THE ROLE OF FOLIC ACID RELATED NUTRITIONAL GENETICS IN COMMON CHRONIC DEGENERATIVE DISORDERS

By

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Statement of Originality

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

However, I acknowledge that the work embodied in this thesis has been done in collaboration with other researchers and has been carried out in part at other institutions. Where necessary, I have indicated within the thesis the extent and type of collaboration, and acknowledged the contributing parties.

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Abbreviations

^{0}C	Degree Celsius
$\overline{x}$	Mean
AD	Alzheimer's disease
ADHD	Attention Deficit Hyperactivity Disorder
AIHW	Australian Institute of Health and Welfare
ANOVA	Analysis of variance
AUD	Australian Dollars
BMI	Body Mass Index
bp	Base pairs
COMT	Catechol-*O*-methyltransferase
CpG	Cytosine-Guanine
CV	Coefficient of variation
CVD	Cardiovascular disease
CβS	Cystathionine β-Synthase
CγL	Cystathionine-γ-lyase
DHFR	Dihydrofolate Reductase
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphate
dTMP	deoxythymidine monophosphate
dUMP	deoxyuridine monophosphate
EDTA	Ethylenediaminetetra-acetic acid
eNOS	endothelial nitric oxide synthase
FAD	Flavin adenine dinucleotide
FFQ	Food Frequency Questionnaire
FMN	Flavin mononucleotide
FSANZ	Food Standards Australia New Zealand
GWAS	Genome-wide association studies
GCPII	Glutamate carboxypeptidase II
H_2PteGlu	Dihydrofolate
H_4PteGlu	Tetrahydrofolate
HADS	Hospital Anxiety Depression Scale/Score
HDL	High Density Lipoprotein
Het	Heterozygote
HPLC	High-performance liquid chromatography
ICPMR	Institute of Clinical Pathology and Medical Research
IVF	*In vitro* fertilisation
LDL	Low Density Lipoprotein
MMSE	Mini mental State Examination
mRNA	Messenger Ribonucleic Acid
MTHFR	Methylenetetrahydrofolate Reductase
MTR	Methionine Synthase
MTRR	Methionine Synthase Reductase
n	Number

NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NHANES	National Health and Nutrition Examination Survey
NHMRC	National Health and Medical Research Council
NMDA	*N*-methyl-d-aspartate
NSW	New South Wales, Australia
NTD	Neural Tube Defects
OR	Odds Ratio
PCFT	Proton Coupled Folate Transporter
PCR	Polymerase Chain Reaction
PLP	Pyridoxal 5' Phosphate
PUFA	Polyunsaturated Fatty Acid
RDI	Recommended Daily Intake
Rec	Recessive
RFC	Reduced Folate Carrier
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
SAH	*S*-adenosylhomocysteine
SAM	S-adenosylmethionine
SBDF	7-Fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid ammonium salt
SD	Standard deviation
SHMT	Serine hydroxymethyltransferase
SNP	Single Nucleotide Polymorphism
TBE	Tris/Borate/EDTA
TCEP	Tris(2-carboxyethyl)phosphine
TS	Thymidylate synthase
TSER	Thymidylate synthase enhancer region
UK	United Kingdom
US	United States
USD	United States Dollars
UV	Ultra Violet
VIC	Victoria, Australia
WHO	World Health Organisation
Wt	Wild-type

Synopsis

Nutrition has long been recognised as having a significant impact on health. In developed countries, there has been a shift away from prevention of overt nutrient deficiency diseases to emphasis on preventing the health complications of nutritional excess. The contemporary burden of chronic disease, in both developed and developing nations, is increasing as society ages and is linked to dietary elements, genetic susceptibility and environmental change. Today's populations largely consume energy-dense nutrient-poor foods, an important component in our contemporary obesogenic environment. This type of diet is often low in essential micronutrients, particularly important B-group vitamins linked to the prevention of a range of chronic diseases.

Folic acid nutritional genetics, the subject of this thesis, influences a broad sphere of clinical conditions. Folic acid has a central role in one-carbon metabolism, a complex nexus responsible for donating methyl units vital for both nucleotide synthesis and provision of *S*-adenosylmethionine. Moderate folate deficiency induces DNA hypomethylation, and via uracil misincorporation, DNA instability; both events are linked to increased cancer risk. Folate deficiency is also associated with potentially vasculo-toxic homocysteine, which accumulates when there is a limited pool of folic acid derived methyl groups. Elevated homocysteine is associated with a range of disorders, most notably increased CVD risk and NTDs. Folate-related one-carbon metabolism contains various polymorphic proteins that modify metabolism and therefore influence disease risk. This dissertation examines four different, common, chronic degenerative disorders that predominately affect ageing populations, with the aim of exploring the relationship between eleven common folate polymorphisms, important indices of folate status, and transsulphuration pathway thiols. This approach employed regression models based on the *a priori* understanding of possible biochemical, genetic and physiologic relationships. The following reflects what are considered to be the major findings of this study.

An examination of hypertension in an elderly retirement village population (n=229) demonstrated that red cell folate, cysteine and cysteinyl-glycine were predictive of recumbent diastolic blood pressure (*p=0.0326, r^2=0.0202, slope estimate=-0.040; p=0.0001, r^2=01246, slope estimate=-0.232; p=0.0008, r^2=01246, slope estimate=0.141* respectively). As a component within a model containing key genetic factors, the 677C>T MTHFR SNP was associated with recumbent diastolic blood pressure (*p=0.0397, r^2=0.0650, slope estimate=-0.011*). Several folate-related SNPs

were associated with standing systolic blood pressure (r^2=*0.0868* for whole model); these were the 677C>T MTHFR (*p=0.0443, slope estimate=-0.009*), the 19 bp deletion DHFR (*p=0.0157, slope estimate=0.009)* and the 1561C>T GCPII *(p=0.0397, slope estimate=-0.021*) variants. An examination of the depression phenotype was undertaken in this same population. It was shown that a novel relationship exists with the amino-thiol, cysteinyl-glycine, which was negatively associated with depression (*p=0.0046,* r^2*=0.0348, slope estimate=-6.127*).

The third clinical phenotype examined involved a cohort of AD patients (n=93), which was compared to the former retirement village population as a control after selecting subjects whose MMSE score reflected a specified threshold for cognitive function (n=229). The 2756A>G MTR SNP was associated with AD (*p=0.0419,* r^2*=0.0512*), with the G allele considered to be protective (OR=0.60:95%CI;0.39-0.92, *p=0.0260*). An ordinal logistic regression model containing all thiols (r^2*=0.1885*) indicated that higher homocysteine (*p=<0.0001*), higher glutathione (*p=0.0003*) but lower cysteinyl-glycine (*p=<0.0001*) was significantly associated with AD. Ordinal logistic regression also supported the association of AD with lower serum folate (*p=0.0097*, r^2*=0.0181*), lower total dietary folate intake (*p=0.0054,* r^2*=0.0231,*) and lower natural methylfolate intake (*p=<0.0001,* r^2*=0.0581*).

The final phenotype examined involved a cohort of subjects screened for colorectal polyps (n=203). The study had a specific focus on adenomatous polyp occurrence and its possible relationship to folate intake. The 3'UTR 6 deletion TS SNP indicated an association with increased risk for an adenomatous polyp occurrence (*p=0.0073*, r^2*=0.2744*). The 66A>G MTRR SNP was also found to be a positive risk factor for an adenomatous polyp (OR=2.50:95%CI;1.23-5.10, *p=0.0163,* ordinal logistic regression, *p=0.0149*, r^2*=0.2744*). This latter SNP was also associated with adenomatous polyp occurrence in subjects with low folate status (below median red cell folate, OR=3.40:95%CI; 1.32-8.75, *p=0.0164*, ordinal logistic regression, *p=0.0261*, r^2*=0.5799*). In subjects with a high folate status, the 1420C>T SHMT SNP was a positive risk factor (OR=4.56:95%CI; 1.38-15.03, *p=0.0225*). Individuals with a low folate status were also found to have red cell folate levels that predicted adenomatous polyp occurrence (ordinal logistic regression *p=0.0331,* r^2*=0.0548*). Whilst this study has identified various potential associations, the nature of the data and associations found, advocates further examination in larger populations.

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Acknowledgment 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 this statement clearly outlining the extent of collaboration, with whom and under what auspices.

