTHFR A1298C genotype might stem from the intake of different folyl vitamers such as PteGlu.

The A1298C polymorphism is located in exon 7 where the C-terminal regulatory domain and the direct binding site of SAM are encoded. MTHFR A1298C variation could affect enzyme regulation, possibly by SAM, an allosteric inhibitor of MTHFR [71, 493]. By contrast with what happens for methylfolate, the higher intake of PteGlu in subjects with total dietary intake above the RDI supplies abundant $H_4$PteGlu via DHFR. However, declined MTHFR activity due to the A1298C mutation may reduce the conversion of 5,10-$CH_2H_4$PteGlu to 5-$CH_3H_4$PteGlu, therefore enhancing purine biosynthesis in colonic neoplasms, and their major antecedent, AP.

By contrast, subjects with total dietary folate intake below the RDI had a lower level of PteGlu intake, and a higher relative intake of methylfolate intake in their total dietary folate intake when compared to the higher folate intake group. Methylfolate does not need to be metabolized via MTHFR, and can join the methylation pathway via MS. Therefore, in this group, MTHFR A1298C was associated with decreased AP occurrence as reported earlier. Interestingly, MTHFR C677T was not associated with the occurrence of AP in this study. It

might be that the increased total dietary folate intake level and normal blood folate level counteract the effect of the variant allele [494]. However, the A1298C variation is located at the binding site for SAM in the expression product, which may result in direct regulation by SAM. Therefore its ramifications may lead to the significant clinical association observed for AP occurrence.

Total dietary folate intake, and also a variety of other nutrients and folyl vitamers showed an association with the occurrence of AP when analysed according to genotype. Important nutrient interactions were observed with intakes of vitamin C, niacin, riboflavin and pyridoxine. These nutrients are involved in a myriad of redox reactions as NAD, FMN and PLP. Genetic variations resulting in missense mutations possibly change the binding site for these coenzymes within the protein structure [495, 496].

Two mutations of interest are GCPII C1561T and RFC G80A which showed an interaction with vitamin C for the risk of AP (see Table 2.31). As described earlier, the expression products of those two enzymes are responsible for the first step in the uptake of methylfolate in the jejunum, and therefore the level of dietary vitamin C may modulate the reduced and oxidized status of these proteins, as well as the stability of methylfolate itself. Research evidence supports a critical role for vitamin C in the physico-chemical stability of methylfolate, a phenomenon that directly influences its bioavailability [461, 464]. The presence of vitamin C in the gastric system is important for the inter-conversion of 5-$CH_3H_2$PteGlu to 5-$CH_3H_4$PteGlu. This likely optimises the bioavailability of food folate. As a strong reducing agent, vitamin C salvages the 5-$CH_3H_4$PteGlu, and reduces 5-$CH_3H_2$PteGlu (a folyl vitamer unable to enter one-carbon mechanism) to 5-$CH_3H_4$PteGlu, a key folyl vitamer in Hcy re-methylation-methionine metabolism (SAM synthesis) [461]. Therefore, altered expression products of GCPII and RFC by genetic variants, in conjunction with reduced/oxidised status of methylfolate influenced by vitamin C intake, possibly influence 5-$CH_3H_4$PteGlu uptake metabolism and, hence, DNA synthesis and methylation metabolism.

The interesting finding of an association between vitamin C intake and genetic variants in folate uptake metabolism with respect to AP risk once again highlights the importance of nutrient-nutrient and nutrient-gene interactions in disease aetiology. Vitamin C is a common fortified vitamin in our diet, and its intake increases with total dietary folate intake (see Tables 2.9 and 2.10). The presence of extra vitamin C in the diet is likely to lead to an unpredictable effect on the folate-vitamin C interaction, but we know little about its potential outcome in

health terms. Therefore, the precise role of vitamin C in underpinning the reduced status of folate needs to be explained further.

The peculiarities in the associations between the many genetic variations examined here and AP occurrence, and their interactions with total dietary folate intake clearly need to be considered in the post folic acid fortification era. Since the commencement of mandatory folic acid fortification there has been a dramatic drop in the incidence of NTDs [350]. However, several potential side effects have been identified. These include the masking effect of low vitamin $B_{12}$ by elevated folate intake, whereby it may lead to megaloblastic anaemia which is a precursor of the more dangerous pernicious anaemia. Mandatory fortification also leads to a decline in cognitive function in the elderly. In addition, the expansion of folic acid fortification has raised other unexpected issues including an increase in CRC occurrence [391, 497], include increased twin births and IVF success rates [388]. In addition, the antifolate chemotherapeutic dose needed for effective chemotherapy needs increased monitoring [375].

The problems caused by mandatory folic acid fortification may not only be due to the dramatic increase of dietary intake or blood folate levels, but to the properties conferred by the synthetic form of the vitamin that is used for fortification. Mandatory folic acid fortification is a 'blanket intervention' designed to target women of child-bearing age. The main vehicles for fortification are bread, cereal and pasta which are regularly consumed by the entire population. This prevents aetiological risk factors from being taken into account including the characteristics of individuals such as age, genotype, recommended nutritional intake level, undiagnosed disease, medicines, chemicals and living environment. In addition, the potential interaction with other common fortified nutrients such as thiamine, vitamin C and iodine has not been evaluated yet. The findings of this study have confirmed the need to investigate these issues further.

There is no doubt that the efficacy of mandatory folic acid fortification has reduced NTD prevalence. However, the present data suggests that, as a critical risk factor in multifactorial pathoaetiology, increased PteGlu intake may be associated with risk for AP, especially when considered in conjunction with other risk factors. Therefore, a careful approach is required in exploring the molecular level mechanisms that are altered by mandatory folic acid fortification.

**- Biochemical data**

In this study, Hcy was not associated with the occurrence of AP, but was predicted by blood folate, serum $B_{12}$, Cys level, methylfolate, pyridoxine, riboflavin, niacin and natural vitamin C

intake, as previously reported [498]. The potential benefit of enhancing folate status following mandatory folate fortification is the lowering effect on Hcy which may result in the reduced risk of stroke and coronary disease [499]. However, levels of PteGlu intake had no effect on Hcy levels. This could be explained by the relatively low level of PteGlu intake in the current study subjects.

Most of the folic acid-Hcy intervention studies showing a decline in Hcy concentration used high doses of folic acid supplements: 400μg/day [228], 700μg/day [256], 1mg/day [497] and 5mg/day [500]. As described earlier in this section, current study subjects were taking a relatively higher level of total dietary folate and methylfolate (454.5 and 324.7μg/day, respectively), and their PteGlu intake level (128.8μg/day) was lower than the folic acid supplement doses used in the aforementioned studies. This may suggest, for the current study subjects, that a low level of PteGlu intake does not have a significant effect in lowering Hcy concentration. Interestingly, when taking into account the level of total dietary folate intake, the subjects with total dietary folate intake above the RDI showed significantly higher levels of PteGlu intake (209.1μg/day) when compared with subjects with a total folate intake below the RDI (49.19 μg/day). However, once again, the PteGlu intake was not associated with Hcy levels in either of the respective groups, and no significant difference in Hcy levels was observed between the two groups.

An Irish study suggested that, to reduce Hcy levels, 200μg/day of PteGlu was appropriate as a long-term fortification level: higher doses of PteGlu had no increased Hcy level lowering effects [499]. However, in this study, the subjects whose total dietary folate intake was above the RDI had levels of PteGlu intake (209.1μg/day) similar to those in the Irish study and this PteGlu intake had no effect on Hcy levels. No difference existed in Hcy levels for these subjects when compared to those in the subjects with lower PteGlu intake. This may suggest, for the current study population, that a higher level of PteGlu intake via folic acid fortification may be required to obtain a lowering effect on Hcy. However, as a blanket intervention (and lifetime-exposure), an increase of folic acid fortification level may lead to the presence of unmetabolized PteGlu in the plasma and the array of adverse effects described earlier. Moreover, lowering Hcy is not a primary objective of mandatory folic acid fortification. Further investigation with regards to the efficacy of PteGlu on Hcy levels in mandatory fortification programs is required.

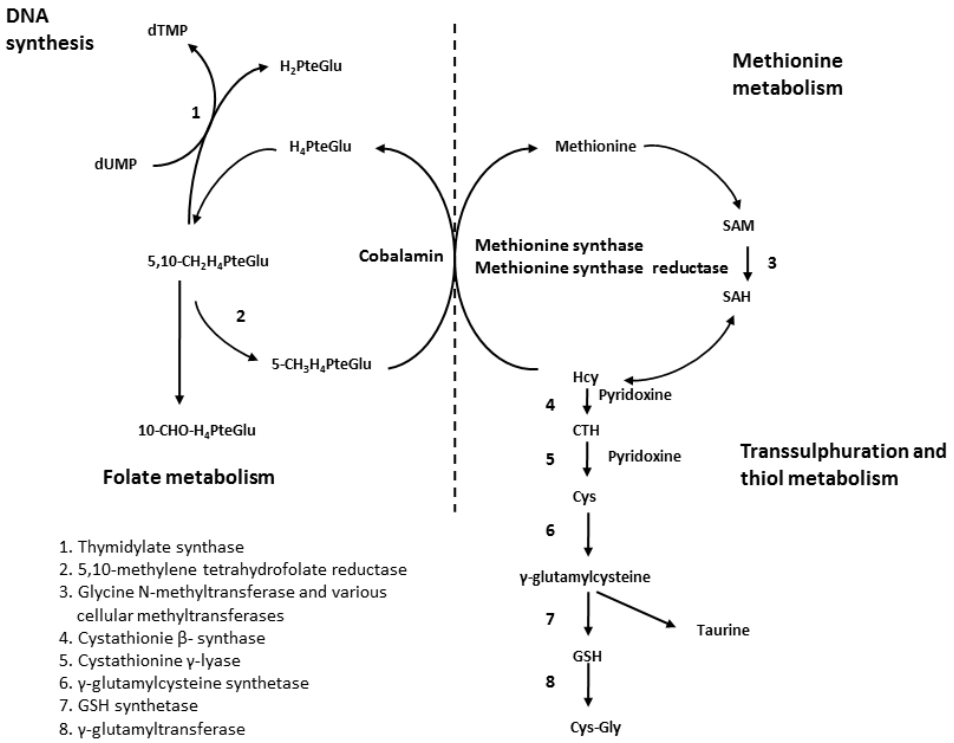



Figure 2.12 Folate, transsulphuration and thiol metabolism

Genetic variations also had an influence on thiol metabolism. The TS 1496del6 variation had an effect on Hcy levels. Similarly, DHFR 19bp del had an association, not only with Hcy levels, but also with Cys and Cys-Gly levels. The relationship between TS polymorphisms and Hcy levels has been studied intensively. As described earlier, altered TS mRNA stability due to the presence of this polymorphism may have an influence on methionine cycles via the substrate 5,10-$CH_2H_4$PteGlu. Altered TS enzyme could affect the conversion of 5,10-$CH_2H_4$PteGlu and dUMP to $H_2$PteGlu and dTMP. As a result trapped 5,10-$CH_2H_4$PteGlu could augment Hcy re-methylation via methionine metabolism (see Figure 2.11) [501-504].

Similarly, 19bp deletion in DHFR has been found to alter DHFR expression and may facilitate Hcy re-methylation [505]. In addition, compound genotypes in the transsulphuration pathway (CTH G1464T– IVS 10-430 C>T and CBS 844ins68-CTH G1464T– IVS 10-430 C>T) were associated with the levels of two intermediates of the transsulphuration pathway: Cys and Cys-Gly. Although the level of cystathionine was not measured in the current study, these polymorphisms are likely to be associated with Cys and Cys-Gly levels via regulated cystathionine production. Interestingly, Cys and Cys-Gly levels showed little association with nutritional intake. These results may suggest that blood Cys and Cys-Gly levels are mainly regulated by genetic variants, rather than nutritional intake.

By contrast, GSH level was associated with methionine and cobalamin intake. GSH is a crucial component in the cellular redox system which is critical for homeostasis and cell defence [506, 507]. Considerable research suggests that decreased GSH levels are associated with various chronic diseases such as diabetes, alcoholic liver disease, cataracts and AIDS [508-510]. Methionine, a major source of sulphur amino acids, has been studied in relation to the GSH-dependent detoxification system [511]. Experimental evidence suggests that restricted intake of dietary methionine decreases ROS production [512, 513] and increases GSH levels in blood, possibly due to an adaptation in sulphur amino acid metabolism by dietary methionine regulation [507, 508]. Furthermore, a high methionine diet showed enhanced ROS production [514]. The inverse relationship between methionine intake and GSH concentration demonstrated in the present study supports this previous research.

Little is known about any possible relationship between dietary cobalamin intake and GSH levels. However, recent experimental observations imply that cobalamin may regulate the oxidative response [515]. In previous studies, GSH levels increased in response to cobalamin therapy. However, there was no evidence of increased serum vitamin $B_{12}$ levels [515-517]. The mechanism of how cobalamin regulates GSH level has not yet been verified, but dietary cobalamin intake may enhance the intracellular uptake of cobalamin, possibly improving GSH status indirectly rather than via cobalamin dependant MS metabolism [517]. If this idea is correct, it may provide a reason why the present study showed that GSH had no association with Cys level, or with genetic variations in MS and MSR.

Although GSH level was not significantly associated with the occurrence of AP, methionine and cobalamin intake predicted GSH concentration in a fashion that was clearly associated with the occurrence of AP according to both gender and total folate intake levels (described earlier in this section). Therefore, the occurrence of AP predicted by methionine and cobalamin intake might possibly be related to GSH. The GSH concentration is relevant to intracellular thiol redox balance [518, 519]. Oxidative stress has been known to be implicated in cancer development and progression [520, 521]. Therefore, elevated GSH concentration is often reported for various types of tumours [518, 522], and this may induce chemo-resistance in neoplastic tissue [523]. This possibly supports the findings of the current study in that the risk of AP occurrence is predicted by methionine and cobalamin intake as a nutritional pathoaetiological factor. This association could also be referred to for further investigations into the role of GSH in carcinogenic mechanisms.

Logistic regression analysis showed that the compound genotype G1364T–IVS 10-430 C>T in CTH also had an effect on GSH levels. This is contradictory, as Cys concentration and pyridoxine were not associated with GSH levels. However, it may suggest that another mechanism for regulation of GSH via the transsulphuration pathway exists. This needs further investigation.

**- Haplotype analysis**

In the LD analysis, three SNPs in MTHFR and two SNPs in CTH were in LD with each other. This showed that the compound genotypes of each gene should be reasonably considered when evaluating the risk of AP and its association with biochemical data. The D` in the current study for each group was quite similar to the HapMap data, however, in the AP cases they were slightly different from other groups for both genes (low LOD). Although haplotypes of polymorphisms in both genes did not predict the occurrence of AP, those differing values possibly suggest altered susceptibility for an AP. Therefore, further study with a larger population sample is required to confirm the results.

Despite coming from two different continents, both HapMap subjects and the controls from the current study had similar Northern and Western European ancestral backgrounds. Even so, they showed dissimilar D` and LOD values. This is likely to suggest a difference in homogeneity between each population. The populations of the two continents, Australia and North America, only began to migrate from Europe approximately 150 and 250 years ago, respectively. This is a very short period in terms of evolutionary aspects. Although there have been migrations constantly between these regions, the altered values in each group imply that the distribution of SNPs is changing in these two geographically separated regions through admixture. Further monitoring of this process is required to consider not only anthropological issues, but also clinical genomics aspects.

## 2.6 Conclusion

The aetiology of AP is multifactorial, being modified by genetic and non-genetic (environmental) influences. Nutritional intake and nutrient related genetic variants show a significant effect on the development and progression of AP. These two factors interact with each other and lead to critical associations underpinning several speculative molecular mechanisms that influence AP aetiology. Furthermore, the total dietary folate intake and the composition of folyl vitamers impact on genetic factors, altering differential susceptibility to AP.

As folate is involved in several critical roles in relation to the human life cycle (*de novo* methionine, dTMP, purine and serine/glycine biosynthesis), for example that underscore reproductive mechanisms right through to the risk of degenerative disorders (including various types of cancer), the complex array of interactions between genetic variants and environmental factors needs further assessment.

## 2.7 Limitations of the study

The present study examining how nutrients and genes interact to modify risk for the occurrence of AP was designed to obtain preliminary data for a larger study as described on p56, thus the study cohort is relatively small. However the data from current study suggest many novel findings that can be implied for further studies.

# III. POTENTIAL EFFECTS OF FOLYL VITAMERS, ANTIOXIDANTS AND THEIR PHOTO-OXIDATIVE DERIVATIVES ON COLON CANCER CELL BIOLOGY: *IN VITRO* APPROACH

## 3.1 Introduction

Folic acid is the generic term for a family of chemical compounds which consist of a pteridine ring, para-aminobenzoic acid and one or more glutamic acid moieties joined together. Many forms of the vitamin exist. These differ by the oxidation status of the pteridine ring, the number of glutamate residues joined together via a series of gamma glutamyl bonds, and the one-carbon substitutions at positions N5 and N10.

It is known that approximately 150 folate coenzymes exist in the cell, and that further forms of folate possibly exist [524]. Due to its good stability and low cost, PteGlu has been used as the vitamer of choice for mandatory folic acid fortification [525]. However, as discussed previously, methylfolate and PteGlu may have differential roles in cellular biochemistry and, hence, disease aetiology. It has been reported that an increased intake of PteGlu may result in multiple adverse effects (see section 1.6.4). Each folate vitamer has its own physicochemical characteristics, although most folate-cell metabolism research has either been carried out using PteGlu alone, or has focused on the pharmacologic action of folate antagonists in cancer chemotherapy. Not much information exists with respect to the specific roles of various folate vitamers (particularly synthetic vitamers versus natural ones) and their interactions with other compounds in cell metabolism. Therefore, it is appropriate to investigate the relative physiological effects of each folate vitamer, and its potential effects on cell proliferation.

In order to achieve these aims, the present study utilises an *in vitro* tissue culture model. Both colon cancer and breast cancer cell lines were treated with various types of folate vitamer, including fully oxidised synthetic PteGlu, fully reduced native 5-$CH_3H_4$PteGlu, partially oxidised native 5-$CH_3H_2$PteGlu and relatively stable reduced 5-$CHOH_4$PteGlu. The impact of antioxidants including DTT, GSH and ascorbic acid on cell growth, together with their effects in combination with the easily oxidised native methylfolate form of the vitamin (5-$CH_3H_4$PteGlu), were also examined.

As mentioned in section 1.2 and Figure 1.2, when PteGlu is exposed to UV it degrades into oxidised derivatives (PCA, 6-FP and p-ABG). Cancer cell lines were cultured in the presence of the oxidative degradation products of PteGlu and 5-$CH_3H_2$PteGlu to investigate whether these have any toxic, or other, effects on cell proliferation.

Finally, the effect of genetic variations on the role of individual vitamers in folate metabolism was investigated in different cell lines. Common genetic variations in folate metabolism,

including those investigated in the AP study, were genotyped in both Caco-2 (colon cancer) and MCF 7 (breast cancer) cell lines.

The experimental objectives were therefore to

1) determine the physico-chemical characteristics of various folate vitamers on cell growth,

2) analyse the interaction of antioxidants with methylfolate in the context of cell growth,

3) evaluate potential adverse effects of folate vitamers and their oxidative degradation products based on concentration, and

4) determine whether methylfolate could have potential as a supplementary vehicle in terms of folic acid fortification.

## 3.2 Materials and methods

### 3.2.1 Chemicals and equipment

The following cell culture media and reagents were purchased from Sigma (Castle Hill, NSW): Roswell Park Memorial Institute 1640 (RPMI 1640, R0883), RPMI 1640 without folate (FFM, R1145), minimum essential medium eagle (MEME, M2279), 200mM L-Glutamine (G7513), 100mM Na-pyruvate (S8636), non-essential amino acids (M7145), 10x phosphate buffered saline (PBS, P5493), trypsin-EDTA solution (T4049), $NaHCO_3$ (S5761), ascorbic acid (A4544), L-GSH (reduced, G6013), DL-DTT (43817), thiazolyl blue tetrazolium bromide (MTT, M2128), sodium hydroxide (NaOH, S8045) and hydrochloric acid (HCl). Foetal bovine serum (FBS) was supplied by SAFC (12007C, Sydney, Australia). Folate vitamers and derivatives including PteGlu, 5-$CH_3H_4$PteGlu, 5-$CH_3H_2$PteGlu, 5-$CHOH_4$PteGlu, p-ABG (Cat. No. 16.701), 6-FP (Cat. No. 11.415) and PCA (Cat. No. 11.403) were purchased from Schirks Laboratory (Jona, Switzerland). Pipette aid was purchased from Drummond (Melbourne, Victoria). Plastic laboratory wares such as 25$cm^2$ and 75 $cm^2$ cell culture flasks, 24-well plates, tubes (15mL, 50mL), disposable pipettes (1, 5, 10, 25, 50mL), syringe filters (0.22µm PES) and a filtering system (0.22µm PES) were all purchased from Corning (CA, USA). The transilluminator was supplied by Bio-Rad (model no. 2000, Gladesville, NSW). Caco-2 (Cat. No. 86010202) and MCF 7 (Cat. No. 86012803) cell lines were obtained from the European Collection of Cell Cultures (ECACC). The water bath was purchased from Clifton (model No. 74121, Weston-super-Mare, UK) while the microscope (Leica microsystem CMS GmbH, 090-135 009-000) and computer were supplied by Leica (North Ryde, NSW). The UV-visible (CARY 50Bio) and microplate (Benchmark Plus)

spectrophotometers were purchased from Varian (Belrose, NSW) and Bio-Rad (Gladesville, NSW), respectively.

### 3.2.2 Cell lines

This research program was designed to ascertain the role of folate as a mediator of cancer risk, particularly for CRC, via genetic and *in vitro* cell culture studies. *In vitro* models of colon cancer (Caco-2) and breast cancer (MCF 7) were also analysed and used for comparison because clinical studies implicate folate as a risk in both colon and breast cancers.

#### 3.2.2.1 Caco-2 cell line

Caco-2 is an immortalized cell line which was originally derived from human colorectal epithelial adenocarcinoma tissue. When Caco-2 cells are grown as a monolayer, cells differentiate structurally. They are functionally similar to the small intestinal epithelium, including the microvilli on the apical membrane, and exhibit tight junctions between neighbouring cells [526, 527]. In *in vitro* assays, a number of studies have reported that Caco-2 cells present better morphological and enterocytic differentiation compared to other colon carcinoma cells. As a result, Caco-2 is the preferred colon cancer cell line for the analysis or investigation of the transport model for candidate drugs and nutrients across the intestinal epithelial cell barrier [528].

#### 3.2.2.2 MCF 7 cell line

MCF 7, human breast adenocarcinoma cells, are the most commonly used cell line in *in vitro* breast cancer models [529]. MCF 7 cells were originally derived from the mammary gland epithelial tissue of a patient who was diagnosed with an invasive ductal carcinoma. MCF 7 is characterized by the presence of metastatic cells, and presents as oestrogen and progesterone receptor positive. Also, MCF 7 cells form interconnected colonies of adherent, tight and desmosomal junctions with polygonal cells that are weakly invasive [530].

### 3.2.3 Methods for cell culture

Caco-2 and MCF 7 cells were delivered from ECACC in frozen vials on dry ice. Cryo tubes containing the cells were immediately moved into a liquid nitrogen tank until defrosted and ready for subculture. All cell culture procedures were carried out under aseptic conditions in a contamination level 2 bio-safety cabinet. Cells were incubated at 37°C in 95% humidity and 5% $CO_2$ at all times.

### 3.2.3.1 Media

Two different types of common culture media, MEME and RPMI 1640 with/without folate, were used. MEME was developed by Harry Eagle [531, 532] and has been used for cultivation of a wide variety of attached cells grown in mono-layers [533]. RPMI 1640 was developed by Moore et. al [534]. The formulation is based on a bicarbonate buffering system and alterations in the amounts of amino acids and vitamins present. RPMI 1640 has also been used for the culture of many types of cultured cells such as human normal and neoplastic leukocytes [535].

Table 3.1 Composition of media for Caco-2 and MCF 7 cells in the current study (g/L) [536, 537]

| | | MEME | RPMI 1640 with folic acid | RPMI 1640 without folic acid (FFM, 10X) |
|---|---|---|---|---|
| Inorganic salts | $CaCl_2 \cdot 2H_2O$ | 0.2 | 0.1 | 1 |
| | $MgSO_4$ (anhydrous) | 0.09767 | 0.04884 | 0.4884 |
| | KCl | 0.4 | 0.4 | 4 |
| | $NaHCO_3$ | 2.2 | 2 | - |
| | NaCl | 6.8 | 6 | 60 |
| | $Na_2HPO_4$ (anhydrous) | - | 0.8 | 8 |
| | $NaH_2PO_4$ (anhydrous) | 0.122 | - | - |
| Amino acids | L-Arginine • HCl | 0.126 | 0.2 | 2 |
| | L-Asparagine • $H_20$ | - | 0.05 | 0.5 |
| | L-Aspartic acid | - | 0.02 | 0.2 |
| | L-Cystine • 2HCl | 0.0313 | 0.0652 | 0.652 |
| | L-Glutamic acid | - | 0.02 | 0.2 |
| | Glycine | - | 0.01 | 0.1 |
| | L-Histidine • HCl • $H_20$ | 0.042 | 0.015 | 0.15 |
| | L-Isoleucine | 0.052 | 0.05 | 0.5 |
| | L-Leucine | 0.052 | 0.05 | 0.5 |
| | L-Lysine • HCl | 0.0725 | 0.04 | 0.4 |
| | L-Methionine | 0.015 | 0.015 | 0.15 |
| | L-Phenylalanine | 0.032 | 0.015 | 0.15 |
| | L-Proline | - | 0.02 | 0.2 |
| | L-Serine | - | 0.03 | 0.3 |
| | L-Threonine | 0.048 | 0.02 | 0.2 |
| | L-Tryptophan | 0.01 | 0.005 | 0.05 |
| | L-Tyrosine • 2Na • $2H_20$ | 0.0519 | 0.02883 | 0.2883 |
| | L-Valine | 0.046 | 0.02 | 0.2 |
| Vitamin | D-Biotin | - | 0.0002 | 0.002 |
| | Choline chloride | 0.001 | 0.003 | 0.03 |
| | Folic acid | 0.001 | 0.001 | - |
| | myo-Inositol | 0.002 | 0.035 | 0.35 |
| | Niacinamide | 0.001 | 0.001 | 0.01 |
| | p-Amino Benzoic Acid | - | 0.001 | 0.01 |
| | D-Pantothenic acid • ½Ca | 0.001 | 0.00025 | 0.0025 |
| | Pyridoxal • HCl | 0.001 | 0.001 | 0.01 |
| | Riboflavin | 0.0001 | 0.0002 | 0.002 |
| | Thiamine • HCl | 0.001 | 0.001 | 0.01 |
| | Vitamin B-12 | - | 0.000005 | 0.00005 |
| Other | Glucose | 1 | 2 | 20 |
| | Phenol red • Na | 0.011 | 0.0053 | 0.053 |
| | Glutathione (reduced) | - | 0.001 | 0.01 |
| Add | L-Glutamine | 0.292 | 0.3 | 3 |
| | $NaHCO_3$ | 2.2 | - | 20 |
| | Non-essential amino acid | 100X | - | - |
| | FBS | | 10% of total volume | |

These two media can be applied to cell culture models without any additives, however, when properly supplemented, they demonstrate wide applicability for supporting cell growth, according to experimental conditions [536]. For instance, optional supplementation of non-essential amino acids to the formulations that incorporate either Hanks' or Eagles' salts for MEME has broadened the usefulness of this medium. The formulation of these media is detailed in Table 3.1.

Following the instructions for ECACC, heat inactivated FBS was used to supplement culture media. The heat inactivation of FBS was carried out at 56°C for 30 minutes using a water bath. All aqueous solutions were prepared with filtered water purified by a Milli-Q system (Millipore, Sydney). Millipore water was sterilized at 121°C for 15 minutes, and then filtrated again with a 0.22 µm PES filtering system. MEME was supplemented with 2mM L-Glutamine, 1% non-essential amino acids and 10% HI-FBS. Normal RPMI 1640 was supplemented with 2mM L-Glutamine and 10% FBS.

FFM was supplied at a 10x concentration. The FFM used for this project was supplemented with 2mM of L-Glutamine, 10% of FBS and 2g of $NaHCO_3$/100mL.

#### 3.2.3.1.1 Preparation of cell lines

According to ECACC, MEME is recommended for Caco-2 and MCF 7 cell cultures. However, RPMI 1640 is the only available form of commercial medium which does not contain folate. As a result, cells were initially defrosted and grown in MEME, and were then cultured in normal RPMI 1640 medium, gradually increasing the ratio of RPMI 1640 to MEME over two weeks. In addition, to prevent the shock from a sudden change of media and to obtain reliable responses from the cell lines, cells were cultured in FFM. To adjust cells to FFM, a similar procedure was performed with normal RPMI 1640 and FFM over a period of three weeks.

#### 3.2.3.1.2 Cell culture

Caco-2 and MCF 7 were initially defrosted in 25$cm^2$ flasks with 5mL of medium. They were then grown with 10mL of medium in a 75 $cm^2$ flask. The medium was changed every other day and cells were passaged every four days before confluence reached approximately 80%. The passage number for the cells used in experiments was between 20 and 32. As both Caco-2 and MCF 7 are adherent cell lines, a trypsinization process was required to detach cells for passage. Flasks containing cells were washed with PBS (half the volume of the medium) to remove the medium and FBS. Then, depending on cell confluence, 2-4 mL of trypsin-EDTA solution was added to detach the cells. After incubation at 37°C for 3-5 minutes, 3-5mL of the appropriate

medium was added. Some of this cell suspension was then transferred to a flask containing new medium (normally at a 1 in 5 ratio).

#### 3.2.3.2 Preparation of folate vitamers and antioxidant standards

Standards for each folate vitamer and antioxidant were prepared as two different types: stock and working standards. From stock standards, serial dilutions were carried out to make working standards. Each working standard was prepared at a concentration 100 times higher than each experimental concentration to allow for the dilution of folate in the media. Typical working standard concentrations were as follows: 1µg/mL, 10µg/mL and 100µg/mL. All standards were filtered (0.22um PES) and dispensed into small aliquots. They were stored at $-84\ ^{\circ}\mathrm{C}$ until required. Details of all stock and working standards are presented in Table 3.2.

##### 3.2.3.2.1 PteGlu standards

PteGlu is insoluble in aqueous solutions at neutral pH (6-7) and so the pH was adjusted to approximately 3 in order to dissolve PteGlu. Two different stock standards for PteGlu, at concentrations of 1mg/mL and 50mg/mL, were prepared and used to make working standards. Additionally, 5mg/mL, 10mg/mL and 25mg/mL working standards for PteGlu were made from a 50mg/mL stock standard.

##### 3.2.3.2.2 5-$CH_3H_4$PteGlu and antioxidant standards

As a preliminary experiment, Caco-2 cells were grown in media with various concentrations of antioxidant to obtain the optimal concentration for cell growth with no cytotoxicity. Stock standards for antioxidants such as ascorbic acid, GSH and DTT were made at concentrations of 1mg/mL, 10mg/mL and 266.7µg/mL, respectively. Each concentration was based on previous research [8, 9]. The level of antioxidant confirmed to be the most appropriate from this preliminary experiment was used for the following experiments which investigated the influence of antioxidants on 5-$CH_3H_4$PteGlu.

5-$CH_3H_4$PteGlu stock standards were made at a concentration of 1mg/mL in the absence or presence of a given antioxidant. 5-$CH_3H_4$PteGlu was dissolved in water (no antioxidant), ascorbic acid (10µg/mL) and GSH solution (10µg/mL). Based on preliminary experimental results, it was decided not to examine the interaction of DTT with 5-$CH_3H_4$PteGlu. From 5-$CH_3H_4$PteGlu stock standard having a concentration of 1mg/mL, a serial dilution was carried out to prepare working standards of the following concentrations: 10µg/mL, 10µg/mL and 100µg/mL.

#### 3.2.3.2.3 5-$CH_3H_2$PteGlu, 5-$CHOH_4$PteGlu and p-ABG standards

Stock standards of 5-$CH_3H_2$PteGlu and 5-$CHOH_4$PteGlu were prepared at a concentration of 1mg/mL with no antioxidant. These stock standards were diluted to the following working standard concentrations: 10µg/mL, 10µg/mL and 100µg/mL. Stock and working standards for p-ABG were prepared in exactly the same way.

#### 3.2.3.2.4 Standards for UV-oxidative degradation products

In order to investigate novel physico-chemical characteristics, and to compare the potential safety of the oxidative degradation of PteGlu and 5-$CH_3H_2$PteGlu, two cancer cell lines were cultured in media containing the photo-oxidative degradation products of these vitamers. The oxidative degradation products of PteGlu are PCA and 6-FP [423, 538, 539]. Each of these was prepared at 11.3mM. It was not possible to calculate the exact proportions of PCA and 6-FP that arise from oxidation of PteGlu [423] and so standards were made at containing equimolar concentrations (2.26µM, 22.6µM, 226µM and 2.26mM). Due to the very low solubility of these chemicals, pH was adjusted using 5M NaOH, thereby the final pH for the 6-FP and PCA standards was 11 and 9, respectively. As a result of the extreme light sensitivity of PCA and 6-FP, all experiments were carried out under dim light [131]. Stock standards of the oxidised degradation derivatives of PteGlu were also characterized spectrophotometrically to identify their signature spectra.

The standards for examining the oxidative degradation products of 5-$CH_3H_2$PteGlu were prepared using the following process. A stock solution of 11.3mM 5-$CH_3H_2$PteGlu was prepared without antioxidant and was adjusted to pH 3 to facilitate the complete degradation of 5-$CH_3H_2$PteGlu. This was filtered and aliquoted into a micro tube. It was then exposed to UV radiation (at a wavelength of 302nm) on a transilluminator for an hour. To avoid direct heat from the transilluminator, tubes were placed on a plastic tripod. When UV exposure was completed, the resulting stock standard was diluted to working standard concentrations (2.26µM, 22.6µM, 226µM and 2.26mM). Stock and working standards were used for cell culture experiments. They were verified to be the pH and photolytically-derived oxidative degradation products of 5-$CH_3H_2$PteGlu by UV-spectrophotometry. Similarly, to confirm the spectra of the degradation derivatives of PteGlu, 11.3mM solution of PteGlu was exposed to UV for an hour and its spectrum was read using a UV-spectophotometer.

Table 3.2 The concentrations of folate vitamer standards and their degradation products

A. Concentration of standards for folate vitamers and their oxidative degradation products

| | | Folate vitamer | | | | | Photo-oxidative degradation products | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Concentration | | PteGlu | 5-$CH_3H_4$ PteGlu | 5-$CH_3H_2$ PteGlu | 5-$CHOH_4$ PteGlu | p-ABG | Concentration | PCA | 6-FP | 5-$CH_3H_2$ PteGlu |
| Low* | 1μg/mL | √ | | √ | √ | √ | 2.26μM | √ | √ | √ |
| | 10μg/mL | √ | √ | √ | √ | √ | 22.6μM | √ | √ | √ |
| | 100μg/mL | √ | √ | √ | √ | √ | 226μM | √ | √ | √ |
| | 1mg/mL | # | # | # | # | # | 2.26mM | √ | √ | √ |
| High* | 5mg/mL | √ | | | | | 11.3mM | # | # | # |
| | 10mg/mL | √ | | | | | | | | |
| | 25mg/mL | √ | | | | | | | | |
| | 50mg/mL | # | | | | | | | | |

*Simple classification to describe the range of folate concentrations, # means stock standard, working standards were prepared 100 times higher than experimental concentration

B. Concentrations of antioxidant standards

| Antioxidants | Stock standard | Working standards | | | |
|---|---|---|---|---|---|
| DTT | 266.7μg/mL | 133μg/mL | 67μg/mL | 33μg/mL | 7μg/mL |
| Ascorbic acid | 10mg/mL | 5mg/mL | 2mg/mL | 1mg/mL | 0.4mg/mL |
| GSH* | 1mg/mL | | | | |

*Only one concentration was used for experiments, working standards were prepared at concentrations 100 times higher than experimental concentration

### 3.2.3.3 Experimental design

As described earlier, previous research has only investigated cell growth for a relatively short period. To investigate the sub-chronic effect of various concentrations of folate vitamers in our *in vitro* model, experiments were designed to measure cell growth over 8 days. Therefore, the optimal starting cell count needed to be determined. Considering cell growth pace, confluency and well size, preliminary experiments suggested that $3x10^3$ cells per well was the most suitable starting cell count for an observation of the growth for 8 days using a 24-well plate. In each well, cells were grown in 500μL of medium. Additionally, a water control (0ng/mL) was included on the plate as a baseline and reference sample. All experiments were done in triplicate.

The following procedure was carried out in order to prepare the experimental plates. Cells cultured in $75cm^2$ flasks were typsinized and resuspended in 5mL of fresh FFM. Some of this suspension was used for passage and cell counting. Cells were counted at least 3 times using a haemocytometer to obtain accurate numbers for the plating. Once the cell numbers within

this suspension had been calculated, the cells were diluted to $3x10^3$ in 30-50µL of medium and seeded into all of the appropriate wells of the 24-well plate except for the blank wells. After seeding cells into each well, the media at the different concentrations of folate vitamers to be analysed, together with the water control were prepared using FFM. The correct volume of media was aliquoted into each well (500µL minus cell suspension volume). 500µL of each medium was added to the blank well (no cell) suspension. Each concentration was analysed in triplicate, including the one blank well (without cells) for reading the absorbance and the MTT assay. The 24-well plates were agitated with care to spread the cells evenly. On the actual plating day, four identical plates were prepared, and the cell growth was read on days 2, 4, 6 and 8 using Alley's modified MTT assay method [540]. The media was changed every other day. Figure 3.1 shows the design of the plate.

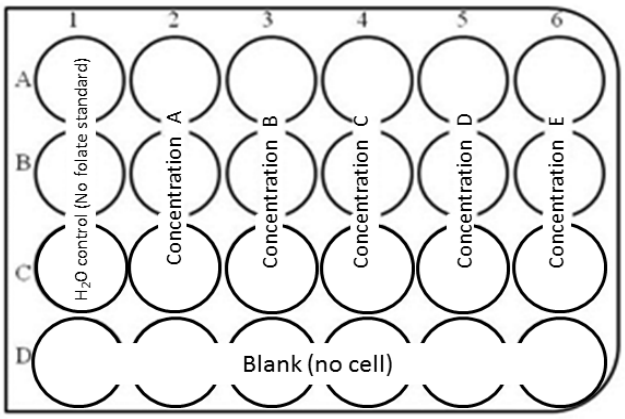



Figure 3.1 24-well plate design for cell culture

### 3.2.3.4 Cell growth measurement

To measure the proliferation of cell lines, an MTT assay was performed. Only mitochondria in living cells can reduce yellow MTT to purple formazan which can be dissolved by an organic solvent such as DMSO, isopropanol or sodium dodecyl sulphate in aqueous solution. The absorbance of this coloured solution is then used to quantify cell growth at a specific wavelength (usually between 500 and 600 nm) using a spectrophotometer. Dead cells do not perform this biochemical reaction and are, hence, not detected [540].

MTT solution was prepared at a concentration of 5mg/mL in PBS. This was then filtered with a 0.22µL PES syringe filter, dispensed in 1.2mL aliquots and stored at -20°C until needed. Before commencing cell growth measurement, the MTT solution was pre-warmed at 37°C for 20 minutes. 100µL of MTT solution was added to each well. The plates were then incubated at

37°C in 95% humidity and 5% $CO_2$ for 3 hours, covered with aluminium foil. Media and MTT solution were carefully removed with a syringe needle and 500µL of DMSO was added into each well to dissolve the purple formazan. The plate was then transferred to a spectrophotometer where it was gently shaken for 10 minutes before the absorbance was read. The absorbance was read at 550nm with a reference filter of 650nm due to the colour of MTT.

#### 3.2.3.5 Measuring the spectra of oxidative degradation products of PteGlu and 5-$CH_3H_2$PteGlu

A review of the literature showed that little is known about the UV-oxidative derivatives of 5-$CH_3H_2$PteGlu. Therefore, to identify oxidative derivatives of the vitamer, and also to confirm the presence of the degradation products of PteGlu, the spectra of PteGlu and 5-$CH_3H_4$PteGlu, and their oxidative degradation products including 6-FP, PCA and p-ABG, were read using a UV-visible spectrophotometer (200-400nm). The concentration of each sample was adjusted, depending on the absorbance range.

#### 3.2.3.6 Genotype determination of cell lines

Sixteen genetic variations - MTHFR C677T, 1208C, G1793A, SHMT C1420T, TS 1496del, TSER 2R3R, TS 3R G>C, GCPII C1561T, CBS 844ins66, DHFR 19bp deletion, RCF G80A, CTH G1208T, C430T, BHMT G595A, MS A2756G and MSR A66G- were analysed for both Caco-2 and MCF 7 cell lines. All of the chemicals and methods used for the genotype analysis have been previously described for the AP study (see section 2.3.1).

### 3.2.4 Statistical analysis

All statistical analysis was performed using SPSS 13.0 for windows (SAS, USA). Student T-test, ANOVA and Tukey's HSD post hoc test was performed to compare the cell growth with a confidence level of 95%. Cell growth change was calculated using the average absorbance of three wells for each concentration, and presented as percentage growth compared to the water control. To examine the variation between each test, the coefficient of variation was calculated, which was below 10% in all cases.

### 3.3 Results

In order to investigate

1) the physicochemical characteristics of folate vitamers,

2) the effects of antioxidants on folate vitamers and

3) antioxidant interactions with methyl folate on cell growth,

two cancer cell lines, Caco-2 (colon cancer) and MCF 7 (breast cancer), were cultured for 8 days in media containing various types and concentrations of folate vitamer, including PteGlu, 5-$CH_3H_4$PteGlu, 5-$CH_3H_2$PteGlu and 5-$CHOH_4$PteGlu, and antioxidants such as DTT, GSH and ascorbic acid. In addition,

4) the effects of the UV-degradation products of 5-$CH_3H_2$PteGlu and PteGlu (i.e. 6-FP and PCA, Figure 1.2) on cell proliferation were studied to examine the potential adverse effects and the relevance of these findings in terms of folic acid fortification.

In this section cell growth differences are given as a percentage relative to the control which was cultured in FFM using water instead of folate standards. To identify the oxidative degradation products of folate vitamers, absorption spectra were obtained within the range 200nm to 400nm. Finally, the genotypes of major genetic variations in folate metabolism were determined to provide a better context for the growth responses of the different cell lines.

Table 3.3 Summary of results: cell growth response to folate vitamers, antioxidants and photo-oxidative degradation products

| | Folate vitamers and antioxidant (concentrations) | Caco-2 | MCF7 |
|---|---|---|---|
| **PteGlu** | Low concentration (10ng/mL, 100ng/mL, 1µg/mL, 10µg/mL) | ↑ | ↑* |
| | High concentration (50µg/mL, 100 µg/mL, 250 µg/mL, 500 µg/mL) | ↑↓* | ↓↑* |
| **5-$CH_3H_4$PteGlu** | **No added antioxidant** (100ng/mL, 1µg/mL, 10µg/mL) | ↓* | ↑* |
| | **GSH** (0ng/mL, 10ng/mL, 100ng/mL, 1µg/mL, 10µg/mL) | ↓* | ↓↑* |
| | **Ascorbic acid** (0ng/mL, 10ng/mL, 100ng/mL, 1µg/mL, 10µg/mL) | ↓↑* | ↑* |
| **5-$CH_3H_2$PteGlu** (10ng/mL, 100ng/mL, 1µg/mL, 10µg/mL) | | ↑* | ↑* |
| **5-$CHOH_4$PteGlu** (10ng/mL, 100ng/mL, 1µg/mL, 10µg/mL) | | ↑* | ↑* |
| **p-ABG** (22.6nM, 226nM, 2.26µM, 22.6µM, 113µM) | | – | – |
| **6-FP** (22.6nM, 226nM, 2.26µM, 22.6µM, 113µM) | | – | ↑↓ |
| **PCA** (22.6nM, 226nM, 2.26µM, 22.6µM, 113µM) | | ↑ | – |
| **UV-5-$CH_3H_2$PteGlu** (22.6nM, 226nM, 2.26µM, 22.6µM, 113µM) | | ↓ | ↑ |

* Statistical significance at a 0.05 confidence level, where there is an ↑ and↓ the sample shows a simplified day dependant cell growth response, ↑ means increased and ↓stands for decreased cell growth, – signifies no particular trend was observed

### 3.3.1 Effects of folate vitamers, antioxidants and photo-oxidative degradation products on cell growth

#### 3.3.1.1 Cell growth response to PteGlu

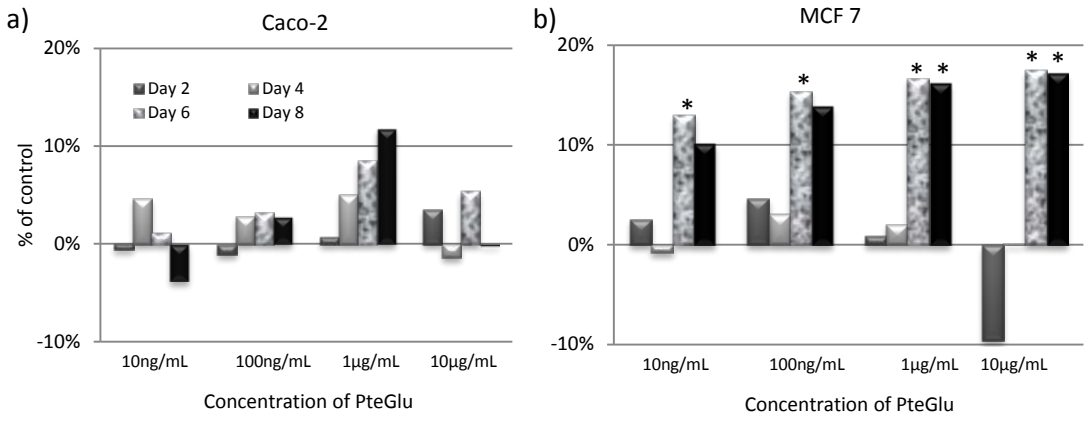



Figure 3.2 The effect of low PteGlu (≤ 10µg/mL) on cell line growth (Asterisks denote significant growth difference compared to the control, $p<0.05$)

Relatively low levels of PteGlu (≤ 10µg/mL) did not have any striking effect on Caco-2 cell growth (see Figure 3.2-a). Caco-2 cells exhibited contrasting responses at PteGlu concentrations of 10ng/mL and 100ng/mL, depending on time. However, none of the concentrations (10ng/mL, 100ng/mL, 1µg/mL, and 10µg/mL) led to a significant difference in growth compared to the control. It is interesting to note that cell growth at 1µg/mL increased up to 12% with time by day 8. However, this finding was not statistically significant.

PteGlu conferred a different growth pattern in MCF 7 cells compared to Caco-2 cells (see Figure 3.2-b). No significant growth difference was observed for low concentrations of PteGlu (≤ 10µg/mL) in Caco-2 cells, whilst the growth of MCF 7 cells was incrementally increased in the later period of the experiment (days 6 and 8). Increasing the concentration of PteGlu induced enhanced cell growth of up to 18% on day 6 ($p<0.0001$) in a dose-dependent manner, with a similar trend being observed for the growth in response to increased PteGlu concentration on day 8 ($p=0.02$). However, there was no statistical difference between growth rates at differing PteGlu concentrations.

Caco-2 cells did not show any significant noteworthy growth in the experiment with relatively low levels of PteGlu. In order to investigate whether higher levels of PteGlu could have an effect on cell growth, cells were cultured in media containing higher concentrations (≥50µg/mL) of PteGlu. Figure 3.3 presents the effects of higher concentrations of PteGlu which resulted in contrasting Caco-2 cell growth, depending on time and concentration (50µg/mL,

100µg/mL, 250µg/mL and 500µg/mL). 50µg/mL of PteGlu enhanced Caco-2 cell growth, with an increase of approximately 36% on day 4, although this was not statistically significant when compared to control cell growth. By contrast, 500µg/mL PteGlu inhibited Caco-2 cell growth by approximately 30% on day 2 ($p<0.005$), but this inhibition was reversed by day 6, and no particular growth trend was shown on day 8.

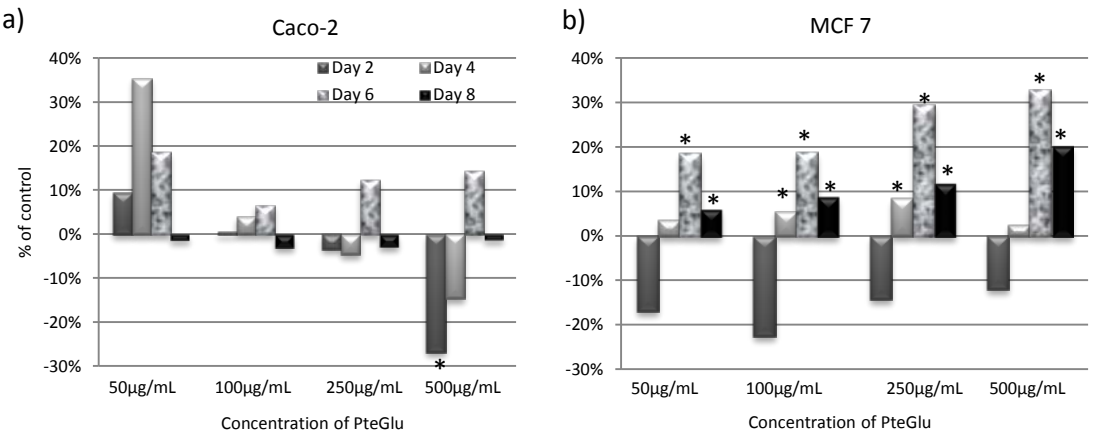



Figure 3.3 The effect of high PteGlu (≥50µg/mL) on cell line growth (Asterisks denotes significant growth difference compared to the control, $p<0.05$)

The higher concentrations of PteGlu yielded contrasting MCF 7 cell growth with time (see Figure 3.3-b). It is of particular interest that the PteGlu concentrations, with the exception of 500µg/mL, did not result in a clear inhibition of growth of Caco-2 cells on day 2. However, for MCF 7 there was a general cell growth suppression with the greatest growth inhibition (-22%) occurring at 100µg/mL on this day. This MCF 7 growth inhibition was not maintained on day 4. All PteGlu concentrations significantly increased growth of MCF 7 cells compared to the control on days 6 and 8 ($p<0.0001$) in a dose-dependent manner. However, no significant cell growth difference was observed between PteGlu concentrations.

### 3.3.1.2 Caco-2 cell growth response to antioxidants

High concentrations of antioxidant could be potentially cytotoxic to cancerous cells [541]. Therefore, to investigate the appropriate level of antioxidant in preserving 5-$CH_3H_4$PteGlu with no cytotoxicity, Caco-2 cells were initially cultured in media containing ascorbic acid and DTT at various concentrations (see Figure 3.4). The growth of Caco-2 cells with GSH was not investigated because it has already been established that GSH is an antioxidant which is minimally toxic at all concentrations, when compared to other antioxidants, and is synthesized

in the cell naturally [523, 542]. DTT and ascorbic acid resulted in diverse growth responses in Caco-2 cells as described below. Day 8 was not included in the analysis for this experiment.

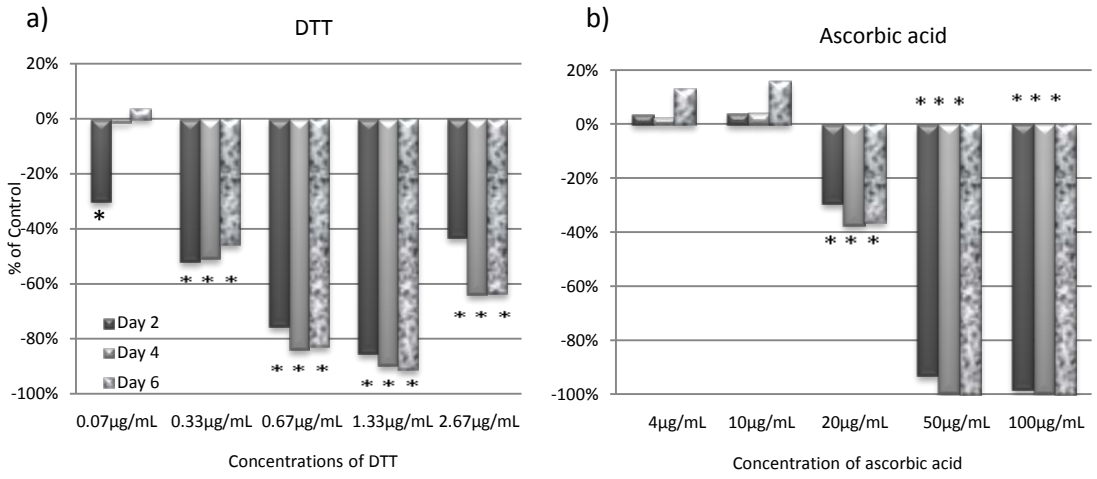



Figure 3.4 The effects of antioxidants (DTT and ascorbic acid) on Caco-2 cell line growth (Asterisks denote significant growth difference compared to the control, $p<0.05$)

#### 3.3.1.2.1 Dithiothreitol

DTT resulted in a significant detrimental cell growth at most concentrations, peaking at a 91% reduction in growth with 1.33µg/mL (see Figure 3.4-a). At 0.07µg/mL, DTT reduced the cell growth significantly on day 2. This phenomenon was reversed on days 4 and 6, and no significant change in growth compared to the control was seen. It is interesting to note that the inhibitory effect of DTT on cell growth was decreased at 2.67µg/mL than at 1.33µg/mL. Due to this inhibitory effect on cell growth, DTT was ruled out as an antioxidant for the 5-$CH_3H_4$PteGlu degradation experiments.

#### 3.3.1.2.2 Ascorbic acid

Ascorbic acid exerted a dose-dependent cytocidal effect on Caco-2 cells (see Figure 3.4-b). At 20µg/mL, 50µg/mL and 100µg/mL, Caco-2 cell growth was significantly inhibited when compared to the growth of the control cells. The top two concentrations, 50µg/mL and 100µg/mL, resulted in a lethal effect on Caco-2 cells. At 4µg/mL and 10µg/mL, ascorbic acid did not show any significant lethal effect on cell growth on days 2 and 4, and there was no statistical difference between the two concentrations on day 6. Therefore, 10µg/mL was selected as an optimal ascorbic acid concentration for preserving 5-$CH_3H_4$PteGlu in subsequent experiments.

### 3.3.1.3 Cell growth response to 5-$CH_3H_4$PteGlu alone and in combination with antioxidants

These experiments were designed to compare the effects of 5-$CH_3H_4$PteGlu and other vitamers on cell growth. In addition, the possible existence of any synergic effects of antioxidants on the stability of 5-$CH_3H_4$PteGlu with respect to cell proliferation was investigated. To achieve these aims, cell lines were grown in the presence of 5-$CH_3H_4$PteGlu, with and without antioxidant. The levels of antioxidant used for these experiments were determined empirically (see section 3.3.1.2).

#### 3.3.1.3.1 The effect of 5-$CH_3H_4$PteGlu in the absence of additional [1] antioxidant

The addition of 5-$CH_3H_4$PteGlu to FFM affected Caco-2 cell growth at all concentrations (100ng/mL, 1µg/mL and 10µg/mL, see Figure 3.5-a). This was observed throughout the whole experimental period (days 2-8) and occurred in a dose-related manner. At 10µg/mL, cell growth was significantly reduced by 11% ($p$=0.002) on day 2. However, this level of inhibition was no longer maintained by day 8.

Figure 3.5-b exhibits the effect of 5-$CH_3H_4$PteGlu in the absence of additional antioxidant on MCF 7 cells. In contrast to the response of Caco-2 cells, 5-$CH_3H_4$PteGlu evidently enhanced proliferation of MCF 7 cells throughout the experimental period. At concentrations of 100ng/mL and 10µg/mL, MCF 7 cells presented significantly enhanced growth on days 6 and 8. This occurred in a dose-dependent manner, when compared to control cells (in both cases $p$=0.003).

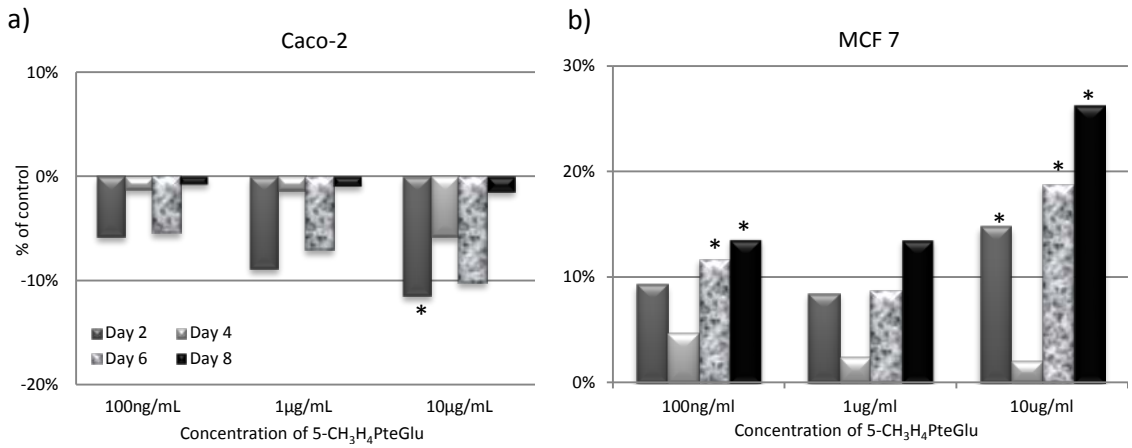



Figure 3.5 The effect of 5-$CH_3H_4$PteGlu on cell lines in the absence of added antioxidant (Asterisks denote a significant growth difference compared to the control, $p$<0.05)

[1] 'Additional' refers to antioxidants over and above the ones found in the proprietary tissue culture medium

#### 3.3.1.3.2 The effect of 5-$CH_3H_4$PteGlu in the presence of GSH

Figure 3.6-a presents the growth patterns of Caco-2 cells cultured in media containing 5-$CH_3H_4$PteGlu in the presence of 10µg/mL of GSH. This concentration of GSH was chosen to be broadly comparable to the effects of ascorbic acid described in the literature [8, 9]. Caco-2 cells showed a similar growth pattern to that of 5-$CH_3H_4$PteGlu in the absence of added antioxidant (see Figure 3.5-a). At concentration of 10ng/mL, no significant change of growth was observed. However, increasing 5-$CH_3H_4$PteGlu levels resulted in a substantial decrease in cell growth. In particular, 1µg/mL and 10µg/mL concentrations of 5-$CH_3H_4$PteGlu significantly inhibited cell growth by 13% ($p$=0.047) and 22% ($p$=0.0001) on day 2, respectively. At all concentrations, this inhibitory effect was reversed on day 6. Similarly, no noticeable growth change was observed on day 8.

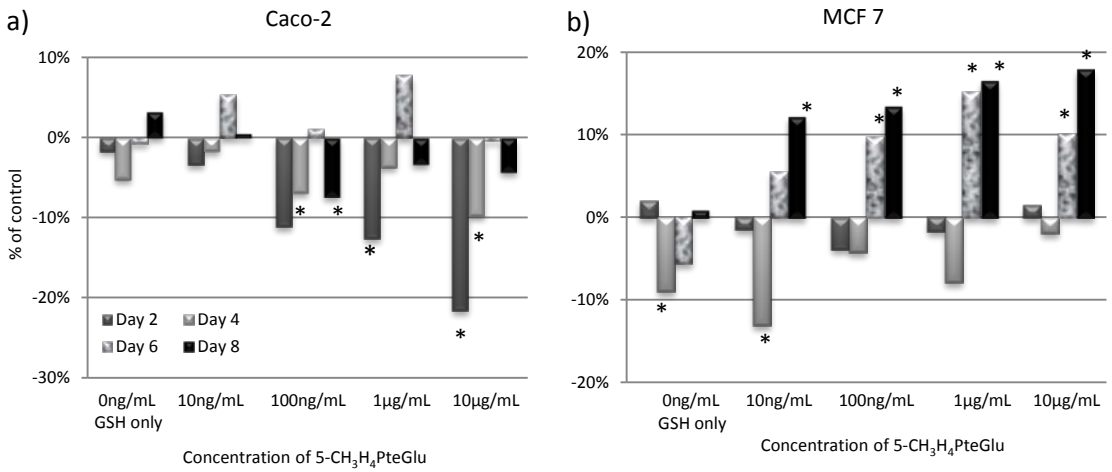



Figure 3.6 The effect of 5-$CH_3H_4$PteGlu in combination with additional GSH (10µg/mL) on cell line growth (Asterisks denote significant growth difference when compared to the control, $p$<0.05)

MCF 7 cell growth in response to 5-$CH_3H_4$PteGlu in the presence of GSH differed from Caco-2 cell growth (see Figure 3.6-b), and from the cell growth of MCF 7 in response to 5-$CH_3H_4$PteGlu with no antioxidant. In the presence of no antioxidant, 5-$CH_3H_4$PteGlu enhanced MCF 7 cell growth at all concentrations throughout the whole experimental period (see Figure 3.5-b). However, 5-$CH_3H_4$PteGlu, in the presence of GSH, inhibited cell growth on days 2 and 4 (especially significant at 10ng/mL on day 4, $p$<0.0001). This decreased cell growth was not maintained on day 6, and the growth of MCF 7 cells increased in a dose-dependent pattern by day 8.

### 3.3.1.3.3 The effect of 5-$CH_3H_4$PteGlu in the presence of ascorbic acid

5-$CH_3H_4$PteGlu, in the presence of ascorbic acid, presented a different growth pattern in Caco-2 cells, when compared to their response to 5-$CH_3H_4$PteGlu with/without GSH (see Figures 3.5-a and 3.6-a). The highest concentration of 5-$CH_3H_4$PteGlu significantly decreased cell growth by 18% ($p$= 0.004) on day 2. However, this inhibitory effect was reversed by day 4. 5-$CH_3H_4$PteGlu with no additional antioxidant, or in the presence of 10µg/mL GSH, generally induced decreased cell growth, while from Figure 3.7-a, it can be seen that 5-$CH_3H_4$PteGlu, in the presence of ascorbic acid, increased Caco-2 cell growth from day 4, peaking at 21% for 1µg/mL ($p$=0.0002).

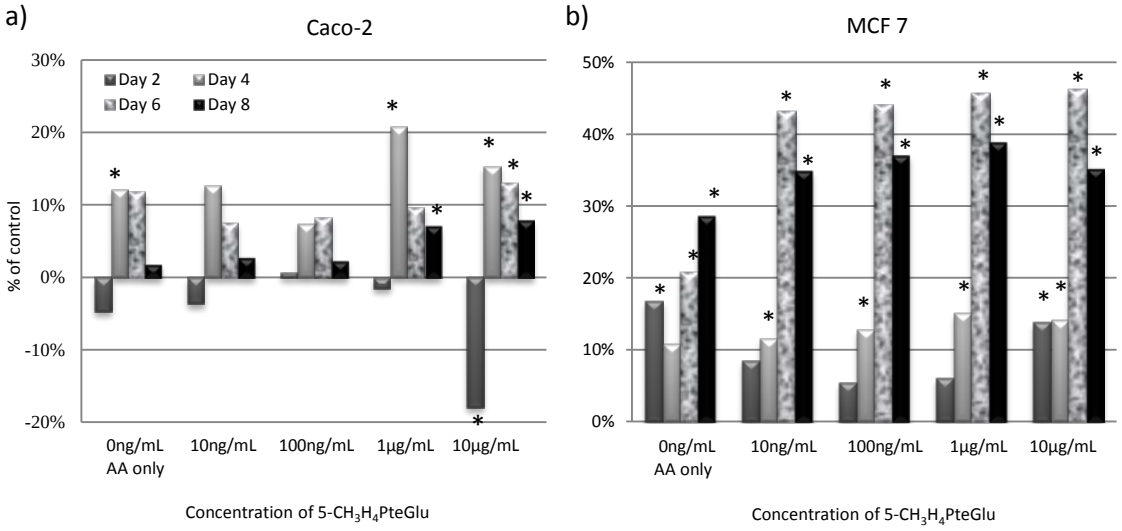



Figure 3.7 The effect of 5-$CH_3H_4$PteGlu in the presence of ascorbic acid (10µg/mL) on cell line growth (AA- ascorbic acid, asterisks denote significant growth difference when compared to the control, $p$<0.05)

The combination of 5-$CH_3H_4$PteGlu with ascorbic acid in the growth media exhibited the most enhanced MCF 7 cell growth (see Figure 3.7-b). MCF 7 cell growth was increased in a dose-dependent pattern, peaking on day 6. At 10µg/mL, cell growth was increased by 46% ($p$<0.0001). However, no growth difference between each concentration was observed. MCF 7 cells showed a differential growth trend when compared to Caco-2 cells. Caco-2 cell growth was generally decreased on day 2, but MCF 7 cells showed a significant increase throughout the whole experimental period (days 4-8).

### 3.3.1.4 Cell growth response to 5-$CH_3H_2$PteGlu with no antioxidant

The partially oxidised folic acid vitamer 5-$CH_3H_2$PteGlu is an intermediate degradation product of 5-$CH_3H_4$PteGlu, and is relatively stable when compared to 5-$CH_3H_4$PteGlu at neutral pH. Not many studies have been carried out to investigate the physicochemical characteristics of this molecule and the effect that it might have on cell proliferation. In this study it was decided to examine the effects of 5-$CH_3H_2$PteGlu on cell proliferation in the two cancer cell lines, Caco-2 and MCF 7.

The addition of different levels of 5-$CH_3H_2$PteGlu resulted in a general increased growth in Caco-2 cells (see Figure 3.8-a). On day 2, Caco-2 cell growth was significantly enhanced by 30% at a concentration of 1µg/mL ($p$=0.043). Interestingly, 1µg/mL induced a better increase in cell growth than 10µg/mL. By day 8 there was no difference in cell growth compared to the control.

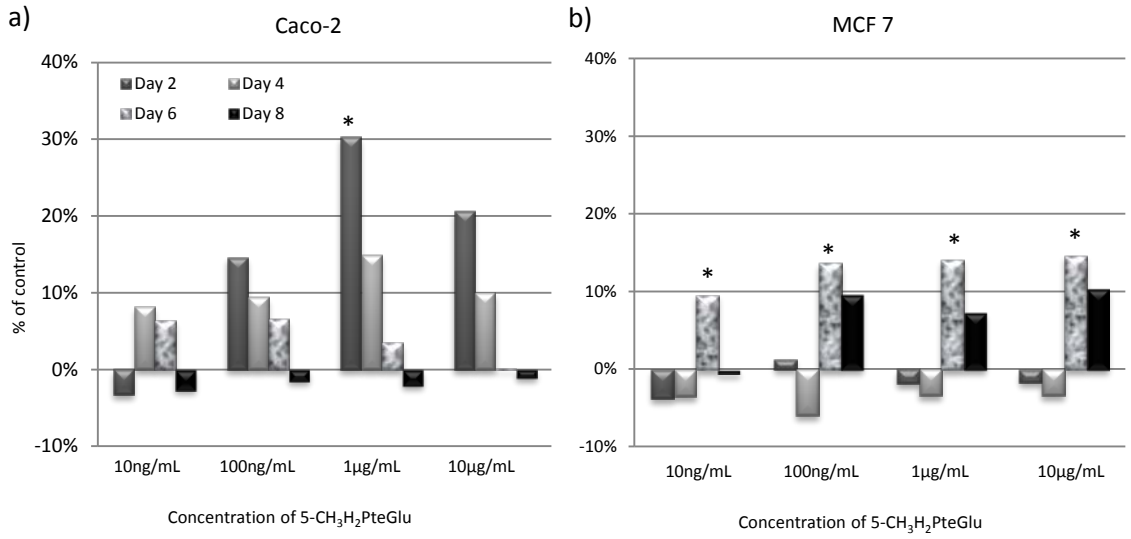



Figure 3.8 The effect of 5-$CH_3H_2$PteGlu on cell line growth (Asterisks denote significant growth difference compared to the control, $p$<0.05)

The presence of 5-$CH_3H_2$PteGlu induced a differential growth trend in MCF 7 cells when compared to Caco-2 cells (see Figure 3.8-b). Caco-2 cell growth was enhanced by 5-$CH_3H_2$PteGlu on day 2, then decreased with time. By contrast, MCF 7 cells exhibited decreased cell growth when compared to the control at days 2 and 4. This decline in cell growth was reversed by day 6 when cell growth was significantly increased by approximately 14% for all concentrations ($p$<0.001). There was no significant differential effect between 5-$CH_3H_2$PteGlu concentrations.

### 3.3.1.5 Cell growth response to 5-CHOH$_4$PteGlu

Figure 3.9-a shows that 5-CHOH$_4$PteGlu induced strikingly similar growth patterns to the effect of 5-CH$_3$H$_2$PteGlu on Caco-2 cells. On day 2 all concentrations of 5-CHOH$_4$PteGlu yielded enhanced cell growth, peaking with an increase of 20% at 1µg/mL ($p$=0.004). The 100ng/mL and 1µg/mL concentrations showed better cell growth than the 10µg/mL concentration, but this was not statistically significant. The enhanced cell growth decreased with time, and reversed on day 6 at most concentrations.

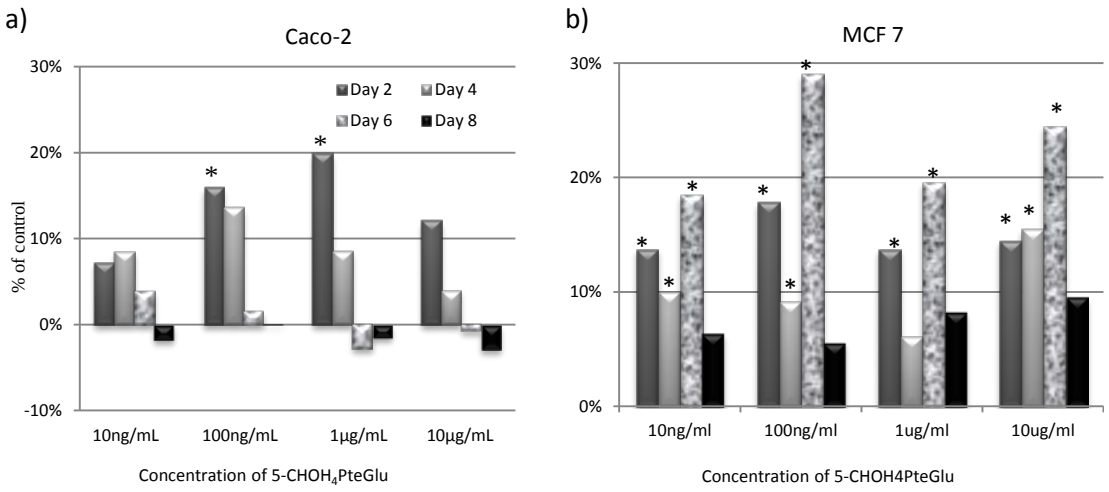



Figure 3.9 The effect of 5-CHOH$_4$PteGlu on cell line growth (Asterisks denote significant growth difference when compared to the control, $p$<0.05)

Although no clear dose-related response was observed, 5-CHOH$_4$PteGlu enhanced MCF 7 cell growth throughout the entire experimental period and at all concentrations (see Figure 3.9-b). Cell growth was significantly enhanced on days 2, 4 and 6, but it was not significantly maintained on day 8. Caco-2 cells showed maximum enhanced growth on day 2, whilst the MCF 7 cell growth increases peaked on day 6.

### 3.3.1.6 Cell growth response to p-ABG

p-ABG is a final excretory product of folate metabolism. The catabolism of folate yields one or more pteridine moieties and p-ABG, which does not have any biological effects in the cell (see the description of the chemical structure of PteGlu in section 1.2)[543, 544]. To confirm these findings, and to obtain further reference data in explaining the effects of folate vitamers and their degradation products on cell proliferation, Caco-2 and MCF 7 cells were cultured in media with differing concentrations of p-ABG.

Although contrasting cell growth change was observed between days 2-4 and 6-8 in the Caco-2 cells, there were no statistically significant results (see Figure 3.5-a). Figure 3.10-b shows that the presence of p-ABG in the growth media did not affect the proliferation of MCF 7 cells. Only minor cell growth changes (<5%) were observed, none of which had statistical significance ($p$>0.05). This suggests that p-ABG does not influence cell growth in MCF 7, as previously confirmed in Caco-2 cells. Based on this result, any effect of p-ABG on cell proliferation was ruled out when analysing the influence of folate vitamers and their derivatives on the growth of either cell line.

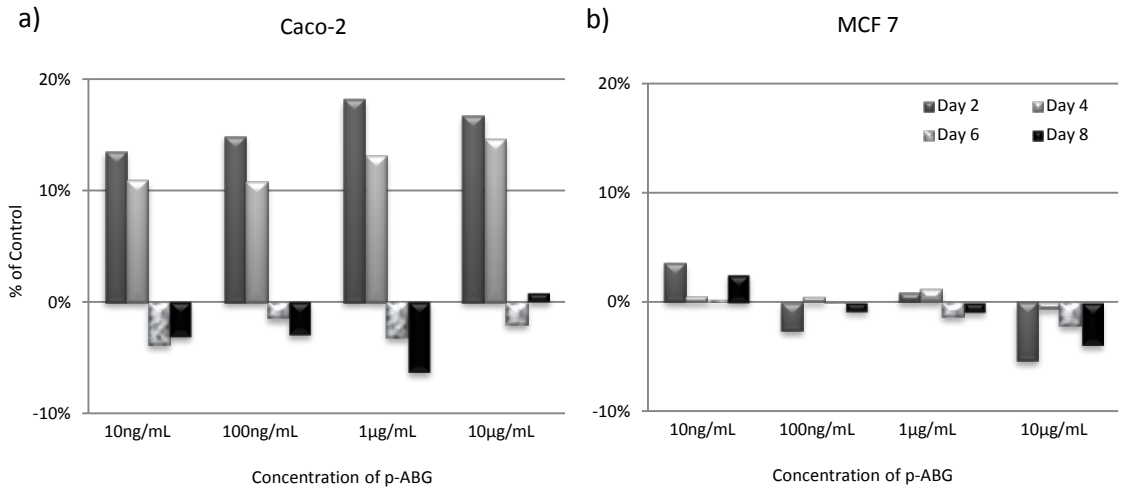



Figure 3.10 The effect of p-ABG on cell line growth

### 3.3.1.7 The effects of UV-catalysed degradation products of folate vitamers on cell growth

When folate vitamers are exposed to UV their chemical structure decomposes and they lose specific biochemical characteristics. Moreover, the degradation products may have unexpected roles in cell metabolism. An experiment was therefore designed to explore the effect of the UV-catalysed degradation of PteGlu on the formation of 6-FP, PCA and p-ABG, as well as the effect of the degradation of 5-$CH_3H_2$PteGlu on cancer cell growth.

#### 3.3.1.7.1 6-formylpterin

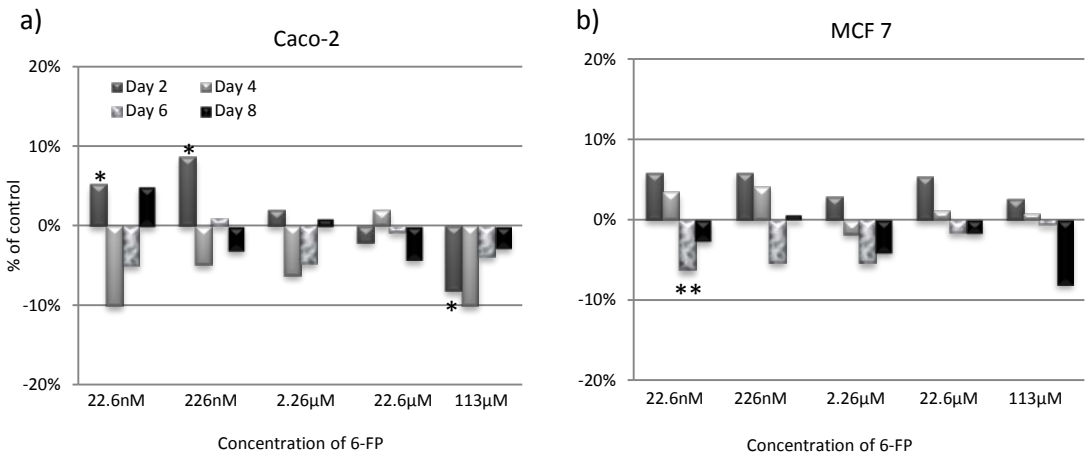



Figure 3.11 The effect of 6-FP on cell line growth (*denotes significant growth difference between concentrations,** stands for significant growth difference when compared to the control, $p<0.05$)

Significant changes in Caco-2 cell growth, with 6-FP present in the culture media, were not exhibited when compared to control samples (see Figure 3.11-a). Significant cell growth differences were only observed between 6-FP concentrations on day 2: 113µM of 6-FP versus 22.6nM and 226nM ($p$ = 0.041 and 0.009, respectively). The addition of 6-FP to the culture media induced a differential growth response in MCF 7 cells, depending on the time and concentration (see Figure 3.11-b), but this, again, was of little significance. At all concentrations, apart from 2.26µM, 6-FP increased cell growth on days 2 and 4. Enhanced cell growth was reversed on day 6; cell growth was significantly decreased by 6% at 22.6nM ($p$ = 0.038). However, this was not maintained on day 8.

#### 3.3.1.7.2 Pterin-6-carboxylic acid

PCA did not induce any statistically significant difference in Caco-2 cell growth, when compared to controls (see Figure 3.12-a). However, on days 2 and 4, PCA increased cell growth at all concentrations. This enhanced growth was decreased on day 6 and was reversed on day 8 in all cases. Similarly, PCA did not have a significant influence on MCF 7 growth (see Figure 3.12-b). There was no particular trend with time or concentration. Only 22.6µM resulted in strikingly enhanced cell growth, compared to 113µM on day 6 ($p$=0.024).

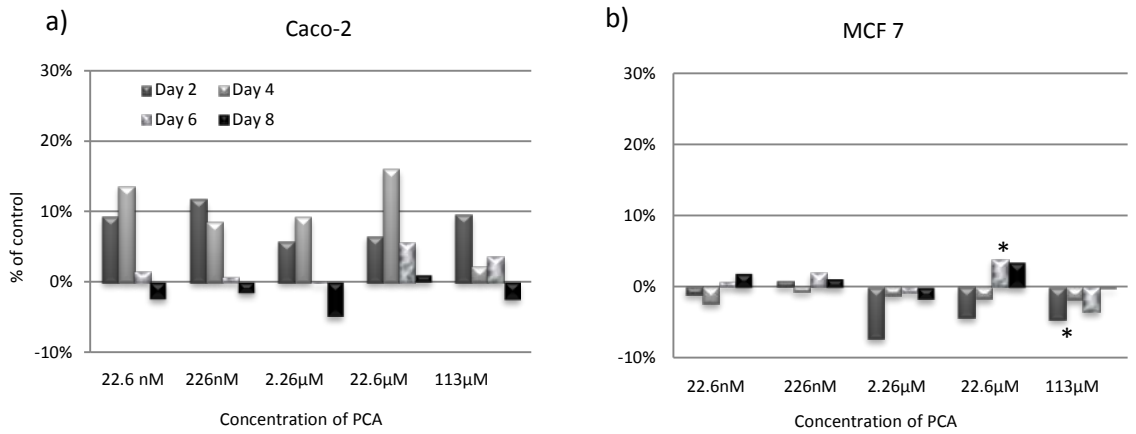



Figure 3.12 The effect of PCA on cell growth (Asterisks denote significant growth difference between concentrations, $p<0.05$)

### 3.3.1.7.3 p-ABG

Oxidation of folyl vitamers generates degradation products at equimolar amounts to its original form. Therefore, to adjust the range of concentrations in line with the other derivatives of the oxidative degradation products of PteGlu and 5-$CH_3H_2$PteGlu, Caco-2 cells and MCF 7 cells were cultured once again in media containing different concentrations of p-ABG.

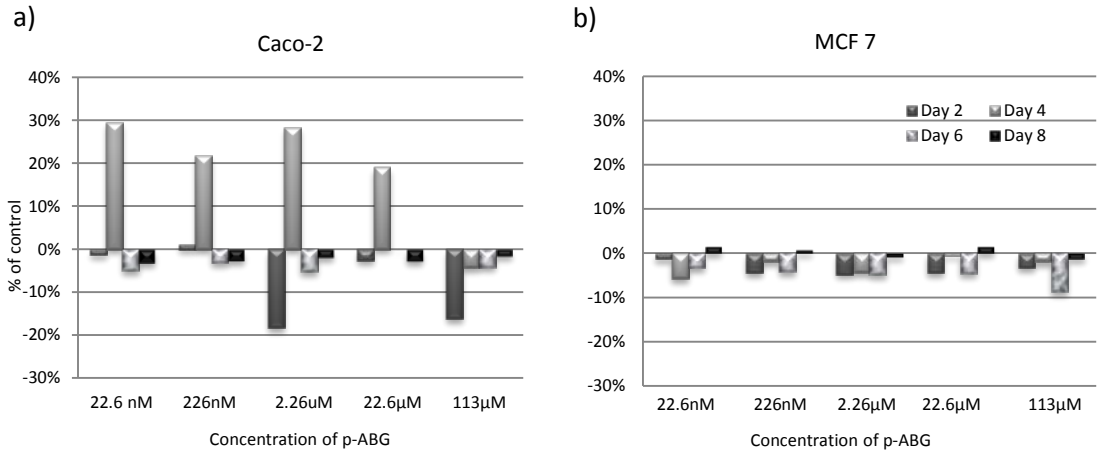



Figure 3.13 The effect of p-ABG on cell growth

Five different concentrations of p-ABG, 22.6nM, 226nM, 2.26µM, 22.6µM and 113µM, were used for this experiment. As described above, p-ABG did not yield any significant growth difference in Caco-2 cells, when compared to controls (see Figures 3.10-a and 3.13-a). On day 2

or day 4 approximately 30% of cell growth difference was observed at most concentrations, but these observations were not statistically significant. As previously observed, p-ABG did not have any effect on MCF 7 cell growth. At all concentrations p-ABG only induced a minor difference in cell growth which was not statistically significant.

### 3.3.1.8 **UV-catalysed degradation products of 5-$CH_3H_2$PteGlu**

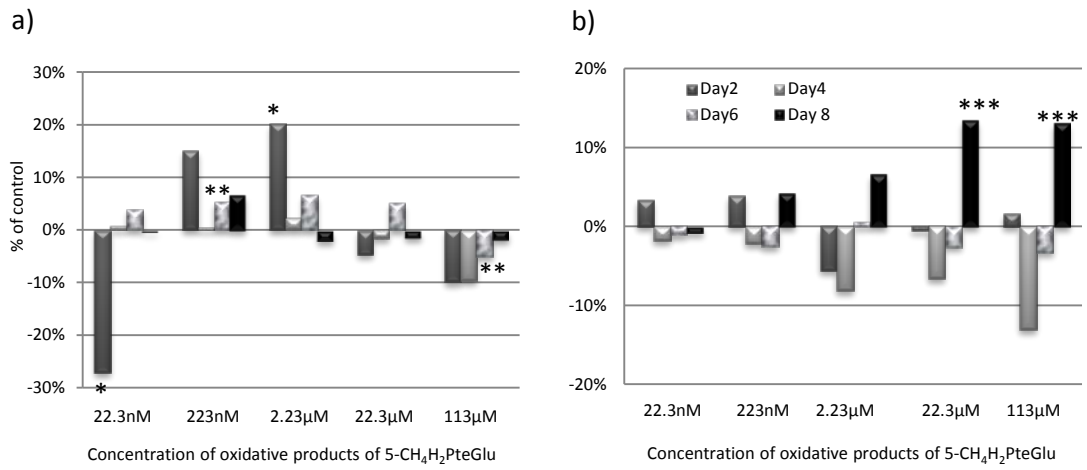



Figure 3.14 The effect of UV-catalysed degradation products of 5-$CH_3H_2$PteGlu on cell growth (*,** and *** denote significant growth difference between two concentrations, $p$<0.05)

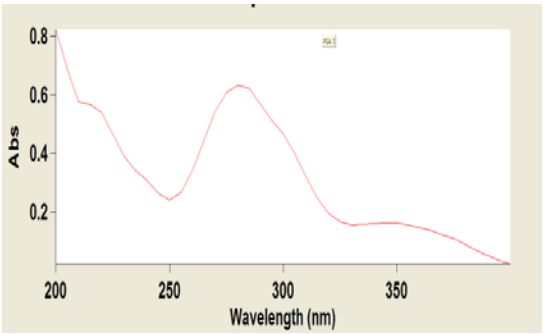
The native form of 5-$CH_3H_2$PteGlu showed an enhanced growth trend which peaked on day 2 and decreased with time. By contrast, the oxidative degradation products of 5-$CH_3H_2$PteGlu did not affect Caco-2 cell growth significantly when compared to the water control (see Figure 3.14-a). Significant cell growth differences were only observed between concentrations: 22.3nM versus 2.23µM, and 223nM versus 113µM. However, no clear trends for this effect were observed in cell growth.

The oxidative degradation products of 5-$CH_3H_2$PteGlu did not result in a statistically significant change in MCF 7 cell growth, except at high concentrations (22.3 and 113 µM) on day 8 ($p$ = 0.001 and $p$ = 0.002, respectively, Figure 3.14-b). Cells did not show any statistically significant growth response on days 2, 4 and 6, although the cell growth response was decreased with concentration.

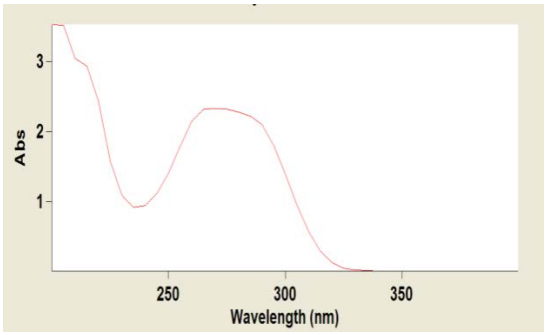
### 3.3.2 Identification of UV-catalysed degradation products of PteGlu and 5-$CH_3H_2$PteGlu

As described above, the UV exposure of PteGlu generates the C9–N10 scission products p-ABG, 6-FP and PCA. However, little is known about the unknown pterin moiety that arises from the UV-degradation of 5-$CH_3H_2$PteGlu. Results from these cell culture experiments suggest that the native form of the vitamin and the UV oxidative degradation products of 5-$CH_3H_2$PteGlu yield clearly differential growth trends. To confirm the presence of the oxidative degradation products of 5-$CH_3H_2$PteGlu, the absorption spectra of p-ABG and 5-$CH_3H_2$PteGlu were scanned within the range of 200-400nm. Scanning was also performed for synthetic PteGlu and the UV-catalysed degradation product PCA. As it has extreme photo-instability, 6-FP was not examined.