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PhD Candidate

CHAPTER 3: B-VITAMIN NUTRITIONAL GENETICS IN THE ELDERLY - A DETAILED STUDY OF HYPERTENSIVE AND DEPRESSIVE PHENOTYPES

I would like to thank and acknowledge various associate investigators and students whose work has been incorporated into this chapter. This study was completed in two stages, for stage 1 I would like to thank Dr Barbara Blades for the recruitment, clinical assessment including blood pressure measurements, HADS, and MMSE data. I would like to thank Dr Virginia Skinner for the continued recruitment in stage 2, and for interviewing, clinical assessments, venepuncture and database management. I would like to thank honours student Lisa Dufficy for work in stage 1, in which she interviewed participants and completed the FFQ's, and dietary folate analysis. Additionally I would like to acknowledge her for genotyping of the first stage cohort for the 80 G>A RFC polymorphism. I would like to recognise honours student Charlotte Naylor for genotyping the 1420C>T SHMT and the 1947G>A COMT polymorphisms for this in the entire cohort of samples covering both stage 1 and 2. I also acknowledge the collaboration with PhD students, Dr Nenad Naumovski (for method development and analysis of the amino-thiols for samples collected in stage 1), and Dr Xiaowei Ng (in sample collection across both stages, and for completion of the genotyping of 677C>T MTHFR, 1298A>C MTHFR, 19bp del DHFR and 1561C>T GCPII polymorphisms in stage 1).

CHAPTER 4: B-VITAMIN NUTRITIONAL GENETICS IN THE ELDERLY - RISK FOR ALZHEIMER'S DISEASE

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CHAPTER 5: B-VITAMIN NUTRITIONAL GENETICS AND OCCURRENCE OF ADENOMATOUS POLYP – A MAJOR ANTECEDENT OF COLORECTAL CANCER

I would like to thank BMedSci student Ron Wai for the initial recruitment, interviewing, and venepuncture of the first 50 subjects recruited into this study. I also acknowledge his completion of the 80G>A RFC polymorphism on those initial subjects as a part of his BMedSci project. This data has been incorporated into the study database and has been used for analysis in this chapter. I would again like to thank Dr Virginia Skinner for her involvement in the recruitment, interviewing, and venepuncture of subjects in this cohort.

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Acknowledgment of Authorship

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

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Principal Supervisor

Journal Papers

LUCOCK, M., YATES, Z., MARTIN, C., CHOI, J. H., **BOYD, L**., TANG, S., NAUMOVSKI, N., ROACH, P. & VEYSEY, M. 2013. Hydrogen sulphide-related thiol metabolism and nutrigenetics in relation to hypertension in an elderly population. *Genes & Nutrition***,** Vol 8, 2:221-229.

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CHAPTER 1

THESIS INTRODUCTION

1. Overview

Folate is an essential B-group vitamin that has long been known to play an important role in maintaining human health. This introductory section will review the socio-economic impact of nutrition in disease prevention, the role of ageing and the burden of common chronic diseases. Furthermore, a detailed examination of folate, including its history, biochemistry, metabolism, and genetic variability of folate-related proteins will be outlined. Also, the impact of folate-related molecular mechanisms in the context of both deprivation and excess intakes will be examined taking account of past and current trends, particularly folic acid fortification programs, that have been instituted and any emerging effects (both positive and negative). The introduction will conclude with a scope that summarises the research undertaken.

1.1. Socio-Economic Impact of Nutrition in Disease Prevention

Nutrition is an important factor in the promotion and maintenance of good health throughout the human lifecycle, and is now increasingly considered to be a key modifiable determinant of chronic disease risk. Scientific evidence supports the view that alterations in diet, both positive and negative, have profound effects on health throughout life. This can be dietary changes that not only affect present health, but which may also play a role in individuals developing long-term degenerative disorders such as cardiovascular disease (CVD), cancer and diabetes that increase morbidity later in life.

Worldwide, there is a growing epidemic of chronic disease. This is not only confined to over nutrition in developed nations, but also related to the double burden of managing communicable disease as well as malnutrition in developing nations [1]. Government food policies are often primarily focused on under nutrition and not on addressing broader issues in the prevention of chronic disease through appropriate nutrition. Chronic diseases like CVD, obesity, diabetes and various types of cancers are influenced by food and nutritional determinants and as such, they are considered to be environmentally determined.

In the last century, the world underwent massive change as a consequence of industrialisation, urbanisation, and economic development. These changes have significantly impacted the health, lifestyle and nutritional status of populations. In developed countries, living standards have improved, food availability has greatly expanded and diversified. Food manufacturing and agricultural production

improvements have created a ‘global food economy’, which has meant that food products have gone from local area production and consumption to food commodities that are traded within the global marketplace. The changes that have occurred to our food supply have altered the types of foods we consume; this has essentially increased the consumption of energy-dense nutrient poor foods - products that are typically high in fat and low in unrefined carbohydrates [2].

In addition to the changing pattern of our food supply, there has also been a major decrease in the amount of physical activity undertaken by populations [3]. This sedentary lifestyle includes leisure time that is physically undemanding, a generalised phasing out of physically challenging manual tasks in the workplace, motorised transport and labour saving devices in the home. This physical inactivity combined with inappropriate dietary patterns and tobacco and alcohol consumption has led to a corresponding increase in the occurrence of chronic degenerative diseases [1].

The past decade has seen a rapid expansion in an array of scientific fields. In particular, focus on human epidemiological studies provides clear evidence of the role of diet and lifestyle in controlling morbidity and premature mortality relating to chronic disease. In addition to this, results from controlled clinical trials have shed light on the mechanisms behind chronic disease, while nutritional interventions have demonstrated effective pathways to reduce risk. There is a growing body of evidence that indicates specific dietary nutrients are able to influence the development and outcome of certain chronic diseases. Before looking at this in detail, it is first necessary to examine the worldwide extent of chronic nutritionally related disease, and the global burden it has created.

1.1.1. The burden of chronic disease and prevention

Chronic diseases are mostly characterised by having multiple risk factors, complex causality, and often result in a prolonged course of illness and almost always give rise to functional impairment or disability of some kind [4]. Most chronic diseases develop over time such as diabetes, but some can be immediately life threatening such as a heart attack or stroke [4]. One account for the increased prevalence of chronic disease is simply improvements in treatment and management that has resulted in people living much further into old age [4]. The chronic diseases considered in this thesis are those that are common and are related to diet and nutrition, they also present the greatest public health burden, either in terms of direct cost to society and government, or in terms of disability-adjusted life years. CVD, cancer and cognitive decline make up the

major focus of this thesis and are hugely relevant chronic degenerative disorders at a global level, although obesity, diabetes and osteoporosis are also considered chronic diseases by the World Health Organisation (WHO), but have not been examined in this thesis [1].

Many WHO reports state that the burden of chronic disease is rapidly increasing worldwide [1]. In 2001, calculations found that chronic diseases contributed approximately 60% of the 56.5 million total reported deaths in the world and approximately 46% of the global burden of disease [5]. The same report projected that the proportion of the burden of chronic diseases is expected to increase to 57% by 2020 [5]. The WHO also cites that worldwide almost half of the total chronic disease deaths are attributable to CVD [1]. In particular, it is noted that obesity and diabetes are beginning to show worrying trends, with high frequencies in populations, all the more startling because increasingly the disease is appearing earlier in life [1].

The United States (US) leads industrialised nations in most health related trends. It is reported that the leading cause of death in North America is CVD with more than 81 million Americans having one or more type of CVD [6]. In addition to this, reports suggest that 74 million Americans are hypertensive and 35 million have high-risk cholesterol concentrations (>240mg/dL) [6]. Second to CVD as a cause of death is cancer; it accounts for approximately 25% of all deaths and it is estimated that one third of these deaths are related to nutritional factors through diet and inappropriate weight management/obesity [7, 8]. Of course, America is not alone; most Western nations in the developed world have similar statistics in terms of the leading causes of death.

In 2007, the major chronic diseases (excluding depression) accounted for 50% of all deaths in Australia [9]. Like the US, CVD (coronary heart disease and stroke) in Australia, was the main contributing pathology. As a disease group, CVD contributed 54% of all male deaths and 59% of all female deaths in 2007 [9]. Second to CVD in total number of deaths is cancer, contributing most prominently through lung, prostate and breast foci [9]. Alzheimer's disease (AD), dementia and diabetes also featured as leading causes of death in 2007 [9]. In addition to these disturbing facts, chronic degenerative diseases in the past have been generally confined to industrialised countries, but this is not the case anymore - developing countries are increasingly suffering from high levels of public health problems associated with chronic degenerative diseases. The concern is that these countries still suffer from malnutrition. To compound this, in places such as sub-Saharan Africa, human immunodeficiency

virus infection / acquired immunodeficiency syndrome, malaria and tuberculosis are the predominate causes of disease [1]. This combination is sometimes referred to as the 'double burden', given both chronic diseases and communicable diseases co-exist in certain populations.