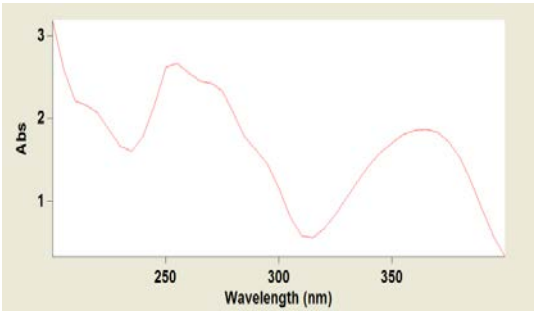
#### 3.3.2.1 UV-spectra of PteGlu and its UV-oxidative degradation products



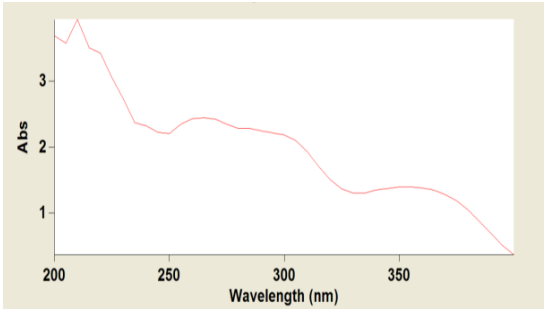

a) PteGlu




b) p-ABG




c) PCA



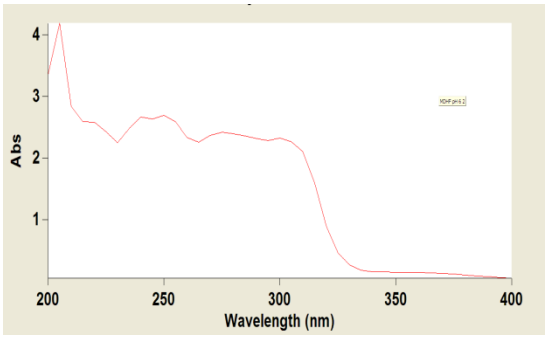

d) Oxidative products of PteGlu to 1hr UV exposure

Figure 3.15 UV-spectra of PteGlu and its oxidative derivatives

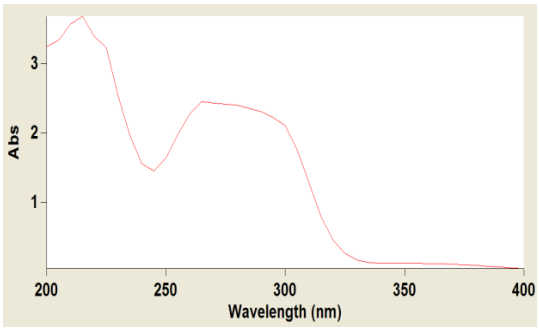
Figure 3.15 provides typical spectral data for PteGlu and p-ABG. PteGlu gave a unique absorption graph with a λmax around 275nm (see Figure 3.15-a), whilst p-ABG showed a broad absorption plateau extending from 250nm to 300nm (see Figure 3.15-b). PCA exhibited a

unique absorption peak between 320nm and 400nm (see Figure 3.15-c). The absorption spectrum obtained from UV-oxidized PteGlu showed a pattern reflecting the presence of a mixture of all of these oxidative derivatives of PteGlu (see Figure 3.15-d).

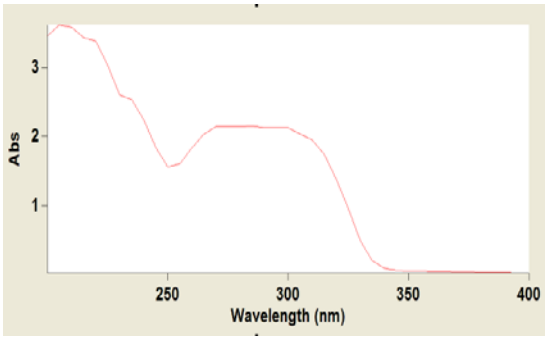
### 3.3.2.2 UV-Spectra of 5-$CH_3H_2$PteGlu and its UV-oxidative degradation products



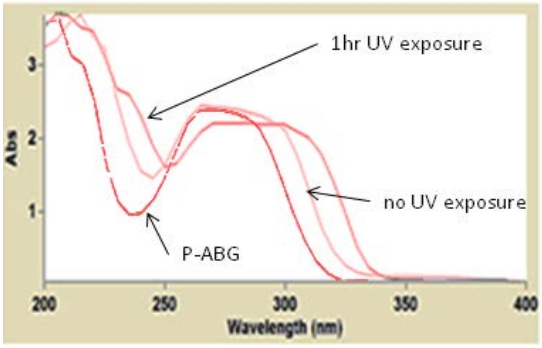

a) 5-$CH_3H_2$PteGlu at pH 6




b) 5-$CH_3H_2$PteGlu at pH 3




c) Effects of 1 hour UV exposure on 5-$CH_3H_2$PteGlu at pH 3




d) Overlaid spectra showing effects of i) no UV exposure, ii) 1 hr UV exposure on 5-$CH_3H_2$PteGlu at pH 3, and iii) p-ABG

Figure 3.16 UV-spectra of 5-$CH_3H_2$PteGlu and its oxidative derivatives

5-$CH_3H_2$PteGlu is fairly stable at neutral pH. Therefore, to facilitate degradation by UV radiation, the 5-$CH_3H_2$PteGlu standard was made at pH 3. Figure 3.16 (a-c) presents UV-spectrophotometric information relating to authentic 5-$CH_3H_2$PteGlu standard (pH 6), oxidised 5-$CH_3H_2$PteGlu (pH 3) and 5-$CH_3H_2$PteGlu (pH 3) following one hour of UV exposure. The unique specific peak of 5-$CH_3H_2$PteGlu at 250nm in neutral pH disappears at pH 3 (see Figure 3.16-a, b and c), which suggests the complete degradation of the vitamer by exposure to low pH and UV treatment.

5-$CH_3H_2$PteGlu without UV exposure (pH 3, Figure 3.16-b) exhibited a similar spectrum to that of p-ABG (see Figure 3.15-b). However, when their spectra were overlaid, a difference between the two compounds was observed (see Figure 3.16-d); notably the slope between 225-250nm. This difference may result from the presence of the pterin moiety within the degradation products milieu following treatment of 5-$CH_3H_2$PteGlu (pH 3). In particular, when comparing the two spectra from oxidised 5-$CH_3H_2$PteGlu at pH 3 (with and without UV exposure), the oxidised degradation product following UV exposure presents a major point of difference at around 250nm and generates a wider plateau extending from 275-320nm. This difference suggests that the unknown pterin moiety in the oxidised degradation product milieu of 5-$CH_3H_2$PteGlu may be denatured into another compound by UV irradiation. Unfortunately it was not possible to identify this benign pterin moiety.

### 3.3.3 Genotype determination of common folate-related genetic variants in Caco-2 and MCF 7 cell lines

Table 3.4 Major genetic variations in folate metabolism genes in the two cancer cell lines, Caco-2 and MCF 7

| Description | Genetic variation | Caco-2 | MCF 7 |
|---|---|---|---|
| Genetic variations in MTHFR | **MTHFR C677T** | **CC** | **TT** |
| | **MTHFR A1298C** | **CC** | **AA** |
| | **MTHFR G1793A** | **GG** | **AG** |
| Genetic variations in folate absorption metabolism | **RFC G80A** | **GA** | **AA** |
| | GCP II C1561T | CC | CC |
| Genetic variations in TS | **TSER 2R3R** | **2R/2R** | **2R/3R** |
| | TS 1496del6 | 6bp/6bp | 6bp/6bp |
| | **TSER 3RG>C** | **.** | **3C/3C** |
| Genetic variations in transsulphuration pathway | CBS 844ins68 | NN | NN |
| | CTH G1364T | GG | GG |
| | CTH IVS 10-430 C>T | TT | TT |
| Genetic variations in serine and $H_4$PteGlu metabolism | **DHFR 19del** | **22** | **12** |
| | **SHMT C1420T** | **CC** | **CT** |
| Genetic variations in methionine and Hcy metabolism | BHMT G595A | GG | GG |
| Genetic variations in cobalamin related MS/MSR metabolism | **MS A2756G** | **AG** | **AA** |
| | MSR A66G | AG | AG |

# SNPs in bold show a different genotype between the two cell lines

Table 3.4 presents the genotypes of sixteen common variants of folate metabolism genes in the two cell lines, Caco-2 and MCF 7. The two cell lines possess very different genetic

variations in folate metabolism. Three SNPs in MTHFR, one of the significant genes in folate metabolism, were found to differ completely between the two cell lines. In addition, differences in the following genetic variations were identified between the Caco-2 and MCF 7 cell lines: RFC G80A, TSER 2R3R, TSER 3RG>C, DHFR 19del, SHMT C1420T and MS A2756G. This may validate the findings of differential growth responses for the two cell lines to the various folate vitamers and their oxidative degradation products.

## 3.4 Discussion

Folic acid has been one of the best studied nutrients in relation to human health over the last couple of decades. Emerging evidence suggests that appropriate folate intake and status are key factors in disease aetiology. However, the latest study suggests that not only folate nutritional intake, but also the form of folate taken, might be risk factors in disease development and progression.

Individual folate vitamers may have differential roles in cell metabolism and disease aetiology together with genetic and physicochemical factors, although any specific influence on cell metabolism is not yet clear. In order to better understand the impacts of the various vitamers on cell proliferation, and to examine their implications, colon and breast cancer cell lines were grown in media containing different folate vitamers and the corresponding cell growth properties were examined.

**-The effects of PteGlu on cell proliferation**

Caco-2 cells were cultured in media containing variable levels of PteGlu. At relatively low concentrations (10ng/mL, 100µg/mL and 1µg/mL), there was no significant difference in cell growth (see Figure 3.2-a). This result may be due to high baseline levels of PteGlu (1µg/mL) in the RPMI 1640 used to culture the cells in the initial stage. Even though Caco-2 cells went through an adaptation period to FFM for 3 weeks prior to the main experiment, high levels of baseline PteGlu within the culture medium may have resulted in a 'threshold effect' for the Caco-2 cells in relation to PteGlu metabolism. This might explain why no significant change in cell growth was observed with a comparatively low folic acid level.

Interestingly, Caco-2 cells showed different growth patterns at higher concentrations (50µg/mL and 500µg/mL) of PteGlu. Caco-2 cell growth was enhanced, peaking on day 4 at 50µg/mL, although this was not statistically significant. At a PteGlu concentration of 500µg/mL, Caco-2 growth was suppressed significantly on day 2 ($p$<0.005), but this suppression effect was no longer apparent on day 6. It may be hypothesized that the effect of high PteGlu concentrations is related to PteGlu metabolism which differs from the metabolism of natural folate coenzymes (see Figure 1.17, section 1.6.3).

PteGlu is a synthetic form of folate which does not exist naturally and needs an additional step to enter into human folate metabolism. This additional step is mediated by DHFR. The main role of DHFR is to catalyse the reduction of $H_2$PteGlu to $H_4$PteGlu. It is also responsible for the

conversion of PteGlu to $H_2$PteGlu, but with a very high *K*m (see Figure 3.17). In addition, $H_2$PteGlu allosterically modulates the activity of MTHFR [132]. PteGlu from fortified foods and supplements is first turned into $H_2$PteGlu and then metabolized into $H_4$PteGlu by DHFR.

Considering the above-mentioned characteristics of $H_2$PteGlu and DHFR, the increased growth of Caco-2 cells at 50µg/mL PteGlu could possibly be explained as follows: $H_2$PteGlu inhibits MTHFR activity and, hence, spare 5,10-$CH_2H_4$PteGlu is shunted towards purine and pyrimidine biosynthesis instead of the Hcy methylation process. In this way PteGlu promotes cell proliferation, and so Caco-2 cells in 50µg/mL PteGlu may show dominant growth when compared to the control cells (see Figure 3.3-a) [132].

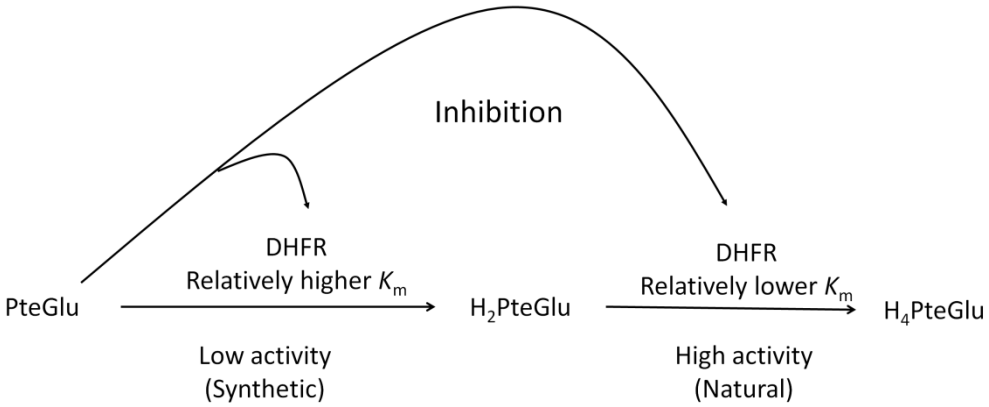



Figure 3.17 Diagram showing the interaction barrier for PteGlu and dihydrofolate reductase (DHFR: dihydrofolate reductase)

The high *K*m of PteGlu for DHFR has also led to the idea that PteGlu may be an antimetabolite for DHFR, conferring competitive substrate inhibition on its activity towards $H_2$PteGlu concentration (see Figure 3.3-a) [131]. In the context of this substrate inhibition, 500µg/mL may be a critical concentration that could result in a decrease of DHFR activity, thereby reducing $H_2$PteGlu product formation and leading to the presence of less $H_4$PteGlu to act as a thymidilate precursor for DNA synthesis. This mechanism would lead to a decrease in cell growth [133].

MCF 7 breast cancer cells showed a different response pattern from Caco-2 cells. MCF 7 cells exhibited only a minor growth difference on days 2 and 4, yet all concentrations showed significantly increased growth when compared to the control on days 6 and 8 (see Figure 3.3-b, $p<0.0001$). It may be speculated that the enzyme activity of MCF 7 is different from that of Caco-2 and, as a result, MCF 7 responded to PteGlu with a different pattern of growth.

Such a discrepancy for DHFR activity has been previously reported. DHFR activity varies between species, with the activity of DHFR in the rat being higher than in humans [131]. Furthermore, approximately 5-fold differences in DHFR activity between individuals have been reported. DHFR also has various inhibition levels depending on the type of antifolate present [545]. This may support the observed variation of DHFR activity for each cell line. Additionally, the ratio of $H_2$PteGlu/PteGlu and the presence of the 19bp del and other polymorphisms of DHFR could possibly affect enzyme activity [125, 127, 546].

The Caco-2 and MCF 7 cell lines used for this study were confirmed to have different DHFR 19del genotypes (22 and 12 for Caco-2 and MCF 7, respectively; see Table 3.4). It has already been reported that the DHFR 19bp del homozygous recessive genotype exhibits altered DHFR mRNA expression compared to the heterozygous genotype [125, 547]. In terms of DHFR activity, these findings could explain the differential cell growth response found between the two cell lines, in relation to the catalytic effect of PteGlu on DHFR in the context of both tissues and genotype. These findings also raise interesting questions in relation to the potential adverse effects of folic acid fortification as a blanket population intervention as they demonstrate the existence of diverse efficacy for DHFR, according to the presence of folate related genetic variants and their potential effects on undiagnosed diseases (this will be discussed later).

**- The effects of 5-$CH_3H_4$PteGlu and antioxidants, and their combined influence on cell proliferation**

5-$CH_3H_4$PteGlu, the most abundant natural form of folate, has been shown to have a chemo-preventive effect on cancer development and progression in *in vitro* models. However, experimental observations suggest that 5-$CH_3H_4$PteGlu has extreme lability in aqueous solutions, depending on the nature of the physical and chemical milieu. Although media for tissue culture contain antioxidants such as GSH, we do not know much about whether the GSH level in culture media is appropriate to arrest the degradation of 5-$CH_3H_4$PteGlu or whether it may generate any additional effects. Therefore, the antioxidants that stabilise the labile form of this folate vitamer need to be considered.

In order to answer these questions, cell line models were used to investigate whether the effect of 5-$CH_3H_4$PteGlu on cell growth is altered by the presence of various antioxidants and, further, whether any differential antioxidant-related interactive effects exist. To accomplish this aim, three different types of antioxidant, DTT, GSH and ascorbic acid, were selected. It is

known that antioxidants have a cytocidal effect on cancer cells [541, 542]. Therefore, as a preliminary experiment, Caco-2 cells were cultured with various concentrations of antioxidant. The results of this experiment were used to set the level of GSH and ascorbic acid in preventing oxidative degradation of the 5-$CH_3H_4$PteGlu standard at 10µg/mL in both cases.

5-$CH_3H_4$PteGlu promoted very interesting growth trends in Caco-2 cells, both in the absence and presence of added antioxidants. 5-$CH_3H_4$PteGlu, with no added antioxidant, affected cell growth in a dose-dependent manner. This verifies previously published reports that suggest that 5-$CH_3H_4$PteGlu may be the active compound in the growth-inhibitory actions of Caco-2 cells [548-550]. Excess 5-$CH_3H_4$PteGlu constrains the CBS gene and SAM synthesis, a mechanism which may be the metabolic turning point of the anti-proliferative effects of folate in colon cancer cells [549]. In addition, a high dose of 5-$CH_3H_4$PteGlu acts as a powerful antioxidant, and may have an influence on the expression of the cytokines which play many important roles in cell metabolism such as tumour suppression and the inhibition of cellular proliferation [551]. However, in terms of the lability of 5-$CH_3H_4$PteGlu, the vitamer may not be the only active compound with effects on cancer cell growth.

Although the stability of 5-$CH_3H_4$PteGlu is enhanced by the presence of cells [9] and GSH in FFM, 5-$CH_3H_4$PteGlu readily oxidises to 5-$CH_3H_2$PteGlu at neutral pH in the absence of an antioxidant (cell culture medium has pH 6.5-7.4)[8]. Additionally, 5-$CH_3H_2$PteGlu further degrades to p-ABG and an unknown pterin moiety. 5-$CH_3H_2$PteGlu is unlikely to re-enter the folate pool in *in vivo* models [552]. However, data from the current study showed that the presence of 5-$CH_3H_2$PteGlu altered cell growth when compared to controls (see Figure 3.8-a). As p-ABG was found to have no significant effects on cell growth (see Figure 3.10-a), this decreased Caco-2 cell growth may have resulted from the presence of a combination of 5-$CH_3H_4$PteGlu, 5-$CH_3H_2$PteGlu and the unknown pterin moiety which arises from the degradation of 5-$CH_3H_2$PteGlu. The cell growth response to 5-$CH_3H_4$PteGlu in conjunction with 10µg/mL of GSH exhibited an interesting trend, and likely provides evidence supporting this hypothesis. 5-$CH_3H_4$PteGlu in the presence of 10µg/mL of GSH resulted in a similar, but improved, inhibition of Caco-2 cell growth to that of 5-$CH_3H_4$PteGlu in the absence of any additional antioxidant (see Figure 3.5-a).

It seems that the preservation of 5-$CH_3H_4$PteGlu by 10µg/mL GSH may result in an enhanced inhibition effect on Caco-2 cells. This is probably not a simple effect arising from the presence of GSH on its own: Caco-2 cells cultured in FFM containing only GSH (no 5-$CH_3H_4$PteGlu) showed a non-significant growth difference when compared to the control sample. Moreover,

5-$CH_3H_4$PteGlu in the presence of 10µg/mL GSH induced a dose-respondent decline in cell growth. This possibly suggests that the presence of additional antioxidant (GSH) in cell culture media may improve the stability of this labile folyl vitamer. Therefore, the addition of an antioxidant should be considered when investigating the efficacy of 5-$CH_3H_4$PteGlu in an *in vitro* model.

Although DTT was not examined for its effect on $CH_3H_4$PteGlu, it gave rise to some interesting results on its own. Increasing DTT concentration affected cell growth significantly, but this lethal effect was decreased at a high concentration (200ng/mL; see Figure 3.4-a). This is most likely because DTT has biphasic toxicity: it destroys cells within a certain range (0.2 to 1.0 mM thiol), but this effect is not seen at higher or lower DTT concentrations [542]. The concentration of DTT inducing cell toxicity in the current study was different from the one used in an earlier report [542]. However, the required concentration may vary depending upon the type of cell line. DTT is a potent reducing agent that has generally been used for cell culture experiments due to its stable 6-member ring structure. However, it has been reported that DTT may alter cell antigen expression [553, 554] and cause cell apoptosis [555]. Unlike DTT, which is a synthetic reducing agent, GSH is an antioxidant that exists in cells naturally. GSH has been reported to have negligible toxic effects at all concentrations [542]. In examining the present data with respect to GSH, the results for DTT and ascorbic acid suggest that, for *in vitro* models, applying the appropriate type and level of antioxidant are critical when examining the efficacy of antioxidants in preventing the degradation of labile compounds such as 5-$CH_3H_4$PteGlu.

A number of studies have been carried out which investigate the physico-chemical role of ascorbic acid with respect to folate. A physiological level of ascorbic acid such as is present in gastric juice prevents the degradation of 5-$CH_3H_4$PteGlu to 5-$CH_3H_2$PteGlu as well as the onward reaction to the C9–N10 scission product p-ABG. In addition, physiological levels of ascorbic acid promote the salvage of 5-$CH_3H_4$PteGlu from 5-$CH_3H_2$PteGlu [8, 9, 556]. However, the present data suggests that ascorbic acid plays a more complicated role with regard to 5-$CH_3H_4$PteGlu and cell proliferation.

The presence of ascorbic acid in cell culture media, in combination with 5-$CH_3H_4$PteGlu, may result in a synergistic effect on cell proliferation through nutrient-nutrient and, possibly, nutrient-gene interactions. 5-$CH_3H_4$PteGlu in the presence of ascorbic acid (10µg/mL) exhibited a contrasting change in Caco-2 cell growth when compared to the results concerning 5-$CH_3H_4$PteGlu in the absence and presence of added GSH (10µg/mL). In the presence of

ascorbic acid, Caco-2 cell growth declined when compared to the control cells on day 2, peaking at a concentration of 10µg/mL. However, this decreased growth was reversed on day 4 with 1µg/mL and 10µg/mL concentrations showing significantly enhanced cell growth when compared to the control from days 4 to 8 (see Figure 3.7-a). This experimental outcome showed that the presence of 10µg/mL of ascorbic acid may play an important role as an antioxidant in preserving 5-$CH_3H_4$PteGlu.

The combination of folate and ascorbic acid may also result in unexpected interactions affecting cell growth. The current research group previously reported that ascorbic acid (vitamin C), either on its own or as a consequence of its interaction with 5-$CH_3H_4$PteGlu, may play a critical role in maintaining RBC folate status [76]. Dietary vitamin C and 5-$CH_3H_4$PteGlu act in synergy to modify RBC folate status, and folate genetic variations such as TSER 2R3R and SHMT C1420T are also involved in this nutrient-nutrient interaction with respect to blood folate levels. In addition, vitamin C on its own may be important in maintaining RBC folate status, taking into account polymorphisms in TS, MTHFR and MSR; critical genes for DNA biosynthesis and methionine metabolism [76]. Also, as described in the AP study, vitamin C intake was associated with risk for AP when taking folate polymorphisms such as GCPII C1561T and RFC A80G into account (see section 2.4.3.3, Table 2.31).

It can therefore be speculated that there may be comparable interactive mechanisms driving interactions between folate and vitamin C in the current cell line models. With this in mind, the interaction between vitamin C and folate metabolism genes may lead to distinctive cell growth trends, setting it apart from the results for 5-$CH_3H_4$PteGlu in the absence or presence of added GSH.

Along with folate, vitamin C is one of the most commonly fortified nutrients in food. In the AP study it was observed that approximately 30% of subjects were taking a synthetic form of vitamin C. The individuals who had a higher folate intake also showed a higher vitamin C intake than subjects who had a lower folate intake. The results from this *in vitro* model may indicate a potential interaction between these two nutrients. Vitamin C, as an abundant antioxidant, likely interacts with other nutrients. Taking all of this into account, the role of ascorbic acid and its interaction with folate may need to be considered as a potentially critical factor in disease aetiology. Its underpinning molecular mechanism will therefore require further explanation.

**-The effects of 5-$CHOH_4$PteGlu on cell growth**

5-$CHOH_4$PteGlu, also known as leucovorin or folinic acid, is a very stable reduced folate vitamer which accounts for 3–10% of total intracellular folate in mammalian cells [557, 558]. Compared to other vitamers, the presence of 5-$CHOH_4$PteGlu in the medium displayed generally enhanced cell growth in both cell lines. It may be speculated that 5-$CHOH_4$PteGlu enhanced both Caco-2 and MCF 7 cell growth via increased DNA synthesis. 5-$CHOH_4$PteGlu is irreversibly catalysed via methenyltetrahydrofolate synthetase (MTHFS) to 5, 10-$CHH_4$PteGlu which is converted to 10-$CHOH_4$PteGlu and becomes the main one-carbon provider for purine synthesis. The formyl group of 10-$CHOH_4$PteGlu is incorporated into the C-2 and C-8 positions of the purine ring.

5-$CHOH_4$PteGlu is not a coenzyme for one-carbon transfer reactions, but may be a form of storage for excess formyl folate which may increase purine synthesis and cell growth, and is possibly a part of the regulation mechanism for the cytoplasmic folate-derived one-carbon pool [559]. Interestingly, Caco-2 cell growth was enhanced when grown in 5-$CHOH_4$PteGlu on days 2 and 4, peaking at 1µg/mL (see Figure 3.9-a), but this enhanced cell growth decreased from day 6. This decreased cell growth difference at higher concentrations and with time may be due to an enzyme inhibitory effect of 5-$CHOH_4$PteGlu and its metabolites. The MTHFS-catalyzed reaction exhibits product inhibition. The product of the MTHFS reaction, 5,10-$CHH_4$PteGlu, exists in chemical equilibrium with 10-$CHOH_4$PteGlu which tightly binds to MTHFS [559]. Therefore, 5-$CHOH_4$PteGlu may not be able to induce consistent cell growth in a dose dependant manner.

5-$CHOH_4$PteGlu is also known to be a slow tight binding inhibitor of SHMT (see Figure 3.18) [557]. SHMT catalyses the conversion of $H_4$PteGlu into 5,10-$CH_2H_4$PteGlu, concomitant with the change of glycine to serine. It also recycles 5,10-$CHH_4$PteGlu into 5-$CHOH_4$PteGlu with low activity. SHMT is involved in the generation of one-carbon units for purine, thymidine and methionine biosynthesis. High levels of 5-$CHOH_4$PteGlu bind to SHMT, and may reduce its activity, leading to less growth change when compared to that at lower concentrations. In addition to inhibiting SHMT, 5-$CHOH_4$PteGlu has also been observed to inhibit other enzymes in one-carbon metabolism, including sarcosine dehydrogenase, dimethylglycine dehydrogenase and DHFR [560].

In the current study, the MCF 7 cell line exhibited a contrasting growth response to 5-$CHOH_4$PteGlu when compared to the responses found in previous research. It has previously

been reported that increased cellular levels of 5-$CHOH_4$PteGlu decrease MCF 7 cell growth via a decline in purine biosynthesis by inhibiting the folate-requiring enzyme AICAR formyltransferase [561]. However, the current results showed that 5-$CHOH_4$PteGlu enhanced MCF 7 growth.

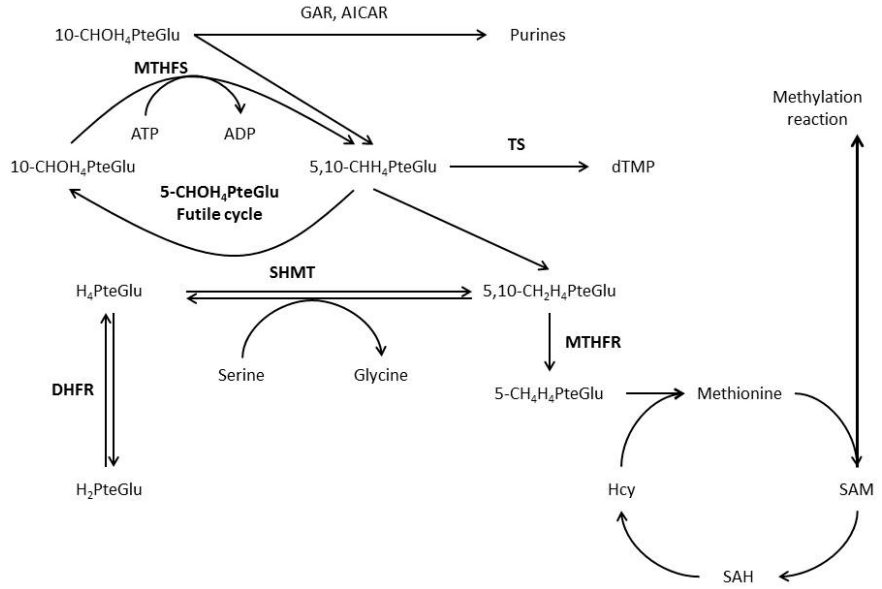



Figure 3.18 Simplified reaction diagram for the 5-$CHOH_4$PteGlu futile cycle that occurs in the cytoplasm (GAR: glycinamide ribonucleotide, AICAR: 5-Aminoimidazole-4-carboxamide riboside, MTHFS: methenyltetrahydrofolate synthetase, TS: thymidylate synthase, DHFR: dihydrofolate reductase, SHMT: Serine hydroxymethyltransferase, MTHFR: Methylenetetrahydrofolate reductase)

The characteristics of cell lines can be altered by various factors including media formation, passage numbers and seeding density [403]. In particular, unlike in previous studies examining the effect of folate vitamers, the MCF 7 cells in this study were cultured in media containing 5-$CHOH_4$PteGlu for a relatively long time (8 days). This may have influenced the adaptation of the cell culture with respect to cell replication, senescence and differentiation [528]. 5-$CHOH_4$PteGlu has been commonly used for over 30 years as an antidote for folic acid antagonists such as MTX and potentiates the effects of 5-FU in cancer chemotherapy. However, the present data showed that 5-$CHOH_4$PteGlu may increase cell growth. This finding should be considered in the mid- to long-term use of 5-$CHOH_4$PteGlu as this may lead to unexpected cancer cell proliferation and/or interaction with other chemotherapy remedies.

The differential responses of the MCF 7 and Caco-2 cell lines with respect to folate vitamers are very interesting. The addition of 5-$CH_3H_4$PteGlu to culture media in the absence of an added antioxidant generated completely contrasting growth trends in the two different cancerous cell lines. Additionally, in the presence of ascorbic acid, MCF 7 cells showed an

improved cell growth of up to approximately 50%, while Caco-2 cell growth was enhanced by a maximum of 25%. This effect was not limited to 5-$CH_3H_4$PteGlu, but was also exhibited by other folate vitamers (PteGlu, 5-$CH_3H_2$PteGlu and 5-$CHOH_4$PteGlu), thereby showing that the MCF 7 and Caco-2 cell lines respond differently to different folate vitamers. This differential cell proliferation response to folate vitamers may be due to a combination of genetic variants in folate metabolism and may therefore be relevant to disease aetiology. For instance, the impacts of the common genetic variations in MTHFR (C677T, A1298C and G1793A), the critical enzyme in metabolism of methyl groups, clearly differ between the Caco-2 and MCF 7 cell lines (see Table 3.4). MCF 7 and Caco-2 also possess different genotypes for the TS gene, the expression product used to generate dTMP as a precursor of DNA.

The specific molecular mechanism of carcinogenesis for each different cell line has not yet been clearly identified. However, numerous folate-related candidate carcinogenic mechanisms possibly exist. Increased folate level possibly prompts the progression of pre-existing neoplasm via purine and pyrimidine synthesis. However, it could also inhibit the development of cancer via repairing impaired DNA stability in the tumour suppression gene [134, 286].

In terms of folic acid fortification, the differential response of each cancer cell line may possibly imply that other adverse effects of folic acid fortification are possible: recall the earlier discussion of the DHFR genotype and enzyme activity. Although it was mainly intended to target women of child-bearing age, the implementation of mandatory folic acid fortification has increased the total dietary folate intake of the entire population as a blanket intervention. The level of mandatory fortification has been adjusted for each country, taking into account the national nutritional intake status of its population and disease risk. However, the roles of different folate vitamers and the diverse characteristics of the population such as the presence of undiagnosed diseases, prescribed medications, and genetic characteristics and their implications were not considered. Therefore, further research with respect to the safety and efficacy of mandatory folate fortification, taking these factors into account, is required.

**-The effects of 5-$CH_3H_2$PteGlu on cell growth**

A review of the literature reveals that the effects of 5-$CH_3H_2$PteGlu on cell proliferation have not previously been studied in an *in vitro* model. Only one *in vivo* study reporting the absorption of 5-$CH_3H_2$PteGlu in humans exists. This study suggests that 5-$CH_3H_2$PteGlu does not actually enter the folate metabolic pool in either normal subjects or anaemic patients, and may not be metabolised in the absence of ascorbic acid in the stomach [552].

In the current study, however, the Caco-2 and MCF 7 cell lines showed an interesting cell growth trend in media containing 5-$CH_3H_2$PteGlu. 5-$CH_3H_2$PteGlu increased Caco-2 cell growth, peaking at a concentration of 1µg/mL on day 2. It also showed a differential growth trend in MCF 7 cells, depending on time, which decreased cell growth on days 2 and 4, and enhanced cell growth on days 6 and 8. This growth change contradicts previous work which suggests that 5-$CH_3H_2$PteGlu does not have a biologically active role in human one-carbon metabolism [552].

The differential results between these two studies could be due to a difference in the experimental model used. Although *in vitro* cell models provide a number of advantages over *in vivo* models, they have limitations due to the availability of only a simplified metabolic system when compared to the whole organism as in human or animal studies (see Table 1.6, section 1.7.1). A larger nutrient pool and a more complex inter-connected metabolism in *in vivo* models may be the cause of the differential results often observed between *in vivo* and *in vitro* models. In addition, other compounds and antioxidants such as GSH which exist in cellular membranes and media may interact with 5-$CH_3H_2$PteGlu and, hence, influence its stability, with possible implications for cell growth.

Various folate vitamers including PteGlu, 5-$CH_3H_4$PteGlu and 5-$CHOH_4$PteGlu have been deployed as folate supplements. Results from the current study suggest that 5-$CH_3H_2$PteGlu can enter the one-carbon metabolic pool in an *in vitro* model. Therefore, in terms of physico-chemical stability, 5-$CH_3H_2$PteGlu may also possess the potential to act as a folate supplement. However, further studies utilising both *in vitro* and *in vivo* models are required to verify its precise uptake mechanism and potential role as one-carbon unit supplier.

**-The effects of photo-oxidative degradation products of PteGlu on cell growth**

Along with other vitamins such as vitamin E, vitamin A and riboflavin, folate is well-known to be a light-sensitive [395]. The degradation of folate by photo-oxidation has been found to have adverse effects on cell survival and reproduction [395], and may also contribute to cancer aetiology [11].

The oxidative degradation products of PteGlu, namely 6-FP, PCA and p-ABG, were thoroughly identified using their UV-spectra (see Figure 3.15). After one hour of UV exposure, oxidised PteGlu did not show a single precise spectrum. Instead, mixed spectra for 6-FP, PCA, p-ABG and the remaining native form of PteGlu were apparent.

Although 6-FP and PCA did not yield significant changes in cell growth in this study, it seems that they may have some influence on cell growth. 6-FP induced elevated cell growth in MCF 7 cells, but this was no longer maintained on day 6 and cell growth was decreased on day 8. PCA increased Caco-2 cell growth, but decreased MCF 7 cell growth (see Figure 3.12). 6-FP has been demonstrated to have many markedly different physiological and pharmacological activities, depending on time and concentrations present [538]. At high concentrations, and in the presence of NADPH or NADH, 6-FP converts molecular oxygen to ROS such as $H_2O_2$ which induce apoptosis in, for example, pancreatic cancer cells [430, 538]. On the other hand, only cancer cell lines have been found to secrete 6-FP into the growth media, and 6-FP has only been found in the urine of cancer patients [397].

Human serum albumin can slow down the oxidation process of folate [562]. However, it is rapidly oxidized in the presence of natural photo-sensitizers (flavins and porphyrins) after UVA and near-UV exposure [563]. PCA is known to induce DNA damage [423]. A high level of PCA in the epidermis may be associated with vitiligo via increased production of $H_2O_2$ [426].

Considering this experimental evidence, both 6-FP and PCA may be attractive candidates for further investigation as potential anti-cancer medicines. However, they also have potential roles as carcinogens. The photo-oxidation of folate is an emerging issue in the post-fortification era. Increased folate levels in the blood and the changed distribution of folate vitamers, including the presence of unmetabolized PteGlu, may accelerate the accumulation of PCA and 6-FP following UV exposure and the photolytic destruction of PteGlu. As described earlier, an increased incidence of CRC has been reported in some of the countries where mandatory folate fortification has been implemented. [391, 392]. The accumulation of increased photo-oxidative products of PteGlu as a result of mandatory folate fortification may contribute to this increase in CRC incidence. However, a clear mechanism for degradation following folate photolysis in cancer aetiology has not been fully elucidated as yet. Therefore, in order to explore the possible adverse effects of folic acid fortification, further investigation is required to comprehensively understand the physico-chemical roles of 6-FP and PCA as patho-aetiological factors.

**-The effects of photo-oxidative degradation products of 5-$CH_3H_2$PteGlu on cell growth**

5-$CH_3H_2$PteGlu is degraded by UV exposure, decomposing into p-ABG and an unknown pterin moiety [551]. As described earlier, however, not enough is known about the properties of this

unknown pterin moiety. Therefore, it is worth speculating on whether these molecules may have an impact on cell proliferation.

The 5-$CH_3H_2$PteGlu-oxidative degradation product did not show any particular cell growth change in Caco-2 cells; only minor cell proliferation changes were observed between each concentration. In MCF 7 cells, when compared to other folate vitamers, the 5-$CH_3H_2$PteGlu-oxidative degradation product induced quite a low rate of growth enhancement (< 15%). This result may imply that, although we could not identify the benign oxidative degradation product(s) of 5-$CH_3H_2$PteGlu, it barely had any influence on colon cancer cell growth, and was a less active compound in terms of breast cancer cell growth when compared to other folate vitamers. Therefore, in terms of stability and safety, this result also supports the possible application of 5-$CH_3H_2$PteGlu as a folate supplement alongside other folyl vitamers.

However, it must be remembered that this is one of very first examinations conducted in relation to the oxidative degradation products of 5-$CH_3H_2$PteGlu in an *in vitro* model. Clearly further study is needed to understand the physiological and pharmacological effects of 5-$CH_3H_2$PteGlu and its oxidative degradation products on cell metabolism before the true relevance of these findings will be known.

### 3.5 Conclusion

Folic acid fortification with PteGlu has resulted in the prevention of congenital disorders and increased blood folate concentrations at a population level. However, as a blanket intervention, there is increased concern with respect to the potential adverse effects that this may have, especially via interaction with genetic and environmental factors. Therefore, it was worth examining the biological effects of this synthetic folate form and a range of other folate vitamers.

Colon and breast cancer cells were cultured in growth media containing various folate vitamers at various concentrations for 8 days, to obtain the sub-chronic effects of these vitamers on cell proliferation. Experimental results show that the particular folate vitamers, the level of folate given and the presence or absence of antioxidants critically influence cell proliferation and, hence, metabolism.

A clearly differential response according to the vitamers given was shown. High levels of PteGlu induced significantly altered cell growth trends. The presence of antioxidants (GSH or ascorbic acid) was decisively shown to preserve 5-$CH_3H_4$PteGlu and also afforded a potentially significant interaction with the vitamer in terms of cell proliferation. In addition, the observations suggest that 5-$CH_3H_2$PteGlu can enter one-carbon metabolism and influence cell growth. Moreover, the oxidative degradation products of 5-$CH_3H_2$PteGlu are fairly stable and were shown to have no significant effect on colon cancer cell proliferation.

The experimental evidence presented here could assist in a further understanding of the physico-chemical characteristics of folyl vitamers in cell growth metabolism, along with their interactions with antioxidants and photo-oxidative degradation products, and the implications for more ambitions *in vivo* model.

# IV. CLOSING REMARKS

The impacts of folate on human disease and health, particularly in relation to cancers and congenital disorders, have been extensively studied. Experimental observations suggest that increased folate intake provides significant health benefits in preventing NTDs [40, 564] and lowering Hcy. These findings have led to the introduction of mandatory folic acid fortification in many countries.

However, emerging evidence suggests that an increased intake of the synthetic form of folate, PteGlu, may be associated with unexpected adverse effects, including an increased risk of CRC [391]. With this in mind, the studies presented in this thesis were designed to re-investigate the effects of folate in cancer aetiology via *in* vivo and *in* vitro models. These studies are unique in the breadth of data presented and the range of interactions investigated. The aetiological role of folate was examined in relation to its interactions with various genetic and nutritional factors including the various vitamers, antioxidants and nutrients related to methyl group metabolism. As AP is a major antecedent for CRC, its occurrence was considered to be of particular relevance.

Findings from the AP study, as an *in vivo* model, imply that RBC folate level, nutritional intake (low methyl folate and high cobalamin intake) and genetic variations (such as CBS 844*ins*68) were associated with AP occurrence either on their own, or in combination with other variables. However, there was no significant association between consumption of PteGlu and the occurrence of AP. Interestingly, it was the level of total dietary folate intake that interacted with genetic variants and predicted AP risk.

To provide an *in vitro* model, colon and breast cancer cell lines were cultured in media containing various folate vitamers at numerous different concentrations, and additionally, the interaction with antioxidants and the effect of oxidative degradation products of folyl vitamers were also investigated. This cell line model demonstrated that each vitamer exhibited critical significance in cancer cell growth, depending on the type of cell line, vitamer concentration, and the presence/absence of antioxidant. In addition, it showed that 5-$CH_3H_2$PteGlu may participate in one-carbon metabolism, something that has previously been questioned.

The level of folic acid for mandatory fortification in Australia (135µg/100g flour) is lower than in other countries such as the United States (140µg) and Canada (150µg). It has been confirmed to be cost-effective for the Australian population, offering substantial long-term benefits such as decreased rates of stroke and CVD [525]. However, increasing research evidence suggests that potential adverse effects exist, and so the use of mandatory folate

fortification remains controversial. The findings from the current studies confirmed that folate vitamers and their intake, interacting with genetic and other milieu factors, may act as a potential risk factor in AP aetiology. Therefore, further research to monitor the dietary intake and blood folate levels of the population, and also to verify the mechanisms underpinning any adverse effects resulting from current mandatory folic acid fortification programs are required. Perhaps, most importantly, given the influence of genetic variations on nutrient metabolism, future nutritional intervention programs, such as mandatory folic acid fortification, should consider the genetic profile of a given population/individual, along with other diverse characteristics such as age, disease status and supplement intake.

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# APPENDIX

Appendix 1: Descriptive data by adenomatous polyp phenotype

| | Control | | Adenomatous polyp | |
|---|---|---|---|---|
| | Mean ± SD | Median (IQR)* | Mean ± SD | Median (IQR) |
| Subjects (female, male) | 164 (99,65) | N/A | 38 (17,21) | N/A |
| Age | 62.6 ± 11.0 | 64 (55-71.8) | 65.7 ± 10.5 | 64 (58.75-74.25) |
| Serum folate (nM) | 21.54 ± 11.90 | 18.0 (12.0-30.0) | 22.13 ± 12.63 | 18 (13-32) |
| Serum folate exclude outlier (nM) | 18.81 ± 9.29 | 17 (11-25) | 19.44 ± 10.40 | 16.5 (12.5-28.5) |
| RBC folate (nM) | 972.4 ± 508 | 862.5 (594.5-1157) | 1030 ± 575.2 | 932.5 (527.5-1387) |
| RBC folate exclude outlier (nM) | 913.66 ± 417.5 | 845.0 (592.25-1120) | 990.5 ± 527.6 | 900 (523-1347) |
| Cys (μM) | 263.88 ± 40.17 | 261.8 (237.4-288.5) | 265.92 ± 39.48 | 264.31 (237.6-290.1) |
| Hcy (μM) | 9.91 ± 2.89 | 9.69 (7.56-11.84) | 10.14 ± 2.72 | 9.55 (8.3-11.73) |
| Gly-Cys (μM) | 23.66 ± 4.88 | 23.17 (20.43-26.72) | 24.41 ± 5.55 | 25 (20-27.73) |
| GSH (μM) | 13.98 ± 5.82 | 12.04 (9.99-17.76) | 13.54 ± 5.06 | 11.41 (9.36-16.86) |
| Serum B12 (pM) | 280.0 ± 151.77 | 250.0 (194.0-321.0) | 280.39 ± 135.17 | 262 (194.25-326.25) |
| Grand total dietary folate intake (μg/day) | 459.23 ± 251.38 | 397.69 (318.81-571.48) | 434.44 ± 181.47 | 404.8 (284.2-512.6) |
| Total dietary methyl folate intake (μg/day) | 323.05 ± 124.14 | 306.7 (242.0-374.6) | 331.54 ± 127.38 | 350.3 (208.2-421.2) |
| Total dietary PteGlu intake (μg/day) | 132.85 ± 212.22 | 79.7 (12.21-150.0) | 111.37 ± 129.94 | 87.5 (15.9-137.9) |
| Total dietary pyridoxine intake (μg/day) | 6.23 ± 9.45 | 2.71 (2.06-4.74) | 4.81 ± 4.33 | 3.12 (2.32-5.94) |
| Total dietary cobalamin intake (μg/day) | 21.94 ± 80.11 | 10.28 (5.56-16.25) | 11.18 ± 6.79 | 9.85 (6.40-15.18) |
| Total dietary methionine intake (g/day) | 2.22 ± 0.93 | 2.01 (1.76-2.46) | 2.55 ± 1.75 | 2.04 (1.68-2.75) |
| Total dietary niacin intake (mg/day) | 33.37 ± 21.52 | 27.09 (21.66-39.15) | 30.69 ± 12.22 | 29.98 (20.55-35.87) |
| Total dietary niacin intake equivalent (mg/day) | 45.54 ±15.44 | 43.05 (36.38-51.79) | 46.43 ±13.02 | 45.36 (37.90-53.42) |
| Total dietary riboflavin intake (mg/day) | 5.06 ± 6.12 | 2.61 (1.89-4.40) | 4.01 ± 4.15 | 2.34 (1.80-4.14) |
| Total dietary vitamin c intake (mg/day) | 259.06 ± 239.44 | 217.7 (136.5-283.8) | 214.96 ± 137.66 | 198.4 (110.8-266.4) |
| Total dietary endogenous vitamin c intake (mg/day) | 204.0 ± 136.0 | 177.1 (124.7-265.7) | 185.6 ±114.8 | 182.3 (99.61-240.5) |

* IQR- interquartile range, 3rd Quartile of a box plot minus the first quartile

Appendix 2: Descriptive data by gender

| | Female | | Male | |
|---|---|---|---|---|
| | Mean ± SD | Median (IQR) | Mean ± SD | Median (IQR) |
| Age | 61.34 ± 10.05 | 63 (55-69) | 65.64 ± 11.76 | 66 (57.75-75) |
| Serum folate (nM) | 21.65 ± 12.17 | 18 (11-31) | 21.65 ± 11.88 | 18 (13-27.25) |
| Serum folate exclude outlier (nM) | 19.43 ± 10.24 | 17 (11-29) | 18.23 ± 8.312 | 16 (12-23) |
| RBC folate (nM) | 970.8 ± 536.9 | 798.5 (575.5-1183) | 1000 ± 500.4 | 918.5 (628-1183) |
| RBC folate exclude outlier (nM) | 915.2 ± 458.1 | 773 (565.5-1155) | 945.9 ± 417.4 | 906 (607-1153) |
| Cys (µM) | 259 ± 40.83 | 257.6 (233.4-285.8) | 271.2 ± 37.88 | 263.5 (243.6-293.4) |
| Hcy (µM) | 9.33 ± 2.761 | 8.875 (7.35-10.62) | 10.78 ± 2.78 | 10.57 (8.783-12.35) |
| Gly-Cys (µM) | 23.77 ± 5.123 | 22.52 (20.65-26.27) | 23.83 ± 4.857 | 23.71 (20.38-27.09) |
| GSH (µM) | 14.35 ± 5.817 | 12.51 (10.14-17.98) | 13.32 ± 5.482 | 11.4 (9.54-16.13) |
| Serum B12 (pM) | 277.9 ± 157.7 | 252 (190-306) | 282.9 ± 136 | 247.5 (195-335) |
| Grand total dietary folate intake (µg/day) | 436.3 ± 197.4 | 394.3 (300.9-534.1) | 478.9 ± 285.7 | 408.6 (341.9-570.2) |
| Total dietary methyl folate intake (µg/day) | 308.6 ± 112.7 | 301.6 (221.1-370.6) | 346.2 ± 136.4 | 336.7 (254.9-403.7) |
| Total dietary PteGlu intake (µg/day) | 124.8 ± 152.8 | 90.41 (16.49-150) | 134 ± 248.8 | 77.91 (4.315-144.1) |
| Total dietary pyridoxine intake (µg/day) | 6.71 ± 10.17 | 2.8 (2.085-6.727) | 4.968 ± 6.207 | 2.737 (2.209-4.207) |
| Total dietary cobalamin intake (µg/day) | 24.84 ± 94.6 | 10.2 (5.562-16.25) | 13.31 ± 14.65 | 10.11 (5.627-15.47) |
| Total dietary methionine intake (g/day) | 2.21 ± 1.126 | 1.969 (1.727-2.46) | 2.383 ± 1.133 | 2.08 (1.8-2.499) |
| Total dietary niacin intake (mg/day) | 32.29 ± 19.19 | 26.65 (19.77-36.77) | 33.64 ± 21.34 | 28.68 (23.18-38.38) |
| Total dietary niacin intake equivalent (mg/day) | 43.19 ± 11.79 | 42.97 (33.4-50.44) | 49.07 ± 17.93 | 44.54 (39.57-55.01) |
| Total dietary riboflavin intake (mg/day) | 5.393 ± 6.729 | 2.723 (1.824-4.619) | 4.149 ± 4.208 | 2.397 (1.889-3.712) |
| Total dietary vitamin c intake (mg/day) | 244.1 ± 241 | 194.5 (133.7-276.9) | 259.5 ± 200.4 | 223.8 (138.2-305.1) |
| Total dietary endogenous vitamin c intake (mg/day) | 191.4 ± 135.4 | 161.3 (119.5-240.7) | 212.9 ± 127.5 | 189.7 (123-277.2) |

Appendix 3: Descriptive data according to total dietary folate intake level by RDI

| | Folate intake above RDI | | Folate intake below RDI | |
|---|---|---|---|---|
| | Mean ± SD | Median (IQR) | Mean ± SD | Median (IQR) |
| Subjects (female, male) | 54, 45 | | 60, 40 | |
| Age | 63.15 ± 10.74 | 63 (56-72) | 63.31 ± 11.39 | 64 (56.25-71.75) |
| Serum folate (nM) | 23.84 ± 12.07 | 21 (13.75-32) | 19.39 ± 11.61 | 16 (10-27) |
| Serum folate exclude outlier (nM) | 20.31 ± 9.058 | 19 (13-26.75) | 17.46 ± 9.558 | 15 (10-23) |
| RBC folate (nM) | 1094 ± 571.5 | 983 (695.5-1309) | 882.1 ± 451.2 | 801.5 (552-1137) |
| RBC folate exclude outlier (nM) | 1017 ± 479.6 | 946.5 (687.5-1203) | 849.1 ± 390.8 | 792 (545.5-1112) |
| Cys (μM) | 259.8 ± 36.36 | 262.6 (236.1-286.8) | 268.8 ± 43.22 | 261.6 (240.3-294.7) |
| Hcy (μM) | 9.605 ± 2.793 | 9.08 (7.53-11.25) | 10.32 ± 2.886 | 10.06 (7.85-12.09) |
| Gly-Cys (μM) | 23.39 ± 4.987 | 22.46 (20.17-26.25) | 24.18 ± 5.081 | 23.78 (20.72-27) |
| GSH (μM) | 13.65 ± 5.782 | 11.4 (9.45-17.57) | 14.1 ± 5.666 | 12.99 (10.06-17.2) |
| Serum B12 (pM) | 285.4 ± 150.1 | 251.5 (194.3-326.5) | 275.6 ± 149.2 | 251 (191-313.8) |
| Grand total dietary folate intake (μg/day) | 605.7 ± 253.8 | 565.7 (453.7-662.8) | 304.8 ± 73.14 | 316.4 (268.4-362) |
| Total dietary methyl folate intake (μg/day) | 394.5 ± 128.7 | 383 (322.6-441.8) | 255.6 ± 69.98 | 251.8 (204.1-305.9) |
| Total dietary PteGlu intake (μg/day) | 209.1 ± 254.7 | 136.4 (51.94-350) | 49.19 ± 46.85 | 49.39 (0.255-90.9) |
| Total dietary pyridoxine intake (μg/day) | 9.226 ± 11.22 | 4.398 (2.847-7.716) | 2.785 ± 2.844 | 2.191 (1.821-2.703) |
| Total dietary cobalamin intake (μg/day) | 21 ± 25.71 | 13.71 (8.904-19.77) | 19.05 ± 99.48 | 8.005 (4.415-11.69) |
| Total dietary methionine intake (g/day) | 2.478 ± 1.065 | 2.214 (1.945-2.693) | 2.088 ± 1.171 | 1.87 (1.603-2.238) |
| Total dietary niacin intake (mg/day) | 41.93 ± 22.55 | 36.27 (29.22-47.99) | 23.88 ± 12.15 | 22.18 (18.26-26.75) |
| Total dietary niacin intake equivalent (mg/day) | 51.78 ± 16.88 | 47.21 (42.62-56.91) | 39.5 ± 9.564 | 38.33 (32.77-45.01) |
| Total dietary riboflavin intake (mg/day) | 7.263 ± 7.163 | 3.587 (2.608-9.903) | 2.494 ± 2.426 | 1.94 (1.629-2.415) |
| Total dietary vitamin C intake (mg/day) | 302.3 ± 197.8 | 264.4 (184.2-367.1) | 179.7 ± 105.2 | 152.2 (110.3-242.5) |
| Total dietary endogenous vitamin C intake (mg/day) | 229.2 ± 121 | 214.4 (145.1-280.4) | 161.9 ± 80.03 | 146.7 (105.7-226.9) |

Appendix 4: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the MTHFR C677T genotypes for control and adenomatous polyp subjects

| | | Wild type (CC) | | Heterozygous type (CT) | | Homo recessive type (TT) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 21.4 (11.90) | 22.71 (13.3) | 21.18 (11.80) | 19.67 (12.83) | 18.71 (12.93) | 26.67 (10.5) |
| | Median (IQR) | 18 (12.0-30.0) | 17 (12.5-36.0) | 20.5 (11.0-31.0) | 15 (10.0-32.0) | 14.5 (9.0-26.25) | 24 (18.0-34.5) |
| Serum folate excluding outliers (nM) | Mean (SD) | 17.82 (7.98) | 19.73 (11.04) | 20.16 (10.05) | 17.86 (11.15) | 16.69 (10.92) | 23 (6.08) |
| | Median (IQR) | 17 (11.0-28.5) | 16 (11.0-30.0) | 19.5 (11.0-28.5) | 14 (9.25-28.25) | 13 (9.0-19.5) | 20 (18.0-29.5) |
| RBC folate (nM) | Mean (SD) | 882.97 (436.8) | 915.24 (438.2) | 979.8 (509.5) | 969.27 (626.8) | 1406.3 (647.1) | 1508.2 (635.2) |
| | Median (IQR) | 804.5 (572.75-1092) | 848 (885.0-2175.3) | 872 (662.5-1155.5) | 793 (497.0-1317) | 1329 (882.3-2038.8) | 1517 (885.0-2175) |
| RBC folate excluding outliers (nM) | Mean (SD) | 838.1 (347.1) | 915.24 (438.2) | 915.5 (409.2) | 859.93 (479.6) | 1322.1 (588.4) | 1508.2 (635.2) |
| | Median (IQR) | 782.5 (562.25-1082) | 848 (545.0-1275) | 868 (607.0-1123.0) | 784.5 (481.2-1215) | 1213 (833.5-2078.8) | 1517 (645.0-2175) |
| Cys (µM) | Mean (SD) | 258.7 (42.7) | 274.4 (46.0) | 267.8 (38.2) | 256.5 (31.2) | 271.9 (35.4) | 264.5 (40.1) |
| | Median (IQR) | 259.3 (233.4-287.0) | 264.3 (244.5-302.4) | 261.8 (238.9-289.4) | 252.1 (231.4-287.9) | 269.5 (240.5-298.42) | 256.9 (234.4-298.5) |
| Hcy (µM) | Mean (SD) | 9.77 (3.22) | 10.6 (2.70) | 9.87 (2.72) | 10.25 (2.99) | 10.95 (1.38) | 8.612 (1.88) |
| | Median (IQR) | 8.875 (7.35-11.90) | 9.83 (8.38-12.47) | 9.69 (7.60-11.56) | 10.15 (7.41-13.39) | 11.31 (9.84-12.19) | 8.69 (7.02-10.17) |
| Cys-Gly (µM) | Mean (SD) | 23.58 (4.29) | 23.97 (3.34) | 23.78 (5.51) | 25.05 (7.86) | 23.52 (4.72) | 24.08 (4.79) |
| | Median (IQR) | 23.46 (20.30-26.71) | 24.47 (20.59-27.04) | 22.65 (10.90-26.52) | 24.77 (18.43-29.39) | 22.79 (19.62-28.83) | 26.92 (18.92-27.83) |
| GSH (µM) | Mean (SD) | 13.26 (5.49) | 11.86 (4.43) | 14.89 (6.43) | 14.9 (3.93) | 13.21 (3.39) | 15 (8.26) |
| | Median (IQR) | 11.38 (9.74-16.20) | 10.38 (8.54-14.46) | 13.15 (9.91-18.35) | 15.89 (10.84-18.88) | 11.79 (10.43-17.29) | 11.25 (9.13-22.75) |
| Serum $B_{12}$ (ρM) | Mean (SD) | 276.81 (119.3) | 236 (87.7) | 288.9 (180.2) | 334.76 (182.3) | 249.79 (148.9) | 270.33 (43.6) |
| | Median (IQR) | 252 (206.0-326.0) | 203 (168.50-309.50) | 249 (186.5-328.25) | 267 (243.0-414.0) | 218.5 (182.0-258.75) | 218.5 (231.5-322.0) |

Appendix 5: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the MTHFR C677T genotypes for control and adenomatous polyp subjects

| | | Wild type (CC) | | Heterozygous type (CT) | | Homo recessive type (TT) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 456.48 (231.9) | 435.21 (172.8) | 437.01 (178.9) | 435.82 (209.8) | 588.19 (525.6) | 428.82 (157.6) |
| | Median (IQR) | 392.4 (314.9-588.12) | 410.5 (291.7-521.4) | 396.2 (320.4-551.3) | 409.1 (262.1-575.6) | 439.8 (337.4-602.3) | 388.8 (312.6-536.1) |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 327.17 (141.4) | 345.04 (125.7) | 313.72 (100.5) | 316.1 (143) | 349 (139.2) | 331.88 (104.7) |
| | Median (IQR) | 301.5 (235.23-372.88) | 376.5 (236.65-424.28) | 307.1 (244.14-377.42) | 279.2 (180.85-421.09) | 341.4 (247.18-381.82) | 350.3 (263.09-398.38) |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 119.3 (145.5) | 88.53 (103.4) | 126.3 (159.1) | 143.06 (165.4) | 239.2 (531.1) | 96.94 (95.48) |
| | Median (IQR) | 78.49 (7.29-148.2) | 87.5 (8.28-139.6) | 83.77 (11.9-150) | 87.5 (38.57-155.8) | 84.41 (39.80-174.7) | 82.8 (17.96-166.1) |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 6.55 (9.684) | 4.86 (4.042) | 5.97 (9.624) | 4.03 (5.423) | 5.94 (7.608) | 4.13 (1.831) |
| | Median (IQR) | 2.685 (2.05-4.37) | 3.317 (2.31-6.20) | 2.775 (2.17-6.39) | 2.847 (2.08-7.01) | 2.868 (2.23-4.67) | 3.783 (2.42-5.86) |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 17.49 (25.23) | 12.42 (8.706) | 28.62 (116.1) | 11.08 (4.966) | 10.45 (9.578) | 7.93 (3.47) |
| | Median (IQR) | 10.34 (5.42-16.81) | 10.21 (6.14-15.51) | 10.56 (6.46-16.58) | 11.02 (6.59-15.69) | 6.755 (4.80-11.95) | 7.516 (5.27-10.46) |
| Total dietary methionine intake (g/day) | Mean (SD) | 2.284 (1.073) | 2.806 (2.191) | 2.173 (0.71) | 2.16 (0.738) | 2.152 (1.113) | 2.796 (2.237) |
| | Median (IQR) | 2.125 (1.783-2.456) | 2.368 (1.679-2.979) | 1.969 (1.775-2.467) | 1.984 (1.643-2.448) | 1.891 (1.639-2.335) | 1.978 (1.75-3.632) |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 47.09 (19.46) | 46.29 (10.59) | 44.18 (10.43) | 47.21 (16.76) | 44.34 (13.26) | 44.9 (10.18) |
| | Median (IQR) | 44.02 (36.11-54.73) | 45.37 (38.23-55.97) | 43 (37.60-49.48) | 44.29 (32.69-55.17) | 42.26 (34.09-54.24) | 48.97 (36.31-52.49) |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 5.12 (6.678) | 4.57 (4.771) | 5 (5.876) | 3.88 (4.214) | 5.24 (4.394) | 2.82 (1.304) |
| | Median (IQR) | 2.475 (1.19-3.95) | 2.63 (2.02-4.33) | 2.792 (1.97-3.98) | 2.087 (1.81-4.12) | 2.437 (1.84-10.29) | 2.398 (1.86-3.35) |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 253.71 (203.4) | 226.86 (126.8) | 266.01 (287.6) | 180.77 (141.7) | 251.03 (119.1) | 267.4 (159) |
| | Median (IQR) | 209 (134.80-300.47) | 224.1 (136.50-284.42) | 208.8 (146.39-279.51) | 147.6 (73.29-240.48) | 223.5 (149.46-298.86) | 221.1 (134.06-452.32) |

Appendix 6: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the MTHFR A1298C genotypes for control and adenomatous polyp subjects

| | | Wild type (AA) | | Heterozygous type (AC) | | Homo recessive type (CC) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 22.9 (12.32) | 22.55 (11.22) | 19.96 (11.1) | 18.57 (12.99) | 21.58 (13.47) | 32.5 (15.37) |
| | Median (IQR) | 20 (12.0-31.5) | 18 (14.5-31.75) | 17 (10.0-25.0) | 14.5 (8.5-24.0) | 20 (9.0-29.25) | 37 (16.25-44.25) |
| Serum folate excluding outliers(nM) | Mean (SD) | 19.79 (9.685) | 21.37 (10.17) | 18.03 (8.946) | 14.17 (7.158) | 16.9 (8.698) | 28.33 (15.82) |
| | Median (IQR) | 18 (12.0-27.0) | 18 (14.0-31.0) | 16 (10.0-23.5) | 13.5 (7.5-18.75) | 16 (9.0-25.50) | 32 (11.0-42.0) |
| RBC folate (nM) | Mean (SD) | 1048.8 (503.4) | 1014.2 (570.4) | 873.9 (473.8) | 1012.14 (639.9) | 1037.83 (664) | 1173.5 (463.3) |
| | Median (IQR) | 944 (696.0-1256) | 906.5 (538.5-1423.5) | 749.5 (563.5-1059.8) | 838 (465.5-1386.8) | 973 (459.75-1353.5) | 1071 (799.0-1650.5) |
| RBC folate excluding outliers (nM) | Mean (SD) | 992.23 (420.7) | 1014.15 (570.4) | 826.1 (387.2) | 897.69 (494.9) | 904.91 (501.8) | 1173.5 (463.3) |
| | Median (IQR) | 929 (689.0-1210.5) | 906.5 (538.5-1423.5) | 736 (554.5-1004.25) | 776 (457.0-1347.5) | 928 (433.0-1142.0) | 1071 (799.0-1650.5) |
| Cys (uM) | Mean (SD) | 266.53 (40.14) | 280.769 (41.91) | 262.81 (42.58) | 250.85 (32.25) | 251.89 (20.95) | 247.99 (29.99) |
| | Median (IQR) | 262.1 (238.6-288.4) | 268.5 (251.9-320.3) | 262.2 (235.03-291.8) | 240 (224.9-287.6) | 257.4 (238.4-266.2) | 260.7 (217.47-265.8) |
| Hcy (uM) | Mean (SD) | 9.75 (2.67) | 10.13 (2.9) | 10.08 (3.259) | 10.9 (2.688) | 10.08 (1.994) | 8.13 (0.764) |
| | Median (IQR) | 9.295 (7.73-11.27) | 9.035 (7.85-12.17) | 9.79 (6.57-12.12) | 10.46 (9.55-13.75) | 10.33 (8.46-11.13) | 8.3 (7.37-8.73) |
| Cys-Gly (uM) | Mean (SD) | 23.42 (5.04) | 24.56 (4.44) | 23.69 (4.796) | 24.56 (7.651) | 25.25 (4.297) | 23.41 (3.61) |
| | Median (IQR) | 22.36 (10.38-26.6) | 25.13 (20.18-27.79) | 23.25 (20.51-26.74) | 22.54 (19.92-27.76) | 25.84 (22.79-27.56) | 23.46 (20.08-26.68) |
| GSH (uM) | Mean (SD) | 14.24 (5.896) | 13.97 (5.184) | 14.07 (6.04) | 13.54 (4.668) | 11.66 (3.305) | 11.86 (6.715) |
| | Median (IQR) | 12.04 (10.50-17.92) | 13.53 (9.72-16.64) | 12.61 (9.59-18.83) | 11.41 (9.48-18.96) | 10.93 (9.10-15.82) | 9.14 (7.71-18.72) |
| Serum $B_{12}$ (pM) | Mean (SD) | 265.11 (123.7) | 300.25 (157.3) | 296.96 (185.4) | 264.714 (108.1) | 281.5 (93.76) | 236 (108.1) |
| | Median (IQR) | 235 (190.0-308.0) | 267.5 (202.3-347.8) | 252.5 (194.3-332.3) | 262 (221.5-292.3) | 273.5 (213.0-331.5) | 197.5 (163.5-347.0) |

Appendix 7: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the MTHFR A1298C genotypes for control and adenomatous polyp

| | | WT (AA) | | Het (AC) | | Rec (CC) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 491.41 (286.4) | 395.0 (187.6) | 440.4 (215.9) | 439.8 (142.2) | 344.4 (105.8) | 613.01 (206.2) |
| | Median (IQR) | 409.8 (343.61-589.1) | 357.2 (270.54-456.4) | 376 (319.7-588.1) | 459.6 (366.9-512.6) | 360.6 (293.8-420.4) | 601.6 (424.9-812.5) |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 328.1 (111.6) | 302.46 (136.6) | 320.39 (141.4) | 344.55 (101.9) | 303.81 (108.8) | 431.37 (130.9) |
| | Median (IQR) | 310.1 (247.8-394.76) | 290.1 (174.2-376.5) | 294.2 (242.4-367.1) | 390.2 (246.0-414.9) | 298.6 (223.9-376.1) | 428.3 (312.4-553.4) |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 167.8 (267.8) | 110.02 (126.7) | 106.6 (130.7) | 95.25 (130.1) | 40.62 (41.93) | 174.64 (163.1) |
| | Median (IQR) | 90.9 (39.67-154.2) | 89.2 (4.73-141.1) | 69.96 (2.38-150) | 68.13 (11.37-107.1) | 42.98 (0.0-81.16) | 124.2 (57.22-342.5) |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 6.63 (9.731) | 4.66 (4.865) | 6.41 (9.855) | 4.31 (2.225) | 2.48 (0.854) | 7.37 (7.053) |
| | Median (IQR) | 2.773 (2.32-5.09) | 2.873 (2.11-5.52) | 2.709 (1.98-6.50) | 3.891 (2.36-6.00) | 2.379 (1.92-3.14) | 4.711 (2.56-14.83) |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 16.7 (24.21) | 9.04 (5.451) | 30.15 (120.2) | 14.6 (7.776) | 10.64 (8.009) | 9.92 (5.653) |
| | Median (IQR) | 10.01 (5.59-15.37) | 8.734(4.08-12.51) | 10.97 (5.79-17.22) | 14.86 (9.91-16.11) | 8.388 (4.17-15.13) | 10.3 (4.38-15.09) |
| Total dietary methionine intake (g/day) | Mean (SD) | 2.25 ( 0.843) | 2.129 (1.331) | 2.197 (1.056) | 3.076 (2.251) | 2.181 (0.677) | 2.805 (1.417) |
| | Median (IQR) | 2.015 (1.852-2.409) | 1.879 (1.441-2.16) | 2.127 (1.697-2.501) | 2.445 (2.083-2.806) | 1.908 (1.791-2.592) | 2.423 (1.697-4.297) |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 45.41 (11.92) | 42.32 (12.26) | 46.47 (19.4) | 50.69 (13.84) | 41.06 (10.46) | 52.05 (8.335) |
| | Median (IQR) | 43.36 (37.7-52.06) | 43.05 (31.28-51.1) | 44.37 (35.23-52.11) | 47.26 (41.75-57.04) | 40.49 (33.5-52.96) | 50.81 (44.96-60.37) |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 4.94 (6.083) | 3.34 (3.465) | 5.59 (6.561) | 4.68 (5.148) | 2.83 (2.497) | 5.02 (3.866) |
| | Median (IQR) | 2.802 (1.90-3.64) | 2.063 (1.60-2.68) | 2.511 (1.95-7.10) | 2.636 (2.15-4.14) | 2.101 (1.53-2.70) | 4.064 (2.01-8.98) |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 237.16 (126.1) | 218.13 (153.5) | 217.73 (301.7) | 210.26 (131.7) | 335.9 (391.8) | 215.54 (95.74) |
| | Median (IQR) | 214.4 (143.3-286.5) | 212.7 (81.5-296.5) | 219.4 (135.2-286.8) | 186.9 (138.9-246.5) | 198.6 (123.9-293.1) | 209.5 (127.9-309.3) |

Appendix 8: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the MTHFR G1793A genotypes for control and adenomatous polyp subjects

| | | Wild type (GG) | | Heterozygous type (GA) | | Homo recessive type (AA) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 21.13 (11.7) | 22.81 (12.62) | 25.79 (14.05) | 10 (4.243) | 22 (11.31) | N/A |
| | Median (IQR) | 18 (9.0-29.0) | 18 (13.25-32.0) | 20.5 (14.75-42.75) | 10 (13.25-32.0) | 22 (14.0-30.0) | |
| Serum folate excluding outliers(nM) | Mean (SD) | 18.62 (9.199) | 20.03 (10.41) | 20.55 (10.76) | 10 (4.243) | 22 (11.31) | N/A |
| | Median (IQR) | 17 (8.10-24.50) | 17.5 (13.0-29.0) | 16 (14.00-25.00) | 10 (7.00-13.0) | 22 (14.0-30.0) | |
| RBC folate (nM) | Mean (SD) | 645.6 (482.8) | 1053.8 (580.7) | 1110.6 (610) | 605 (241.8) | 1962 (760.8) | N/A |
| | Median (IQR) | 862.5 (589.25-1143) | 982.5 (538.5-1414.2) | 827.5 (671.75-1519) | 605 (434.0-776.0) | 1962 (2535-2500) | |
| RBC folate excluding outliers (nM) | Mean (SD) | 901.82 (411.4) | 1012.49 (532.7) | 1003.8 (479.5) | 605 (241.8) | 1424 | N/A |
| | Median (IQR) | 847.5 (580.8-1109) | 965 (532.0-1373.0) | 812 (654.5-1314) | 605 (434.0-776.0) | 1424 | |
| Cys (µM) | Mean (SD) | 263.9 (40.48) | 265.87 (40.24) | 264.13 (41.36) | 266.7 (37.8) | 261.1 (24.12) | N/A |
| | Median (IQR) | 261.7 (237.6-288.6) | 261.3 (233.5-288.9) | 267.6 (232.9-291.4) | 266.7 (239.9-293.4) | 261.1 (244.1-278.2) | |
| Hcy (µM) | Mean (SD) | 10.03 (2.925) | 10.1 (2.812) | 9.06 (2.535) | 10.81 (0.495) | 7.71 (0.198) | N/A |
| | Median (IQR) | 9.79 (7.59-11.98) | 9.07 (8.30-12.03) | 8.765 (6.74-11.70) | 10.81 (10.46-11.16) | 7.71 (7.57-7.85) | |
| Cys-Gly (µM) | Mean (SD) | 23.81 (4.792) | 24.6 (5.46) | 21.39 (5.261) | 21.67 (8.613) | 28.31 (4.808) | N/A |
| | Median (IQR) | 23.2 (20.43-26.75) | 25 (15.58-27.76) | 21.56 (19.92-23.54) | 21.67 (15.58-27.76) | 28.31 (24.91-31.71) | |
| GSH (µM) | Mean (SD) | 14.43 (5.898) | 13.48 (5.064) | 9.83 (3.121) | 14.51 (7.106) | 10.82 (1.775) | N/A |
| | Median (IQR) | 12.41 (10.37-18.22) | 11.41 (9.28-16.56) | 9.435 (7.74-12.64) | 14.51 (9.48-19.53) | 10.82 (9.56-12.07) | |
| Serum $B_{12}$ (pM) | Mean (SD) | 276.87 (153.2) | 280.22 (138.9) | 306.71 (147.4) | 283.5 (26.16) | 322.5 (84.15) | N/A |
| | Median (IQR) | 247 (190.0-318.0) | 253.5 (192.75-328.75) | 267 (197.5-357.75) | 283.5 (265.0-302.0) | 322.5(263.0-382.0) | |

Appendix 9: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the MTHFR G1793A genotypes for control and adenomatous polyp subjects

| | | Wild type (GG) | | Heterozygous type (GA) | | Homo recessive type (AA) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 450.8 (241.6) | 433.74 (186.3) | 557.7 (345.1) | 447 (53.64) | 383.6 (14.58) | N/A |
| | Median (IQR) | 394.7 (315.5-571.0) | 397.3 (282.0-530.3) | 446.2 (338.6-654.8) | 447 (409.1-484.9) | 383.6 (373.2-393.9) | |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 319.0 (117.7) | 331.61 (129.7) | 366.1 (184.2) | 330.2 (108.7) | 312.8 (43.13) | N/A |
| | Median (IQR) | 301.8 (240.84-373.32) | 350.3 (206.32-421.29) | 356.4 (279.15-386.55) | 330.1 (253.25-407.03) | 312.8 (282.34-343.33) | |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 131.5 (211.5) | 111.1 (133.3) | 155.9 (240) | 116.9 (55.1) | 70.72 (28.55) | N/A |
| | Median (IQR) | 79.92 (9.88-150) | 87.5 (13.95-136.3) | 69.97 (14.42-187.5) | 116.9 (77.91-155.8) | 70.72 (50.53-90.9) | |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 5.81 (8.376) | 4.93 (4.423) | 11.16 (17.19) | 2.77 (0.107) | 2.92 (0.874) | N/A |
| | Median (IQR) | 2.697 (2.03-4.65) | 3.558 (2.29-6.46) | 3.386 (2.40-10.66) | 2.771 (2.69-2.85) | 2.923 (2.30-3.54) | |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 21.09 (83.5) | 10.95 (6.905) | 33.45 (38.32) | 15.41 (1.102) | 4.05 (0.84) | N/A |
| | Median (IQR) | 10.2 (5.56-15.72) | 9.663 (6.00-15.05) | 17.48 (9.60-40.26) | 15.41 (14.63-16.19) | 4.047 (3.45-4.64) | |
| Total dietary methionine intake (g/day) | Mean (SD) | 2.518 (0.758) | 2.552 (1.798) | 2.955 (1.887) | 2.505 (0.337) | 1.844 (0.086) | N/A |
| | Median (IQR) | 1.992 (1.754-2.383) | 1.997 (1.674-2.702) | 2.566 (2.945) | 2.505 (2.743) | 1.844 (1.783-1.905) | |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 44.61 (12.95) | 45.78 (13.05) | 56.4 (30.59) | 58.13 (4.186) | 37.87 (6.314) | N/A |
| | Median (IQR) | 42.97 (36.13-51.24) | 45.09 (36.69-52.63) | 51.03 (41-88-59.04) | 58.13 (55.17-61.09) | 37.87 (33.4-42.33) | |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 4.82 (5.87) | 4.08 (4.255) | 7.95 (8.327) | 2.81 (0.295) | 2.37 (0.099) | N/A |
| | Median (IQR) | 2.52 (1.87-3.95) | 2.267 (1.79-4.19) | 3.11 (2.21-16.49) | 2.808 (2.60-3.02) | 2.371 (2.30-2.44) | |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 248.11 (224.1) | 219.62 (139.4) | 385.97 (361.7) | 131.02 (81.64) | 176.27 (16.79) | N/A |
| | Median (IQR) | 217.7 (135.54-278.68) | 210.9 (112.59-270.50) | 279.1 (136.43-508.40) | 131 (73.29-188.75 | 176.3 (164.39-188.14) | |

Appendix 10: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the combined MTHFR C677T-A1298C-G1793A genotypes for control and adenomatous polyp subjects (1 of 3)

| | | CT/AA/GG | | CC/AC/GG | | CC/AA/GG | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 22.5 (11.39) | 21.9 (12.71) | 18.8 (9.988) | 20 (13.39) | 24.6 (13.16) | 19.3 (10.42) |
| | Median (IQR) | 21.5 (11.8-30.3) | 21.5 (10.8-35) | 16 (11-24.5) | 15 (9-30) | 20 (12.5-35) | 16.5 (13.3-23.5) |
| Serum folate excluding outliers(nM) | Mean (SD) | 20.2 (9.128) | 21.9 (12.71) | 17.1 (7.585) | 15.8 (8.329) | 19.5 (9.012) | 19.3 (10.42) |
| | Median (IQR) | 19.5 (11-27.5) | 21.5 (10.8-35) | 15 (10-24) | 14.5 (8.5-22.5) | 17.5 (12-28.5) | 16.5 (13.3-23.5) |
| RBC folate (nM) | Mean (SD) | 962.6 (490.8) | 739.8 (406.3) | 746.2 (279.6) | 792.7 (448.6) | 963.2 (339.7) | 886 (411) |
| | Median (IQR) | 868.5 (656-1156.3) | 653.5 (380.8-1127) | 714 (500-899) | 714 (440-1373) | 982 (631.8-1255.8) | 741.5 (551.5-1274.3) |
| RBC folate excluding outliers (nM) | Mean (SD) | 885.7 (354.9) | 739.8 (406.3) | 746.2 (279.6) | 792.7 (448.6) | 963.2 (339.7) | 886 (411) |
| | Median (IQR) | 857.5 (606-1090.5) | 653.5 (380.8-1127) | 714 (500-899) | 714 (440-1373) | 982 (631.8-1255.8) | 741.5 (551.5-1274.3) |
| Cys (µM) | Mean (SD) | 272.5 (40.7) | 265.8 (25.65) | 264.5 (47.76) | 255.1 (47.76) | 252.4 (40.97) | 315 (45.09) |
| | Median (IQR) | 261.8 (239-295.4) | 265.6 (239-287.3) | 262.2 (235.5-299.3) | 251.1 (225.1-287.1) | 247.2 (216.5-272.1) | 325.3 (273.6-351.1) |
| Hcy (µM) | Mean (SD) | 10 (2.86) | 10.6 (3.539) | 10.8 (3.765) | 12.1 (2.489) | 8.8 (2.61) | 11.1 (2.833) |
| | Median (IQR) | 9.69 (8.1-14.1) | 10.7 (6.9-14.2) | 10.13 (7.3-13.7) | 11.3 (9.8-14.7) | 8.18 (6.9-10.1) | 10.76 (8.7-13.7) |
| Cys-Gly (µM) | Mean (SD) | 23.8 (5.781) | 24.6 (5.8) | 24.1 (4.512) | 12.4 (4.011) | 23.1 (4.275) | 25 (2.898) |
| | Median (IQR) | 22.17 (20.4-26.5) | 24.77 (19.1-30.4) | 23.78 (20.7-26.8) | 22.14 (20.1-27.4) | 22.44 (20.3-27.2) | 25 (22.3-27.7) |
| GSH (µM) | Mean (SD) | 15.2 (6.742) | 14.7 (3.402) | 14.3 (6.22) | 11.7 (4.333) | 13.6 (5.402) | 12 (3.35) |
| | Median (IQR) | 12.41 (10.9-20) | 14.85 (11.2-17.8) | 12.35 (9.9-10.2) | 10.55 (8.8-15.2) | 12.03 (10.4-17.5) | 13.17 (8.5-15) |
| Serum $B_{12}$ (pM) | Mean (SD) | 257.5 (130.5) | 359.6 (228.9) | 262.8 (125.1) | 223.1 (81.61) | 281.8 (96.12) | 251 (95.52) |
| | Median (IQR) | 215 (179.3-278.3) | 320.5 (180.3-447.8) | 252 (194-304.5) | 236 (122-289) | 251 (212.5-331) | 218.5 (170.3-349) |

Appendix 11: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the combined MTHFR C677T-A1298C-G1793A genotypes for control and adenomatous polyp subjects (2 of 3)

| | | CT/AC/GG | | TT/AA/GG | | CC/CC/GG | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 20.3 (11.58) | 20 (15.23) | 19.5 (13.14) | 26.7 (10.5) | 19.4 (13.65) | 32.5 (15.37) |
| | Median (IQR) | 20 (10-29.5) | 15 (9-33.5) | 16 (10.0-29.5) | 24 (18-34.5) | 18 (9-27) | 37 (16.3-44.3) |
| Serum folate excluding outliers(nM) | Mean (SD) | 19.4 (10.65) | 13.8 (6.99) | 17.3 (11.15) | 23 (6.08) | 15.2 (8.424) | 28.3 (15.82) |
| | Median (IQR) | 20 (10-27) | 14 (7-20.3) | 14.5 (9.5-21.3) | 20 (18-29.5) | 13.5 (8.3-23.2) | 32 (11-42) |
| RBC folate (nM) | Mean (SD) | 972.8 (565) | 1482.2 (755.7) | 1473 (621.5) | 1508 (635.2) | 701 (318.9) | 1174 (463.3) |
| | Median (IQR) | 926.5 (577-1129) | 1322 (843-2202) | 1445 (957-2051) | 1517 (885-2175) | 578 (413-1018) | 1071 (799-1651) |
| RBC folate excluding outliers (nM) | Mean (SD) | 911.7 (481) | 1227.8 (574.3) | 1387 (563.5) | 1508 (635.2) | 701 (318.9) | 1174 (463.3) |
| | Median (IQR) | 906 (574-1116) | 1252 (674-1758) | 1329 (944-2026) | 1517 (885-2175) | 578 (413-1018) | 1071 (799-1650.5) |
| Cys (μM) | Mean (SD) | 260 (34.34) | 237.6 (35.68) | 276.5 (33.15) | 264.5 (40.06) | 251.1 (22.95) | 248 (29.99) |
| | Median (IQR) | 261.6 (241.8-287.1) | 223.9 (214-275) | 273.9 (242.4-302) | 256.9 (234.4-298.5) | 259.7 (231-266.9) | 260.7 (217.5-266) |
| Hcy (μM) | Mean (SD) | 9.7 (2.557) | 9.5 (3.271) | 11 (1.43) | 8.6 (1.88) | 11 (2.021) | 8.1 (0.764) |
| | Median (IQR) | 9.85 (7.5-11.9) | 9.1 (6.6-12.8) | 11.38 (9.7-12.2) | 8.69 (7-10.2) | 10.58 (9.6-12.7) | 8.3 (7.4-8.7) |
| Cys-Gly (μM) | Mean (SD) | 24.1 (4.524) | 27.5 (11.4) | 22.9 (4.47) | 24.1 (4.79) | 24.4 (4.21) | 23.4 (3.61) |
| | Median (IQR) | 23.45 (20.9-27.2) | 24.02 (19.1-39.3) | 22.38 (19.6-27.1) | 26.92 (18.9-27.8) | 25.94 (21.3-26.7) | 23.46 (20.1-26.7) |
| GSH (μM) | Mean (SD) | 15.4 (6.11) | 15.4 (4.49) | 12.9 (3.43) | 15 (8.26) | 12 (3.75) | 11.9 (6.72) |
| | Median (IQR) | 14.54 (9.6-19.3) | 16.12 (10.9-19) | 11.72 (10.3-17.7) | 11.25 (9.1-22.8) | 10.46 (7.7-15.9) | 9.14 (7.7-18.7) |
| Serum $B_{12}$ (pM) | Mean (SD) | 333.9 (246.5) | 315.4 (146.3) | 251.7 (154.9) | 270.3 (43.64) | 284.1 (116.1) | 236 (108.1) |
| | Median (IQR) | 261 (194.3-346.5) | 259 (237.5-421.5) | 212 (174-261.5) | 259 (231.5-322) | 292 (174-343) | 197.5 (163.5-347) |

Appendix 12: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the combined MTHFR C677T-A1298C-G1793A genotypes for control and adenomatous polyp subjects (3 of 3)

| | | CT/AC/AG | | CC/AC/AG | CC/CC/AA | CC/CC/AG | TT/CC/GG | CT/AA/AG |
|---|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | Control | Control | Control (n=1) | Control (n=1) |
| Serum folate (nM) | Mean (SD) | 24.8 (15.61) | 10 (4.24) | 20.8 (12.7) | 22 (11.31 ) | 35 (14.14) | 9 | 42 |
| | Median (IQR) | 16 (12.5-41.5) | 10 (7-13) | 17 (12.8-28.5) | 22 (14-30) | 35 (25-45) | | |
| Serum folate excluding outliers(nM) | Mean (SD) | 19.8 (12.45) | 10 (4.24) | 16 (5.15) | 22 (11.31 ) | 25 | 9 | 42 |
| | Median (IQR) | 15.5 (11.3-32.5) | 10 (7-13) | 16 (11.5-20.5) | 22 (14-30) | 25 | | |
| RBC folate (nM) | Mean (SD) | 1115 (483.7) | 605 (241.8) | 947.5 (769) | 1962 (760.8) | 1542 (635.7) | 540 | 1208 |
| | Median (IQR) | 843 (747-1618) | 605 (434-776) | 665.5 (533-1234) | 1962 (1424-2500) | 1542 (1092-1991) | | |
| RBC folate excluding outliers (nM) | Mean (SD) | 1115 (483.7) | 605 (241.8) | 637 (126.9) | 1424 | 1542 (635.7) | 540 | 1208 |
| | Median (IQR) | 843 (747-1618) | 605 (434-776) | 620 (521-761.5) | 1424 | 1542 (1092-1991) | | |
| Cys (µM) | Mean (SD) | 270 (44.01) | 266.7 (37.8) | 260.1 (56.3) | 261.1 (24.12) | 259.8 (6.58) | 221.6 | 270.8 |
| | Median (IQR) | 261.1 (233-314.9) | 266.7 (240-293.4) | 273.3 (203.6-310) | 261.1 (244.1-278.2) | 259.8 (255.1-264.4) | | |
| Hcy (µM) | Mean (SD) | 9.9 (3.31) | 10.8 (0.5) | 8 (2.48) | 7.7 (0.2) | 10 (1.88) | 10.2 | 8.9 |
| | Median (IQR) | 10.04 (6.7-13.0) | 10.81 (10.5-11.2) | 7.05 (6.1-10.5) | 7.7 (7.6-7.9) | 10 (9.6-12.7) | | |
| Cys-Gly (µM) | Mean (SD) | 21.9 (9.6) | 21.7 (8.61) | 20.8 (2.28) | 28.3 (4.81) | 21.9 (2.4) | 30 | 21.4 |
| | Median (IQR) | 23.06 (12.3-30.3) | 21.67 (15.6-27.8) | 20.82 (18.6-22.9) | 28.3 (24.9-31.7) | 21.95 (20.3-23.6) | | |
| GSH (µM) | Mean (SD) | 10.6 (4.97) | 14.51 (7.11) | 9.6 (2.3) | 10.8 (1.78) | 9.5 (2.72) | 16.1 | 8.6 |
| | Median (IQR) | 11.31 (5.5-15) | 14.51 (9.5-19.5) | 9.41 (7.6-11.7) | 10.82 (9.6-12.1) | 9.5 (8.6-11.4) | | |
| Serum $B_{12}$ (pM) | Mean (SD) | 308.6 (116.8) | 283.5 (26.16) | 315.2 (210.1) | 322.5 (84.15) | 259.5 (34.65) | 225 | 341 |
| | Median (IQR) | 281 (218.5-412.5) | 283.5 (265-302) | 221 (176.5-481.8) | 322.5 (263-382) | 259.5 (235-284) | | |