In developing countries, 79% of deaths are attributed to chronic disease; these diseases typically affect people aged between 45 and 65, and alarmingly, China and India collectively have a burden of CVD greater than all other industrialised nations combined [1, 10]. India is a seminal example of these changes - at present the nation faces a combination of communicable disease and chronic disease, with chronic disease just exceeding that of communicable disease [1]. Reflecting this, Murray and Lopez *et al.* [11] report that globally, communicable disease will still occupy critical importance up until the year 2020. A different example of the shift in patterns of disease is with obesity. In parts of Asia, Latin America and Africa, the prevalence of obesity has doubled over the past decade [1]. This is commensurate with rapidly changing diets and lifestyles in these countries and although seemingly paradoxical, it's perhaps not surprising that food security and poor nutrition exists in the same countries where chronic diseases are emerging as a major epidemic. Additionally, major concerns surround children in developing countries; the prevalence of overweight children has increased dramatically in the past twenty years, and this cuts across nations with differing dietary patterns ranging from India and Mexico to Nigeria and Tunisia [1, 12].

What is also very important to consider is the interaction between early life, and adult-life chronic disease development; predominantly low-birth weight. Major research has established that a low-birth weight followed by subsequent adult obesity imparts a significantly higher risk for CVD and diabetes; this is often referred to as the 'Barker Hypothesis'. In 1995, Barker [13], established that adults who were 9-pound babies had a lower risk of coronary heart disease than those who were 8-pound babies. The risk of heart disease was highest in people with the lowest birth weight and the risk fell progressively across the range of birth weights [13]. Unfortunately, even in contemporary Western societies, mothers often consume diets that are unbalanced in macronutrients and deficient in essential micronutrients resulting in babies who are poorly nourished. Many girls and young women in developing countries are chronically malnourished, but also in industrial countries there are many women who are excessively thin or by contrast overweight but are still undernourished. In either event this results in poor foetal growth and ultimately a low-birth weight, and, according to the

hypothesis, this results in a trend towards higher risk of adult-onset chronic disease [13, 14].

What is now becoming clearer, is that chronic degenerative diseases are no longer disorders of ‘affluence’, particularly given their emergence in developing countries and in poorer populations within industrialised nations [1]. What is also concerning is the rate at which change is taking place; half a century ago current industrialised nations saw a much slower rate of change in disease patterns compared to today’s developing nations in which chronic disease has impacted much quicker than ever before [15]. These rapid changes in the burden of disease create a major public health threat which requires effective action, particularly given chronic diseases are largely preventable. To this end, industrialised nations have developed primary prevention strategies aimed at reducing the incidence of chronic disease. This approach is undoubtedly the most cost effective way of dealing with what is a major healthcare problem linked in to an increasingly ageing population [1].

Prevention strategies have included the development of ‘risk-factor’ identifiers; these identifiers are often categorised as behavioural, biomedical, environmental, genetic and demographic risk factors. Within these categories there are risks that are non-modifiable; such as age, gender, family history, and genetic susceptibility whereas the all other groups contain factors that can be modifiable [16]. Behavioural risk factors include poor diet, physical inactivity, excessive tobacco and alcohol consumption. Biomedical risk-factors include having hypertension, dyslipidaemia, being overweight or obese and having impaired glucose tolerance [16]. There is increasing evidence that chronic disease risk factors can begin as early as foetal life and continue into old age, and that adult development of chronic disease reflects a lifetime of exposure to physical and social environments that are damaging [1, 13, 17, 18].

The largest and most important modifiable risk-factor is poor diet and nutrition. The single biggest problem affecting diet has been global change during the latter half of the 20th century. Arguably, before this, the first important event was the domestication of plant and animal food sources during the Neolithic period - a process that facilitated the development of civilisations, and the second event was the development of industrial scale food processing in the nineteenth century. This resulted in a major modification from a traditional diet which had a larger amount of non-processed plant and animal based foods to one that is highly manufactured resulting in foods that are energy-dense, predominately from cereal grains, but also, which contain high amounts saturated fat and sugars [1]. Diet is critical to prevention strategies, but it is only one

risk factor, the shift that occurred in dietary habits has been exacerbated by a second shift involving increasingly sedentary behaviours. Physical inactivity has swept the globe and is now recognised as an increasingly important determinant of health and thus risk factor for chronic degenerative diseases [1]. These two changes alone justify the need for an integrated approach to tackling the rise in chronic disease.

So far, progress in the prevention of chronic disease has been slow, there is often a long period between effective interventions and measurable outcomes, even then outcomes are often undervalued. Other factors like commercial pressures and inadequate funding affect the overall success of health based prevention measures [1]. An eloquent example of this occurred in Finland. Between the early 1970's and 1995 rates of coronary heart disease dropped significantly, after analysis of the risk factors (smoking, hypertension and hyperlipidaemia), diet was found to be the biggest factor in the reduction, which operates by reducing plasma cholesterol and blood pressure [1, 19]. The contribution of medical intervention like anti-lipid and hypotensive drugs was negligible [1].

Another example of how diet can effectively impact chronic disease rates has occurred in the Republic of Korea. Despite major economic and social changes, fat intake and obesity rates, and overall prevalence of chronic disease remains low [20, 21]. This is almost entirely due to the maintenance of traditional diets in which vegetables are the main food source. This example demonstrates that a country's economic development and social progression does not necessarily have to lead to poorer health outcomes for the residents of that nation. This is particularly important given observations of dietary patterns and physical inactivity as risk factors have shown an ability to travel across countries and become transferable from one population to another, analogous to an infectious disease [1]. However, the Republic of Korea has remained relatively immune to this phenomenon despite changes in other neighbouring Asian countries i.e. China. Clearly, the cultural identity of a population extends to the diet, and this can underpin a nation's health, and its growth and development. Unfortunately, national identity can also negatively influence health risk factors via social, cultural, political and economic factors unless measures are taken to make the environment a health-promoting one.

1.1.2. Gene-nutrient interactions, genetic susceptibility and evolutionary discordance

Since the early 1990's there have been significant advances in basic research into the prevention and or control of common chronic diseases. However, with the mapping of

the human genome, any discussion of common chronic disease needs to consider genetic influences and variability. Gene expression has been shown to be influenced by nutrients from the diet and by physical activity; it is thought that these factors have helped shape the human genome and resulting phenotypes over the course of hominin evolution [1]. Because of this, genes provide a window for determining a person's susceptibility to disease, but it will ultimately be the environment a person is exposed to which will determine which susceptible people go on to develop any such illness [1]. Given the recent dramatic changes in our food supply and lifestyle, both within industrialised and developing nations, this may result in the phenotypic expression of underlying genetic traits that promote chronic disease risk [1].

Researching the effects of both genes and nutrition together has created a whole new area of scientific interest; sometimes referred to as nutritional genomics or nutrigenomics. Nutritional genomic research aims to examine the interaction between the human diet and genes, its influence on metabolism and subsequent susceptibility to development of common diseases [22]. The promise of nutritional genomics is a deeper understanding of nutrition at a molecular level, which someday may help to design diets that maximise an individual's potential for health and wellness [22]. This could be, for example, beneficial for patients with enzymatic deficiencies, or in people with a genetic predisposition to multifaceted diseases, or in patients already suffering from such illness; a tailor-made diet may help reduce severity of disease symptoms or help prevent further diseases from occurring [22]. An examples of nutritional genomics research might include findings that dietary intake combined with specific genotypes raises cholesterol levels [23].

A key piece of research central to this thesis is the prominent relationship between the B-group vitamin folic acid and CVD; nutritional genomic research has revealed that a common single gene mutation reduces the activity of an enzyme involved in folate metabolism. The variant expression product methylenetetrahydrofolate reductase (MTHFR) is associated with a moderate (~ 20%) increase in serum homocysteine; a toxic thiol, which in turn results in a higher risk for both ischaemic heart disease and deep vein thrombosis [24]. Genetic variability in folate metabolism is discussed at length on page 52.

Targeted dietary advice for susceptible people, subgroups and populations is desirable, but it is not always feasible for use in national/international public health policies [1]. It also appears that environmental changes and not changes in genetic susceptibility are to blame for the escalating rates of chronic disease which are polygenic in nature [1].

Humans have evolved to survive on a wide variety of foods, although recent changes in dietary patterns have had demonstrable impact on our genetically heterogeneous species. It is worth noting that from an evolutionary perspective, the success of our hominin ancestors most often related to reproductive efficiency early in the lifecycle, and did not consider healthy eating in terms of lifelong health and chronic disease prevention, since this is most often only relevant after reproduction has occurred and hence does not influence species survival [1]. It is necessary to add a caveat to this last statement, because in humans at least, alloparenting ensures that older, post-reproductive members of a population can still contribute to the overall success/survival of younger members. Despite this caveat, the main point still stands [25].