Appendix 13: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the combined MTHFR C677T-A1298C-G1793A genotypes for control and adenomatous polyp subjects (1 of 3)

| | | CT/AA/GG | | CC/AC/GG | | CC/AA/GG | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 426 (125.5) | 423.5 (264.5) | 433.1 (200.2) | 429.7 (151.4) | 514.9 (239.6) | 323.1 (46.02) |
| | Median (IQR) | 407.9 (307.7-508.4) | 319.2 (194.5-663.8) | 368 (267.3-584.7) | 488.2 (285.3-539.1) | 443.4 (349.6-691) | 325 (277.9-366) |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 308.2 (96.55) | 314 (184.1) | 312.6 (132.7) | 370.6 (113.1) | 353.5 (121) | 257.7 (94.18) |
| | Median (IQR) | 304.9 (229.6-374.6) | 230 (166.4-464.3) | 278.9 (227.4-358.9) | 402.8 (285.3-421.4) | 331.4 (277.6-424.7) | 236.6 (174.1-366) |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 132.5 (138.9) | 153.3 (172.3) | 108 (144.7) | 59.13 (68.93) | 167.1 (166) | 65.4 (68.96) |
| | Median (IQR) | 90.41 (22.9-150.2) | 95.4 (21.9-320.9) | 56.6 (0-172.7) | 27 (3.6-90.9) | 101 (67.2-259.4) | 52.17 (0-143.4) |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 4.7 (4.531) | 5.5 (7.394) | 6.1 (9.843) | 4.1 (2.531) | 7.8 (10.52) | 4.1 (2.862) |
| | Median (IQR) | 2.85 (2.4-4.7) | 2.413(1.5-6.5) | 2.697 (1.6-4.3) | 3.799 (2.3-5.7) | 2.753 (2.1-6.2) | 2.797 (2.1-6.4) |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 13.4 (15.73) | 8.4 (4.963) | 12.1 (10.55) | 15 (11.08) | 21.6 (32.62) | 11.1 (7.703) |
| | Median (IQR) | 10.01 (6.4-14.1) | 7.937 (3.6-13.7) | 9.654 (5.6-15.8) | 10.21 (9.6-15.6) | 11.22 (5.3-17.4) | 10.02 (4.2-16) |
| Total dietary methionine intake (g/day) | Mean (SD) | 2.26 (0.779) | 1.823 (0.521) | 2.106 (0.627) | 3.608 (3.112) | 2.287 (0.811) | 1.871 (0.768) |
| | Median (IQR) | 1.971 (1.864-2.467) | 1.689 (1.467-1.959) | 2.183 (1.57-2.464) | 2.476 (2.368-2.893) | 2.123 (1.867-2.326) | 1.673 (1.223-2.464) |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 44.1 (9.713) | 41.81 (15.5) | 45.66 (16.6) | 48 (10.31) | 48.07 (14.23) | 40.45 (10.87) |
| | Median (IQR) | 42.64 (37.60-46.61) | 39.02 (30.59-50.43) | 43.84 (32.51-54.0) | 47.32 (36.09-55.69) | 46.03 (38.42-55.39) | 41.23 (29.96-47.53) |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 3.9 (3.824) | 3.3 (3.52) | 4.8 (5.312) | 4.9 (5.585) | 6 (8.442) | 4 (5.069) |
| | Median (IQR) | 2.792 (2-3.5) | 1.755 (1.2-5.9) | 2.45 (1.9-6.7) | 2.641 (2.4-4.2) | 2.91 (1.9-4.3) | 2.081 (1.5-5.3) |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 223.5 (136.8) | 194.3 (191.7) | 22.98 (160.3) | 255.2 (171.5) | 243.3 (103.9) | 200.6 (92.78) |
| | Median (IQR) | 185.6 (119.5-270) | 109.5 (58.5-309.6) | 216.7 (126.3-280.9) | 239.4 (160.5-319.8) | 255 (147.2-311.1) | 232 (100.1-270.8) |

Appendix 14: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the combined MTHFR C677T-A1298C-G1793A genotypes for control and adenomatous polyp subjects (2 of 3)

| | | CT/AC/GG | | TT/AA/GG | | CC/CC/GG | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 415.5 (187.6) | 451.1 (174) | 593.8 (546.7) | 428.8 (157.6) | 287.9 (98.01) | 613 (206.2) |
| | Median (IQR) | 375.5 (320.4-600) | 421.6 (312.4-604.4) | 425.8 (325.7-615.9) | 388.8 (312.6-536.1) | 312.1 (191.4-347.9) | 601.6 (425-812.5) |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 311.8 (116.2) | 313.9 (95.62) | 336.2 (136.1) | 331.9 (104.7) | 250.9 (95.02) | 431.4 (130.9) |
| | Median (IQR) | 308.5 (244.1-372) | 335.8 (214.8-402) | 339.8 (244.8-374.6) | 350.3 (263.1-398.4) | 232.1 (191.4-314.9) | 428.3 (312.4-553.4) |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 103.7 (120.3) | 137.2 (206) | 257.6 (548.2) | 96.9 (95.48) | 36.9 (49.69) | 174.6 (163.1) |
| | Median (IQR) | 65.28 (2.5-144.1) | 58.35 (22.5-291.2) | 90.9 (44.9-199.5) | 82.8 (18-166.1) | 0 (0-90.9) | 124.2 (57.2-342.5) |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 6.1 (10.24) | 5.2 (2.06) | 6.1 (7.9) | 4.1 (1.83) | 2 (0.622) | 7.4 (7.05) |
| | Median (IQR) | 2.43 (1.8-6.8) | 5.47 (3.2-7.1) | 2.73 (2.2-5.1) | 3.78 (2.4-5.9) | 2.06 (1.4-2.6) | 4.7 (2.6-14.8) |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 52.6 (194.2) | 13.7 (3.22) | 10.7 (9.94) | 7.9 (3.47) | 10.5 (6.71) | 9.9 (5.66) |
| | Median (IQR) | 11 (4.4-17.6) | 15.1 (10.3-16.4) | 5.64 (4.6-12.9) | 7.52 (5.3-10.5) | 10.9 (4-15.4) | 10.3(4.4-15.1) |
| Total dietary methionine intake (g/day) | Mean (SD) | 1.933 (0.561) | 2.56 (0.96) | 2.11 (1.147) | 2.8 (2.24) | 2.15 (0.83) | 2.81 (1.42) |
| | Median (IQR) | 1.89 (1.6-2.28) | 2.1(2.01-3.34) | 1.88 (1.63-2.15) | 1.98 (1.75-3.63) | 1.87 (1.76-2.29) | 2.42 (1.70-4.3) |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 42.48 (11.33) | 51.49 (20.44) | 43.64 (13.53) | 44.9 (10.18) | 38.39 (11.45) | 52.05 (8.34) |
| | Median (IQR) | 41.7 (34.43-49.23) | 44 (40.67-66.06) | 41.75 (33.84-51.53) | 48.97 (36.31-52.49) | 37.18 (25.96-51.52) | 50.8 (44-96-60.37) |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 5.7 (7.39) | 5.2 (6.1) | 4.9 (4.36) | 2.7 (1.304) | 2.1 (1.54) | 5 (3.87) |
| | Median (IQR) | 2.41 (1.7-5.2) | 2.19 (1.8-10) | 2.387 (1.8-10.4) | 2.398 (1.9-3.3) | 1.63 (1.2-2.5) | 4.06 (2.0-9.0) |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 335.2 (446.7) | 179.1 (52.02) | 251 (123.9) | 267.5 (1.3) | 168.3 (86.7) | 215.5 (95.7) |
| | Median (IQR) | 220.1 (155-292.2) | 174.6 (130.2-230.2) | 221.5 (145.7-321.6) | 221.1 (134.1-452.3) | 145.6 (110-235.4) | 209.5 (127.9-309.2) |

Appendix 15: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the combined MTHFR C677T-A1298C-G1793A genotypes for control and adenomatous polyp subjects (3 of 3)

| | | CT/AC/AG | | CC/AC/AG | CC/CC/AA | CC/CC/AG | TT/CC/GG | CT/AA/AG |
|---|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | Control | Control | Control (n=1) | Control (n=1) |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 460.7 (123.2) | 447 (53.64) | 562.1 (413.8) | 383.6 (14.58) | 418 (6.68) | 514.7 | 1296 |
| | Median (IQR) | 487.8 (344.6-563.2) | 447 (409.1-484.9) | 406.3 (287.9-890.9) | 383.6 (373.2-393.9) | 418 (413.3-422.7) | | |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 353.4 (47.12) | 330.1 (108.7) | 369 (293.4) | 312.8 (43.13) | 374.3 (4.997) | 514.7 | 396.1 |
| | Median (IQR) | 354 (308.4-398.1) | 330.1 (253.2-407) | 272.1 (183.6-506.6) | 312.8 (282.3-343.3) | 374.3 (370.8-377.9) | | |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 107.3 (123.1) | 116.9 (55.1) | 109.8 (130.3) | 70.7 (28.55) | 43.7 (11.67) | 0 | 899.9 |
| | Median (IQR) | 75 (5.8-225) | 116.9 (77.9-155.8) | 86.96 (11.6-172.9) | 70.72 (50.5-90.9) | 43.69 (35.4-51.9) | | |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 4.4 (2.41) | 2.8 (0.11) | 11 (12.69) | 2.9 (0.874) | 2.8 (0.658) | 4.1 | 62.5 |
| | Median (IQR) | 3.17 (2.4-7) | 2.771 (2.7-2.8) | 4 (2.3-24) | 2.923 (2.3-3.5) | 2.793 (2.3-3.3) | | |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 16.1 (4.639) | 15.41 (1.1) | 40.6 (47.54) | 4 (0.84) | 19 (14.21) | 7.9 | 106.1 |
| | Median (IQR) | 14.75 (12.1-20.8) | 15.41 (14.6-16.2) | 20.81 (6.3-80.6) | 4.05 (3.5-4.6) | 18.96 (8.9-29) | | |
| Total dietary methionine intake (g/day) | Mean (SD) | 2.59 (0.52) | 2.51 (0.34) | 3.5 (2.889) | 1.844 (0.086) | 2.38 (0.548) | 2.69 | 2.7 |
| | Median (IQR) | 2.596 (2.11-3.06) | 2.51 (2.27-2.74) | 2.48 (1.76-5.1) | 1.84 (1.78-1.91) | 2.379 (1.99-2.77) | | |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 51.78 (10.1) | 58.13 (4.19) | 63.65 (47.1) | 37.87 (6.314) | 47.4 (8.55) | 53.45 | 53.99 |
| | Median (IQR) | 49.42 (44.21-60.54) | 58.13 (55.17-61.09) | 50.96 (32.92-87.97) | 37.87 (33.4-42.33) | 47.4 (41.35-53.44) | | |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 6.8 (6.42) | 2.8 (0.3) | 8.4 (9.47) | 2.4 (0.099) | 2.3 (0.737) | 9.9 | 22.6 |
| | Median (IQR) | 3.43 (2.1-13.1) | 2.8 (2.6-3) | 3.15 (2.1-18.2) | 2.37 (2.3-2.4) | 2.25 (1.7-2.8) | | |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 222.2 (84.96) | 131 (81.64) | 262 (201.3) | 176.3 (16.79) | 1125 (379) | 250.9 | 471.2 |
| | Median (IQR) | 276.3 (131-286.4) | 131 (73.3-188.8) | 146.8 (135.8-445.5) | 176.3 (164.4-188.1) | 1125 (856.7-1393) | | |

Appendix 16: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each DHFR 19del genotypes for control and adenomatous polyp subjects

| | | WT/WT | | WT/del | | del/del | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 22.66 (12.67) | 22.22 (13.08) | 21.14 (11.61) | 19.21 (9.815) | 20.65 (11.53) | 28.67 (16.55) |
| | Median (IQR) | 21 (10.5-31.0) | 16.5 (13.75-33.25) | 18 (12.0-29.0) | 18 (10.75-28.25) | 17 (11.0-24.0) | 32(10.0-45.0) |
| Serum folate excluding outliers(nM) | Mean (SD) | 19.81 (10.4) | 19.38 (10.78) | 18.45 (8.822) | 19.21 (9.815) | 18.04 (8.639) | 20.5 (13.77) |
| | Median (IQR) | 19 (9.0-29.0) | 15.5 (13.25-27.50) | 17 (12.0-24.0) | 18 (10.75-28.25) | 16 (11.0-23.0) | 19.5 (8.0-34.0) |
| RBC folate (nM) | Mean (SD) | 1043 (559.2) | 1140.5 (583.8) | 944.4 (510.4) | 696.9 (601) | 925.7 (403.9) | 839.8 (503) |
| | Median (IQR) | 952 (617-1170) | 1047 (609.3-1462) | 814 (592-1172) | 784.5 (482.8-1352) | 878 (610-1076) | 601.5 (479-1423) |
| RBC folate excluding outliers (nM) | Mean (SD) | 921.5 (379) | 1061 (489.7) | 903.9 (449.5) | 969.93 (601) | 925.68 (403.9) | 839.83 (503) |
| | Median (IQR) | 879 (588-1137) | 1000 (583.5-1375) | 805 (586.5-1159) | 784.5 (482.8-1352) | 878 (610-1076) | 601.5 (479-1423) |
| Cys (μM) | Mean (SD) | 274.2 (37.55) | 276.1 (36.62) | 258.9 (42.6) | 252.76 (32.3) | 258.1 (35.76) | 270.3 (55.97) |
| | Median (IQR) | 271.7 (242.6-294) | 269.9 (246.2-310) | 260.6 (230.7-285.5) | 259.1 (225-277.8) | 258.6 (239.8-287.7) | 251.4 (233.9-309.4) |
| Hcy (μM) | Mean (SD) | 10.32 (2.837) | 10.54 (2.792) | 9.78 (3.121) | 10.03 (2.695) | 9.54 (2.307) | 9.52 (2.989) |
| | Median (IQR) | 9.84 (7.85-12.07) | 9.84 (8.33-12.74) | 9.105 (7.44-11.76) | 9.59 (8.44-12.07) | 9.34 (7.44-11.36) | 9.12 (6.91-11.74) |
| Cys-Gly (μM) | Mean (SD) | 24.69 (5.684) | 25.41 (6.91) | 23.56 (4.473) | 24.27 (4.392) | 22.08 (3.931) | 22.51 (4.581) |
| | Median (IQR) | 23.33 (20.54-27.05) | 25.5 (20.34-27.77) | 23.27 (20.84-26.08) | 24.77 (20.54-273.4) | 21.28 (19.2-24.83) | 20.46 (19.42-27.56) |
| GSH (μM) | Mean (SD) | 14.7 (6.615) | 13.1 (6.256) | 14.08 (5.49) | 15.07 (4.499) | 12.45 (5.011) | 11.45 (2.071) |
| | Median (IQR) | 13.12 (10.55-17.68) | 9.48 (8.54-16.42) | 11.88 (10.39-18.26) | 16.03 (10.84-19.32) | 10.49 (9.07-15.08) | 10.9 (9.87-13.91) |
| Serum $B_{12}$ (pM) | Mean (SD) | 284.7 (155.9) | 274.5 (100) | 280.5 (169.9) | 289.71 (187.7) | 270.74 (84.11) | 276.3 (96.07) |
| | Median (IQR) | 245 (185-334) | 257.5 (201-326.2) | 249 (194-310) | 246.5 (180.5-363.5) | 252 (207-336) | 279.5 (212-344.3) |

Appendix 17: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each DHFR 19del genotypes for control and adenomatous polyp subjects (2of 2)

| | | WT/WT | | WT/del | | del/del | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 439.5 (177.8) | 432.8 (137.1) | 455.9 (280.8) | 479.2 (239.5) | 498.7 (277.5) | 335.0 (114.9) |
| | Median (IQR) | 393.9 (319.7-565.7) | 416 (338.4-491.4) | 402.2 (300.9-581) | 442.7(274.1-703.6) | 406.7 (331.1-603.2) | 350.6 (238.1-408.8) |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 327.13 (129.2) | 314.64 (98.48) | 312.53 (108.6) | 367.54 (162.3) | 342.23 (150.5) | 298.21 (112.2) |
| | Median (IQR) | 322.1 (232.1-377.9) | 309 (220.5-407.4) | 298.6 (242.5-372.9) | 384.4 (178.6-484.8) | 324.6 (245.9-383.2) | 338.8 (161.7-385.6) |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 104.8 (129.8) | 118.11 (118.6) | 146.5 (260.7) | 134.67 (160) | 156.51 (185.4) | 36.83 (47.17) |
| | Median (IQR) | 64.93 (3.03-115.8) | 90.9 (33.14-148) | 75 (12.60-149.4) | 82.71 (20.25-193.7) | 93.56 (34.08-223.8) | 13.64 (0.52-93.71) |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 6.31 (9.363) | 4.19 (2.404) | 5.74 (8.318) | 6.25 (6.362) | 7.34 (12.1) | 3.32 (1.985) |
| | Median (IQR) | 2.875 (2.23-4.64) | 3.323 (2.26-5.86) | 2.666 (2.01-4.37) | 4.99 (2.16-6.76) | 2.59 (2.16-6.36) | 2.713 (2.16-4.29) |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 15.15 (20.23) | 11.47 (5.463) | 28.97 (113.2) | 10.56 (4.751) | 15.65 (20.06) | 11.8 (13.4) |
| | Median (IQR) | 9.699 (5.06-15.04) | 11 (6.82-15.51) | 10.89 (5.64-16.5) | 9.96 (5.95-15.20) | 10.73 (5.64-16.99) | 7.11(4.87-16.5) |
| Total dietary methionine intake (g/day) | Mean (SD) | 2.26 (0.917) | 2.19 (0.742) | 2.16 (0.712) | 2.16 (0.868) | 2.33 (1.337) | 4.55 (3.624) |
| | Median (IQR) | 1.933 (1.76-2.62) | 2.05 (1.66-2.54) | 2.018 (1.71-2.46) | 1.965 (1.70-2.39) | 2.14 (1.86-2.45) | 3.001 (1.8-8.12) |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 44.53 (15.04) | 47.15 (14.01) | 44.8 (12.49) | 45.61 (13.48) | 48.98 (21.57) | 46.18 (10.49) |
| | Median (IQR) | 40.51 (35.31-53.45) | 44.15 (39.29-53.69) | 43.75 (37.81-50.44) | 46.28 (36.36-55.3) | 45.77 (38.56-53.44) | 49.74 (33.77-54.05) |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 5.11 (6.204) | 4.79 (5.245) | 4.99 (6.426) | 3.63 (3.282) | 5.14 (5.352) | 2.57 (0.89) |
| | Median (IQR) | 2.476 (2.88-4.58) | 2.481 (1.77-4.65) | 2.488 (1.86-3.69) | 2.278 (1.70-4.54) | 2.87 (1.95-5.01) | 2.359 (1.90-3.12) |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 312.2 (352) | 209.32 (117) | 240.71 (173.9) | 244.64 (173.1) | 218.04 (111.2) | 162.62 (99.92) |
| | Median (IQR) | 253.2 (138.2-310.2) | 189.9 (112.8-256.5) | 192.6 (134.8-290.8) | 213 (87.87-325.9) | 186.1 (145.4-275.7) | 193.9 (49.89-245.2) |

Appendix 18: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of RFC G80A genotypes for control and adenomatous polyp subjects

| | | Wild type (GG) | | Heterozygous type (GA) | | Homo recessive type (AA) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 22.93 (13.04) | 21.36 (13.44) | 20.96 (11.11) | 20.98 (11.15) | 20.42 (11.69) | 27 (14.37) |
| | Median (IQR) | 20 (12.0-32.0) | 17 (12.0-32.5) | 18 (12.0-29.0) | 17.5 (13.25-30.75) | 20 (10.0-30.0) | 25.5 (12.25-43.0) |
| Serum folate excluding outliers(nM) | Mean (SD) | 18.79 (9.589) | 19.54 (12.06) | 18.81 (8.794) | 18.73 (9.331) | 18.84 (10.14) | 21 (10.79) |
| | Median (IQR) | 17 (11.25-24.75) | 16 ( 11.0-29.0) | 17 (12.0-24.0) | 17 (13.0-27.0) | 17 (10.0-29.0) | 18 (11.0-32.5) |
| RBC folate (nM) | Mean (SD) | 999.25 (492.3) | 1056 (687) | 983.4 (531.2) | 967.3 (475.1) | 901.3 (490.2) | 1110.8 (608.9) |
| | Median (IQR) | 908.5 (694.2-1149) | 924 (509-1465) | 814 (601-1180) | 1019 (507.3-1321) | 812 (487.5-1141) | 879 (601.5-1708) |
| RBC folate excluding outliers (nM) | Mean (SD) | 943.67 (404.2) | 944.9 (569.3) | 919 (436.1) | 967.3 (475.1) | 851.3 (403.8) | 1110.8 (608.9) |
| | Median (IQR) | 885.5 (688.5-1121) | 848 (486-1302) | 787 (594.3-1149) | 1019 (507.3-1321) | 811.5 (484.3-1104) | 879 (601.5-1708) |
| Cys (μM) | Mean (SD) | 259.8 (40.94) | 265.11 (40.29) | 272.5 (40.61) | 249.75 (28.58) | 251.5 (34.26) | 291.31 (43.49) |
| | Median (IQR) | 256.7 (233.5-286) | 260.1 (225.4-294.7) | 271.9 (247.8-295.1) | 252.1 (223.6-277) | 246 (233.4-271.4) | 278.7 (262.8-321.6) |
| Hcy (μM) | Mean (SD) | 9.4 (2.654) | 10.69 (2.815) | 10.34 (2.851) | 10.04 (2.809) | 9.83 (3.297) | 9.55 (2.682) |
| | Median (IQR) | 8.94 (7.35-11.23) | 10.46 (8.35-12.32) | 10.06 (8.51-12.08) | 9.035 (8.4-13.1) | 9.285 (7.52-11.29) | 9.21 (7.21-11.28) |
| Cys-Gly (μM) | Mean (SD) | 23.62 (4.475) | 28.08 (5.734) | 23.79 (5.175) | 20.61 (3.689) | 23.46 (5.012) | 25.05 (4.169) |
| | Median (IQR) | 22.84 (20.47-26.72) | 26.92 (25.0-29.47) | 23.32 (20.43-26.78) | 19.94 (17.85-23.85) | 22.28 (20.51-26.32) | 25.71 (20.73-27.83) |
| GSH (μM) | Mean (SD) | 13.67 (4.773) | 12.76 (4.088) | 14.49 (6.635) | 12.76 (4.511) | 13.38 (5.602) | 15.79 (6.814) |
| | Median (IQR) | 12.03 (10.14-16.83) | 11.25 (9.36-15.97) | 12.51 (9.80-18.4) | 11.33 (8.99-16.97) | 11.79 (9.93-15.28) | 14.88 (10.76-20.6) |
| Serum $B_{12}$ (pM) | Mean (SD) | 285.4 (130.5) | 291.29 (196.5) | 273.9 (154.8) | 282.13 (78.12) | 284.06 (180.8) | 104.88 (104.9) |
| | Median (IQR) | 259 (197-342) | 249.5 (162.2-323.8) | 240 (189.5-307.5) | 266 (233-347.3) | 245 (184.0-312.5) | 245 (194.7-316) |

Appendix 19: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of RFC G80A genotypes for control and adenomatous polyp subjects

| | | Wild type (GG) | | Heterozygous type (GA) | | Homo recessive type (AA) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 456.7 (307.6) | 387.4 (153.7) | 444.5 (204.9) | 409.6 (160.6) | 495.7 (241.6) | 566.5 (221.7) |
| | Median (IQR) | 387.8 (327.9-493.2) | 373.6 (281.1-498.5) | 406.6 (312.8-518.1) | 401.6 (277.3-497.8) | 425.8 (315.8-624.1) | 489.4 (399.5-808.7) |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 323.3 (114.6) | 284.39 (112) | 311.3 (112.7) | 347.31 (147.2) | 348.29 (159.2) | 382.48 (89.97) |
| | Median (IQR) | 293.2 (249.8-366.2) | 276.6 (178.9-383.1) | 328.7 (220.9-373.4) | 349.1 (231.5-421.3) | 306.7 (255.3-397.2) | 420 (348.2-429.9) |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 129.9 (281.2) | 102.97 (131.2) | 128.5 (169.9) | 82.4 (96.74) | 147.43 (158) | 184.05 (170.6) |
| | Median (IQR) | 80.84 (14.80-142.3) | 82.71 (2.85-143.4) | 75 (15.94-134.1) | 69.14 (13.95-109.5) | 95.24 (4.89-279.4) | 113.6 (51.10-372.3) |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 6.91 (10.93) | 4.37 (2.758) | 5.91 (9.015) | 4.01 (1.928) | 5.76 (7.669) | 7.19 (8.285) |
| | Median (IQR) | 2.57 (2.07-5.57) | 2.85 (2.15-6.90) | 2.709 (2.17-4.44) | 3.891 (2.31-5.51) | 3.038 (2.07-5.19) | 3.01 (2.36-14.29) |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 18.48 (24.27) | 12.77 (9.595) | 27.42 (117) | 10.42 (4.636) | 15.81 (22.65) | 9.94 (4.338) |
| | Median (IQR) | 9.222 (5.79-15.29) | 9.915 (5.44-16.33) | 10.28 (5.48-16.00) | 10.51(5.10-14.89) | 10.34 (5.79-15.29) | 7.972 (6.60-15.09) |
| Total dietary methionine intake (g/day) | Mean (SD) | 2.27 (0.905) | 2.57 (2.35) | 2.13 (0.697) | 2.56 (1.61) | 2.35 (1.328) | 2.5 (0.6) |
| | Median (IQR) | 2.122 (1.73-2.59) | 1.944 (1.63-2.44) | 1.965 (1.81-2.43) | 2.024 (1.49-2.86) | 2.125 (2.35-1.75) | 2.595 (1.84-3.05) |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 45.73 (13.92) | 42.89 (8.595) | 43.26 (11.39) | 46.99 (17.83) | 50.25 (23.25) | 51.51 (4.659) |
| | Median (IQR) | 43.66 (36.61-54.84) | 44.15 (33.74-48.58) | 42.31 (35.39-49.26) | 43.42 (33.54-61.58) | 45.04 (38.95-51.66) | 51.95 (46.72-55.40) |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 5.24 (6.608) | 5.01 (5.948) | 4.58 (5.417) | 2.55 (1.471) | 5.81 (6.793) | 5.19 (3.497) |
| | Median (IQR) | 2.488 (1.88-4.19) | 2.342 (1.57-5.63) | 2.451 (1.56-3.71) | 2.063 (1.79-2.92) | 2.874 (2.02-7.56) | 4.33 (2.27-9.19) |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 294.54 (329.5) | 181.74 (134.7) | 255.09 (196.7) | 207.41 (132.3) | 206.6 (89.95) | 288.2 (143.2) |
| | Median (IQR) | 217.7 (136-310.1) | 154 (98.76-227.7) | 219.4 (149.4-294.5) | 202.4 (96.51-264.6) | 209 (126.3-270.5) | 290.7 (206-416.7) |

Appendix 20: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of GCP II C1561T genotypes for control and adenomatous polyp subjects

| | | Wild type (CC) | | Heterozygous type (CT) | | Homo recessive type (TT, n=0) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 21.76 (11.94) | 22.58 (13.21) | 18.83 (11.62) | 20.14 (10.3) | N/A | N/A |
| | Median (IQR) | 19 (12.0-30.0) | 18 (13.0-32.0) | 15 (9.0-27.75) | 16 (14.0-28.0) | | |
| Serum folate excluding outliers(nM) | Mean (SD) | 19 (9.347) | 19.26 (10.61) | 16.45 (8.595) | 20.14 (10.3) | N/A | N/A |
| | Median (IQR) | 17 (11.0-25.0) | 17 (11.0-30.0) | 14 (9.0-24.0) | 16 (14.0-28.0) | | |
| RBC folate (nM) | Mean (SD) | 988.1 (499.6) | 1034 (558.3) | 776.8 (592.8) | 1010 (693.5) | N/A | N/A |
| | Median (IQR) | 881.5 (611.5-1170) | 965 (560-1373) | 584 (476.3-795) | 558 (497-1575) | | |
| RBC folate excluding outliers (nM) | Mean (SD) | 935.9 (419.7) | 985.9 (496) | 620.1 (250.1) | 1010 (693.5) | N/A | N/A |
| | Median (IQR) | 869 (608.5-1144) | 932.5 (548.5-1335) | 548 (475-744) | 558 (497-1575) | | |
| Cys (μM) | Mean (SD) | 263.4 (40.31) | 257.6 (31.96) | 268.5 (40.01) | 300.6 (51.61) | N/A | N/A |
| | Median (IQR) | 261.8 (237-287.3) | 256.9 (233.5-275.6) | 270.7 (240-306.5) | 300 (269.2-337.6) | | |
| Hcy (μM) | Mean (SD) | 9.91 (2.95) | 9.76 (2.337) | 10.01 (2.231) | 11.74 (3.812) | N/A | N/A |
| | Median (IQR) | 9.59 (7.54-11.69) | 9.07 (8.30-11.38) | 9.825 (8.09-12.02) | 12.67 (7.89-14.95) | | |
| Cys-Gly (μM) | Mean (SD) | 23.71 (4.832) | 24.23 (5.844) | 23.19 (5.533) | 25.16 (4.497) | N/A | N/A |
| | Median (IQR) | 23.23 (20.64-26.77) | 25 (19.98-27.68) | 21.03 (19.90-25.69) | 25.43 (20.52-28.72) | | |
| GSH (μM) | Mean (SD) | 13.84 (5.695) | 13.88 (5.354) | 15.52 (7.156) | 12.14 (3.686) | N/A | N/A |
| | Median (IQR) | 12.03 (9.99-16.96) | 11.41 (9.39-18.08) | 13.64 (9.74-20.41) | 12.61 (8.23-15.84) | | |
| Serum $B_{12}$ (pM) | Mean (SD) | 279.05 (149.7) | 287.94 (143.7) | 291.92 (183.4) | 247 (88.2) | N/A | N/A |
| | Median (IQR) | 251 (196-320) | 265 (195-325) | 239 (158.5-370.5) | 242 (165-330) | | |

Appendix 21: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of GCP II C1561T genotypes for control and adenomatous polyp subjects

| | | Wild type (CC) | | Heterozygous type (CT) | | Homo recessive (TT, n=0) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 458.2 (248.3) | 451.7 (195.7) | 447.4 (299.7) | 358.1 (57.74) | N/A | N/A |
| | Median (IQR) | 403.1 (321-571) | 421.6 (280.9-575.6) | 353.6 (267.3-560.8) | 376.5 (298.1-400.4) | | |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 319.4 (114.1) | 339.3 (131.1) | 369 (216) | 297 (111.3) | N/A | N/A |
| | Median (IQR) | 307.6 (240.8-374.6) | 338.8 (224.2-427.2) | 294.5 (261.1-443.7) | 361.8 (180.9-376.5) | | |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 137 (217.8) | 122.7 (138.9) | 78.4 (114.5) | 61.12 (64.12) | N/A | N/A |
| | Median (IQR) | 79.92 (15.01-150.4) | 90.9 (23.71-136.35) | 28.15 (0-90.9) | 38.57 (0.69-142.7) | | |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 6.32 (9.535) | 4.83 (4.662) | 5.19 (8.612) | 4.75 (2.631) | N/A | N/A |
| | Median (IQR) | 2.75 (2.06-4.94) | 2.74 (2.28-5.67) | 2.391 (1.84-3.83) | 3.799 (3.01-6.87) | | |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 22.57 (83.1) | 11.05 (6.928) | 14.07 (16.96) | 11.77 (6.643) | N/A | N/A |
| | Median (IQR) | 10.28 (5.56-16.50) | 10 (5.39-15.08) | 8.8 (5.04-14.89) | 9.377 (6.59-15.62) | | |
| Total dietary methionine intake (g/day) | Mean (SD) | 2.19 (0.755) | 2.61 (1.91) | 2.66 (2.147) | 2.3 (0.741) | N/A | N/A |
| | Median (IQR) | 2.01 (1.76-2.46) | 2.064 (1.67-2.48) | 2.041 (1.65-2.93) | 1.945 (1.87-3.0) | | |
| Total dietary niacin intake equivalent (g/day) | Mean (SD) | 44.95 (12.62) | 46.05 (13.53) | 52.94 (35.66) | 48.13 (11.2) | N/A | N/A |
| | Median (IQR) | 43.66 (37.18-51.79) | 45.35 (36.09-52.84) | 41.85 (35.71-54.32) | 51.19 (39.5-58.07) | | |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 5.15 (6.226) | 4.06 (4.118) | 3.92 (4.691) | 3.8 (4.617) | N/A | N/A |
| | Median (IQR) | 2.719 (1.90-4.48) | 2.599 (1.78-4.21) | 2.198 (1.39-2.97) | 2.124 (1.94-2.33) | | |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 261 (247.7) | 224.7 (145.7) | 238.8 (83.69) | 171.8 (89.78) | N/A | N/A |
| | Median (IQR) | 207.3 (136.5-283.8) | 194.8 (112.5-304.4) | 257.1 (143.2-291.2) | 201.9 (63.1-240.4) | | |

Appendix 22: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the combined RFC G80A- GCPII C1561T genotypes for control and adenomatous polyp subjects (1 of 2)

| | | GA/CC | | GG/CC | | AA/CC | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 21.3 (11.2) | 20.8 (11.41) | 22.25 (13.1) | 19.9 (14.41) | 21.96 (11.81) | 31.5 (13.77) |
| | Median (IQR) | 18 (12.0-29.25) | 18 (13.0-32.0) | 19 (12.0-32.0) | 14.5 (8.5-33.0) | 22.5 (10.0-30.75) | 34 (17.75-45) |
| Serum folate excluding outliers(nM) | Mean (SD) | 19.08 (8.88) | 19.07 (9.59) | 18.2 (9.59) | 17.11 (12.1) | 20.19 (10.24) | 24.75 (11.56) |
| | Median (IQR) | 17.5 (12.0-24.75) | 17.5 (12.25-28.25) | 14 (11.0-24.0) | 13 (8.0-25.0) | 21 (10.0-30.0) | 25.5 (13.25-35.5) |
| RBC folate (nM) | Mean (SD) | 1005 (532.6) | 1000 (472.6) | 977.5 (460) | 924.3 (663.4) | 966.6 (501.4) | 1305.2 (580.8) |
| | Median (IQR) | 879 (609.3-1192) | 1138 (514-1322) | 908.5 (694.3-1139) | 812 (423.5-1178) | 841.5 (546.3-1245) | 1169 (777.8-1902) |
| RBC folate excluding outliers (nM) | Mean (SD) | 937.52 (436.3) | 1000.2 (472.6) | 947.6 (410.5) | 749.2 (387.6) | 909.85 (409) | 1305.2 (580.8) |
| | Median (IQR) | 814 (607-1169) | 1138 (514-1322) | 893 (691-1113) | 776 (407-1047) | 840 (539-1175) | 1169 (777.8-1902) |
| Cys (μM) | Mean (SD) | 270.7 (40.62) | 246.4 (27.39) | 259.1 (41.57) | 259.9 (36.26) | 252.89 (34.62) | 275.1 (30.15) |
| | Median (IQR) | 271.7 (245.8-292.4) | 251.7 (218.3-264.2) | 256.7 (233.5-285.8) | 255.6 (226.4-286.8) | 247.2 (233.4-273.9) | 268.6 (255.4-297.5) |
| Hcy (μM) | Mean (SD) | 10.3 (2.91) | 9.62 (2.5) | 9.36 (2.64) | 10.9 (2.112) | 9.93 (3.56) | 8.5 (1.84) |
| | Median (IQR) | 9.98 (8.48-12.07) | 9 (8.30-11.16) | 8.94 (7.32-11.23) | 10.88 (8.79-12.17) | 9.7 (7.49-11.49) | 8.265 (6.9-10.02) |
| Cys-Gly (μM) | Mean (SD) | 23.85 (5.24) | 20.68 (3.86) | 23.29 (4.05) | 28.79 (6.614) | 24.14 (5.24) | 24.67 (3.4) |
| | Median (IQR) | 23.33 (20.71-26.75) | 19.95 (17.83-24.28) | 22.65 (20.47-26.72) | 27.31 (25.07-29.82) | 23.17 (21.71-27.18) | 25.71 (20.62-27.82) |
| GSH (μM) | Mean (SD) | 14.24 (6.3) | 13.25 (4.38) | 13.62 (4.76) | 13.08 (4.405) | 13.22 (5.783) | 16.1 (8.01) |
| | Median (IQR) | 12.41 (9.835-18.34) | 13.17 (9.2-17.2) | 12.03 (10.14-16.8) | 11.03 (9.57-18.21) | 11.72 (9.81-14.37) | 14.13 (9.74-14.37) |
| Serum $B_{12}$ (pM) | Mean (SD) | 269.4 (143.9) | 278.9 (79.78) | 285.8 (133.2) | 302.6 (230.6) | 290.4 (192) | 286 (98.62) |
| | Median (IQR) | 243.5 (196.8-302.3) | 265 (232-353) | 259 (197-338) | 230 (146.36-360) | 248.5 (185.5-313.8) | 268.5 (200.3-358.5) |

Appendix 23: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the combined RFC G80A- GCPII C1561T genotypes for control and adenomatous polyp subjects (2 of 2)

| | | GG/CT | | AA/CT | | GA/CT | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 32 (9.1) | 25 (11.6) | 11.8 (6.54) | 13.5 (3.54) | 13 (3.61) | 14 |
| | Median (IQR) | 29.5 (25.25-41.25) | 23 (15.0-37.0) | 9 (7.5-17.5) | 13.5 (11.0-16.0) | 14 (9.0-16.0) | |
| Serum folate excluding outliers(nM) | Mean (SD) | 27.67 (3.22) | 25 (11.6) | 11.8 (6.54) | 13.5 (3.54) | 13 (3.61) | 14 |
| | Median (IQR) | 29 (24.0-30.0) | 23(15.0-37.0) | 9 (7.5-17.5) | 13.5 (11.0-16.0) | 14 (9.0-16.0) | |
| RBC folate (nM) | Mean (SD) | 1282 (855.6) | 1385.3 (722.8) | 535.4 (168.6) | 527.5 (43.13) | 505.3 (104.3) | 474 |
| | Median (IQR) | 998 (660-2188) | 1376 (693.3-2087) | 481 (418-680) | 527.5 (497-558) | 480 (416-620) | |
| RBC folate excluding outliers (nM) | Mean (SD) | 876 (330.4) | 1385.25 (722.8) | 535.4 (168.6) | 527.5 (43.13) | 505.33 (104.3) | 474 |
| | Median (IQR) | 744 (632-1252) | 1376 (693.3-2086) | 481 (418-680) | 527.5 (497-558) | 480 (416-620) | |
| Cys (μM) | Mean (SD) | 267.5 (37.2) | 279 (55.84) | 244.9 (35.6) | 339.9 (48.99) | 308.97 (18.63) | 286.6 |
| | Median (IQR) | 264.4 (233.6-304.5) | 294.7 (217-325.3) | 240.2 (217.2-275) | 339.9 (305.3-374.5) | 311 (289.5-326.6) | |
| Hcy (μM) | Mean (SD) | 9.86 (3.21) | 10.11 (4.83) | 9.37 (1.85) | 12.67 (2.70) | 11.26 (1.28) | 14.75 |
| | Median (IQR) | 8.86 (7.47-13.26) | 8.4 (6.37-15.56) | 8.86 (7.93-11.07) | 12.67 (10.76-14.58) | 11.85 (9.79-12.14) | |
| Cys-Gly (μM) | Mean (SD) | 27.33 (7.74) | 26.2 (1.996) | 20.35 (1.92) | 26.21 (7.76) | 22.41 (3.98) | 19.92 |
| | Median (IQR) | 25.43 (21.34-35.21) | 26.92 (23.94-27.73) | 20.82 (18.43-22.04) | 26.21 (20.72-31.7) | 20.43 (19.81-27.0) | |
| GSH (μM) | Mean (SD) | 14.27 (5.64) | 11.91 (3.76) | 14.11 (5.2) | 14.88 (1.3) | 19.52 (12.15) | 7.37 |
| | Median (IQR) | 12.77 (9.82-20.22) | 11.25 (8.52-15.96) | 12.35 (9.62-19.48) | 14.88 (13.96-15.8) | 17.83 (8.31-32.43) | |
| Serum $B_{12}$ (pM) | Mean (SD) | 279.8 (101.5) | 263 (78.68) | 248.6 (104.2) | 173.5 (96.87) | 380.33 (362.1) | 330 |
| | Median (IQR) | 292.5 (176.3-370.5) | 277.5 (182.3-329.3) | 229 (159-348) | 173.5 (105-242) | 189 (154-798) | |

Appendix 24: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the combined RFC G80A- GCPII C1561T genotypes for control and adenomatous polyp subjects (1 of 2)

| RFC GCP | | GA/CC | | GG/CC | | AA/CC | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 452.2 (205.3) | 407.9 (166.1) | 463.6 (317.6) | 399.6 (181.4) | 473.4 (201.9) | 647.9 (189.3) |
| | Median (IQR) | 408.6 (317.3-544.2) | 394.1 (276.1-501) | 391.2 (327.9-510.5) | 390.3 (244.5-552.2) | 414.8 (309-624.9) | 611.6 (473.4-849.4) |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 314.6 (112.8) | 342.4 (151) | 323.5 (116.3) | 288.1 (118) | 323.4 (116.8) | 417.1 (41.93) |
| | Median (IQR) | 329.8 (221.8-374.6) | 335.8 (224.2-421.1) | 297.4 (246.4-366.2) | 276.6 (178.9-403.9) | 302.8 (245.2-392.4) | 428.3 (394.4-438.6) |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 132.8 (172.1) | 87.03 (98.3) | 136.3 (290.8) | 111.4 (151) | 150 (160.1) | 230.8 (171.7) |
| | Median (IQR) | 75 (25.0-138.7) | 79.92 (16.84-112) | 80.84 (17.82-148.9) | 82.71 (2.68-149.4) | 97.57 (3.96-289.7) | 195.8 (77.9-421.5) |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 6.09 (9.17) | 4.02 (1.995) | 7.28 (11.26) | 3.66 (2.60) | 5.06 (6.525) | 8.78 (9.15) |
| | Median (IQR) | 2.795 (2.32-4.51) | 3.98 (2.28-5.53) | 2.7 (2.07-6.01) | 2.44 (1.97-6.01) | 2.88 (1.94-6.31) | 3.632 (2.4-18.9) |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 28.28 (119.5) | 10.07 (4.58) | 19.13 (25.02) | 12.76 (10.46) | 14.79 (22.55) | 10.64 (4.83) |
| | Median (IQR) | 10.34 (5.54-16.23) | 10 (4.0-14.63) | 9.22 (5.67-18.51) | 9.92 (4.15-16.33) | 10.31 (5.42-15.48) | 10.3 (6.32-15.58) |
| Total dietary methionine intake (g/day) | Mean (SD) | 2.15 (0.699) | 2.54 (1.66) | 2.29 (0.94) | 2.9 (2.74) | 2.08 (0.44) | 2.3 (0.55) |
| | Median (IQR) | 1.98 (1.84-2.45) | 1.98 (1.43-2.74) | 2.16 (1.69-2.65) | 2.32 (1.63-2.46) | 2.08 (1.74-2.29) | 2.2 (1.77-2.85) |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 43.72 (11.36) | 45.93 (17.93) | 46.02 (14.37) | 43.57 (8.87) | 45.99 (12.29) | 50.47 (4.52) |
| | Median (IQR) | 42.58 (35.48-49.68) | 42.83 (32.69-61.09) | 44.37 (36.61-55.65) | 44.83 (33.74-49.28) | 44.53 (38.36-51.38) | 51.77 (45.22-53.69) |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 4.72 (5.49) | 2.59 (1.516) | 5.47 (6.8) | 4.93 (6.23) | 5.62 (6.96) | 6.27 (3.38) |
| | Median (IQR) | 2.56 (1.89-3.98) | 2.1 (1.78-3.02) | 2.72 (1.88-5.01) | 2.48 (1.49-6.05) | 2.86 (1.95-6.71) | 4.85 (3.84-10.50) |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 256.1 (200.2) | 208.9 (136.8) | 298.7 (341.4) | 181.56 (155) | 200.2 (85.94) | 336.2 (111.1) |
| | Median (IQR) | 206 (151.3-294.4) | 219.9 (91.05-272.5) | 199.9 (135.2-311.4) | 144.1 (98.76-207.67) | 208.1 (126.3-262.5) | 329.8 (228.2-452.3) |