Nutritional genomics research has given rise to another concept called ‘evolutionary discordance’ which is relatively new but considers a much larger picture of why chronic disease is plaguing many nations of the world [2, 26, 27]. The concept of ‘evolutionary discordance’ states that there is discordance between our ancient, genetically determined biology and the nutritional, cultural and activity patterns of today’s industrialised nations and this may help explain the alarming rates of chronic disease seen globally [2]. In essence, a mismatch exists between our “stone-age’ genes and our contemporary lifestyles – particularly Western diets

Over time, evolution signifies on-going interactions between a species genome and the environment, positive and negative genetic traits may be selected relative to their concordance or discordance with environmental selective pressures [2, 28]. If the environment stays the same, genetic selection favours the optimal average for that population, but when environmental changes occur, evolutionary discordance arises [2]. Over time, this discordance can be changed into directional genetic selection, which moves the average population genome to a new set point, causing limited problems [2]. However, when discordance arises rapidly in an affected genotype, the result, phenotypically, is usually reduced reproductive success and disease increased morbidity and mortality [2]. Like other species, the human genome is genetically adapted to survive the environment of our recent ancestors. The problem is that the environment of modern human ancestors was hugely different to the environment we live in today. Part of this evolutionary discordance concept specifically blames the profound changes of our diet, the environment and to our lifestyles that occurred approximately 10,000 years ago when agriculture and animal husbandry first began [2, 29]. The advent of agriculture produced novel foods that were introduced as staples that the human genome had little evolutionary experience of [2]. These food products

and rapid changes to overall dietary patterns meant the human genome could not adjust, and because of this inability to adapt, diseases of civilisation, including chronic diseases central to this thesis, have emerged.

1.1.3. The industrial era - post agriculture diets & health ramifications

Dietary patterns can change over time; diets are influenced by many things such as supply of food staples, prices, incomes, cultural traditions, religious beliefs, individual preferences, as well as environmental and social factors. As described earlier, traditional diets are being replaced by foods that are energy-dense, high in saturated fat and contain added sugars. Before the development of farming and agriculture, dietary sources would have consisted of wild plants and animals, which required little processing and maintain original nutrient content. Changes during the 20th century have led to the development of processed foods – which permits quantitative and qualitative food and nutrient combinations not previously encountered over the course of human history, but which now dominate typical Western diets [2].

Contrasted below (table 1-1), are types of foods that would have generally been unavailable to pre-agricultural humans, but these food types are classed as today's food staples and make up the majority of Western diets. Using mixtures from the food groups, processed contemporary foods include; ice cream, chocolate, pizza, snack foods, soft drinks, mayonnaise, salad dressing and bakery products like cakes and cookies to name a few. This shift away from nutrient-dense foods such as lean meats seafood, fruits and vegetables, has resulted in a decline in dietary vitamin and mineral density. This has far reaching health implications, as it can promote the development of overt or marginal vitamin deficiency, and can therefore be an important aetiological factor in various chronic degenerative diseases [2].

This shift in dietary intakes can be considered as a causal factor in the rise of diet-related chronic disease, given that hunter-gathers and non-western populations have a lower prevalence of these diseases. In Australia, these diseases represent the single largest cause of morbidity and mortality [4], and as described earlier, they occur in epidemic proportions in all current western nations, with up to 50 to 65% of the adult populations suffering from one or another of these chronic diseases.

These novel foods groups, introduced gradually during the industrial era, have changed food consumption; in Australia the National Nutrition Survey reported that 35-36% of energy for adult persons comes from cereal and cereal products, while meat and

Table 1-1: Current food staples - environment & consumption in pre and post agriculture eras (information sourced from Cordain *et al.* [2] Origins and evolution of the Western diet: health implications for the 21st century).

Food Group		Pre- Agriculture	Post- Agriculture
Dairy Products	Environment	Wild Animals – inability to capture and milk animals such as sheep, cows and goats.	Domestication of livestock, mechanisation of dairy farming producing high yields of milk globally.
	Consumption	Nil Exception of human breast milk.	High consumption of cow's milk, low fat varieties, butter, cream, cheese and yoghurts.
Cereals	Environment	Wild cereal grains - small, difficult to harvest, and minimally digestible without processing (grinding) and cooking. Use of stone tools, grain contained the entire contents the germ, bran, and endosperm.	Hectares of grain fields farmed, mechanised steel roller mills and automated sifting devices. Nutritional characteristics diminished with removed of germ and bran leaving flour comprised mainly of endosperm [30, 31].
	Consumption	Rarely consumed as year round staples by most worldwide hunter-gatherers, except by certain groups living in arid and marginal environments [32, 33]	85.3% of the cereals consumed in the current American diet are highly processed refined grains of uniformly small particulate size [34, 35].
Refined Sugars	Environment	Honey was first source of sugar and only seasonally available in particular climates.	Crystalline sucrose production appears, ability to manufacture high-fructose corn syrup in mass quantities, containing mixture of fructose and glucose.
	Consumption	Honey was highly favoured, high consumption existed in the short months of supply, evidenced in Aboriginals; it was only a minor dietary component over the course of the year, no crystalline forms of sugar.	Increased sugar consumption reflects a large worldwide trend that has occurred in all Western nations, examples: per capita refined sucrose consumption in England rose from 6.8 kg in 1815 to 54.5 kg in 1970 [36].
Vegetable Oils	Environment	Impossible ability to extract oils from nuts and seeds without mechanisation.	Industrialisation/mechanisation of the oil-seed industry use of non-traditional oils - cottonseed, In 1897 hydrogenation is developed creating margarine and shortening products by solidifying vegetable oils, hydrogenation process produces novel trans fatty acid isomers that never existed in foods before [37].
	Consumption	Plant based oils such as olive oil did exist in small amounts and were used, but other oils were used for non-food purposes such as illumination, lubrication, and medicine [38].	Striking increase in the use of vegetable oils has occurred; between 1909 and 1999 in the US per capita consumption of margarine increased 410% and salad and cooking oils increased 130% [34].
Domestic Fatty meats	Environment	All animal meats consumed by humans were derived from wild animals, with varying body fat % different ages and gender. Meat was affected seasonally in a cyclic waxing and waning manner with changing availability of food sources and the photoperiod [39, 40].	Advent of animal husbandry, provisioning of domesticated animals with stored plant foods. The ability to slaughter the animal at peak body fat %, (obese cattle 30%) increased practice of feeding grain (corn primarily) fattening cattle in feedlots producing 545-kg animals in 14 months (short lifespan), ability to exhibited "marbled meat" not seen in wild/ free-range animals [41, 42].
	Consumption	Hunting was a key part of life, meat intake would have been considerable, meat consumed was high in monounsaturated and polyunsaturated fatty acids. Seasonal cyclic depletion of saturated fatty acids mean a year-round intake would have not been possible for pre-agricultural humans preying on wild mammals.	Meat containing high amounts of saturated fatty acids, lower proportion of n–3 fatty acids, high n–6 fatty acids, represents a recent component of human diets[43], annual meat consumption per capita in Australia in 1968-69 was 85.9kg in 2005-06 this increased to 112 kg [44].
Alcohol	Environment	Evidence of use for medicinal purposes and some small consumptions within various population-groups, however seasonal fluctuations in fruit availability and limited liquid storage capacity.	Highly refined distilled alcoholic beverages are now produced globally as an industry, producing predominately beer, wine and liquor /spirits. Products have increasing amount of pure alcohol, with scientific methods being applied to produce high quality beverages such as wine, an ability to produce alcoholic beverages is linked to the economic development of a country.
	Consumption	Fermented fruit drinks such as wine and beers would have made an insignificant contribution to total energy in diet [45].	In 2004-05 Australia's average intake was 9.8 litres of alcohol per person [46]. Increased social acceptability and drinking with meals have increased intakes.
Salt	Environment	No evidence that Palaeolithic people undertook salt extraction or took interest in inland salt deposits, even though it was present.	High global production manufactured from sea water using evaporation, multiple purification and re-crystallisation with added chemicals and anticaking agents. It is used extensively as perseverative in most processed foods and home cooking.
	Consumption	Hunter-gatherers living in coastal areas may have dipped food in seawater or used dried seawater salt, inland hunter-gatherers add no or little salt to their food on a daily basis [47].	Today 90% of the salt in the typical Western diet comes from manufactured salt that is added to the food supply. The total quantity of sodium included in the typical Western diet amounts to 3271 mg/day [35].

poultry foods represent 13-15% and dairy products 11-12% of total energy [48]. The unfortunate rise of these novel food products has resulted in lower fruit and vegetable consumption. In the self-reported data from the 2007–08 National Health Survey, it was shown that just over 51% of the population consumed two or more serves of fruit per day and only 9% consumed five or more serves of vegetables per day, which is the recommended serving size [49]. Fruit and vegetable consumption is strongly linked to good health and the prevention of chronic disease [50]. Inadequate intake was estimated by Begg *et al.* [51] to be responsible for 2.1% of the total burden of disease in Australia. Importantly, fruits and vegetables are nutritional 'superfoods', with most containing a vast array of essential vitamins and minerals, antioxidants and phytochemicals, as well as supplying the diet with the majority of its fibre requirement. Pre-agricultural diets are known to have consisted of a large proportion of fruits and vegetables [2]. The reduction in their consumption in the post-agricultural/industrial era as well as the rise of these new food groups can be attributed to the rising epidemic of poor health and chronic disease. This shift has adversely affected key nutritional characteristics in the human body; the most important of these relates to micronutrient concentrations.

Micronutrients are vital because they are responsible for helping the body produce and utilise enzymes, hormones and other substances essential for growth and development. They are only needed in minuscule amounts but a lack of them can represent a major threat to health. Refined foods such as those listed in table 1-1 are relatively devoid of vitamins and minerals, particularly refined vegetable oils, sugars and grains. Eating foods that contain for example refined sugar reduces the total micronutrient density of an entire diet by displacing more nutrient-dense foods [2]. This not only relates to refined sugars but the other two big food groups; refined grains and milk, as they have low nutrient density so the displacement of fruit, vegetables, and lean meats by these additional two food staples lowers the overall micronutrient bulk in the diet [2]. Therefore, wild animal and plant foods consumed by hunter-gathers would have maintained high micronutrient concentrations and so the introduction of dairy foods and cereal grains as staples has caused the average micronutrient content of the diet to decline [2]. This has been hastened by milling grain for the production of bread flour which is devoid of the micronutrient-dense bran and germ [30]. This shift has far reaching health implications as the replacement of nutrient-rich for more nutrient-poor foods results in a decline in dietary vitamin and mineral density. The consequences of this are not only promotion of the development of vitamin deficiency diseases but also the development of more chronic degenerative diseases [2].

Increasing knowledge about vitamin deficiency diseases has led to governments developing public health policies that aim to mandate fortification of the food supply with certain vitamins to counteract deficiency syndromes and specific disorders, and to ensure that populations meet minimum dietary requirements. Examples of fortification programs in place in Australia include wheat bread flour containing folic acid and thiamine and iodised salt being added during bread production [52]. Other examples include the addition of vitamin D to margarine and oil spreads and the fluoridation of water supplies [53, 54]. Other methods used to increase micronutrient density include the manufacture of functional foods; these are foods that have been manufactured with an added bioactive compound that may reduce risk of disease, often vitamins and minerals are used, examples include calcium added to fruit juices and iron added to breakfast cereals.

In recent years, this gradual removal of natural/unprocessed nutritious foods from our diet has increased vitamin and mineral deficiencies; a typical Western diet can be insufficient in vitamin B_6, vitamin A, vitamin E, vitamin D, magnesium, calcium, iodine, iron, selenium, zinc and folate [55-58]. Meeting recommended dietary intakes is difficult when consuming energy-dense nutrient-poor foods; however the traditional overt vitamin deficiency diseases that were more common 50 to 100 years ago such as scurvy, rickets and pellagra are no longer seen [59]. Despite this, increasingly, marginal vitamin deficiencies are being linked to conditions such as cancer and CVD, particularly in respect of the B group vitamin folate; an essential micronutrient responsible for a broad range of biochemical reactions as well as for healthy foetal development and growth [60]. In Australia, it is estimated that the current intake of folic acid by females of child-bearing age (16–44 years) is 108 µg per day [61], which is well below the recommended 400 µg per day level. Likewise mild iodine deficiency has also been reported in Australia and vitamin D and iron intakes are also of concern [62]. Although the focus of this dissertation is on micronutrients, specifically the B-group vitamin folate, there have been other important modifications from the post-agricultural environment that have resulted in other negative health ramifications. Summarised below are changes that include; altered macronutrient and fatty acid compositions, increased glycaemic load, pH imbalances, altered sodium-potassium ratios and reduced fibre consumption [2].

Macronutrient composition: Unlike the finer roles of micronutrients, macronutrients also have a key role to play in nutrition and health, particularly because they often supply the micronutrients to the diet. Energy supplied in the diet comes from carbohydrates,

fat and protein, with alcohol also contributing a small amount of dietary energy; in Australia statistics put the macronutrient profile at 46.0%, 32.5% 17.1% and 3.7% respectively [48]. There is a growing body of evidence that a major imbalance in the relative proportions of macronutrients can increase risk of chronic disease. Although difficult to determine, comparison of studies of agrarian diets suggest dietary protein is elevated to between 19-35% of energy, but maybe as high as 50%, which reduces the proportion of carbohydrate utilised for energy [2, 63]. This may be important in relation to CVD risk, as there is evidence that higher protein diets can improve metabolic control in patients with diabetes, reduce plasma triacylglycerol levels, total/ low density lipoprotein (LDL), and cholesterol, while increasing high density lipoprotein (HDL) cholesterol levels [64, 65]. Additionally, a low intake of dietary protein has been associated with high blood pressure [66, 67]. Protein has a greater thermic effect and provides greater satiety value when compared to carbohydrates or fats [68]. Therefore, higher protein diets may assist in weight management and control of obesity [2]. Intakes similar to the diets of pre-agriculture humans (19-35% of total energy) might therefore aid in the reduction of chronic diseases such as CVD and obesity.

Fatty acid composition: Western diets today contain high amounts of saturated fatty acids, with the major sources coming from blends of seed oils, such as vegetable oil, which includes palm and canola oil. These are contained in food products such as margarines and a wide variety of bakery foods. Additional saturated fatty acids in the diet come from high fat meats and dairy products such as milk, cheese, creams, and butters. However, these foods were not present in human diets prior to the industrial revolution and the introduction of animal husbandry. The fatty acid composition of pre-historical diets was high in monounsaturated and polyunsaturated fatty acids (PUFA); high levels of saturated fatty acids could not have been achieved all year round because of the lean nature of tissues from wild animals [2]. In addition to the reduction in good fatty acids and the increase in saturated fatty acid, the industrial revolution created the hydrogenation process, which introduced *trans* elaidic acid (*trans* fatty acid). This acid formed from vegetables oils is known to elevate risk of CVD by increasing both total and LDL blood cholesterol levels [2, 69, 70].

The rise in both saturated and *trans* fatty acid intakes has also resulted in a decrease in beneficial fats; specifically too little omega-3 PUFA compared with omega-6 PUFA. This imbalance began with the rise in consumption of cereal grain products, and vegetable fat intake, which both contain high concentrations of omega-6 PUFA, and no omega-3 PUFA. But this change in ratio has also been exacerbated by grain feeding

livestock; grain fed animals produce meat with high omega-6 PUFA concentrations. This is particularly important given that research has determined omega-3 PUFAs reduce risk of CVD [71]. They achieve this through reducing blood pressure, reduced growth of artery plaques, reduced platelet aggregation, and reduced serum triacylglycerol levels. They may also play a role in preventing inflammatory and autoimmune diseases [71, 72]. Although a decrease in dietary fat intake is important for reducing CVD risk, it is now thought that the quality of fat is more important than the quantity of fat consumed. Hunter-gatherer diets of wild animals and plants would have had a ratio of omega-6 to 3 PUFA of 2:1 or 3:1. However, today omega-6 PUFA intake has significantly increased where the ratio is as high as 20:1 [73]. This change in quality of fatty acid maybe a component in the rise of chronic disease, particularly heart disease.

Glycemic index: This index is used to assess the blood glucose raising potential of a food based on both the quality and quantity of dietary carbohydrate [74]. Refined grains and sugars sustain much higher glycemic loads than unprocessed fruits, vegetables and wild plant foods like those available to contemporary and extinct hunter-gatherers, which typically exhibit low glycemic indices [2, 75]. A considerable portion of today's diets contain sugars such as high-fructose corn syrup, sucrose, glucose and fusions of all three, as well as refined cereal grains. These food types all have extremely high glycemic indexes. Food products containing these ingredients promote insulin resistance in the body through a number of hormonal and physiologic changes [76, 77]. This is normally evidenced through chronic hyperglycemia and hyperinsulinemia; conditions that are the primary metabolic defects in 'metabolic syndrome' [78]. Diseases associated with metabolic syndrome and insulin resistance include; obesity, cancers, CVD, hypertension and type-2 diabetes [79-81]. Additionally, elevated LDL cholesterol plus reduced HDL cholesterol is evident [82]. Given the dominance of high glycemic foods in our current diets, and the fact that consumption of these foods was rare in hunter-gatherer societies, a considerable burden of chronic disease may be attributable to this recent historical shift from low to high glycemic loads in our diets.