Appendix 25: Mean (SD) and median (IQR) for folate and other nutrients related to one-carbon metabolism intake within each of the combined RFC G80A-GCPII C1561T genotypes for control and adenomatous polyp subjects (1 of 2)

| | | GG/CT | | AA/CT | | GA/CT | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP (n=1) |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 367.3 (87.72) | 356.9 (43.93) | 620.7 (410.5) | 322.4 (76.45) | 265.3 (87.7) | 434.4 |
| | Median (IQR) | 362.4 (287.0-452.4) | 364.6 (311.5-394.6) | 588.1 (309.0-948.7) | 322.4 (268.4-376.5) | 272.6 (174.2-349.1) | |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 320.9 (103.3) | 275.1 (111.2) | 487.9 (286.2) | 278.7 (138.3) | 235 (98.91) | 421.4 |
| | Median (IQR) | 273.9 (261.9-427.0) | 285.5 (166.8-372.8) | 339.8 (284.5-765.4) | 278.7 (180.9-376.5) | 181.7 (174.2-349.1) | |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 46.34 (51.47) | 81.84 (73.49) | 132.8 (162) | 43.75 (61.87) | 30.3 (52.48) | 12.99 |
| | Median (IQR) | 47.24 (0.90-90.9) | 90.66 (10.2-144.7) | 49.01 (3.65-303.8) | 43.75 (0-87.5) | 0 (0-90.9) | |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 2.12 (0.388) | 6.15 (2.615) | 9.69 (12.66) | 2.44 (1.245) | 1.78 (0.28) | 3.8 |
| | Median (IQR) | 2.22 (1.72-2.43) | 6.17 (3.62-8.66) | 4.09 (2.84-19.34) | 2.437 (1.56-3.32) | 1.77 (1.51-2.07) | |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 9.8 (5.582) | 12.78 (8.4) | 21.54 (24.97) | 7.83 (1.74) | 7.31 (6.612) | 15.62 |
| | Median (IQR) | 8.65 (5.12-15.62) | 10.18 (6.70-21.46) | 14.1 (6.55-40.25) | 7.83 (6.59-9.06) | 4.18 (2.85-14.91) | |
| Total dietary methionine intake (g/day) | Mean (SD) | 1.99 (0.161) | 1.75 (0.35) | 3.86 (3.07) | 3.11 (0.162) | 1.57 (0.41) | 2.89 |
| | Median (IQR) | 2.01 (1.83-2.13) | 1.91 (1.38-1.945) | 3.14 (1.78-6.3) | 3.11 (2.99-3.22) | 1.6 (1.153-1.96) | |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 41.86 (4.513) | 41.2 (8.87) | 74.09 (49.68) | 54.63 (4.87) | 32.47 (5.93) | 62.85 |
| | Median (IQR) | 42.43 (37.46-45.72) | 40.8 (33.01-49.8) | 56.83 (36.3-120.5) | 54.63 (51.19-58.07) | 35.67 (25.62-36.11) | |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 2.15 (0.656) | 5.18 (6.05) | 6.93 (6.36) | 1.92 (0.281) | 1.27 (0.142) | 2.01 |
| | Median (IQR) | 2.13 (1.53-2.79) | 2.27 (2.0-11.28) | 2.97 (2.30-13.55) | 1.92 (1.73-2.12) | 1.26 (1.13-1.41) | |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 238.9 (51.12) | 182.2 (80.95) | 242.6 (113.9) | 144.2 (163) | 232.5 (92.53) | 185 |
| | Median (IQR) | 257.1 (185.2-274.3) | 212.7 (97.79-236.2) | 276.1 (126.7-341.6) | 144.2 (28.95-259.5) | 240.7 (136.1-320.7) | |

Appendix 26: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the TSER 2R3R genotypes for control and adenomatous polyp subjects

| | | 2R/2R | | 2R/3R | | 3R/3R | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 22.78 (12.35) | 29.5 (12.29) | 19.65 (11.26) | 20.18 (12.51) | 23.98 (12.37) | 23.91 (13.4) |
| | Median (IQR) | 20 (12.5-27.5) | 29 (18.25-41.25) | 17 (10.0-30.0) | 17.5 (10.75-28.25) | 21 (13.0-34.0) | 20 (13-37) |
| Serum folate excluding outliers(nM) | Mean (SD) | 18.48 (8.078) | 24.33 (8.145) | 18.29 (9.855) | 17.7 (10.08) | 20.21 (9.226) | 21.8 (12.04) |
| | Median (IQR) | 17 (12-24) | 28 (15-30) | 16 (10-27) | 16.5 (10.25-21.5) | 20 (13-27) | 17 (12.5-33.25) |
| RBC folate (nM) | Mean (SD) | 983.05 (457.7) | 1207.3 (408.6) | 934.27 (507.9) | 1019.82 (553.8) | 1039.1 (552.4) | 1035 (699.2) |
| | Median (IQR) | 913 (651.5-1162) | 1305 (779-1538) | 798 (563.0-1123) | 932.5 (527.5-1394) | 928 (651.5-1560) | 793 (505-1177) |
| RBC folate excluding outliers (nM) | Mean (SD) | 983.05 (457.7) | 1207.3 (408.6) | 872.46 (407.9) | 1019.82 (553.8) | 934.74 (400.1) | 888.5 (530) |
| | Median (IQR) | 913 (651-1162) | 1305 (779-1538) | 760.5 (558.5-1089) | 932.5 (527.5-1394) | 897.5 (617.5-1132) | 676.5(497.3-1150) |
| Cys (μM) | Mean (SD) | 271 (34.5) | 234.4 (24.56) | 258 (37.92) | 266 (32.4) | 270.2 (45.94) | 268.1 (52.41) |
| | Median (IQR) | 265.1 (253.5-289) | 234.4 (217-251.7) | 257.6 (233-284.8) | 262.8 (248.2-289.1) | 261.6 (239-305.3) | 254.9 (228.3-298.9) |
| Hcy (μM) | Mean (SD) | 9.28 (2.22) | 7.53 (1.64) | 9.98 (3.249) | 10.29 (2.668) | 10.31 (2.637) | 9.97 (2.686) |
| | Median (IQR) | 9.03 (7.34-11.29) | 7.53 (6.37-8.69) | 9.075(7.56-12.08) | 9.695 (8.73-11.88) | 10.1 (8.18-12.04) | 9.255 (8.14-11.81) |
| Cys-Gly (μM) | Mean (SD) | 22.69 (6.026) | 22.38 (6.428) | 24.12 (4.534) | 24.62 (3.824) | 23.74 (4.486) | 23.71 (7.943) |
| | Median (IQR) | 21.48 (19.34-25.08) | 22.38 (17.83-26.92) | 23.51 (21.43-26.83) | 25.39 (20.62-27.74) | 23.45 (20.27-26.72) | 21.43 (19.37-25.90) |
| GSH (μM) | Mean (SD) | 12.5 (5.876) | 10.05 (1.704) | 14.78 (5.686) | 14.64 (5.89) | 13.75 (5.976) | 14.05 (3.411) |
| | Median (IQR) | 11.02 (9.28-13.69) | 10.05(8.84-11.25) | 13.1 (10.55-18.76) | 13.53 (9.40-19.46) | 12.03 (9.78-17.18) | 11.41 (9.13-15.96) |
| Serum $B_{12}$ (pM) | Mean (SD) | 273.84 (124.1) | 276.75 (32.44) | 263.96 (144.6) | 265.46 (149.3) | 313.61 (180.8) | 327.55 (120.2) |
| | Median (IQR) | 259 (195.5-329) | 271.5 (249.8-309) | 229 (189.0-285) | 235 (185.5-272.5) | 262 (193-345) | 325 (242-395) |

Appendix 27: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake each of the TSER 2R3R genotypes for control and adenomatous polyp subjects

| | | 2R/2R | | 2R/3R | | 3R/3R | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 468.3 (340) | 428.7 (115) | 468.4 (230) | 388.8 (171) | 440 (205) | 542.9 (190) |
| | Median (IQR) | 403 (356.7-466) | 399 (337.9-548.9) | 394 (313-595.2) | 378 (267.1-485.8) | 401 (295-578) | 501 (376.5-719.4) |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 326.3 (95.9) | 365.9 (44.6) | 338.3 (133) | 311.1 (130) | 296.8 (127) | 373.7 (136) |
| | Median (IQR) | 307 (258.6-378.4) | 380 (319.9-397.9) | 328 (251.8-385.1) | 311 (178.9-408.5) | 275 (206.1-372.9) | 421 (253.3-464.1) |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 146 (340) | 62.88 (85.2) | 126 (167) | 93.61 (112) | 136 (142) | 166.7 (170) |
| | Median (IQR) | 74.6 (23.43-126) | 31.1 (6.45-151) | 81.8 (5.16-149) | 82.7 (5.76-129.3) | 90.4 (21.09-207) | 112 (27-255.3) |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 5.83 (9.5) | 5.38 (3.18) | 6.43 (9.96) | 3.97 (3.5) | 6.29 (8.76) | 6.59 (5.86) |
| | Median (IQR) | 2.71 (2.22-4.19) | 5.06 (2.57-8.5) | 2.8 (2.06-4.61) | 2.6 (2.21-5.47) | 2.61 (1.97-6.92) | 4.56 (3.32-7.01) |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 14.36 (18.5) | 9.8 (4.09) | 29.61 (113) | 10 (7.8) | 15.28 (19.4) | 14.46 (4.49) |
| | Median (IQR) | 9.2 (5.29-14.21) | 8.97 (6.46-13.98) | 10 (5.64-16.81) | 9.54 (3.98-13.51) | 10.8 (5.29-17.77) | 14.6 (9.62-16.08) |
| Total dietary methionine intake (g/day) | Mean (SD) | 2.17 (0.7) | 3.45 (2.57) | 2.33 (1.11) | 2.31 (1.92) | 2.1 (0.71) | 2.66 (1.08) |
| | Median (IQR) | 2.06 (1.72-2.43) | 2.28 (1.98-6.08) | 2.08 (1.8-2.54) | 1.92 (1.55-2.3) | 1.95 (1.74-2.35) | 2.45 (1.73-3.22) |
| Total dietary niacin intake (mg/day) | Mean (SD) | 44.29 (10.4) | 47.41 (3.9) | 47.68 (18.4) | 42.38 (11.4) | 43.02 (12.9) | 53.74 (15.9) |
| | Median (IQR) | 44.5 (36.26-53.04) | 47.2 (43.9-51.11) | 43 (37.79-53.45) | 43 (33.77-49.29) | 42.1 (34.55-49.85) | 55.7 (44.29-61.74) |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 3.73 (5.3) | 6.04 (7.6) | 5.02 (6.21) | 2.79 (2.19) | 6.27 (6.52) | 5.93 (5.17) |
| | Median (IQR) | 2.39 (1.81-2.97) | 2.39 (2.00-13.72) | 2.51 (1.85-4.48) | 2.14 (1.61-2.72) | 2.9 (2.01-9.36) | 3.68 (2.12-10.52) |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 302.2 (275) | 197.4 (49.9) | 254.4 (264) | 227.6 (159) | 234.9 (152) | 212.9 (110) |
| | Median (IQR) | 200(140.9-328.8) | 200 (151.4-240.4) | 221 (136.5-282.7) | 198 (101.9-308.3) | 213 (124.2-286.4) | 220 (140.7-264.4) |

Appendix 28: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the TS 1496del6 genotypes for control and adenomatous polyp subjects

| | | 6bp/6bp | | 6bp/0bp | | 0bp/0bp | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 21.87 (12.19) | 21.69 (15.99) | 21.6 (11.68) | 23.83 (11.07) | 19 (12.08) | 18.57 (10.08) |
| | Median (IQR) | 18.5 (11.75-30.25) | 15 (8.0-40.5) | 20 (12.0-29.0) | 20 (13.75-32.0) | 15(10.0-28.5) | 16 (13.0-27.0) |
| Serum folate excluding outliers (nM) | Mean (SD) | 19.23 (9.838) | 14.7 (10.27) | 18.72 (8.751) | 22.59 (10.03) | 16.64 (9.32) | 18.57 (10.08) |
| | Median (IQR) | 17 (10.0-26.25) | 13 (6.5-19.75) | 17 (11.5-23.0) | 20 (13.5-31.5) | 14 (10.0-24.0) | 16 (13.0-27.0) |
| RBC folate (nM) | Mean (SD) | 940.6 (475.8) | 1061 (671.7) | 992.7 (528.5) | 1148 (555.1) | 1057 (610) | 669.9 (263.7) |
| | Median (IQR) | 809.5 (597.5-1106) | 900 (477-1570) | 891 (599-1164) | 1118 (616.3-1436) | 859.5 (615.5-1440) | 505 (474-848) |
| RBC folate excluding outliers (nM) | Mean (SD) | 920.4 (443.8) | 941 (536.9) | 904.1 (390.4) | 1148 (555.1) | 925.8 (426.8) | 669.9 (263.7) |
| | Median (IQR) | 805 (579-1092) | 816 (458.5-1505) | 873 (592.7-1121) | 1118 (616.2-1436) | 834 (593-1424) | 505 (474-848) |
| Cys (μM) | Mean (SD) | 266.3 (41.4) | 242.8 (19.54) | 258.1 (39.33) | 274.8 (47.06) | 286.9 (27.95) | 281.2 (32.81) |
| | Median (IQR) | 264.7 (240-289) | 245.3 (223.6-262.8) | 255.6 (233.5-277.5) | 267 (245.6-302.4) | 292.8 (267-304.2) | 286.6 (240-305.3) |
| Hcy (μM) | Mean (SD) | 9.73 (2.614) | 9.46 (2.626) | 9.76 (2.874) | 9.89 (2.52) | 12.42 (4.037) | 11.64 (3.076) |
| | Median (IQR) | 9.64 (7.66-11.36) | 8.78(7.08-11.56) | 9.075 (7.44-11.54) | 9.275 (8.30-10.93) | 12.04(9.42-14.75) | 11.73 (9.07-14.58) |
| Cys-Gly (μM) | Mean (SD) | 23.45 (5.492) | 26.57 (7.284) | 23.88 (4.164) | 23.67 (3.909) | 23.69 (5.12) | 22.81 (5.423) |
| | Median (IQR) | 22.36 (20.18-26.8) | 25.83 (20.56-29.59) | 23.27 (20.99-26.64) | 23.24 (20.14-27.74) | 23.25 (19.68-28.17) | 24.28 (17.92-25.25) |
| GSH (μM) | Mean (SD) | 13.52 (5.178) | 13.89 (5.057) | 14.5 (6.014) | 14.27 (5.47) | 13.88 (8.937) | 11.61 (4.408) |
| | Median (IQR) | 12.51 (9.42-17.79) | 12.57 (9.87-19.21) | 11.94(10.39-17.52) | 13.57 (9.40-16.95) | 10.8 (9.37-15.03) | 9.48 (8.55-15.80) |
| Serum $B_{12}$ (pM) | Mean (SD) | 255.9 (104.5) | 311.3 (198.2) | 307.5 (187.9) | 262.2 (80.81) | 269.2 (149.6) | 269.7 (111.6) |
| | Median (IQR) | 247(185.5-318.5) | 243 (191.0-367.5) | 254 (199.0-325.0) | 262 (209.25-326.75) | 242.5 (144.5-396.5) | 265 (195.0-330.0) |

Appendix 29: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the TS 1496del6 genotypes for control and adenomatous polyp subjects

| | | 6bp/6bp | | 6bp/0bp | | 0bp/0bp | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 484.3 (287.1) | 386.4 (201.2) | 442 (220.9) | 446 (148.1) | 400.4 (156.2) | 493.8 (224.7) |
| | Median (IQR) | 404.8 (350.3-588.6) | 324.8 (246.4-478.3) | 382.6 (302.5-573) | 405.4 (369.8-548.2) | 371.5 (264.2-554.5) | 434.4 (269-693.2) |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 337 (103.3) | 291.4 (102.2) | 313.8 (144.2) | 348.5 (126.2) | 291.3 (114.6) | 362 (169.1) |
| | Median (IQR) | 336.7(261.5-394.5) | 285.3 (188.6-390.2) | 294.9 (229.9-364.4) | 362.1 (257.9-425.7) | 266.4 (209.8-336.1) | 421.1 (181.2-430) |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 144 (270.4) | 95.05 (165) | 125 (139.8) | 95.9 (68.84) | 109 (147.3) | 181.4 (170.9) |
| | Median (IQR) | 75(6.31-150.0) | 23.71 (2.13-96.51) | 90.9(26.01-148.9) | 95.4 (35.68-96.51) | 50.15 (8.15-177.4) | 87.5 (79.92-393.8) |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 6.12 (9.785) | 4.69 (4.394) | 6.82 (9.821) | 4.43 (2.442) | 3.42 (1.641) | 6.02 (7.609) |
| | Median (IQR) | 2.796 (2.32-4.74) | 2.702 (2.25-7.07) | 2.487 (1.94-6.48) | 3.939 (2.40-5.94) | 3.52 (2.00-4.08) | 3.799 (2.18-4.51) |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 15.2 (18.13) | 11.05 (9.345) | 31.78 (118.7) | 10.7 (5.412) | 7.35 (4.089) | 12.56 (4.912) |
| | Median (IQR) | 10.4 (5.34-17.24) | 9.524 (4.88-13.28) | 10.81 (5.69-16.73) | 9.663 (6.32-14.05) | 5.793 (4.39-10.21) | 14.98 (6.59-15.69) |
| Total dietary methionine intake (g/day) | Mean (SD) | 2.17 (0.645) | 3.04 (2.751) | 2.32 (1.197) | 2.19 (0.82) | 1.98 (0.501) | 2.57 (0.955) |
| | Median (IQR) | 2.062(1.8-2.54) | 1.959(1.63-2.76) | 2.015 (1.73-2.45) | 2.037 (1.66-2.43) | 1.927 (1.65-2.3) | 2.743 (1.73-3.0) |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 45 (11.38) | 41.5 (8.768) | 46.7 (19.06) | 44.53 (11.13) | 41.2 (14.87) | 60.54 (15.61) |
| | Median (IQR) | 44.55(35.84-52.63) | 44 (33.41-47.16) | 43.01 (37.69-54.16) | 46.35 (37.02-53.42) | 38.57 (33.64-43.46) | 61.09(45.35-71.5) |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 4.67 (5.208) | 3.96 (4.339) | 5.75 (7.285) | 3.94 (4.488) | 3.44 (3.188) | 4.29 (3.366) |
| | Median (IQR) | 2.719 (2.01-3.85) | 2.352 (1.80-3.60) | 2.631 (1.82-5.39) | 2.267 (1.76-3.82) | 2.476 (1.66-3.14) | 3.017(1.73-7.12) |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 292.3 (304.9) | 210 (147.6) | 224.1 (149.9) | 234 (143.8) | 249.3 (155.2) | 173.4 (107.1) |
| | Median (IQR) | 243.1 (151.5-294.3) | 194.8 (95.61-280.2) | 208.1 (118.3-265.6) | 212.7 (131.9-274.4) | 175.8 (126.3-388.6) | 185 (73.29-272.5) |

Appendix 30: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the TSER 3RG>C genotypes for control and adenomatous polyp subjects (1of 2)

| | | 3RC/2R | | 3RG/2R | | 3RC/3RC | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 19.8 (11.35) | 24.8 (14.56) | 19.4 (11.32) | 15.9 (5.862) | 27.3 (12.7) | 20.8 (16.21) |
| | Median (IQR) | 17 (9.8-3) | 20 (12-40.5) | 16 (10-28) | 16 (11.5-19) | 22.5 (16-43.3) | 13.5 (11.5-37.3) |
| Serum folate excluding outliers (nM) | Mean (SD) | 18.7 (10.2) | 20.7 (12.24) | 17.7 (9.49) | 15.9 (5.862) | 21.3 (8.304) | 12.7 (1.528) |
| | Median (IQR) | 16.5 (9.3-29.8) | 17.5 (10.5-33) | 16 (10-23) | 16 (11.5-19) | 20 (16-29) | 13 (11-14) |
| RBC folate (nM) | Mean (SD) | 915.3 (473.6) | 1262 (567.2) | 960.7 (558.7) | 785.4 (365.2) | 1060 (373.8) | 1155 (936.8) |
| | Median (IQR) | 760.5 (555-1099) | 1320 (774-1757) | 811 (574.5-1171) | 776 (453-1051.5) | 1031 (769.8-1388) | 826 (495-2144) |
| RBC folate excluding outliers (nM) | Mean (SD) | 880 (413.6) | 1262 (567.2) | 861.4 (406) | 785.4 (365.2) | 1060 (373.8) | 708.7 (336.3) |
| | Median (IQR) | 744 (553.5-1088) | 1320 (774-1757) | 798 (568-1091) | 776 (453-1051.5) | 1031 (769.8-1388) | 558 (474-1094) |
| Cys (µM) | Mean (SD) | 256.9 (39.75) | 246.5 (26.63) | 259.8 (35.8) | 283.2 (29.96) | 267.4 (51.83) | 296.8 (73.11) |
| | Median (IQR) | 258.2 (226.1-284.3) | 249.8 (220.1-265.8) | 257.1 (233.4-285.7) | 281.4 (254.7-310.9) | 251.9 (235.9-315) | 286.6 (229.4-374.5) |
| Hcy (µM) | Mean (SD) | 9.5 (2.81) | 8.5 (1.752) | 10.6 (3.742) | 11.7 (2.571) | 10.1 (2.685) | 11.3 (3.232) |
| | Median (IQR) | 8.87 (7.5-11.6) | 8.585 (7.1-9.8) | 9.79 (7.9-13.4) | 11.45 (9-14.4) | 9.98 (8.1-12) | 10.76 (8.4-14.8) |
| Cys-Gly (µM) | Mean (SD) | 23.4 (4.592) | 23.4 (2.862) | 25.1 (4.346) | 25.1 (4.316) | 24.3 (5.388) | 28.2 (13.63) |
| | Median (IQR) | 22.44 (20.6-25.6) | 23.91 (20.3-26) | 24.8 (22-27.1) | 27.59 (22.1-27.8) | 25.73 (18.9-27) | 20.72 (19.9-43.9) |
| GSH (µM) | Mean (SD) | 14.5 (5.166) | 14.8 (5.351) | 15.2 (6.421) | 15 (6.821) | 11.9 (4.886) | 12.4 (4.499) |
| | Median (IQR) | 13.47 (10.3-18.4) | 15.08 (9.7-19.7) | 13.05 (10.6-19.8) | 13.17 (9-19.5) | 10.13 (8.1-15.9) | 13.96 (7.4-16) |
| Serum $B_{12}$ (pM) | Mean (SD) | 260.5 (100.7) | 246.9 (76.4) | 268.8 (191.4) | 226.2 (82.75) | 304.4 (118.5) | 359 (149.1) |
| | Median (IQR) | 241 (187-311) | 235 (190.5-283.5) | 217 (193.5-271) | 224 (158.5-265) | 263.5 (209.8-445.5) | 309 (253.5-514.5) |

Appendix 31: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the TSER 3RG>C genotypes for control and adenomatous polyp subjects (2 of 2)

| | | 3RG/3RC | | 3RG/3RG | |
|---|---|---|---|---|---|
| | | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 20.2 (10.7) | 30.8 (8.221) | 27.2 (15.35) | 19 (15.87) |
| | Median (IQR) | 19 (12-40.5) | 31.5 (22.8-38) | 27 (14-40.5) | 13 (7-37) |
| Serum folate excluding outliers (nM) | Mean (SD) | 18.9 (9.292) | 30.8 (8.221) | 22.8 (13.5) | 19 (15.87) |
| | Median (IQR) | 18 (12-22.8) | 31.5 (22.8-38) | 25.5 (9-33.8) | 13 (7-37) |
| RBC folate (nM) | Mean (SD) | 930.6 (587.8) | 1257 (658.1) | 1389.6 (914.6) | 577.3 (190.1) |
| | Median (IQR) | 768.5 (553-1065.3) | 1160 (705.5-1905.3) | 1189 (592.5-2287) | 505 (434-793) |
| RBC folate excluding outliers (nM) | Mean (SD) | 756.2 (253.4) | 1257 (658.1) | 1112 (775.6) | 577.3 (190.1) |
| | Median (IQR) | 735 (534.3-943.8) | 1160 (705.5-1905.3) | 1095 (388.3-1852.8) | 505 (434-793) |
| Cys (µM) | Mean (SD) | 278.3 (37.81) | 255.8 (53.99) | 243.8 (54.24) | 255.9 (29.66) |
| | Median (IQR) | 280.3 (244.2-307.7) | 247.4 (208.7-311.4) | 254.1 (187.5-289.9) | 240 (237.6-290.1) |
| Hcy (µM) | Mean (SD) | 10.5 (2.676) | 8.6 (1.048) | 10.6 (2.881) | 10.4 (3.712) |
| | Median (IQR) | 9.85 (8.2-12.4) | 8.35 (7.8-9.7) | 10.6 (7.9-13.4) | 11.16 (6.4-13.8) |
| Cys-Gly (µM) | Mean (SD) | 23.9 (3.664) | 23.4 (3.376) | 20.5 (2.669) | 19.6 (5.045) |
| | Median (IQR) | 23.3 (21.2-27.2) | 23.04 (20.4-26.9) | 20.9 (17.9-22.7) | 17.92 (15.6-25.3) |
| GSH (µM) | Mean (SD) | 14.4 (4.618) | 13.3 (3.764) | 19.1 (12.31) | 10 (1.198) |
| | Median (IQR) | 12.99 (10.5-18.2) | 13.69 (9.5-16.6) | 15.31 (9.9-32.1) | 9.48 (9.2-11.4) |
| Serum $B_{12}$ (pM) | Mean (SD) | 335 (227.2) | 293.5 (118.6) | 260.8 (190.9) | 331 (116.2) |
| | Median (IQR) | 267 (203.5-333.5) | 328.5 (172.8-379.3) | 190 (131-426) | 302 (232-459) |

Appendix 32: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the TSER 3RG>C genotypes for control and adenomatous polyp subjects (1 of 2)

| | | 3RC/2R | | 3RG/2R | | 3RC/3RC | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Total grand dietary folate intake (μg/day) | Mean (SD) | 526 (260.6) | 428.2 (168.4) | 383 (139.5) | 360.3 (170) | 384.5 (167.6) | 472.2 (159.8) |
| | Median (IQR) | 461.9 (348-640.9) | 405.4 (282-500) | 353 (279.1-466.4) | 351.9 (217.3-480.0) | 364 (256.3-494) | 405.4 (371.7-639.5) |
| Total dietary methyl folate intake (μg/day) | Mean (SD) | 364.4 (143) | 337.3 (96.26) | 299.5 (108) | 291.5 (166.1) | 283.1 (95.17) | 320.6 (97.08) |
| | Median (IQR) | 341.9 (278.3-410.6) | 348.8 (266.6-423.6) | 294.2 (215.2-356.4) | 209.2 (163.4-384.7) | 264.4 (219-372) | 327.8 (223.8-410.1) |
| Total dietary PteGlu intake (μg/day) | Mean (SD) | 152.1 (191.2) | 90.9 (109.7) | 86.73 (115.3) | 107.6 (122.3) | 108.9 (109.5) | 151.6 (237.4) |
| | Median (IQR) | 89.26 (9.9-255.4) | 74.63 (22.3-101.6) | 72.31 (0.27-93.24) | 87.5 (1.8-139.5) | 84.81 (15.7-156.2) | 51.95 (3.2-399.6) |
| Total dietary pyridoxine intake (μg/day) | Mean (SD) | 8.3 (12.21) | 4.9 (4.392) | 3.7 (3.927) | 3.1 (1.662) | 6.8 (11.34) | 4.1 (2.099) |
| | Median (IQR) | 3.033 (2.3-7.1) | 2.721 (2.3-6.5) | 2.425 (2-3.5) | 2.422 (1.8-5.0) | 3.067 (1.9-7.2) | 3.558 (2.4-6.2) |
| Total dietary cobalamin intake (μg/day) | Mean (SD) | 22.3 (29.46) | 8.7 (4.166) | 40.4 (175.5) | 12.5 (10.99) | 14.5 (13.41) | 12.7 (4.072) |
| | Median (IQR) | 12.05 (6.9-19.6) | 9.542 (4.3-10.8) | 8.318 (4.6-14.5) | 10.98 (4.2-15.6) | 10.53 (4.6-20.9) | 12.45 (9.1-16.5) |
| Total dietary methionine intake (g/day) | Mean (SD) | 2.455 (1.318) | 2 (0.533) | 2.14 (0.684) | 2.81 (2.959) | 2.16 (0.91) | 2.55 (0.685) |
| | Median (IQR) | 2.158 (1.87-2.66) | 1.952 (1.67-2.35) | 2 (1.74-2.45) | 1.885(1.4-2.34) | 1.931(1.64-2.26) | 2.67(1.84-3.14) |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 50.35 (21.77) | 42.72 (8.287) | 43.76 (11.16) | 43.32 (15.02) | 42.9 (12.59) | 49.45 (13.72) |
| | Median (IQR) | 43.83 (37.98-53.99) | 44.41 (36.69-47.91) | 42.76 (36.93-50.55) | 42.09 (30.43-54.01) | 43.49 (34.45-49.53) | 51.18 (35.51-61.66) |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 6.4 (7.55) | 3.1 (2.541) | 3 (2.3) | 2.6 (1.804) | 7.7 (7.493) | 5.4 (7.025) |
| | Median (IQR) | 3.235 (2-9.7) | 2.145 (1.8-3.8) | 2.259 (1.8-3.3) | 2.332 (1.6-2.7) | 3.453 (1.9-12.9) | 2.069 (1.7-12.5) |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 284.7 (327.5) | 263 (172.2) | 209.8 (111.4) | 197 (141.6) | 224.7 (154.6) | 183.2 (54.47) |
| | Median (IQR) | 243.1 (153.2-294.5) | 220.6 (128.3-333.2) | 197.7 (119.9-275) | 188.8 (49.2-288.5) | 199.3 (121.8-276.7) | 166.3 (142.4-240.9) |

Appendix 33: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the TSER 3RG>C genotypes for control and adenomatous polyp subjects (2 of 2)

| | | 3RG/3RC | | 3RG/3RG | |
|---|---|---|---|---|---|
| | | Control | AP | Control | AP |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 493.9 (225.9) | 572.8 (203.6) | 438.8 (228.8) | 597.5 (250.9) |
| | Median (IQR) | 427.8 (321.1-625.6) | 629.3 (358.3-730.9) | 473.4 (235.9-624.3) | 501 (409.1-882.3) |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 322.4 (152.9) | 430.9 (193.1) | 244.2 (120.8) | 368.2 (99.62) |
| | Median (IQR) | 274.6 (195.8-405) | 488.1 (230.6-574.0) | 283 (121.3-347.8) | 421.1 (253.2-430.2) |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 147.7 (158.2) | 134.9 (94.48) | 194.5 (191) | 229.3 (196.7) |
| | Median (IQR) | 90.9 (20.8-325) | 128.7 (48.3-227.9) | 99.9 (36.4-400) | 155.8 (79.9-452.1) |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 6.7 (6.837) | 6.6 (2.007) | 2.7 (1.071) | 10 (11.34) |
| | Median (IQR) | 2.663 (1.9-12.2) | 6.197 (4.8-8.6) | 2.316 (1.8-3.8) | 4.163 (2.8-23.1) |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 17.9 (25.41) | 15.5 (6.594) | 7.5 (4.612) | 15.5 (0.748) |
| | Median (IQR) | 11.64 (8.2-18.1) | 13.71 (10.6-22.1) | 8.366 (3-11.7) | 15.69 (14.6-16.1) |
| Total dietary methionine intake (g/day) | Mean (SD) | 2.12 (0.527) | 2.58 (1.499) | 1.77 (0.41) | 2.91 (1.26) |
| | Median (IQR) | 2.078(1.77-2.52) | 2.189 (1.42-4.12) | 1.871(1.38-2.12) | 2.743(1.74-4.24) |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 45.04 (14.1) | 49.78 (13.39) | 35.02 (4.952) | 64.75 (21.47) |
| | Median (IQR) | 43.75 (36.14-54.11) | 53.26 (35.83-60.23) | 35.22 (30.44-39.51) | 61.09 (45.35-87.82) |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 5.9 (5.989) | 6.5 (5.31) | 2.2 (0.909) | 5.9 (4.049) |
| | Median (IQR) | 2.885 (2.3-9.0) | 4.47 (2.9-12) | 2.161 (1.4-3.1) | 4.12 (3.0-10.5) |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 238.1 (147.8) | 248.7 (156.1) | 262.2 (185.3) | 205 (125) |
| | Median (IQR) | 209 (133.6-294.3) | 244.2 (103.4-398.4) | 283.8 (94-419.5) | 219.9 (73.3-321.9) |

Appendix 34: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the SHMT C1420T genotypes for control and adenomatous polyp subjects

| | | Wild type (CC) | | Heterozygous type (CT) | | Homo recessive type (TT) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 20.22 (11.74) | 19.33 (12.33) | 23.43 (12.31) | 21.33 (12.65) | 21.07 (10.74) | 34.6 (6.229) |
| | Median (IQR) | 17 (10.0-29.5) | 16.5 (10.75-21.33) | 20 (14.0-33.0) | 16 (13-30) | 20 (11.0-27.0) | 32 (29.5-41.0) |
| Serum folate excluding outliers(nM) | Mean (SD) | 18 (9.464) | 16.13 (8.57) | 19.83 (9.218) | 17.69 (8.882) | 19.36 (8.776) | 34.6 (6.229) |
| | Median (IQR) | 16 (10.0-24.0) | 14.5 (10.25-20.0) | 18 (13.0-26.25) | 16 (13.0-23.0) | 19.5 (10.75-25.5) | 32 (29.5-41.0) |
| RBC folate (nM) | Mean (SD) | 920.7 (454.1) | 845.4 (584) | 1066 (586.5) | 1210 (610.2) | 867.4 (392.9) | 1155 (113.1) |
| | Median (IQR) | 845 (580.25-1104) | 597.5 (464.0-1088.5) | 947 (691.0-1257.0) | 1181 (776-1566) | 768 (595-1040) | 1142 (1069-1247) |
| RBC folate excluding outliers (nM) | Mean (SD) | 882.2 (385) | 845.4 (584) | 969.2 (465.2) | 1118 (513.7) | 867.4 (392.9) | 1155 (113.1) |
| | Median (IQR) | 828.5 (579.5-1092) | 597.5 (464.0-1088.5) | 893 (620.0-1208.0) | 1138 (708-1462) | 768 (595-1040) | 1142 (1069-1247) |
| Cys (μM) | Mean (SD) | 259.7 (41.51) | 267.2 (38.23) | 271 (38.93) | 263.2 (41.56) | 256.8 (34.29) | 267.5 (50.72) |
| | Median (IQR) | 254.1 (234.5-285.5) | 261.3 (245.5-278.2) | 267.5 (244.7-291.3) | 262.6 (223.6-296.4) | 263.3 (220.5-289.9) | 270.7 (217.5-314.3) |
| Hcy (μM) | Mean (SD) | 9.84 (2.859) | 10.47 (2.973) | 10.14 (3.041) | 10.24 (2.754) | 9.4 (2.499) | 8.52 (0.371) |
| | Median (IQR) | 9.495 (7.58-11.81) | 10.11 (8.18-13.18) | 9.84 (7.56-11.92) | 10.15 (7.87-12.24) | 8.955 (7.25-10.96) | 8.35 (8.30-8.90) |
| Cys-Gly (μM) | Mean (SD) | 23.83 (4.421) | 23.08 (4.704) | 23.99 (5.561) | 26.94 (7.069) | 21.16 (3.949) | 23.73 (2.895) |
| | Median (IQR) | 23.19 (20.92-26.69) | 22.14 (19.94-27.77) | 23.78 (20.67-26.91) | 25.39 (23.80-28.75) | 20.59 (17.85-22.88) | 24.11 (20.87-26.21) |
| GSH (μM) | Mean (SD) | 14.15 (5.901) | 13.24 (5.642) | 13.88 (5.988) | 14.12 (4.432) | 13.43 (4.891) | 13.42 (5.145) |
| | Median (IQR) | 12.75 (9.83-17.79) | 11.41 (9.13-16.56) | 11.72 (10.32-15.93) | 13.6 (10.21-19.10) | 11.38 (9.39-19.23) | 12.66 (9.03-18.57) |
| Serum $B_{12}$ (pM) | Mean (SD) | 277 (168.8) | 280.4 (163.3) | 288.3 (128.8) | 273.9 (114.9) | 261.8 (146.2) | 299.8 (94.07) |
| | Median (IQR) | 229 (194.5-285.0) | 245 (185.25-326.25) | 265 (201-339) | 265 (195-289) | 207 (156-291) | 332 (209.5-374) |

Appendix 35: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the SHMT C1420T genotypes for control and adenomatous polyp subjects

| | | Wild type (CC) | | Heterozygous type (CT) | | Homo recessive type (TT) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 455.3 (276.3) | 401.2 (178.2) | 463.4 (220.9) | 438.3 (181.8) | 463.9 (238.1) | 542.4 (185.7) |
| | Median (IQR) | 387.8 (323.3-571.3) | 385.3 (272.6-478.3) | 407.6 (339.5-567.9) | 400.4 (281-503.8) | 445.1 (272.5-588.5) | 575.6 (354.3-713.94) |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 311.1 (99.35) | 313.1 (120) | 344.6 (144.8) | 321 (93.06) | 301 (151.2) | 429.3 (211.3) |
| | Median (IQR) | 300.2 (242.4-365.9) | 337.3 (170.1-422.82) | 322.3 (264.9-403.1) | 361.8 (224.2-407) | 277.9 (188.5-358.8) | 475.7 (213.4-622.05) |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 141.3 (251.6) | 88.12 (108.2) | 114.1 (161) | 117.3 (154.4) | 162.9 (153.2) | 177.5 (122.3) |
| | Median (IQR) | 84.67 (11.28-150) | 48.66 (2.68-137.9) | 73.66 (5.95-120.8) | 79.92 (16.84-90.9) | 93.24 (51.66-300) | 136.4 (106-269.6) |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 5 (6.027) | 4.4 (3.699) | 6.8 (11.24) | 5.2 (5.493) | 10.82 (14.93) | 5.15 (2.852) |
| | Median (IQR) | 2.711 (2.09-4.29) | 3.558 (2.29-5.49) | 2.733 (2.06-5.73) | 2.695 (2.22-7.01) | 2.412 (1.99-17.13) | 4.512 (2.62-7.99) |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 24.63 (108.2) | 11.24 (8.135) | 19.29 (28.27) | 10.38 (4.645) | 17.85 (20.08) | 13.38 (7.855) |
| | Median (IQR) | 10.72 (5.88-15.79) | 9.809 (5.97-14.75) | 10.2 (5.20-17.19) | 9.524 (5.81-15.69) | 10.34 (5.01-20.33) | 13.71 (6.63-19.97) |
| Total dietary methionine intake (mg/day) | Mean (SD) | 2.2 (0.702) | 2.88 (2.357) | 2.24 (1.14) | 2.24 (0.732) | 2.27 (1.088) | 2.29 (1.381) |
| | Median (IQR) | 2.081 (1.79-2.47) | 2.137 (1.66-2.94) | 1.991 (1.67-2.33) | 2.089 (1.74-2.48) | 2.062 (1.52-2.89) | 1.885 (1.45-3.35) |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 45.19 (11.94) | 44.06 (12.21) | 46.17 (18.64) | 47.58 (13.37) | 44.87 (18.98) | 51.52 (15.66) |
| | Median (IQR) | 43.83 (38.4-51.1) | 43.46 (33.77-55.83) | 42.31 (35.56-53.99) | 45.35 (42.83-52.37) | 40.88 (31.37-56.34) | 48.15 (38.11-66.62) |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 4.56 (5.038) | 2.9 (2.169) | 5.4 (6.905) | 4.77 (5.352) | 6.46 (8.133) | 5.72 (5.255) |
| | Median (IQR) | 2.723 (1.94-3.90) | 2.463 (1.80-2.82) | 2.406 (1.86-4.62) | 2.203 (1.73-4.21) | 2.52 (1.87-9.27) | 3.683 (1.77-10.69) |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 221.8 (170.6) | 207.4 (133.3) | 314.8 (315) | 212.1 (125.4) | 236.4 (162.7) | 250.8 (207.2) |
| | Median (IQR) | 188.1 (132.5-268.8) | 189.9 (77.06-308.3) | 247.8 (155.7-367.1) | 201.9 (140.7-240.5) | 157.1 (113.5-339.9) | 224.1 (84.34-430.7) |

Appendix 36: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the CBS 844*ins*68 genotypes for control and adenomatous polyp subjects

| | | Wild type (NN) | | Heterozygous type (NI) | | Homo recessive type (II) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control (n=1) | AP |
| Serum folate (nM) | Mean (SD) | 21.36 (11.77) | 21.78 (12.81) | 23.26 (13.29) | 23 (12.77) | 14 | N/A |
| | Median (IQR) | 18 (12.0-30.0) | 17 (13.0-32.0) | 20 (11.0-30.0) | 18 (16.0-31.0) | | |
| Serum folate excluding outliers(nM) | Mean (SD) | 19 (9.513) | 19.92 (11.36) | 17.47 (7.482) | 18.11 (7.474) | 14 | N/A |
| | Median (IQR) | 17 (11.0-25.0) | 15 (12.0-31.0) | 18 (11.0-24.0) | 18 (13.5-23.0) | | |
| RBC folate (nM) | Mean (SD) | 956.8 (480.2) | 946 (519.7) | 1111 (685.3) | 1237 (675.2) | 550 | N/A |
| | Median (IQR) | 852.5 (611-1143) | 900 (514-1181) | 1001 (539-1315) | 1373 (558-1820) | | |
| RBC folate excluding outliers (nM) | Mean (SD) | 912.1 (407.2) | 886.3 (424.9) | 947.6 (508.8) | 1237 (675.2) | 550 | N/A |
| | Median (IQR) | 845 (609-1109) | 846.5 (511.7-1178) | 928 (529.5-1272) | 1373 (558-1820) | | |
| Cys (µM) | Mean (SD) | 262.6 (40.6) | 259.3 (35.25) | 272.5 (38.26) | 279.9 (46) | 258.81 | N/A |
| | Median (IQR) | 261.6 (237-286.7) | 260.1 (227-288) | 273.2 (241-301) | 268.6 (249.4-310.9) | | |
| Hcy (µM) | Mean (SD) | 9.83 (2.811) | 10.37 (2.871) | 10.44 (3.472) | 9.68 (2.462) | 11.19 | N/A |
| | Median (IQR) | 9.495 (7.51-11.86) | 9.84 (8.33-13.04) | 9.775 (7.83-11.53) | 9.275 (7.51-11.30) | | |
| Cys-Gly (µM) | Mean (SD) | 23.57 (5.01) | 24.78 (5.908) | 24.11 (4.042) | 23.63 (4.919) | 26.78 | N/A |
| | Median (IQR) | 22.84 (20.38-26.71) | 25.25 (19.94-27.75) | 23.54 (20.94-26.85) | 22.88 (20.14-27.65) | | |
| GSH (µM) | Mean (SD) | 14.04 (5.98) | 14.45 (5.59) | 13.22 (4.6) | 11.63 (3.191) | 20.88 | N/A |
| | Median (IQR) | 12.21 (10.0-17.74) | 13.89 (9.28-19.20) | 10.9 (9.88-16.25) | 20.88-14.42 | | |
| Serum $B_{12}$ (pM) | Mean (SD) | 274.7 (139.5) | 289.4 (152.5) | 316 (227.7) | 258.2 (79.94) | 357 | N/A |
| | Median (IQR) | 249 (195-318) | 265 (190-332) | 242 (187-382) | 357 (203-321) | | |