Acid-base imbalance: All foods that enter systemic circulation after digestion become either an acid or base. Meat, seafood, eggs, cereal grains milk, and salt are all net-acid producing products, whereas base-producing foods include fresh fruit, vegetables, and nuts. Given the imbalance in food staple consumption, a typical Western diet yields a net acid load [83]. Over time, this can result in pathogenic metabolic acidosis. Metabolic acidosis can worsen with age and greatly affect proper functioning of the

kidneys [84]. Diets absent in energy-dense, nutrient-poor foods are net-base yielding, thus ancient pre-agriculture diets were always net-base [85]. Research has determined that a net-base diet can help prevent cancer, hypertension, stroke, osteoporosis, kidney stones, age-related renal failure, and a range of degenerative disorders [86-90].

Altered sodium-potassium intakes: Currently, modern dietary intakes of sodium are significantly higher than potassium intakes [35]. This is due to the increased use of manufactured sodium chloride in processed foods, while dairy products, refined grains, sugars and vegetable oils are devoid of any potassium [2]. In addition to this, there has been a reduction in consumption of potassium rich foods such as fruit and vegetables. This displacement has caused a 400% decline in the potassium intakes among populations with such diets; simultaneously a 400% increase in sodium ingestion has occurred [2, 91, 92]. This imbalance may be contributing to the rise in various diseases given there has been no previous evolutionary adaptation under these conditions. High dietary intakes of sodium and low potassium intakes may trigger or exacerbate a variety of chronic diseases such as gastrointestinal tract cancers, cardiovascular ailments, asthma and osteoporosis [93-96].

Reduced fibre consumption: A typical Western diet does not contain enough fibre. Refined grains, sugars, vegetable oils, dairy products and alcohol are lacking in fibre, but in terms of energy contribution, these food groups can constitute almost half of the total energy consumed [2]. Also, refined grains contain less fibre than whole grains, yet these refined grains represent 85% of the grains consumed in industrialised nations [2]. Once again, the displacement of fibre-rich plant fruit and vegetables by novel dietary foodstuffs reflects a change that is contra-natural to human metabolism. Indeed, most traditional hunter-gatherer diets would have always been high in fibre. Health implications attributed to high fibre diets, particularly soluble fibres, include; a decrease in plasma cholesterol levels beyond the reductions attributed to a lower saturated fat intake, also high fibre consumption is linked with slow gastric emptying which can reduce appetite and aid in weight management [97].

To summarise, Australia, like most Western countries, has significant rates of diet-related chronic diseases that contribute to morbidity and mortality. Over the past 20 years, research has determined that no one single factor is the cause for increased rates, evidence now tells us that chronic disease aetiology consists of multifactorial dietary elements, genetic susceptibility, and environmental changes. CVD for example, is not simply a consequence of consuming excess saturated fat, but more likely reflects a diet vastly different from hunter-gatherers, which contains mostly new novel foods

such as refined sugars, grains, and vegetable oil, along with fatty meats, dairy products, and added salt.

This diet combined with low fruit and vegetable consumption may explain the epidemic proportions of CVD and other chronic diseases in Western countries. This type of diet is low in essential micronutrients particularly the important B-group vitamins, which have been linked to the prevention of a range of chronic diseases. Not only is the lack of micronutrients an enormous problem, so are other changes that include altered fatty acid and macronutrient compositions, glycemic index of foods, low fibre consumption and imbalances in sodium-potassium intakes and acid base changes. Each of these factors can be linked to certain underlying causes of chronic disease; furthermore, our ancient genome has simply not yet adjusted to the new nutritional environments that contemporary society exposes us too. An environment of energy-dense nutrient-poor foods combined with affluent lifestyles and little physical activity has combined to create a context which is far removed from the Palaeolithic life and diet of our hunter-gatherer ancestors, as well as the few extant ones that still survive.

1.1.4. The ageing process and chronic degenerative diseases

The plague of chronic diseases that is afflicting the world globally is closely linked with the drastic changes in our post-industrial era food staples and lifestyles. Additionally, during the past century, modern research and medical advancement has also helped populations to live longer. This has resulted in a major demographic shift that is generally confined to developed nations of the world. Older people are currently defined as those aged 65 years and above, a definition that is very different to the one that would have prevailed during the last century, when 65 years of age and above often exceeded the average life expectancy. Today, the majority of elderly people live in the developed world and Australia is no exception.

One of the most serious challenges facing Australia is how to plan for a rapidly ageing population. Despite increased speculation about how public and private institutions might respond, there is as yet little agreement on the causes, dynamics, and magnitude of the ageing phenomenon. The health and living conditions of Australians have greatly improved during this past century; the average life expectancy has increased by more than 20 years, from around 55 years for males and 59 years for females at the turn of the nineteenth century to more than 79 and 84 years, respectively, in 2005-2010 [98, 99]. Also during the past several decades, the number and proportion of the population aged 65 years and over has risen considerably. In

2009, more than 2.9 million (13.3% of the population) people were aged 65 years or over, this is compared with under 1.1 million (8.3%) in 1971 [9]. The increase in the population aged 85 years and over has been even more obvious, with the number of people increasing more than fivefold over the same period [9]. The number of centenarians has also risen substantially, from just 200 in 1971 to more than 3,700 in 2009 [9].

Ageing is an inexorable multidimensional biological process that results in physical, psychological and social changes; chronic diseases associated with ageing are generally the result of pathogenic changes in physiological systems and physical structures that manifest during senescence [100-102]. For example, a decline in elasticity of the large arteries can predispose an ageing individual to systolic hypertension [101]. As discussed previously, a wide-range of conditions may be categorised as chronic degenerative diseases. Generally, they are ageing-related and are normally the leading causes of mortality among older adults in developed countries. Stand out conditions include CVD, cancer and diabetes, but other non-fatal conditions with a high prevalence and burden of morbidity and disability in late life include osteoarthritis, osteoporosis, and cognitive decline or AD [100, 101, 103]. Complicating matters is the problem that older adults also frequently suffer comorbidity; i.e. multiple coexisting chronic disorders [101, 104, 105]. Risk factors associated with these chronic diseases include a multitude of diet, genetic and environmental influences which throughout life course contribute to disease risk in old age [101, 106]. As described earlier, there is now substantial evidence that many age-associated chronic diseases may be programmed in utero and in infancy, and that pre adult influences are further altered by exposures in adulthood which may produce eventual disease [101, 107, 108].

In the past, it was thought that risk factors associated with diet and lifestyle would not significantly improve disease outcomes in older people. There has been this idea that someone in their 80's wouldn't greatly benefit from dietary changes in order to reduce weight for example, and that they have 'earned' the right to maintain unhealthy behaviours because they have reached 'old age' [1]. Epidemiological evidence now suggests that older people can greatly benefit from eating a healthy diet, which is as large and as varied as possible in order to maintain their weight and particularly to continue undertaking physical exercise. For example, Liu *et al.* [109] reported among older women, atherosclerotic disease was approximately 20 to 30% lower if they consumed between 5 and 10 servings of fruits and vegetables per day when compared

to women who ate 2 to 5 servings per day. Therefore, it is likely that as elderly people have a higher cardiovascular risk, they can potentially gain greatly from risk factor modification. In fact, changes in risk factors for many chronic diseases can be reversible at any age [1, 110]. Unfortunately, little attention is paid to this age group in terms of primary prevention, despite the fact that it is known intervention aimed at supporting individuals to live healthier lifestyles will often lead to increased independence and reduce the burden on health care systems [1].

In Australia, the 2004-05 National Health Survey found that over 7 million people have at least one chronic condition, this increases with age as does the proportion of people suffering from more than one condition [111]. Eighty per cent of Australians over 65 have reported suffering from one or more chronic condition [4]. This may be partly because 61% of all adult Australians are either overweight or obese, and a reported 85% of Australians do not consume enough vegetables which puts many of them at risk of developing chronic diseases [4, 111]. Figures for the years 2000 to 2001 published in a government report into chronic disease in Australia found that these diseases are a drain on the health system, a staggering 70% of total health expenditure is allocated to the treatment and management of chronic diseases [112].

Also unfortunately, some sub-populations are more affected than others. Compared with other Australians, Aboriginal and Torres Strait Islander populations have higher mortality from diabetes (14 times higher), chronic kidney disease (8 times higher) and heart disease (5 times higher) [4, 113]. In terms of mortality in Australia, coronary heart disease (also known as ischaemic heart disease, heart attack and related disorders) was the leading specific cause of death in both sexes [62]. The top ten leading causes of death in all ages in 2009 are tabulated below (table 1-2). When examined by age, however, in the over 65's, cancer and other tumours, and CVD were the two most common causes of death. The third condition following on from these was deaths attributed to respiratory system diseases, while in the over 85 age group, dementia and AD contributed almost 1 in 10 deaths (4^{th} place) [9, 114]

Given the prominence of CVD, AD and colorectal cancer amongst the major causes of death in Australia, these specific clinical phenotypes are being studied in depth as part of this dissertation. In addition, a fourth clinical phenotype focusing on depression in the over 65's is also examined. The sections below review these four chronic degenerative conditions in more detail and give background to their role in society beginning with the leading cause of death, CVD.

Table 1-2: The ten leading underlying specific causes of death, all ages, 2009

	Males		Females	
		% of all deaths		% of all deaths
1	Coronary heart disease	16.7	Coronary heart disease	15.3
2	Lung cancer	6.6	Cerebrovascular diseases (stroke)	9.8
3	Cerebrovascular diseases (stroke)	6.2	Dementia and Alzheimer disease	8.0
4	Chronic obstructive pulmonary disease	4.4	Lung cancer	4.4
5	Prostate cancer	4.3	Breast cancer	4.1
6	Dementia and Alzheimer disease	3.9	Chronic obstructive pulmonary disease	4.0
7	Colorectal cancer	3.1	Diabetes	3.0
8	Blood and lymph cancer (including leukaemia)	3.0	Heart failure and complications and ill-defined heart diseases	2.8
9	Diabetes	2.9	Disease of the kidney and urinary system	2.7
10	Suicide	2.3	Colorectal cancer	2.6

Data sourced from [114] reported in Australia's Health 2012 – The thirteenth biennial report of the Australian Institute Health and Welfare [62].

Cardiovascular Disease: CVD can be characterized as a group of multifactorial conditions covering all disease and conditions of the heart and blood vessels; it is closely associated with atherosclerosis, hypertension, and thrombosis [115]. All these pathologic entities are known to be closely related to both genetic factors and environmental influences, with a strong relationship between diet composition and CVD risk well established [116]. As demonstrated above, it is the single greatest killer of Australians, it affects more than 3.4 million Australians or one in every six people [49, 99]. It kills another person every 12 minutes and accounts for over 45,000 deaths in Australia every year [115]. This continues to generate a considerable burden on the population; in relation to direct health care expenditure, CVD is the most expensive health condition costing approximately 5.9 billion dollars (AUD) of the total allocated health system expenditure in 2004-05 [117]. The incidence of CVD increases with age, and the number of people with CVD is likely to increase in the future as the number of older Australians increases [115].

CVD is complex in nature, with multiple causes attributable to it; atherosclerosis for example, is a key element in its pathogenesis. This is a complex combination of lipid transport and metabolism disorders which results in chronic inflammation of arteries [118]. Unlike some other multifactorial conditions, especially cancer, atherosclerosis is a CVD risk factor that can be detected at a preclinical stage by relatively simple quantitative assessment of blood lipid profiles. Permanently elevated plasma levels of total cholesterol, LDL cholesterol, and triglycerides predispose to the development of atherosclerotic plagues, whereas increased HDL cholesterol levels appear to be

protective [116]. Atherosclerosis is very important in the development of CVD, and it is known that diet and lifestyle factors can help to alleviate this biomedical risk.

Another very important clinical risk factor linked to CVD is hypertension. This is defined by an elevated blood pressure; the normal dividing line between being normotensive and hypertensive is having a blood pressure of ≥140/90 mm Hg [119]. Higher levels of blood pressure are strongly associated with increasing rates of CVD. Observational studies also show that the lower the blood pressure, the lower the risk of stroke, coronary heart disease, chronic kidney disease, heart failure and death [120, 121]. Hypertension is responsible for more deaths and disease than any other biomedical risk factor worldwide [122]. Unfortunately, individuals with hypertension usually don't have symptoms and it is a disease that is often under-diagnosed. Data from the 1999-2000 Australian Diabetes, Obesity and Lifestyle Study indicated that 30% or 3.7 million Australians over the age of 25 years were hypertensive [62].

Sodium, fatty acids, energy and alcohol consumption are some of the most important dietary determinants of blood pressure [115]. Additionally, leading a sedentary lifestyle is also linked with higher blood pressure [115]. It is now well understood that numerous genetic factors are involved in blood pressure regulation, and some genetic patterns can be responsible for raising blood pressure [123]. Polymorphic genes implicated in blood pressure regulation such as the genes encoding endothelium-associated factors such as endothelial nitric oxide synthase (*eNOS*), endothelin-1 and prostacyclin synthase have also been associated with the pathogenesis of hypertension and CVD in a protective manner [116, 123].

Another important factor in vascular function and health is folate-dependent one-carbon metabolism involving regulation of homocysteine. Homocysteine is an important sulphur amino acid that can reach toxic levels in the blood when certain vitamins are deficient. High blood homocysteine concentration is a condition called hyperhomocysteinemia, and is now regarded as an independent risk factor in the development of CVD as well as other vascular problems such as venous thrombosis [124, 125]. Nutritional disorders that potentially lead to hyperhomocysteinemia particularly in persons with underlying genetic predispositions, are deficiencies of folate, vitamin B_{12} and vitamin B_6 [124]. Selhub *et al.* [126] estimated that 67% of cases of hyperhomocysteinemia are the result of inadequate B-vitamin status. It is for this reason that as part of this thesis, CVD (in terms of a hypertensive phenotype) is being investigated in relation to its connection with B-group vitamins, specifically folate. This

avenue of enquiry is a major theme within the present dissertation and includes not just nutritional, but key nutrigenetic correlates.

Alzheimer's disease: In 2011, it was estimated that approximately 300,000 Australians had dementia, of which AD is the most common form, occurring for up to 75% of all dementia cases [127]. The risk of developing it doubles every five years over age 65, such that by age 90 there is a 1 in 3 chance of developing the disease [128]. At the moment there is no cure and very little known about prevention [127]. AD is a progressive, physical disease which attacks the brain; it can result in impaired memory, thinking and behaviour [127]. AD is only one of the disorders that cause dementia; many other illnesses can also result in dementia. These include Parkinson's disease, and even stroke. Dementia simply means the symptom of a deterioration of mental abilities resulting from an unspecified disease or disorder of the brain [129].

The disease is named after the German physician, Alois Alzheimer, who in 1907 first described it. As brain cells die, the substance of the brain shrinks [129]. Abnormal material builds up as "tangles" in the centre of the brain cells and "plaques" outside the brain cells, disrupting messages within the brain, damaging connections between brain cells [129]. This leads to the eventual death of the brain cells and prevents the recall of information. Memory of recent events is the first to be affected, but as the disease progresses, long-term memory is also lost. The disease also affects many of the brain's other functions, and consequently, many other aspects of behaviour are disturbed [130].

It has been reported that individuals with AD often have low blood levels of vitamin B_{12}, and folate [131, 132] and high levels of homocysteine [128, 133]. Over the past 15 years multiple studies have investigate this link, but unfortunately results haven't always been consistent, Morris *et al.* [134] reported that out of ten studies specifically investigating folate status in relation to AD [130, 133, 135-142], six linked low folate status to a higher AD prevalence or incidence [130, 136, 138-140, 142]. Hyperhomocysteinemia, however, has been significantly associated with a subsequent AD diagnosis [133]. It is possible that the current rolling out of mandatory folic acid fortification programs is impeding our understanding of the relationship between folate status, homocysteine level, and occurrence of AD. (See section 1.3.4 below for further discussion on this point)

As displayed in table 1-2, AD is the third leading cause of death in women and the sixth leading cause in men. In Australia, an epidemic is already well underway, and will

increase rapidly as the first of the post-war baby boomers turn 65 in 2011. It is predicated that by the year 2020 there will be around 400,000 baby boomers suffering from Alzheimer's dementia, and by 2050 the figure is likely to be closer to a million [143]. The baby boomer bulge in Australia's demographic profile means that in the coming decades we will see an acceleration of the impacts of ageing on dementia prevalence greater than anything ever previously seen. In fact there are predictions that Alzheimer's dementia will be the fastest growing source of major disease burden, which will overtake CVD by the year 2023 [144]. Furthermore, AD has a significantly greater requirement for healthcare in terms of direct cost and in effective and appropriate dementia care when compared to CVD [144]. Therefore, prevention, research and early intervention could potentially save billions of dollars in Australian healthcare expenditure and help ensure individuals enjoy a better quality of late-stage life i.e. improve the compression of morbidity.