Appendix 37: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the CBS 844*ins*68 genotypes for control and adenomatous polyp subjects

| | | Wild type (NN) | | Heterozygous type (NI) | | Homo recessive type (II) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control (n=1) | AP |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 462.4 (264.5) | 447.3 (199.3) | 440.6 (128.7) | 402.8 (130.3) | 363 | N/A |
| | Median (IQR) | 394.8 (313.5-588.3) | 421.6 (285-575.6) | 408.3 (349.13-485.7) | 377.2 (276.07-468.4) | | |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 320.7 (125.2) | 335.2 (135.2) | 338.6 (121.1) | 322.6 (111.1) | 363 | N/A |
| | Median (IQR) | 306.7 (237.6-376.8) | 335.8 (209.2-421.4) | 306.1 (266.12-370.8) | 362.5 (181.2-412.9) | | |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 137.9 (222.8) | 124.1 (145.2) | 102 (110) | 80.21 (78.38) | 0 | N/A |
| | Median (IQR) | 79.92 (13.16-150) | 90.9 (16.84-142.7) | 72.31 (3.57-148.2) | 87.5 (0.69-127) | | |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 6.52 (9.968) | 5.41 (4.934) | 4.3 (3.812) | 3.35 (1.646) | 2.57 | N/A |
| | Median (IQR) | 2.75 (2.05-4.74) | 3.984 (2.37-7.01) | 2.467 (2.22-6.73) | 2.847 (2.18-4.56) | | |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 23.51 (85.43) | 12.31 (7.438) | 10.84 (5.327) | 8.41 (3.873) | 8.97 | N/A |
| | Median (IQR) | 10.2 (5.56-16.84) | 10.98 (8.41-15.62) | 10.73 (4.67-14.91) | 6.886 (5.39-13.02) | | |
| Total dietary methionine intake (g/day) | Mean (SD) | 2.24 (0.974) | 2.64 (2.036) | 2.05 (0.41) | 2.32 (0.681) | 2.66 | N/A |
| | Median (IQR) | 2.01 (1.76-2.46) | 1.984 (1.67-2.45) | 1.991 (1.84-2.3) | 2.21 (1.94-2.99) | | |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 45.58 (16.24) | 45.6 (14.15) | 44.59 (7.47) | 48.46 (9.986) | 56.84 | N/A |
| | Median (IQR) | 42.76 (36.11-52.42) | 44.82 (34.13-52.84) | 44.67 (40.82-47.07) | 51.19 (40.36-56.25) | | |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 5.13 (6.297) | 4.53 (4.783) | 4.66 (4.905) | 2.74 (1.307) | 2.72 | N/A |
| | Median (IQR) | 2.511 (1.87-4.48) | 2.594 (1.80-4.12) | 2.874 (1.90-3.52) | 2.124 (1.73-4.21) | | |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 262.9 (248) | 221.4 (146.2) | 231 (173.3) | 199 (119) | 243 | N/A |
| | Median (IQR) | 219.4 (136.8-290.2) | 188.8 (112.9-272.5) | 165.9 (126.3-278.7) | 201.9 (91.05-259.5) | | |

Appendix 38: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the MTHFR 677- CBS 844*ins*68 genotypes for control and adenomatous polyp subjects (1of 2)

| | | CC/NN | | CT/NN | | TT/NN | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 21.1 (11.7) | 20.9 (12) | 22.1 (11.6) | 20.9 (13.3) | 19.2 (13.4) | 32.5 (17.7) |
| | Median (IQR) | 17.5 (12-29.3) | 16 (11.8-31.5) | 20.5 (11.3-31) | 15 (11.5-34) | 16 (9-29.5) | 32.5 (20-45) |
| Serum folate excluding outliers (nM) | Mean (SD) | 17.8 (8.13) | 20.9 (12) | 20.6 (10.3) | 18.9 (11.6) | 17 (11.3) | 20 |
| | Median (IQR) | 16.5 (12-24) | 16 (11.8-31.5) | 19.5 (11-29) | 14 (10.8-30.8) | 14 (9-21.3) | |
| RBC folate (nM) | Mean (SD) | 852 (377) | 860.4 (404) | 988.6 (516) | 1046.8 (640) | 1322 (588) | 805 (226) |
| | Median (IQR) | 798 (592.5-1080) | 816 (488.5-1168) | 872 (683.3-1165) | 1094 (509.5-1319) | 1213 (833.5-2024) | 805 (645-965) |
| RBC folate excluding outliers (nM) | Mean (SD) | 827 (317) | 860.4 (404) | 914.3 (400) | 925.7 (488) | 1322 (588) | 805 (226) |
| | Median (IQR) | 783 (592.3-1074) | 816 (488.5-1168) | 868 (681.5-1115) | 944 (507.3-1283) | 1213 (833.5-2024) | 805 (645-965) |
| Cys (μM) | Mean (SD) | 257 (43) | 259.2 (38.6) | 267.5 (38.5) | 253.4 (29.7) | 270.5 (36.7) | 289.4 (53.3) |
| | Median (IQR) | 257 (231.-285.7) | 260 (225.1-290.6) | 262 (239.9-287.5) | 252 (226.6-283.5) | 265 (240-302) | 289 (251.7-327.1) |
| Hcy (μM) | Mean (SD) | 9.6 (3.04) | 11.1 (3.06) | 9.9 (2.75) | 9.7 (2.91) | 11.1 (1.39) | 10.1 (1.95) |
| | Median (IQR) | 8.86 (7.2-11.9) | 10.1 (8.4-14.7) | 9.55 (4.6-11.6) | 9.46 (6.9-12.7) | 11.4 (10.2-12.2) | 10.1 (8.7-11.5) |
| Cys-Gly (μM) | Mean (SD) | 23.6 (4.49) | 24.6 (3.4) | 23.7 (5.68) | 25.3 (7.78) | 22.9 (4.4) | 22.8 (7.06) |
| | Median (IQR) | 23.2 (20.2-26.8) | 25.3 (21-27.3) | 22.4 (20.1-25.9) | 24.8 (19.4-28.3) | 22.4 (19.6-27.1) | 22.8 (17.8-27.8) |
| GSH (μM) | Mean (SD) | 13.4 (5.7) | 12.5 (5.15) | 15 (6.61) | 15.4 (3.91) | 13.3 (3.53) | 18.7 (14) |
| | Median (IQR) | 11.4 (9.6-17) | 10.2 (8.7-17.5) | 13.3 (10.2-18.3) | 16.1 (11.2-19.5) | 11.7 (10.3-17.7) | 18.7 (8.8-28.6) |
| Serum $B_{12}$ (pM) | Mean (SD) | 275 (121) | 223.6 (89.8) | 279.2 (155) | 354.9 (184) | 249.6 (155) | 269 (15.6) |
| | Median (IQR) | 252 (205-318.8) | 191 (156.8-315.8) | 247 (187.6-326.8) | 267 (251-436.5) | 212 (174-261.5) | 259 (248-270) |

Appendix 39: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the MTHFR 677- CBS 844*ins*68 genotypes for control and adenomatous polyp subjects (2 of 2)

| | | CT/NI | | CC/NI | | TT/NI | | CC/II |
|---|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP | Control |
| Serum folate (nM) | Mean (SD) | 22.8 (13.7) | 11.5 (6.36) | 25.1 (13.9) | 27 (16.6) | 13 | 23.8 (6.75) | 14 |
| | Median (IQR) | 22 (10.5-31.5) | 11.5 (7-16) | 21 (12.8-41.3) | 18 (13.5-45) | | 23 (18-30.3) | |
| Serum folate excluding outliers (nM) | Mean (SD) | 17.3 (8.15) | 11.5 (6.36) | 18.5 (7.64) | 15 (3.61) | 13 | 23.8 (6.75) | 14 |
| | Median (IQR) | 18.5 (9.5-24.8) | 11.5 (7-16) | 18 (10.8-25.5) | 16 (11-18) | | 23 (18-30.3) | |
| RBC folate (nM) | Mean (SD) | 923.4 (487) | 465.5 (44.5) | 1172 (757) | 1047 (537) | 2500 | 1860 (401) | 550 |
| | Median (IQR) | 842 (534.3-1191) | 466 (434-497) | 1090 (519.5-1816) | 848 (596.5-1596) | | 1862 (1488-2230) | |
| RBC folate excluding outliers (nM) | Mean (SD) | 923.4 (487) | 465.5 (44.5) | 982.3 (576) | 1047 (537) | N/A | 1860 (401) | 550 |
| | Median (IQR) | 842 (534.3-1191) | 466 (434-497) | 928 (511-1292) | 848 (596.5-1596) | | 1862 (1488-2230) | |
| Cys (μM) | Mean (SD) | 269.6 (38.4) | 272.6 (46.2) | 273.9 (42.8) | 301.9 (49.5) | 287.6 | 247.9 (27.5) | 258.8 |
| | Median (IQR) | 273 (232.2-303.7) | 273 (240-305.1) | 269 (248-306.5) | 288 (259.9-351.1) | | 257 (217-269.9) | |
| Hcy (μM) | Mean (SD) | 9.9 (2.68) | 12.9 (2.42) | 11.2 (4.46) | 9.6 (1.78) | 9.7 | 7.6 (1.26) | 11.2 |
| | Median (IQR) | 9.85 (7.5-11.4) | 12.9 (11.2-14.6) | 9.48 (8.1-13.4) | 9.55 (8-11.2) | | 7.66 (6.4-8.9) | |
| Cys-Gly (μM) | Mean (SD) | 24.3 (4.64) | 23.6 (11.4) | 23.1 (2.87) | 22.9 (3.28) | 30.4 | 24.9 (4.29) | 26.8 |
| | Median (IQR) | 23.3 (10.7-29.8) | 23.6 (15.6-31.7) | 23.6 (21.2-25.7) | 20.8 (20.5-26.3) | | 26.9 (20-27.8) | |
| GSH (μM) | Mean (SD) | 15 (5.55) | 12.6 (4.47) | 11.4 (2.86) | 10.7 (2.87) | 11.9 | 12.5 (3.88) | 20.9 |
| | Median (IQR) | 13.1 (9.8-20.6) | 12.6 (9.5-15.8) | 11 (9.7-14.2) | 10.6 (7.9-13.6) | | 11.3 (9.4-16.9) | |
| Serum $B_{12}$ (pM) | Mean (SD) | 351.5 (300) | 203.5 (139) | 279.1 (117) | 266 (83.8) | 252 | 276 (54.4) | 357 |
| | Median (IQR) | 250 (148.6-497) | 204 (105-302) | 269 (199-370.5) | 242 (199-344.5) | | 278 (226.5-324) | |

Appendix 40: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the MTHFR 677- CBS 844*ins*68 genotypes for control and adenomatous polyp subjects (1 of 2)

| | | CC/NN | | CT/NN | | TT/NN | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Total grand dietary folateintake (µg/day) | Mean (SD) | 463 (245) | 451.6 (198) | 437.8 (187) | 451 (221) | 574.3 (544) | 400 (106) |
| | Median (IQR) | 392 (314-604.2) | 422 (288-578) | 397 (307.7-562) | 422 (246-634) | 426 (325.7-551.7) | 400 (324.8-475) |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 327 (149) | 341.9 (139) | 314.6 (104) | 331.3 (147) | 319.7 (89.6) | 320 (26.6) |
| | Median (IQR) | 300 (230.2-380) | 341 (223-426) | 315 (242-380) | 336 (189-425.6) | 340 (244.8-374.6) | 320 (301-338.8) |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 126 (152) | 107.3 (116) | 127 (163) | 146.4 (178) | 254.6 (550) | 80 (79.6) |
| | Median (IQR) | 79.1 (8.6-150) | 101 (14-145) | 83.8 (12.8-150) | 79.9 (22.5-247.9) | 90.9 (44.9-199.5) | 80 (23.7-136.3) |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 7 (10.2) | 5.8 (4.48) | 6.1 (10.2) | 5.5 (5.72) | 6.1 (7.89) | 2.4 (0.001) |
| | Median (IQR) | 2.75 (2-4.5) | 4.73 (2.4-8.4) | 2.83 (2.1-5.1) | 4.16 (2.2-7.1) | 2.73 (2.2-5.3) | 2.42 (2.4-2.4) |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 18.5 (26.7) | 14.4 (9.58) | 31.3 (125) | 11.2 (5.1) | 10.5 (9.97) | 7.5 (1.26) |
| | Median (IQR) | 10.6 (5.4-17.4) | 12.3 (9.7-15.6) | 10.3 (6.8-16.8) | 11 (6.6-15.9) | 5.64 (4.6-12.9) | 7.52 (6.6-8.4) |
| Total dietary methionine intake (g/day) | Mean (SD) | 2.28 (1.14) | 2.91 (2.6) | 2.22 (0.74) | 2.05 (0.73) | 2.16 (1.16) | 4.85 (3.45) |
| | Median (IQR) | 2.11 (1.76-2.46) | 2.12 (1.67-2.79) | 1.98 (1.81-2.56) | 1.96 (1.61-2.18) | 1.88 (1.63-2.45) | 4.85 (2.41-7.29) |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 46.65 (20.6) | 44.62 (11.4) | 44.66 (10.9) | 45.8 (17.6) | 43.2 (13) | 50 (4.05) |
| | Median (IQR) | 43.6 (33.7-53.8) | 45.1 (34.6-53.6) | 42.8 (37.7-51.9) | 44 (32.6-51.7) | 41.8 (33.8-50) | 50 (47.1-52.8) |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 5.4 (7.05) | 5.3 (5.53) | 4.8 (5.83) | 4.1 (4.5) | 5.4 (4.52) | 2.7 (0.08) |
| | Median (IQR) | 2.46 (1.9-5.2) | 2.64 (2.1-8.8) | 2.79 (1.9-3.8) | 2.09 (1.7-5.6) | 2.39 (1.8-10.4) | 2.65 (2.6-2.7) |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 255 (201) | 228 (146) | 275.5 (307) | 201 (142) | 242 (119) | 314.3 (235) |
| | Median (IQR) | 233 (135-308) | 224 (119-306) | 209 (152-281) | 175 (95.6-256.5) | 222 (146-270.7) | 314 (148-480.2) |

Appendix 41: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the MTHFR 677- CBS 844*ins*68 genotypes for control and adenomatous polyp subjects (2 of 2)

| | | CT/NI | | CC/NI | | TT/NI | | CC/II |
|---|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP | Control |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 431.9 (120) | 338.7 (99.5) | 410.6 (87.4) | 396 (93) | 768.5 | 443.3 (192) | 363 |
| | Median (IQR) | 396 (342-520.7) | 339 (268.4-409.1) | 434 (309.1-476.6) | 376 (316-486) | | 389 (301-639.7) | |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 308.4 (84) | 217 (51.2) | 327.6 (73.6) | 352.5 (99.5) | 729.2 | 338 (134) | 363 |
| | Median (IQR) | 291 (245-360.5) | 217 (181-253.2) | 306 (283.4-367.8) | 376 (272-421.2) | | 369 (202.2-442.2) | |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 123.5 (138) | 122 (48.3) | 83 (71.2) | 43.5 (44.7) | 39.3 | 105.4 (113) | 0 |
| | Median (IQR) | 86.1 (2.6-202.4) | 122 (87.5-155.8) | 71.4 (15.7-139) | 39 (0-89.2) | | 82.8 (102-223.3) | |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 5.3 (4.91) | 2.2 (0.91) | 3.1 (1.82) | 2.5 (0.55) | 3.5 | 5 (1.63) | 2.6 |
| | Median (IQR) | 2.63 (2.2-7.5) | 2.2 (1.6-2.8) | 2.32 (2.1-4) | 2.34 (2-3) | | 5.04 (3.4-6.5) | |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 11.5 (6.28) | 10.6 (5.68) | 10.1 (4.57) | 7.7 (3.44) | 10.3 | 8.1 (4.4) | 9 |
| | Median (IQR) | 12.4 (4.6-15.9) | 10.6 (6.6-14.6) | 9.82 (5.2-15) | 6.89 (4.9-11) | | 7.59 (4.2-12.6) | |
| Total dietary methionine intake (g/day) | Mean (SD) | 1.89 (0.4) | 2.87 (0.18) | 2.24 (0.38) | 2.55 (0.72) | 2.08 | 1.77 (0.4) | 2.66 |
| | Median (IQR) | 1.85 (1.58-2.17) | 2.87 (2.74-3) | 2.16 (1.93-2.39) | 2.78 (1.84-3.14) | | 1.94 (1.37-1.99) | |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 41.2 (6.04) | 56.14 (7) | 47.03 (6.47) | 50.28 (7.75) | 59.4 | 42.36 (11.9) | 56.84 |
| | Median (IQR) | 43.8 (35-45.46) | 56.1 (51.19-61.09) | 46.5 (41.6-53.4) | 52.7 (42.2-57.16) | | 45.2 (29.9-52) | |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 6 (6.38) | 2.4 (0.91) | 3.2 (2.02) | 2.9 (1.36) | 2.9 | 2.8 (1.68) | 2.7 |
| | Median (IQR) | 2.75 (1.9-11.5) | 2.37 (1.7-3) | 2.63 (1.9-3.5) | 2.12 (1.8-4.3) | | 2.07 (1.7-4.5) | |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 205 (88.1) | 51.1 (31.4) | 246.6 (252) | 222.1 (72.9) | 367 | 244.1 (147) | 243 |
| | Median (IQR) | 201 (126.2-286.6) | 51.1 (29-73.3) | 151 (125.6-248.8) | 239 (154-282) | | 221 (119-392.4) | |

Appendix 42: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the CTH G1364T genotypes for control and adenomatous polyp subjects

| | | Wild type (GG) | | Heterozygous type (GT) | | Homo recessive type (TT) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP (n=1) |
| Serum folate (nM) | Mean (SD) | 22.28 (12.4) | 22.4 (13.4) | 21.17 (11.7) | 22.53 (12.1) | 19.3 (9.78) | 10 |
| | Median (IQR) | 20 (12.0-31) | 17.5 (13.25-32) | 18 (11-30) | 18 (13.0-33.5) | 17.5 (14.5-20.25) | |
| Serum folate excluding outliers(nM) | Mean (SD) | 18.56 (8.98) | 18.41 (10) | 19.29 (9.99) | 21.13 (11) | 16.44 (3.97) | 10 |
| | Median (IQR) | 16 (11.5-24) | 16 (13-30.75) | 17.5 (10.25-25.75) | 18 (13.0-30.75) | 17 (13.0-19.5) | |
| RBC folate (nM) | Mean (SD) | 991.1 (515) | 1040 (596) | 970.6 (505) | 1060 (559) | 854.8 (521) | 342 |
| | Median (IQR) | 840 (607-1189) | 1047 (538.5-1423.50 | 878 (594-1144) | 900 (574.5-1400) | 690 (471-1194) | |
| RBC folate excluding outliers (nM) | Mean (SD) | 924.5 (413) | 962.7 (500) | 911.8 (412) | 1060 (559) | 854.8 (521) | 342 |
| | Median (IQR) | 823 (595.8-1142) | 1000 (532-1317) | 872 (590-1116) | 900 (574.5-1400) | 690 (471-1194) | |
| Cys (µM) | Mean (SD) | 265.6 (43.9) | 265.5 (44.1) | 261.8 (39.1) | 266.5 (36.7) | 267.6 (20) | 264.2 |
| | Median (IQR) | 263 (233.7-293.9) | 259 (232-291.4) | 260 (237.2-286) | 266 (234.5-290.9) | 267 (252.4-286.3) | |
| Hcy (µM) | Mean (SD) | 9.83 (2.95) | 10.34 (2.82) | 10.07 (2.95) | 9.64 (2.54) | 9.36 (2.19) | 14.03 |
| | Median (IQR) | 9.78 (7.41-12) | 9.59 (8.31-12.17) | 9.59 (8.36-11.87) | 9.28 (7.52-11.34) | 8.77 (7.46-11.43) | |
| Cys-Gly (µM) | Mean (SD) | 23.97 (5.95) | 24.89 (6.83) | 23.15 (3.58) | 24.43 (3.47) | 25.37 (5.21) | 16.4 |
| | Median (IQR) | 23 (20.51-27.36) | 23.4 (20.18-29.04) | 22.8 (20.28-26.04) | 25.4 (20.13-27.63) | 25.9 (19.53-29.64) | |
| GSH (µM) | Mean (SD) | 13.68 (6.35) | 11.51 (3.6) | 14.49 (5.45) | 15.61 (5.79) | 12.41 (4.97) | 17.2 |
| | Median (IQR) | 11.4 (9.57-16.67) | 9.85 (8.86-15.34) | 13.2 (10.41-18.52) | 14.9 (11.08-19.64) | 11 (10.02-13.65) | |
| Serum $B_{12}$ (pM) | Mean (SD) | 272.5 (132) | 274.8 (172) | 284.9 (167) | 294.5 (76.4) | 292.7 (163) | 152 |
| | Median (IQR) | 254 (201-320) | 243 (166.7-298.5) | 222 (185.5-330.5) | 273 (235-331) | 257 (223.5-300) | |

Appendix 43: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the CTH G1364T genotypes for control and adenomatous polyp subjects

| | | Wild type (GG) | | Heterozygous type (GT) | | Homo recessive type (TT) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP (n=1) |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 448 (197.1) | 410.5 (166.5) | 444 (215.9) | 479 (189.5) | 661 (602.4) | 164.5 |
| | Median (IQR) | 396.2 (333-528) | 385.3 (277.3-521.4) | 390 (296-581) | 475 (330-548) | 486.3 (371.4-634) | |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 337 (133.3) | 319.4 (148) | 308 (115.5) | 356 (94.67) | 345 (123.5) | 164.5 |
| | Median (IQR) | 324.4 (248-396) | 276.6 (187-417.8) | 296.8 (232-363.4) | 397.3 (312.01-421.2) | 325.1 (255.5-411) | |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 114 (146.1) | 107.1 (128) | 127 (152.3) | 123 (136.5) | 316 (624.9) | 0 |
| | Median (IQR) | 71.14 (12.6-112) | 87.5 (24.53-134) | 89.91 (4.18-179) | 90.9 (9.74-166.9) | 95.4 (64.1-223) | |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 5.72 (7.542) | 3.76 (1.951) | 6.87 (11.23) | 6.26 (5.868) | 4.68 (4.571) | 1.23 |
| | Median (IQR) | 2.666 (2.06-4.35) | 2.794 (2.20-5.52) | 2.888 (2.06-5.52) | 3.984 (2.39-8.08) | 2.999 (2.34-5.09) | |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 29.02 (118.9) | 10.65 (7.859) | 17.19 (22.92) | 12.24 (5.351) | 10.65 (5.47) | 4 |
| | Median (IQR) | 10.34 (5.5-17.19) | 9.402 (4.91-14.4) | 10.07 (5.63-15.76) | 13.02 (8-15.66) | 9.753 (5.54-14.7) | |
| Total dietary methionine intake (mg/day) | Mean (SD) | 2.25 (1.119) | 2.91 (2.289) | 2.19 (0.775) | 2.2 (0.689) | 2.25 (0.5) | 1.33 |
| | Median (IQR) | 2.078 (1.8-2.36) | 2.077 (1.65-3.05) | 1.954 (1.71-2.52) | 2.01 (1.76-2.46) | 2.14 (1.93-2.55) | |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 45.54 (16.76) | 45.93 (12.34) | 45.5 (14.8) | 48.4 (13.14) | 45.8 (11.55) | 23.1 |
| | Median (IQR) | 44.02 (36.13-51.24) | 44.83 (35.2-56.11) | 42.1 (36.91-54.98) | 47.2 (39.9-52.8) | 45.25 (35.98-51.22) | |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 4.63 (5.94) | 3.16 (3.307) | 5.46 (6.439) | 5.2 (4.878) | 4.84 (4.924) | 0.86 |
| | Median (IQR) | 2.475 (1.89-3.47) | 2.159 (1.60-2.95) | 2.789 (1.85-5.80) | 2.63 (2.03-7.88) | 2.469 (2.25-6.28) | |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 251 (149.1) | 182 (123.2) | 266.63 (307.1) | 262.3 (143.9) | 256.1 (95.56) | 67.3 |
| | Median (IQR) | 230.8 (151-295) | 161.5 (94.7-241) | 181 (120.1-279) | 239.4 (172.7-330) | 260.9 (171.2-315) | |

Appendix 44: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the CTH IVS 10-430 C>T genotypes for control and adenomatous polyp subjects

| | | Wild type (CC) | | Heterozygous type (CT) | | Homo recessive type (TT) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 22.17 (11.99) | 23.48 (12.93) | 20.88 (12.1) | 23.54 (12.29) | 20.23 (10.98) | 10.5 (6.952) |
| | Median (IQR) | 20 (11.75-30.25) | 18 (13.0-34.0) | 17 (12.0-30.0) | 18 (15.0-34.0) | 14 (11.5-28.5) | 9 (4.75-17.75) |
| Serum folate excluding outliers(nM) | Mean (SD) | 19.31 (9.361) | 19.89 (10.06) | 18.2 (9.48) | 21.75 (10.93) | 18.17 (8.43) | 10.5 (6.952) |
| | Median (IQR) | 18 (10.25-25.0) | 17 (12.5-29.0) | 15.5 (11.0-24.0) | 17.5 (15.0-30.75) | 14 (10.75-26.0) | 9 (4.75-17.75) |
| RBC folate (nM) | Mean (SD) | 941.9 (480.9) | 997 (551.9) | 1025 (545.9) | 1269 (567.2) | 937.8 (528.4) | 429 (162.5) |
| | Median (IQR) | 869 (603.5-1115) | 965 (505.5-1345) | 852.5 (592.2-1283) | 1181 (820.5-1734) | 885 (575-1046) | 466 (260.7-559) |
| RBC folate excluding outliers (nM) | Mean (SD) | 906.1 (423) | 921.8 (442.4) | 947.6 (437.4) | 1269 (567.2) | 807.7 (253.5) | 428.7 (162.5) |
| | Median (IQR) | 843 (595-1092) | 870.5 (501.2-1282) | 847 (586-1191) | 1181 (820.5-1734) | 848.5 (557.5-1007) | 466 (260.7-559.5) |
| Cys (μM) | Mean (SD) | 264 (38.85) | 266.2 (35.35) | 259.5 (42.34) | 258.5 (38.05) | 284 (34.71) | 279.4 (65.75) |
| | Median (IQR) | 261.6 (238.9-287) | 264.2 (239.5-293.4) | 255.4 (236.7-284.9) | 255.9 (223.1-285) | 289.4 (267.4-310.8) | 259.1 (231.5-347.7) |
| Hcy (μM) | Mean (SD) | 9.99 (2.845) | 10.22 (2.77) | 9.87 (3.056) | 9.04 (2.646) | 9.65 (2.557) | 11.97 (2.032) |
| | Median (IQR) | 9.715 (7.64-11.78) | 9.07 (8.35-11.45) | 9.545 (7.55-12.0) | 8.495 (6.74-11.26) | 9.7 (7.28-10.68) | 11.54 (10.27-14.09) |
| Cys-Gly (μM) | Mean (SD) | 24.11 (5.066) | 24.51 (6.416) | 22.83 (4.497) | 23.59 (3.845) | 24.7 (5.18) | 25.57 (4.813) |
| | Median (IQR) | 23.02 (20.34-26.73) | 24.28 (19.95-27.73) | 23.24 (20.43-26.23) | 25.13 (19.08-26.51) | 23.16 (20.82-30.05) | 25.81 (21.08-29.82) |
| GSH (μM) | Mean (SD) | 14.22 (5.746) | 14.38 (5.703) | 13.73 (6.134) | 12.56 (4.47) | 13.61 (5.166) | 11.56 (1.677) |
| | Median (IQR) | 12.75 (10.17-16.32) | 13.89 (9.41-18.96) | 11.32 (9.45-18.23) | 10.38 (8.93-16.71) | 11.95 (10.27-16.13) | 11.03 (10.32-13.32) |
| Serum $B_{12}$ (pM) | Mean (SD) | 262.2 (110.4) | 286.1 (107.7) | 311.4 (205) | 255.3 (76.04) | 258 (77.32) | 331.7 (344.2) |
| | Median (IQR) | 248.5 (188.5-311.2) | 288 (197.5-342.5) | 250 (199.7-338.2) | 243 (213.5-271.5) | 252 (200.5-336) | 182 (122.0-691.2) |

Appendix 45: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the CTH IVS 10-430 C>T genotypes for control and adenomatous polyp subjects

| | | Wild type (CC) | | Heterozygous type (CT) | | Homo recessive type (TT) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 465.5 (287.7) | 444.83 (178.7) | 436.23 (172.1) | 454.92 (186.3) | 523.05 (298.8) | 313.34 (179.5) |
| | Median (IQR) | 380.9 (306-584.7) | 409.1 (291.67-539.7) | 403.5 (337.4-515.1) | 410.5 (302.9-546.2) | 404.8 (330.5-700.8) | 274.2 (167.4-498.4) |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 314.79 (121.4) | 330.23 (145) | 313.73 (89.03) | 341.6 (86.55) | 422.05 (219.5) | 305.69 (169.4) |
| | Median (IQR) | 297.9 (232.8-372) | 338.8 (193.1-424.3) | 310.9 (241.9-377.3) | 361.8 (269.1-411.9) | 322.1 (268.2-554.6) | 274.2 (164.7-478.2) |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 152.4 (250.6) | 129.94 (139.2) | 111.1 (156) | 113.32 (124.6) | 101 (138.8) | 7.643 (13.01) |
| | Median (IQR) | 90.9 (13.85-152.6) | 99.9 (25.98-144) | 61.36 (5.13-145.3) | 79.92 (31.1-162.4) | 39.29 (8.73-125.8) | 1.785 (0.0-21.14) |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 5.84 (8.487) | 4.52 (3.698) | 6.64 (10.88) | 5.83 (5.632) | 7 (9.113) | 3.04 (1.941) |
| | Median (IQR) | 2.611 (2.0-4.56) | 2.847 (2.35-6.12) | 2.8 (2.27-5.62) | 4.163 (2.35-7.0) | 3.519 (2.17-6.74) | 2.474 (1.55-5.09) |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 16.15 (22.57) | 10.85 (5.558) | 32.26 (127.9) | 10.48 (4.568) | 13.21 (16.13) | 15.23 (15.97) |
| | Median (IQR) | 9.807 (5.53-15.65) | 10.21 (6.20-15.26) | 11.47 (5.55-17.22) | 10 (6.38-15.28) | 9.198 (5.86-10.78) | 9.338 (4.83-31.51) |
| Total dietary methionine intake (g/day) | Mean (SD) | 2.221 (0.822) | 2.25 (0.808) | 2.15 (0.691) | 2.45 (1.626) | 2.56 (2.015) | 4.45 (4.165) |
| | Median (IQR) | 1.965 (1.79-2.46) | 2.21 (1.71-2.76) | 2.012 (1.74-2.47) | 1.959 (1.68-2.28) | 2.081 (1.63-2.37) | 2.796 (1.77-8.76) |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 45.5 (14.42) | 47.17 (12.79) | 43.99 (10.61) | 45.85 (13.96) | 53.02 (32.52) | 44.46 (14.48) |
| | Median (IQR) | 42.52 (36.26-54.20) | 48.15 (36.53-55.71) | 43.05 (37.54-51.24) | 44.01 (39-47.22) | 43.83 (33.36-56.41) | 44.91 (30.98-57.48) |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 5.15 (6.609) | 4.23 (4.222) | 5.05 (5.722) | 4.22 (4.684) | 4.52 (4.657) | 2.15 (0.767) |
| | Median (IQR) | 2.608 (1.86-4.55) | 2.352 (1.86-4.33) | 2.851 (1.91-3.90) | 2.203 (1.65-4.69) | 2.317 (1.93-6.36) | 2.382 (1.34-2.72) |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 262.13 (285.1) | 225.32 (162) | 256.59 (161.8) | 215.53 (99.1) | 249.7 (216.5) | 158.7 (120.7) |
| | Median (IQR) | 192.6 (136.7-287.3) | 194.8 (82.17-288.5) | 240.7 (128.8-279.9) | 201.9 (130.6-280.2) | 184.2 (128.6-338.7) | 168.9 (43.97-263.2) |

Appendix 46: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the CTH G1364T-IVS 10-430 C>T genotypes for control and adenomatous polyp subjects (1 of 2)

| | | GT/CC | | GG/CC | | GG/CT | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 20.1 (11.31) | 23.4 (13) | 25.1 (13.4) | 24.9 (13.42) | 19.8 (11.75) | 26.2 (13.67) |
| | Median (IQR) | 19.5 (10-28.5) | 19 (12.5-37) | 23 (13-38) | 22.5 (13.8-35.3) | 16.5 (11.5-25.8) | 19.5 (15.8-42.8) |
| Serum folate excluding outliers(nM) | Mean (SD) | 19.5 (9.879) | 21 (11.19) | 19.9 (9.615) | 19.9 (9.342) | 17 (8.517) | 22.4 (11.28) |
| | Median (IQR) | 18 (10-26.5) | 18 (12-32) | 20 (11-29) | 17 (13.3-30.8) | 15 (10-23) | 17 (15.5-32) |
| RBC folate (nM) | Mean (SD) | 960 (516.7) | 866.1 (395.1) | 947.8 (402) | 1193.3 (641.2) | 1050.9 (610.5) | 1190.5 (469) |
| | Median (IQR) | 906 (595-1092) | 754 (504-1226) | 893 (657.5-1201) | 1140 (523.3-1549.3) | 819.5 (577-1446.8) | 1091 (797.3-1650.3) |
| RBC folate excluding outliers (nM) | Mean (SD) | 897.2 (418.6) | 866.1 (395.1) | 947.8 (402) | 1048.1 (474.8) | 947.4 (483.4) | 1190.5 (469) |
| | Median (IQR) | 875 (588-1090) | 754 (504-1226) | 893 (657.5-1361.8) | 1138 (514.5-1388) | 801.5 (571-1361.8) | 1091 (797.3-1650.3) |
| Cys (μM) | Mean (SD) | 262.4 (36.95) | 271.6 (38.78) | 266 (47.95) | 259.8 (34.49) | 259.6 (42.63) | 263.1 (47.86) |
| | Median (IQR) | 260.6 (235.5-287) | 273.9 (236-301.4) | 261.7 (233.8-289.5) | 262.1 (232-192.4) | 257.5 (234.4-289.3) | 255.9 (222.4-310.9) |
| Hcy (μM) | Mean (SD) | 10.1 (2.612) | 9.7 (2.415) | 10 (3.49) | 10.4 (3.128) | 9.8 (2.683) | 8.7 (2.373) |
| | Median (IQR) | 9.715 (8.6-11.9) | 9.275 (8.1-11.3) | 9.86 (7-12) | 8.98 (8.3-13.7) | 9.875 (7.5-12) | 8.495 (6.5-11) |
| Cys-Gly (μM) | Mean (SD) | 22.7 (3.491) | 24.6 (3.348) | 26.4 (6.539) | 25.5 (8.999) | 22.1 (5.246) | 23.1 (3.905) |
| | Median (IQR) | 22.13 (20.1-25.4) | 25.72 (20.1-27.6) | 23.33 (21.8-30.1) | 22.52 (19.9-30.7) | 21.75 (18.9-24.8) | 23.77 (19-26.4) |
| GSH (μM) | Mean (SD) | 14.4 (4.764) | 16.1 (6.273) | 15.1 (7.797) | 11.9 (4.506) | 13.1 (5.827) | 10.8 (3.687) |
| | Median (IQR) | 13.54 (10.5-18.2) | 14.93 (11.1-20.1) | 11.46 (9.9-17.8) | 9.445 (8.6-15.9) | 11.14 (9.2-17.9) | 9.1 (8.6-14.5) |
| Serum $B_{12}$ (pM) | Mean (SD) | 264.5 (114) | 298.1 (76.84) | 246.6 (77.34) | 287.6 (133) | 304.1 (180.4) | 215.5 (48.4) |
| | Median (IQR) | 225.5 (187-321.8) | 305 (234-349) | 254 (185.5-310.5) | 276.5 (193.5-363.5) | 261 (217-333.5) | 219 (167.5-261.8) |

Appendix 47: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the CTH G1364T-IVS 10-430 C>T genotypes for control and adenomatous polyp subjects (2 of 2)

| | | GT/CT | | GG/TT | | TT/CC | | GT/TT | TT/CT |
|---|---|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP | Control | Control |
| Serum folate (nM) | Mean (SD) | 22.1 (12.74) | 21.3 (11.56) | 21 (11.54) | 10.5 (6.952) | 19.6 (10.33) | 10 | 16 (8.485) | 17 |
| | Median (IQR) | 17 (11.5-30.5) | 18 (13-31) | 14 (13.0-30.0) | 9 (4.8-17.8) | 18 (13-20.5) | | 16 (10-22) | |
| Serum folate excluding outliers(nM) | Mean (SD) | 19.5 (10.57) | 21.3 (11.56) | 18.6 (8.809) | 10.5 (6.952) | 16.4 (4.241) | 10 | 16 (8.485) | 17 |
| | Median (IQR) | 16 (11-29.3) | 18 (13-31) | 14 (12.3-27.8) | 9 (4.8-17.8) | 17.5 (11.5-19.8) | | 16 (10-22) | |
| RBC folate (nM) | Mean (SD) | 993.7 (489.3) | 1336.1 (669.8) | 964.4 (548.8) | 428.8 (162.5) | 819.9 (539.7) | 342 | 792 (537.4) | 1169 |
| | Median (IQR) | 868 (653-1232) | 1322 (793-2148) | 885 (610-1009) | 466 (260.8-559.5) | 689 (462-998) | | 792 (412-1172) | |
| RBC folate excluding outliers (nM) | Mean (SD) | 939.9 (401.6) | 1336.1 (669.8) | 810.8 (215.3) | 428.8 (162.5) | 819.9 (539.7) | 342 | 792 (537.4) | 1169 |
| | Median (IQR) | 862.5 (623-1193) | 1322 (793-2148) | 848.5 (592.5-1003) | 466 (260.8-559.5) | 689 (462-998) | | 792 (412-1172) | |
| Cys (μM) | Mean (SD) | 258.9 (43.68) | 254..0 (32.12) | 285.2 (36.47) | 279.4 (65.75) | 267.1 (21.21) | 264.2 | 276.1 | 271.7 |
| | Median (IQR) | 245 (237-285) | 254 (223-285) | 291 (258-312) | 259 (231-347.7) | 264.3 (251-287) | | | |
| Hcy (μM) | Mean (SD) | 10 (3.524) | 9.4 (3.213) | 9.9 (2.587) | 12 (2.032) | 9.5 (2.305) | 14.03 | 7.5 | 8.5 |
| | Median (IQR) | 9.4 (7.8-11.2) | 8.75 (6.7-12.8) | 9.97 (7.2-11.5) | 11.54 (10.3-14.1) | 9.03 (7.4-11.5) | | | |
| Cys-Gly (μM) | Mean (SD) | 23.5 (3.383) | 24.1 (4.3) | 24 (4.902) | 25.6 (4.813) | 25.1 (5.443) | 16.4 | 31.6 | 27.9 |
| | Median (IQR) | 23.78 (21.3-26.6) | 25.38 (19.8-27.3) | 22.34 (20.2-30) | 25.81 (21.1-29.8) | 23.99 (19.3-30.4) | | | |
| GSH (μM) | Mean (SD) | 14.1 (6.259) | 14.4 (4.937) | 12.5 (3.669) | 11.6 (1.677) | 11 (2.559) | 17.2 | 25.1 | 24.8 |
| | Median (IQR) | 11.35 (10.3-19.2) | 14.13 (9.8-19.2) | 11.9 (10-14.9) | 11.03 (10.3-13.3) | 10.91 (9.8-12.7) | | | |
| Serum $B_{12}$ (pM) | Mean (SD) | 321.7 (233.4) | 289.4 (81.67) | 256.9 (76.72) | 331.8 (344.2) | 299.1 (171.6) | 152 | 264 (113.1) | 235 |
| | Median (IQR) | 218 (184.5-403) | 267 (234-325) | 252 (206-328) | 182 (122-691.3) | 258 (219.5-303.5) | | 264 (184-344) | |

Appendix 48: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the CTH G1364T-IVS 10-430 C>T genotypes for control and adenomatous polyp subjects (1 of 2)

| | GT/CC | | GG/CC | | GG/CT | |
|---|---|---|---|---|---|---|
| | Control | AP | Control | AP | Control | AP |
| Total grand dietary folate intake (µg/day) | 433.2 (217.3) | 432.2 (167.1) | 455.1 (205.3) | 485.5 (179.8) | 407 (108.1) | 350.1 (67.44) |
| | 373.2 (287.7-565.7) | 405.8 (294.9-489.7) | 384.9 (314-632.5) | 438.7 (333-696.9) | 396.2 (343.6-443.3) | 359.5 (277.9-413.2) |
| Total dietary methyl folate intake (µg/day) | 313.9 (131.4) | 334.4 (103.3) | 309 (101.8) | 342.6 (181.1) | 325 (92.34) | 289.9 (68.74) |
| | 288.2 (229.2-373.2) | 369.5 (254-415) | 308.2 (237.9-366) | 266.2 (199.2-505.4) | 327.1 (240.1-396) | 287.6 (243.4-347.6) |
| Total dietary PteGlu intake (µg/day) | 122.3 (137.7) | 97.8 (123.9) | 146.1 (160.1) | 175.1 (149.7) | 89.2 (131.1) | 60.2 (45.23) |
| | 89.91 (0.5-157.1) | 84.41 (0.5-138.6) | 90.9 (47.5-143.6) | 119.5 (90.1-215.3) | 51.24 (2.6-93.7) | 48.46 (22-99.7) |
| Total dietary pyridoxine intake (µg/day) | 5.9 (8.876) | 5.3 (4.949) | 6 (8.874) | 4.1 (1.981) | 5 (5.217) | 3.7 (2.109) |
| | 2.73 (2.1-4.6) | 2.85 (2.4-7.7) | 2.434 (1.9-4.8) | 3.679 (2.5-5.8) | 2.733 (2.4-4.4) | 2.463 (2.3-5.9) |
| Total dietary cobalamin intake (µg/day) | 15.5 (19.31) | 11.7 (6.674) | 19 (30.46) | 10.7 (4.259) | 43.8 (177.9) | 7.5 (4.485) |
| | 8.97 (5.6-15.3) | 11.62 (5.7-15.8) | 10.1 (5.4-18.9) | 10.34 (6.8-14.7) | 10.97 (5.3-17.2) | 6.376 (3.8-10.9) |
| Total dietary methionine intake (g/day) | 2.29 (0.831) | 2.14 (0.546) | 2.07 (0.887) | 2.45 (1) | 2.26 (0.732) | 2.64 (2.304) |
| | 1.976 (1.82-2.64) | 2.239 (1.69-2.53) | 1.884 (1.62-2.19) | 2.216 (1.82-3.01) | 2.163 (1.87-2.6) | 1.867 (1.41-3.39) |
| Total dietary niacin intake equivalent(mg/day) | 47.05 (15.75) | 47.19 (10.15) | 42..41 (12.25) | 49.56 (13.77) | 44.7 (8.452) | 40.86 (7.24) |
| | 42.32 (38.36-56.46) | 49.79 (38.44-53.42) | 43.14 (34.24-49.53) | 49.67 (39.71-61.25) | 44.53 (38.11-51.52) | 43.42 (35.71-45.81) |
| Total dietary riboflavin intake (mg/day) | 4.9 (5.832) | 4.4 (4.332) | 5.6 (8.369) | 4.4 (4.417) | 3.7 (3.017) | 1.8 (0.482) |
| | 2.745 (1.9-4.6) | 2.476 (2.0-5.8) | 2.516 (1.7-3.5) | 2.675 (1.7-5.1) | 2.52 (1.9-3.5) | 1.649 (1.4-2.3) |
| Total dietary vitamin C intake (mg/day) | 280.9 (363.8) | 267.9 (167.8) | 229.3 (100.7) | 198.5 (156.7) | 264.7 (154.9) | 170.3 (59.81) |
| | 181 (121.7-278.2) | 239.9 (154.5-373.3) | 228.4 (151.7-291.8) | 171.2 (86.6-236.2) | 230.8 (161.3-282) | 161.5 (110.8-240.5) |

Appendix 49: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the CTH G1364T-IVS 10-430 C>T genotypes for control and adenomatous polyp subjects (2 of 2)

| | GT/CT | | GG/TT | | TT/CC | | GT/TT | TT/CT |
|---|---|---|---|---|---|---|---|---|
| | Control | AP | Control | AP | Control | AP | Control | Control |
| Total grand dietary folate intake (µg/day) | 464.8 (220.2)<br>408.3 (297.9-607.4) | 544.8 (212.6)<br>501 (400.4-719.4) | 556.3 (314.6)<br>409.8 (358.7-768.5) | 313.3 (179.5)<br>274.2 (167.4-498.4) | 680.8 (635.5)<br>487.8 (366.9-642.4) | 164.5 | 340.2 (53.67)<br>340.2 (302.3-378.2) | 484.9 |
| Total dietary methyl folate intake (µg/day) | 298.8 (84.7)<br>306.1 (241.1-346.4) | 385.9 (77.94)<br>402.8 (361.8-430.2) | 446.8 (231.1)<br>394.3 (260.7-594.5) | 305.7 (169.4)<br>274.2 (164.7-478.2) | 338 (128.7)<br>301.5 (253.5-392.8) | 164.5 | 285.7 (13.96)<br>285.7 (275.8-295.5) | 409.9 |
| Total dietary PteGlu intake (µg/day) | 134.9 (179.8)<br>81.75 (13.2-203.3) | 158.8 (155.3)<br>90.9 (38.6-255.3) | 109.4 (148.9)<br>39.29 (10.7-149.2) | 7.6 (13.01)<br>1.785 (0-21.1) | 342.8 (656.7)<br>99.9 (53.2-295.5) | 0 | 54.6 (67.62)<br>54.56 (6.7-102.4) | 75 |
| Total dietary pyridoxine intake (µg/day) | 8.6 (14.73)<br>3.038 (1.8-6.8) | 7.7 (7.139)<br>4.561 (4.0-9.0) | 7.4 (9.837)<br>3.519 (2.3-6.3) | 3 (1.941)<br>2.474 (1.6-5.1) | 4.9 (4.807)<br>3.003 (2.2-5.8) | 1.23 | 4.5 (3.795)<br>4.518 (1.8-7.2) | 2.99 |
| Total dietary cobalamin intake (µg/day) | 20.7 (28.62)<br>13.14 (6.2-18.1) | 13 (2.869)<br>13.71 (10.0-15.7) | 13.9 (17.59)<br>8.366 (5.8-11.3) | 15.2 (15.97)<br>9.338 (4.8-31.5) | 10.8 (5.785)<br>10.03 (5.3-15.6) | 4 | 9.7 (0.711)<br>9.701 (9.2-10.2) | 9.47 |
| Total dietary methionine intake (g/day) | 2.05 (0.65)<br>1.951 (1.57-2.44) | 2.29 (0.895)<br>1.959 (1.74-2.48) | 2.73 (2.16)<br>2.214 (1.97-2.38) | 4.45 (4.165)<br>2.796 (1.78-8.76) | 2.3 (0.504)<br>2.156 (1.97-2.66) | 1.335 | 1.63 (0.063)<br>1.627 (1.58-1.67) | 1.81 |
| Total dietary niacin intake equivalent(mg/day) | 43.3 (12.78)<br>40.37 (33.9-50.8) | 50.1 (17.32)<br>45.35 (39.5-50.8) | 56.8 (34.18)<br>44.37 (42.6-59.4) | 44.5 (14.48)<br>44.91 (31.0-57.5) | 46.16 (12.2)<br>47.88 (35.6-53.0) | 23.1 | 32.4 (1.315)<br>32.43 (31.5-33.4) | 42.62 |
| Total dietary riboflavin intake (mg/day) | 6.6 (7.466)<br>2.969 (1.8-10.1) | 6.3 (5.741)<br>4.12 (2.2-10.5) | 5 (4.919)<br>2.36 (2.1-9.9) | 2.1 (0.767)<br>2.382 (1.3-2.7) | 5.2 (5.116)<br>2.488 (2.3-7.2) | 0.86 | 1.7 (0.163)<br>1.741 (1.6-1.9) | 2.02 |
| Total dietary vitamin C intake (mg/day) | 248 (173.9)<br>240.7 (119.9-286.1) | 254.3 (113.4)<br>219.9 (160.5-321.9) | 266.5 (232.8)<br>190.5 (113.7-367.1) | 158.7 (120.7)<br>168.9 (44-263.2) | 256.1 (101.4)<br>265.7 (163.4-338.4) | 67.3 | 157.1 (2.114)<br>157.1 (155.7-158.6) | 256 |

Appendix 50: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the BHMT G595A genotypes for control and adenomatous polyp subjects

| | | Wild type (GG) | | Heterozygous type (GA) | | Homo recessive type (AA, n=0) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP (n=0) | Control | AP |
| Serum folate (nM) | Mean (SD) | 21.47 (11.86) | 22.13 (12.63) | 24.25 (15.2) | N/A | N/A | N/A |
| | Median (IQR) | 18 (12.0-30.0) | 18 (13.0-32.0) | 25 (9.5-38.25) | | | |
| Serum folate excluding outliers(nM) | Mean (SD) | 18.65 (9.11) | 19.44 (10.4) | 24.25 (15.2) | N/A | N/A | N/A |
| | Median (IQR) | 17 (11.0-24.25) | 16.5 (12.5-28.5) | 25 (9.5-38.25) | | | |
| RBC folate (nM) | Mean (SD) | 975.1 (511.4) | 1030 (575.2) | 867 (383.9) | N/A | N/A | N/A |
| | Median (IQR) | 868.5 (594.5-1157) | 932.5 (527.5-1387) | 735.5 (601.5-1264) | | | |
| RBC folate excluding outliers (nM) | Mean (SD) | 914.9 (419.4) | 990.5 (527.2) | 867 (383.9) | N/A | N/A | N/A |
| | Median (IQR) | 847.5 (592.3-1120) | 900 (523.0-1347.5) | 735.5 (601.5-1264) | | | |
| Cys (μM) | Mean (SD) | 264 (40.34) | 265.9 (39.48) | 259.9 (39.05) | N/A | N/A | N/A |
| | Median (IQR) | 262.1 (237.4-288.5) | 261.3 (237.6-290) | 244.1 (235.3-300.2) | | | |
| Hcy (μM) | Mean (SD) | 9.9 (2.89) | 10.14 (2.72) | 10.32 (3.33) | N/A | N/A | N/A |
| | Median (IQR) | 9.69 (7.56-11.84) | 9.55 (8.3-11.73) | 9.525 (7.63-13.82) | | | |
| Cys-Gly (μM) | Mean (SD) | 23.66 (4.89) | 24.41 (5.553) | 23.98 (4.98) | N/A | N/A | N/A |
| | Median (IQR) | 23.17 (20.49-26.69) | 25 (20.0-27.73) | 23.46 (19.63-28.86) | | | |
| GSH (μM) | Mean (SD) | 13.89 (5.82) | 13.54 (5.07) | 17.15 (5.94) | N/A | N/A | N/A |
| | Median (IQR) | 12.03 (10.0-17.61) | 11.41 (9.36-16.86) | 17.86 (11.11-22.48) | | | |
| Serum $B_{12}$ (pM) | Mean (SD) | 281.5 (152.9) | 280.4 (135.2) | 219.5 (82.63) | N/A | N/A | N/A |
| | Median (IQR) | 251 (195.0-321.0) | 262 (194.2-326.2) | 210 (145.0-303.5) | | | |

Appendix 51: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the BHMT G595A genotypes for control and adenomatous polyp subjects

| | | Wild type (GG) | | Heterozygous type (GA) | | Homo recessive type (AA, n=0) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP (n=0) | Control | AP |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 459.4 (254.2) | 434.4 (181.5) | 454.1 (98.7) | N/A | N/A | N/A |
| | Median (IQR) | 394.8 (316.4-578.2) | 404.7 (284.19-512.62) | 444.3 (364.2-553.7) | | | |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 322.5 (125.5) | 331.5 (127.4) | 343.1 (49.98) | N/A | N/A | N/A |
| | Median (IQR) | 306.1 (240.8-374.6) | 350.3 (208.23-421.16) | 351.5 (0.758-384.3) | | | |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 133.4 (214) | 111.4 (129.9) | 111 (142.1) | N/A | N/A | N/A |
| | Median (IQR) | 79.7 (13.16-150.4) | 87.5 (15.88-137.9) | 71.97 (0.76-260.2) | | | |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 6.32 (9.55) | 4.81 (4.33) | 2.66 (1.367) | N/A | N/A | N/A |
| | Median (IQR) | 2.71 (2.06-4.94) | 3.16 (2.32-5.94) | 2.58 (1.41-4.0) | | | |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 22.34 (81.1) | 11.18 (6.79) | 6.03 (3.73) | N/A | N/A | N/A |
| | Median (IQR) | 10.28 (5.64-16.50) | 9.86 (6.4-15.18) | 4.97 (3.22-9.89) | | | |
| Total dietary methionine intake (g/day) | Mean (SD) | 2.23 (0.93) | 2.55 (1.75) | 1.93 (0.33) | N/A | N/A | N/A |
| | Median (IQR) | 2.01 (1.78-2.46) | 2.04 (1.68-2.75) | 1.79 (1.72-2.28) | | | |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 45.36 (15.31) | 46.43 (13.02) | 52.45 (21.55) | N/A | N/A | N/A |
| | Median (IQR) | 43.05 (36.13-51.79) | 45.36 (37.90-53.42) | 43.43 (39.54-74.39) | | | |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 5.08 (6.19) | 4.01 (4.15) | 4.05 (2.33) | N/A | N/A | N/A |
| | Median (IQR) | 2.61 (1.88-3.95) | 2.34 (1.80-4.14) | 3.38 (2.27-6.51) | | | |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 259.7 (242.4) | 215 (137.7) | 234.2 (37.38) | N/A | N/A | N/A |
| | Median (IQR) | 209 (136.1-290.8) | 198.4 (110.8-266.4) | 234.3 (197.9-270.3) | | | |

Appendix 52: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the MS A2756G genotypes for control and adenomatous polyp subjects

| | | Wild type (AA) | | Heterozygous type (AG) | | Homo recessive type (GG) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 20.41 (11.67) | 20.11 (11.76) | 24.02 (12.33) | 27.5 (13.21) | 20.33 (10.02) | 29.0 (22.63) |
| | Median (IQR) | 17 (11.5-27) | 17 (11.5-29.5) | 22 (14-33) | 27 (14.8-40.8) | 21 (10-30) | 29 (13-45) |
| Serum folate excluding outliers(nM) | Mean (SD) | 17.93 (9.06) | 18.19 (9.79) | 20.68 (9.68) | 25.0 (12.06) | 20.33 (10.02) | 13 |
| | Median (IQR) | 17 (10-24) | 16 (10.8-27.3) | 20.5 (11.3-29.8) | 22 (14-37) | 21 (10-30) | 13 |
| RBC folate (nM) | Mean (SD) | 940.2 (495.9) | 1015.1 (615.97) | 1025.6 (495.3) | 1123.0 (502.7) | 1229.7 (1121.8) | 869.5 (317.5) |
| | Median (IQR) | 823 (583-1165) | 874 (507.3-1401) | 931 (689-1150) | 1158 (616.8-1517) | 814 (375-2500) | 869.5 (645-1094) |
| RBC folate excluding outliers (nM) | Mean (SD) | 895.6 (425.3) | 960.1 (553.2) | 965.4 (401.5) | 1123.0 (502.7) | 594.5 (310.4) | 869.5 (317.5) |
| | Median (IQR) | 805 (579-1144.5) | 848 (505-1322) | 929 (688-1110.5) | 1158.5 (616.8-1517.8) | 594.5 (375-814) | 869 (645-1094) |
| Cys (µM) | Mean (SD) | 262.7 (42.07) | 263.9 (36.17) | 268.4 (34.91) | 276.5 (56.04) | 226.67 (54.11) | 251.74 |
| | Median (IQR) | 264.2 (232.9-289.7) | 262.8 (231.9-292.6) | 261.7 (242.5-285.5) | 273.4 (231.3-309.4) | 244.05 (166-269.9) | 251.74 |
| Hcy (µM) | Mean (SD) | 9.95 (3.0) | 10.42 (2.65) | 9.97 (2.64) | 9.29 (3.24) | 7.92 (3.6) | 8.69 |
| | Median (IQR) | 9.495 (7.51-1.91) | 9.98 (8.36-12.17) | 9.85 (8.14-11.43) | 8.93 (6.3-11.76) | 7.85 (4.36-11.56) | 8.69 |
| Cys-Gly (µM) | Mean (SD) | 23.27 (4.98) | 25.14 (5.92) | 24.42 (4.71) | 22.58 (2.88) | 23.33 (3.84) | 17.83 |
| | Median (IQR) | 22.48 (20.36-26.09) | 25.38 (20.19-27.80) | 23.78 (21.12-27.15) | 21.63 (20.12-25.65) | 24.91 (18.95-26.13) | 17.83 |
| GSH (µM) | Mean (SD) | 13.85 (6.06) | 14.26 (5.36) | 14.22 (5.42) | 11.48 (3.20) | 14.18 (6.77) | 8.84 |
| | Median (IQR) | 11.99 (9.99-16.48) | 13.53 (9.43-18.52) | 12.38 (9.97-17.96) | 10.98 (8.86-14.54) | 11.02 (9.56-21.95) | 8.84 |
| Serum $B_{12}$ (pM) | Mean (SD) | 273.70 (149.3) | 279.9 (149.9) | 293.1 (160.96) | 282.25 (99.52) | 286.0 (85.51) | 279.0 (12.7) |
| | Median (IQR) | 245 (190-308) | 245.5 (192.8-324) | 254 (195-339) | 274 (189.5-347.3) | 258 (218-382) | 279 (270-288) |

Appendix 53: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the MS A2756G genotypes for control and adenomatous polyp subjects

| | | Wild type (AA) | | Heterozygous type (AG) | | Homo recessive type (GG) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 458.1 (283.1) | 426.9 (187.2) | 464.6 (178.8) | 482.3 (183.7) | 410.4 (62.1) | 347.4 (31.9) |
| | Median (IQR) | 385.7 (304.0-574.6) | 404.8 (270.5-529.6) | 409.7 (349.1-612.2) | 422.4 (380.9-557.6) | 376.0 (373.2-482.1) | 347.4 (324.8-370.1) |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 318.9 (125.8) | 322.4 (141.3) | 332.4 (124.0) | 373.8 (76.32) | 311.4 (80.42) | 290.1 (15.52) |
| | Median (IQR) | 306.7 (235.2-373.2) | 350.3 (180.9-417.6) | 307.6 (242.3-394.3) | 394.7 (289.5-427.9) | 282.3 (249.4-402.4) | 290.1 (279.2-301.1) |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 131.8 (243.6) | 116.1 (131.2) | 137.1 (135.1) | 108.4 (146.9) | 99.05 (24.46) | 57.31 (47.51) |
| | Median (IQR) | 69.96 (9.04-110.9) | 87.5 (11.62-144.71) | 99.9 (31.4-200) | 74.63 (13.95-127.2) | 90.9 (79.7-126.5) | 57.31 (23.7-90.9) |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 5.821 (9.532) | 4.745 (3.441) | 7.305 (9.532) | 5.694 (7.115) | 3.001 (1.091) | 2.253 (0.237) |
| | Median (IQR) | 2.666 (2.039-4.196) | 4.073 (2.26-6.832) | 3.994 (2.206-7.28) | 3.029 (2.381-5.051) | 2.439 (2.305-4.258) | 2.253 (2.085-2.42) |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 24.28 (97.14) | 11.76 (7.407) | 17.82 (19.20) | 9.76 (5.01) | 7.31 (3.653) | 8.844 (0.616) |
| | Median (IQR) | 9.807 (5.107-15) | 10.6 (6.602-15.35) | 11.64 (7.872-20.84) | 9.383 (4.307-15.48) | 5.809 (4.641-11.47) | 8.844 (8.409-9.28) |
| Total dietary methionine intake (g/day) | Mean (SD) | 2.164 (0.926) | 2.513 (1.78) | 2.364 (0.943) | 2.196 (0.695) | 4.465 (3.991) | 4.465 (3.991) |
| | Median (IQR) | 1.992 (1.721-2.452) | 2.050 (1.711-2.469) | 2.123 (1.866-2.609) | 2.024 (1.686-2.864) | 1.905 (1.754-2.156) | 4.465 (1.643-7.287) |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 44.79 (15.94) | 46.66 (13.88) | 47.14 (14.80) | 47.29 (11.16) | 45.38 (3.43) | 39.85 (10.27) |
| | Median (IQR) | 42.52 (36.25-51.66) | 44.56 (36.94-54.59) | 45.04 (36.13-54.95) | 46.76 (40.21-56.73) | 44.71 (42.33-49.09) | 39.85 (32.58-47.11) |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 4.727 (6.388) | 4.378 (4.527) | 5.713 (5.568) | 3.201 (3.085) | 5.994 (6.267) | 2.092 (0.709) |
| | Median (IQR) | 2.391 (1.793-3.579) | 2.615 (1.852-4.364) | 2.975 (2.095-9.434) | 2.051 (1.54-3.692) | 2.451 (2.301-13.23) | 2.092 (1.591-2.594) |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 255.7 (266.7) | 205.9 (138.8) | 267.3 (178.3) | 264.4 (146.1) | 241.5 (68.27) | 144.6 (5.4310 |
| | Median (IQR) | 194.5 (136.3-278.0) | 198.4 (81.50-270.5) | 230.8 (133.7-373.4) | 239.9 (177.2-306.3) | 265.7 (164.4-294.3) | 144.6 (140.7-148.4) |

Appendix 54: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the MSR A66G genotypes for control and adenomatous polyp subjects

| | | Wild type (AA) | | Heterozygous type (AG) | | Homo recessive type (GG) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 20.56 (11.42) | 19.6 (14.76) | 21.67 (12.18) | 23.4 (11.39) | 22.16 (12.03) | 21.78 (13.63) |
| | Median (IQR) | 18 (11-29) | 15 (10-31.5) | 18 (12-30) | 22 (14-32) | 20 (12-30) | 17.5 (10.75-33) |
| Serum folate excluding outliers(nM) | Mean (SD) | 18.53 (9.28) | 13.25 (4.65) | 18.29 (8.86) | 23.4 (11.39) | 19.93 (10.10) | 17.13 (9.33) |
| | Median (IQR) | 16 (11-23.75) | 14 (8.5-17.25) | 16 (11-24.5) | 22 (14-32) | 18 (11-26.5) | 16 (10-20) |
| RBC folate (nM) | Mean (SD) | 1046 (516.0) | 1267.8 (656.2) | 955.1 (531.8) | 1133.3 (486.8) | 938.6 (461.5) | 878.2 (610.9) |
| | Median (IQR) | 947 (607-1201) | 1181 (764-1815) | 801.5 (591.5-1105.75) | 1138 (793-1459) | 875 (570-1172) | 640 (465.5-1344.8 |
| RBC folate excluding outliers (nM) | Mean (SD) | 1007.8 (463.5) | 1267.8 (656.2) | 871.6 (400.6) | 1133.3 (486.8) | 903.1 (399.9) | 782.8 (471.6) |
| | Median (IQR) | 939 (603.5-1192) | 1181 (764-1815) | 772.5 (580.3) | 1138 (793-1459) | 861.5 (559-1162.2) | 635 (457-1141) |
| Cys (µM) | Mean (SD) | 268.19 (37.5) | 263.8 (33.7) | 261.4 (43.11) | 269.2 (50.4) | 264.6 (37.82) | 263.1 (26.68) |
| | Median (IQR) | 264.3 (241.8-285.3) | 263.8 (239.9-287.6) | 258.8 (230.9-293.4) | 264.9 (218-302.4) | 261.8 (240.8-288.4) | 261.3 (239.5-286.6) |
| Hcy (µM) | Mean (SD) | 10.87 (3.54) | 10.36 (1.14) | 9.57 (2.79) | 9.36 (2.79) | 9.76 (2.40) | 10.85 (2.73) |
| | Median (IQR) | 10.42 (8.15-12.03) | 10.36 (9.55-11.16) | 9.15 (7.35-11.85) | 8.65 (7.36-11.0) | 9.03 (8.13-10.8) | 10.46 (8.69-14.03) |
| Cys-Gly (µM) | Mean (SD) | 23.9 (4.68) | 17.89 (3.26) | 23.76 (5.38) | 23.88 (3.20) | 23.38 (4.25) | 25.78 (6.89) |
| | Median (IQR) | 23.64 (21.45-26.7) | 17.89 (15.6-20.2) | 23.23 (20.5-26.8) | 24.64 (20.5-26.9) | 22.33 (20.17-26.81) | 26.16 (19.95-29.47) |
| GSH (µM) | Mean (SD) | 13.99 (6.68) | 10.02 (0.76) | 13.87 (6.15) | 12.79 (4.18) | 14.16 (4.65) | 14.72 (5.91) |
| | Median (IQR) | 11.03 (10.46-15.58) | 10.02 (9.48-10.55) | 11.85 (9.41-18.46) | 11.33 (9.13-16.41) | 13.68 (10.37-17.53) | 13.89 (10.21-18.96) |
| Serum $B_{12}$ (pM) | Mean (SD) | 282.7 (154.6) | 271.2 (30.52) | 288.78 (168.5) | 271.4 (83.04) | 262.2 (115.5) | 290.4 (183.5) |
| | Median (IQR) | 235 (189-327) | 288 (238.5-295.5) | 252 (198-328) | 259 (224-325) | 251 (182-314) | 256.5 (167-364.8) |

Appendix 55: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the MSR A66G genotypes for control and adenomatous polyp subjects

| | | Wild type (AA) | | Heterozygous type (AG) | | Homo recessive type (GG) | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Total grand dietary folate intake (µg/day) | Mean (SD) | 397.5 (137.8 ) | 420.9 (50.04) | 487.0 (239.3) | 466.8 (199.8) | 461.2 (328.8) | 411.2 (191.5) |
| | Median (IQR) | 379.5 (300.0-463.6) | 409.1 (385.2-462.7) | 408.6 (319.7-625.7) | 394.1 (298.1-693.2) | 398.8 (347.9-486.8) | 398.4 (266.8-548.2) |
| Total dietary methyl folate intake (µg/day) | Mean (SD) | 288.9 (83.79) | 338.0 (68.65) | 335.9 (146.6) | 349.1 (152.8) | 328.5 (104.3) | 315.1 (120.0) |
| | Median (IQR) | 301.5 (205.1-357.1) | 361.8 (266.2-397.9) | 299.3 (239.9-394.3) | 376.5 (209.2-430.2) | 329.8 (254.9-372.5) | 319.9 (178.6-422.8) |
| Total dietary PteGlu intake (µg/day) | Mean (SD) | 108.6 (111.5) | 82.95 (48.42) | 142.4 (174.6) | 139.2 (133.2) | 136.0 (314.5) | 96.06 (142.9) |
| | Median (IQR) | 90.9 (25.5-131.6) | 90.9 (38.57-123.4) | 81.75 (12.99-229.1) | 112.0 (58.35-145.4) | 74.64 (0.875-125.9) | 32.98 (2.678-110.7) |
| Total dietary pyridoxine intake (µg/day) | Mean (SD) | 4.757 (8.352) | 4.253 (2.521) | 8.266 (11.59) | 5.734 (5.158) | 3.943 (3.965) | 4.202 (4.019) |
| | Median (IQR) | 2.441 (2.011-4.022) | 2.847 (2.212-6.996) | 3.14 (2.099-7.531) | 4.512 (3.005-5.528) | 2.711 (2.133-3.992) | 2.558 (1.834-6.008) |
| Total dietary cobalamin intake (µg/day) | Mean (SD) | 11.27 (10.01) | 9.642 (3.286) | 32.68 (113.7) | 11.51 (5.802) | 12.35 (15.06) | 11.34 (8.329) |
| | Median (IQR) | 9.936 (4.327-13.71) | 9.377 (7.337-12.08) | 11.28 (5.637-20.33) | 11.02 (5.806-15.08) | 10.03 (6.346-14.54) | 9.663 (5.945-15.57) |
| Total dietary methionine intake (g/day) | Mean (SD) | 2.119 (0.715) | 2.240 (0.502) | 2.323 (1.108) | 2.191 (1.041) | 2.136 (0.706) | 2.934 (2.326) |
| | Median (IQR) | 1.974 (1.764-2.257) | 2.089 (1.794-2.760) | 2.127 (1.754-2.604) | 1.885 (1.670-2.064) | 1.968 (1.793-2.474) | 2.391 (1.729-2.918) |
| Total dietary niacin intake equivalent (mg/day) | Mean (SD) | 42.96 (10.81) | 45.74 (11.23) | 47.52 (18.85) | 49.02 (15.81) | 44.28 (11.47) | 44.47 (11.10) |
| | Median (IQR) | 42.31 (35.56-46.85) | 42.83 (36.04-56.9) | 43.75 (36.13-54.95) | 46.35 (38.5-58.07) | 43.83 (36.78-53.45) | 47.16 (33.77-53.42) |
| Total dietary riboflavin intake (mg/day) | Mean (SD) | 3.924 (4.843) | 2.644 (1.014) | 6.375 (7.527) | 4.147 (3.772) | 3.733 (3.358) | 4.275 (4.987) |
| | Median (IQR) | 2.387 (1.741-3.304) | 2.203 (1.892-3.616) | 2.807 (1.927-9.348) | 2.332 (1.801-5.258) | 2.52 (1.888-3.477) | 2.473 (1.769-3.175) |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 278.8 (373.1) | 179.1 (71.74) | 255.7 (159.9) | 212.3 (104.7) | 247.9 (212.3) | 227.1 (174.6) |
| | Median (IQR) | 188.1 (126.3-265.7) | 201.9 (107.1-239.9) | 235.4 (134.8-314.9) | 223.5 (112.5-272.5) | 199.9 (149.5-275.1) | 186.9 (75.56-312.7) |

Appendix 56: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the combined MS A2756G-MSR A66G genotypes for control and adenomatous polyp subjects (1 of 2)

| | | AA/AG | | AA/GG | | AG/AG | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Serum folate (nM) | Mean (SD) | 19.65 (11.13) | 21.73 (10.49) | 21.85 (12.75) | 20.29 (13.59) | 25.52 (13.55) | 28 (14.17) |
| | Median (IQR) | 16 (11-27) | 18 (14-31) | 18 (12-29) | 17 (9.75-31.5) | 22 (14-37) | 29.5 (13.75-40.75) |
| Serum folate excluding outliers(nM) | Mean (SD) | 17.49 (8.602) | 21.73 (10.49) | 18.66 (9.926) | 16.17 (9.418) | 19.95 (9.599) | 28 (14.17) |
| | Median (IQR) | 15 (10-24) | 18 (14-31) | 17 (11-24.5) | 14.5 (9.25-20) | 17 (12.5-30) | 29.5 (13.75-40.75) |
| RBC folate (nM) | Mean (SD) | 886.6 (518.5) | 1159 (474.7) | 925.3 (431.9) | 843.3 (647.1) | 1087 (550.4) | 1064 (588.1) |
| | Median (IQR) | 749.5 (561.5-1024) | 1142 (848-1459) | 906 (570-1172) | 597.5 (423.5-1081) | 990 (687-1292) | 896.5 (616.8-1677) |
| RBC folate excluding outliers (nM) | Mean (SD) | 783.6 (325.3) | 1159 (474.7) | 925.3 (431.9) | 715.8 (455.3) | 1033 (481.8) | 1064 (588.1) |
| | Median (IQR) | 736 (557-885) | 1142 (848-1459) | 906 (570-1172) | 560 (407-870.5) | 986.5 (685.5-1202) | 896.5 (616.8-1677) |
| Cys (µM) | Mean (SD) | 255.6 (40.6) | 268.5 (44.25) | 264.5 (41.89) | 262.2 (31.15) | 274.7 (41.75) | 271.2 (71.57) |
| | Median (IQR) | 256.7 (221.5-290.1) | 275.6 (218-302.4) | 265.1 (240.1-291.9) | 261.3 (234.5-280.3) | 262.1 (243.6-311.8) | 248.9 (218.8-345.9) |
| Hcy (µM) | Mean (SD) | 9.542 (2.77) | 9.957 (2.871) | 9.9 (2.62) | 10.72 (2.645) | 9.815 (2.715) | 7.86 (2.183) |
| | Median (IQR) | 9.06 (7.215-11.92) | 8.98 (8.14-12.24) | 9.34 (8.04-11.6) | 10.46 (8.61-13.18) | 9.84 (7.535-11.62) | 7.365 (6.07-10.15) |
| Cys-Gly (µM) | Mean (SD) | 23.19 (5.355) | 23.9 (3.506) | 22.88 (4.216) | 26.83 (6.784) | 24.57 (5.495) | 23.84 (2.737) |
| | Median (IQR) | 22.49 (20.49-25.54) | 24.64 (19.96-27.12) | 21.99 (20.01-26.71) | 27.59 (21.45-29.7) | 23.62 (20.53-28.24) | 23.9 (21.18-26.45) |
| GSH (µM) | Mean (SD) | 13.64 (6.096) | 12.81 (4.717) | 13.15 (4.504) | 15.74 (5.679) | 13.92 (6.277) | 12.75 (3.005) |
| | Median (IQR) | 11.8 (9.31-19.7) | 10.33 (8.828-17.51) | 12.8 (9.998-14.9) | 15.8 (11.03-19.25) | 12.03 (9.345-17.94) | 12.68 (9.87-15.69) |
| Serum $B_{12}$ (pM) | Mean (SD) | 277.5 (157.2) | 268.8 (68.18) | 269.4 (125.8) | 293.1 (206.1) | 312.7 (191.4) | 278.5 (128.8) |
| | Median (IQR) | 252 (196-320) | 265 (224-325) | 258 (196-314) | 225.5 (144.5-403.5) | 255 (208-351) | 250.5 (176-409) |

Appendix 57: Mean (SD) and median (IQR) values for major parameters in folate metabolism within each of the combined MS A2756G-MSR A66G genotypes for control and adenomatous polyp (2 of 2)

| MS/MSR | | AA/AA | | AG/GG | | AG/AA | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP (N=1) |
| Serum folate (nM) | Mean (SD) | 20.08 (11.57) | 13.33 (5.686) | 22.36 (10.53) | 21 (9.644) | 22.31 (11.51) | 45 |
| | Median (IQR) | 18 (10.5-26.5) | 15 (7-18) | 23 (10-30) | 17 (14-32) | 20 (11.5-32.5) | |
| Serum folate excluding outlier (nM) | Mean (SD) | 17.91 (9.199) | 13.33 (5.686) | 22.36 (10.53) | 21 (9.644) | 20.42 (9.681) | N/A |
| | Median (IQR) | 16 (10-23) | 15 (7-18) | 23 (10-30) | 17 (14-32) | 18 (11.25-29.25) | N/A |
| RBC folate (nM) | Mean (SD) | 1067 (525.2) | 1291 (916.4) | 836.5 (290.1) | 1119 (572.3) | 1058 (503.1) | 1373 |
| | Median (IQR) | 947 (600-1507) | 1181 (434-2257) | 811 (544-1057) | 1317 (474-1566) | 998 (796-1132) | |
| RBC folate excluding outlier (nM) | Mean (SD) | 1067 (525.2) | 1291 (916.4) | 836.5 (290.1) | 1119 (572.3) | 937.5 (266.9) | 1373 |
| | Median (IQR) | 947 (600-1507) | 1181 (434-2257) | 811 (544-1057) | 1317 (474-1566) | 964.5 (755-1112) | |
| Cys (μM) | Mean (SD) | 275.2 (44.46) | 240 | 266.7 (28.14) | 286.6 | 255.9 (20.02) | 287.6 |
| | Median (IQR) | 273.9 (241.8-292.8) | | 262.8 (245.5-285.3) | | 254.2 (237.6-267.5) | |
| Hcy (μM) | Mean (SD) | 10.91 (3.888) | 11.16 | 9.578 (1.878) | 14.75 | 10.76 (3.216) | 9.55 |
| | Median (IQR) | 10.27 (7.6-12.45) | | 8.91 (8.608-10.51) | | 10.42 (8.21-12.03) | |
| Cys-Gly (μM) | Mean (SD) | 24.08 (5.446) | 15.58 | 24.52 (4.458) | 19.92 | 23.94 (3.13) | 20.19 |
| | Median (IQR) | 23.33 (20.9-27) | | 23.05 (20.57-29.29) | | 24.35 (21.75-26.69) | |
| GSH (μM) | Mean (SD) | 15.43 (7.928) | 9.48 | 17.04 (3.894) | 7.37 | 11.8 (3.243) | 10.55 |
| | Median (IQR) | 12.04 (10.58-19.3) | | 16.98 (15.19-18.52) | | 10.7 (9.46-12.89) | |
| Serum $B_{12}$ (pM) | Mean (SD) | 271.5 (166.5) | 259.7 (36.94) | 229.6 (72.85) | 285 (98.53) | 306.2 (139.2) | 289 |
| | Median (IQR) | 215 (186.5-281) | 243 (234-302) | 225 (166-297) | 330 (172-353) | 275 (194-410) | |

Appendix 58: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the combined MS A2756G-MSR A66G genotypes for control and adenomatous polyp (1 of 2)

| | | AA/AG | | AA/GG | | AG/AG | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP |
| Grand total dietary folate (µg/day) | Mean (SD) | 480.6 (258.8) | 449 (191.3) | 468.2 (380.7) | 413.3 (209.6) | 499.5 (206.8) | 515.8 (244.7) |
| | Median (IQR) | 394.4 (302.3-625.4) | 377.2 (276.1-693.2) | 385.7 (318.6-482.8) | 415.5 (239.6-552.2) | 409.8 (363.0-633.1) | 402.3 (380.9-764.3) |
| Total dietary methyl folate (µg/day) | Mean (SD) | 325.9 (149.6) | 347.2 (177.2) | 319.7 (110.9) | 300.7 (124.7) | 352.5(144.3) | 354.1 (65.88) |
| | Median (IQR) | 294.2 (224.7-373.2) | 376.5 (181.2-464.1) | 314.6 (247.4-368.3) | 312.0 (170.1-412.1) | 303.8 (260.7-407.6) | 356.1 (289.5-416.7) |
| Total dietary PteGlu (µg/day) | Mean (SD) | 136.3 (190.2) | 131.1 (111.3) | 153 (362.3) | 112.5 (158.1) | 156.2 (146.4) | 161.7 (201.5) |
| | Median (IQR) | 69.96 (7.29-150) | 112 (79.92-145.4) | 62.13 (0.875-150.4) | 58.44 (0-149.4) | 115.8 (38.53-241.7) | 97.35 (14.59-373.2) |
| Total dietary pyridoxine (µg/day) | Mean (SD) | 8.588 (13.08) | 4.692 (2.058) | 3.746 (3.841) | 4.6 (4.5) | 7.805 (8.539) | 8.6 (9.752) |
| | Median (IQR) | 2.685 (2.028-7.531) | 4.512 (3.005-5.528) | 2.711 (1.947-3.148) | 2.558 (1.613-7.054) | 4.285 (2.429-7.716) | 4.393 (2.709-18.7) |
| Total dietary cobalamin (µg/day) | Mean (SD) | 41.31 (140.7) | 11.75 (6.092) | 9.687 (5.556) | 11.89 (9.161) | 17.16 (17.31) | 10.84 (5.711) |
| | Median (IQR) | 10.97 (5.776-20.33) | 11.02 (5.806-14.98) | 8.762 (5.093-13.84) | 9.915 (5.945-15.71) | 11.28 (5.558-20.88) | 12.07 (4.926-15.54) |
| Total dietary methionine (g/day) | Mean (SD) | 2.257 (1.157) | 2.197 (1.167) | 2.166 (0.789) | 2.816 (2.304) | 2.468 (1.033) | 2.173 (0.722) |
| | Median (IQR) | 2.155 (1.671-2.535) | 1.885 (1.474-2.01) | 1.992 (1.783-2.541) | 2.391 (1.729-2.606) | 2.081 (1.866-3.079) | 1.899 (1.686-2.934) |
| Total dietary niacin equivalents (mg/day) | Mean (SD) | 46.48 (20.04) | 49.82 (18.09) | 44.03 (12.73) | 43.93 (10.51) | 49.60 (16.96) | 46.82 (8.16) |
| | Median (IQR) | 42.59 (35.84-52.63) | 44.01 (36.09-61.74) | 42.62 (34.31-55.84) | 46.01 (33.77-53.42) | 45.79 (39.95-60.49) | 45.36 (40.21-54.5) |
| Total dietary riboflavin (mg/day) | Mean (SD) | 6.391 (8.566) | 4.21 (3.765) | 3.725 (3.543) | 4.919 (5.519) | 6.091 (5.193) | 3.974 (4.37) |
| | Median (IQR) | 2.511 (1.813-8.765) | 2.63 (1.824-5.258) | 2.52 (1.846-3.394) | 2.62 (1.769-5.958) | 3.479 (2.36-10.37) | 1.963 (1.54-8.418) |
| Total dietary vitamin C (mg/day) | Mean (SD) | 243.4 (148.7) | 211.2 (112.4) | 239.2 (228.0) | 209 (169.7) | 277.4 (182.4) | 215.4 (94.91) |
| | Median (IQR) | 224.1 (134.8-310.3) | 223.5 (112.5-272.5) | 181.9 (141.6-271) | 174.6 (64.35-312.7) | 243.1 (125.4-414.8) | 217.0 (122.8-306.3) |

Appendix 59: Mean (SD) and median (IQR) values for folate and other nutrients related to one-carbon metabolism intake within each of the combined MS A2756G-MSR A66G genotypes for control and adenomatous polyp (2 of 2)

| | | AA/AA | | AG/GG | | AG/AA | |
|---|---|---|---|---|---|---|---|
| | | Control | AP | Control | AP | Control | AP (n=1) |
| Grand total dietary folate (µg/day) | Mean (SD) | 393.7 (132.1) | 410.4 (10.64) | 448.2 (104) | 430.3 (147.4) | 405.9 (157.6) | 503.8 |
| | Median (IQR) | 379.5 (296.9-427.8) | 409.1 (400.41-421.58) | 413.1 (367.1-514.7) | 434.4 (280.9-575.6) | 404.8 (291.1-544.2) | 503.8 |
| Total dietary methyl folate (µg/day) | Mean (SD) | 302.4 (84.34) | 332.7 (69.62) | 359.1 (83.93) | 387.1 (109.9) | 268.1 (84.1) | 412.89 |
| | Median (IQR) | 314.2 (220.9-373.5) | 361.8 (253.25-383.01) | 349.1 (301.8-412.8) | 421.4 (264.1-475.7) | 298 (178.8-332.8) | 412.89 |
| Total dietary PteGlu (µg/day) | Mean (SD) | 91.26 (101.0) | 77.66 (67.7) | 89.09 (107.4) | 43.24 (49.1) | 137.9 (130.5) | 90.9 |
| | Median (IQR) | 77.91 (25.97-93.24) | 38.57 (38.57-155.8) | 74.64 (0-102.1) | 16.84 (12.99-99.9) | 102.1 (1.86-249.9) | 90.9 |
| Total dietary pyridoxine (µg/day) | Mean (SD) | 2.913 (1.345) | 5.613 (2.399) | 4.685 (4.578) | 2.939 (0.781) | 8.483 (13.95) | 2.34 |
| | Median (IQR) | 2.42 (2.054-3.287) | 6.87 (2.847-7.122) | 3.17 (2.206-4.398) | 2.74 (2.276-3.799) | 2.912 (1.812-7.441) | 2.34 |
| Total dietary cobalamin (µg/day) | Mean (SD) | 8.779 (5.19) | 11.18 (2.994) | 21.03 (28.06) | 9.757 (5.838) | 16.48 (14.79) | 5.394 |
| | Median (IQR) | 8.904 (4.038-12.88) | 9.524 (9.377-14.63) | 11.37 (8.862-17.36) | 9.708 (3.945-15.62) | 11.76 (7.787-21.07) | 5.394 |
| Total dietary methionine (g/day) | Mean (SD) | 1.973 (0.424) | 2.259 (0.425) | 2.069 (0.432) | 2.034 (0.835) | 2.396 (1.064) | 2.7767 |
| | Median (IQR) | 1.909 (1.704-2.143) | 2.089 (1.945-2.743) | 1.953 (1.778-2.418) | 1.984 (1.224-2.893) | 2.177 (1.816-2.424) | 2.7767 |
| Dietary niacin equivalents (mg/day) | Mean (SD) | 42.36 (8.825) | 47.81 (11.62) | 45.21 (7.668) | 46.10 (17.85) | 43.64 (14.49) | 52.71 |
| | Median (IQR) | 41.35 (37.25-46.74) | 42.83 (39.5-61.09) | 45.18 (39.62-50.33) | 48.15 (27.32-62.85) | 44.92 (32.64-46.96) | 52.71 |
| Total dietary riboflavin (mg/day) | Mean (SD) | 2.657 (1.524) | 2.471 (0.473) | 3.885 (3.035) | 1.832 (0.381) | 6.473 (7.685) | 4.215 |
| | Median (IQR) | 2.331 (1.674-2.939) | 2.203 (2.194-3.017) | 2.665 (1.9-4.404) | 2.014 (1.393-2.087) | 2.975 (2.043-9.754) | 4.215 |
| Total dietary vitamin C intake (mg/day) | Mean (SD) | 302.5 (449.1) | 171.9 (87.53) | 281.9 (172.2) | 338.1 | 234.1 (184.6) | 239.38 |
| | Median (IQR) | 188.1 (139.8-258.9) | 201.9 (73.29-240.5) | 250.9 (180.5-300.5) | 240.52 (185-588.8) | 165.9 (100.3-331.1) | 239.38 |

Appendix 60: Mean (SD) and median (IQR) values for major parameters in folate metabolism and other nutrients related to one-carbon metabolism intake within each of the combined MS A2756G-MSR A66G genotypes for control and adenomatous polyp subjects

| | | GG/GG | | GG/AA | | GG/AG | | GG/GG | | GG/AA | | GG/AG |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Control (n=1) | AP (n=1) | Control (n=1) | AP (n=1) | Control (n=1) | | Control (n=1) | AP (n=1) | Control (n=1) | AP (n=1) | Control (n=1) |
| Serum folate (nM) | Mean (SD) | | | | | | Grand total dietary folate (µg/day) | 373.24 | 324.81 | 375.98 | 370.05 | 482.1 |
| | Median (IQR) | 30 | 45 | 10 | 13 | 21 | | | | | | |
| Serum folate excluding outlier (nM) | Mean (SD) | | | | | | Total dietary methyl folate (µg/day) | 282.34 | 301.1 | 249.44 | 279.15 | 402.41 |
| | Median (IQR) | 30 | . | 10 | 13 | 21 | | | | | | |
| RBC folate (nM) | Mean (SD) | | | | | | Total dietary PteGlu (µg/day) | 90.9 | 23.71 | 126.54 | 90.9 | 79.7 |
| | Median (IQR) | 2500 | 645 | 375 | 1094 | 814 | | | | | | |
| RBC folate excluding outlier (nM) | Mean (SD) | | | | | | Total dietary pyridoxine (µg/day) | 2.305 | 2.42 | 2.439 | 2.085 | 4.258 |
| | Median (IQR) | . | 645 | 375 | 1094 | 814 | | | | | | |
| Cys (µM) | Mean (SD) | | | | | | Total dietary cobalamin (µg/day) | 4.641 | 8.409 | 5.809 | 9.28 | 11.47 |
| | Median (IQR) | 244.1 | 251.7 | 270 | . | 166 | | | | | | |
| Hcy (µM) | Mean (SD) | | | | | | Total dietary methionine (g/day) | 1.9052 | 7.2873 | 2.1557 | 1.6427 | 1.7536 |
| | Median (IQR) | 7.85 | 8.69 | 11.56 | . | 4.36 | | | | | | |
| Cys-Gly (µM) | Mean (SD) | | | | | | Dietary niacin equivalents (mg/day) | 42.33 | 47.11 | 49.09 | 32.58 | 44.71 |
| | Median (IQR) | 24.91 | 17.83 | 18.95 | . | 26.13 | | | | | | |
| GSH (µM) | Mean (SD) | | | | | | Total dietary riboflavin (mg/day) | 2.301 | 2.594 | 2.451 | 1.591 | 13.23 |
| | Median (IQR) | 9.56 | 8.84 | 11.02 | . | 21.95 | | | | | | |
| Serum $B_{12}$ (pM) | Mean (SD) | | | | | | Total dietary vitamin C (mg/day) | 164.39 | 148.4 | 265.68 | 140.72 | 294.33 |
| | Median (IQR) | 382 | 270 | 258 | 288 | 218 | | | | | | |