Colorectal Cancer: In industrialised countries, cancer is second only to CVD as a cause of death, but it is a condition that was virtually non-existent a few hundred years ago. Cancer is categorised by abnormal cells that are not destroyed by normal metabolic processes but instead proliferate and spread out of control [145]. Cancers are distinguished by the specific type of cell involved and the location in the body in which the disease begins. In the case of colorectal cancer, which is often referred to as bowel cancer, abnormal cells form in the large intestine, which can grow for many years without causing any symptoms. Colorectal cancer is the fourth most common cancer in men and the third most common cancer in women worldwide [146, 147], in Australia it is the third cancer-related cause of death, behind lung, and prostate cancer for men and breast cancer for women (table 1-2) [62]. In more than 80% of cases there is no known heritable association and the rapid increase in incidence is thought to reflect changing dietary and physical activity patterns [7]. This particularly explains the rapid rise of rates in economically transitioning countries throughout the world [148].

Colorectal cancer may be preceded by adenomatous polyp, which may become invasive cancer if undetected. These colonic adenomatous polyps are associated with genetic mutations identical to those found in colorectal cancer. Colorectal adenomatous polyps are benign, slow growing growths of colonic tissue; approximately 90% of these at detection are less than 1cm in diameter and confer a small chance of becoming malignant [149, 150]. However, if an adenomatous polyp is larger > 2cm in diameter, individuals have between an 11 and 50% risk of going on to develop cancer [151]. Prevention and early detection of colorectal adenomatous polyps is vital in order

to reduce mortality from colorectal cancer. Currently adenomatous polyps are detected by colonoscopies and treatment requires a polypectomy, or in the case of more extreme cases, colonic resection [150].

Currently, two major non modifiable risk factors are associated with the development of colorectal cancer; increasing age and male gender [152, 153]. Lifestyle risk factors also include increased body fatness with a higher waist circumference/waist to hip ratio [154, 155] and there is strong evidence for a dose-dependent relationship with alcohol consumption, which appears to be stronger in men than in women [156, 157]. But diet is also clearly an important factor in the development of this cancer, with reduced consumption of vegetables, fibre and increased consumption of red meat, processed meats, and animal fats all key dietary changes associated with colorectal cancer development [158-161]. As detailed earlier, modern day diets increasingly consist of energy-dense nutrient-poor foods and thus micronutrients are no longer being consumed at the appropriate levels. This is of concern because it is known that B vitamin deficiencies lead to an elevated rate of deoxyribonucleic acid (DNA) damage and an altered methylation of DNA (Cytosine-Guanine (CpG) methylation), both of which are important risk factors for cancer of any kind (see further information on these mechanisms in section 1.3 on page 73) [162]. A particularly relevant focus with this clinical phenotype is folate (status and genetics), which is required for synthesis of DNA. There is evidence that decreased availability of folate results in DNA that is 'fragile' and more susceptible to damage [163]. Also, deficiency of vitamin B_{12} traps folate in a form that is unusable by the body leading to a further reduction in cellular DNA synthesis, and therefore deficiencies in either vitamin can result in a diminished capacity for both synthesis and methylation of DNA [164].

Results from epidemiologic studies involving both colorectal adenomatous polyps and colorectal cancer indicate a significant inverse association between folate status and colorectal neoplasia, either high dietary folate intakes or high biomarkers of folate intake are consistently associated with decreased risk [165-168]. However, recently this association has been questioned with attenuated findings occurring when total folate consumption (folate from foods and supplements) is considered [169-171]. This topic which also encompasses issues related to fortification is of significant contemporary interest. This is evident by the number of articles on colorectal cancer, associated genetics, and the relationship this disease has with B-vitamins. This topic is elaborated on in detail below (section 1.3.4 on page 88).

Depression: Although in a different category to the previously discussed chronic disorders, mental health problems, particularly depression are not normally considered to be a major disease burden. However, the WHO does consider depression to be the leading cause of disability as measured by years lived with disability, and the fourth leading contributor to the overall global burden of disease [11, 172]. The WHO predicts that by the year 2020 depression will contribute the largest share to the burden of disease in the developing world and the second largest worldwide [11]. The economic costs of this increase are likely to be high [11]. In the 2012 Australia Health Report, anxiety and depression are listed as the second leading cause of disease burden behind coronary heart disease [62]. A separate 2007 government report into mental health found that an estimated 1 in 5 Australians aged between 16 and 85 years experienced one or more of the common mental disorders, these were mood disorders such as depression, anxiety disorders and substance abuse disorders [173].

Depression is a mood disorder characterised by feelings of sadness, loss of interest or pleasure in nearly all activities, feelings of hopelessness and suicidal thoughts or self-blame [174]. It is understood to be a condition that can come and go throughout certain stages of the life cycle, and is driven either by genetic and biological factors or is a response to major life events [174]. Furthermore, recent reviews have highlighted the considerable prevalence of depression in people diagnosed with chronic conditions such as CVD and cancers. Depression has been shown to predict future cardiac events in people with coronary artery disease, with suggestions that individuals being treated for depression are 2.3 times more likely to have a heart attack [174, 175]. Also, reports suggest that psychiatric disorders which usually involve depressive may inflict up to half of all people under medical treatment for cancer [176]. Cancer patients suffering from depression are frequently under-diagnosed and therefore under-treated [177]. The diagnosis of depression is clouded by common symptoms shared by both disorders such as loss of appetite, insomnia and loss of energy; these may be secondary to either cancer or depression [174].

Depression can affect people at all ages, although it affects a significant number of older people, but in either event, it is often not well recognised or detected. The prevalence of depressive disorders can decrease with age, particularly among those who live in the general community, among those in residential aged care however, depressive symptoms and disorders are common [178]. Depression in older people is often under diagnosed, as symptoms such as sadness, sleep and appetite problems or mood changes may all be dismissed as a ‘normal’ part of ageing [179, 180]. These

symptoms are also confused with other conditions such as dementia and it has been suggested that depression may be twice as common as dementia [181]. Social isolation and loneliness commonly accompany depression in adults over the age of 65, and older people generally find it difficult to talk about feeling sad or depressed. If depression is developed later in life, it is mostly likely associated with physical health problems, whereas the origins of depression earlier in life may be due to life experiences, or personality and genetic traits [174].

It is often assumed that poor memory and concentration in older people is due to age-related changes in the brain, rather than being connected to depression. Over the past 40 years, several lines of investigation have shown that the chemistry and function of both the developing and the mature brain are influenced by diet. The notion that constituents of the normal diet can influence brain function is not novel, since there is a strong association between the developing brain in utero and folate status [182, 183], and similarly a close relationship between B-vitamin status and dementia [131, 132, 184]. Other micronutrients also have a clear role in neurochemistry, and figure 1-1 below shows some of these correlates. Unfortunately, the highest incidence of folate deficiency as measured by serum and red cell folate concentrations occurs in elderly populations, this high incidence also corresponds with plasma homocysteine rises which may contribute to the overall ageing process [185, 186].

There are many studies that have formed the consensus view that lower folate levels are associated with depression and that this relationship holds after adjustment for possible confounders including socioeconomic status and lifestyle factors, including body mass index (BMI), smoking and alcohol consumption [187-191]. This association however, may operate in two ways, folate deficiency may lead to depression, but folate deficiency may result from poor appetite and reduced dietary intake in depressed people. There are many mechanisms that could link low folate status to depression, they may include folates role in neurotransmitter synthesis, or a reduction in methylation reactions leading to raised homocysteine levels, or possibly direct effects on the central nervous system [192]. There is some evidence that folic acid may be an effective treatment for depression [193, 194], one example is a double-blind, placebo-controlled trial of 15 mg methylfolate daily. Over 6 months folate-treated depressed patients significantly improved clinical and social recovery [195].

B-vitamins are clearly important, particularly for the synthesis of biogenic amine neurotransmitters via provision of *de novo* methyl groups. However, B-vitamin related genetics may also be important in the pathoaetiology of affective disorders with several

variant genes affecting risk. B-group vitamins, especially folate, are closely linked to an array of chronic health conditions. These are detailed in section 1.2 below, which gives a more complete story for folate and its metabolic and genetic associations in relation to disease development.

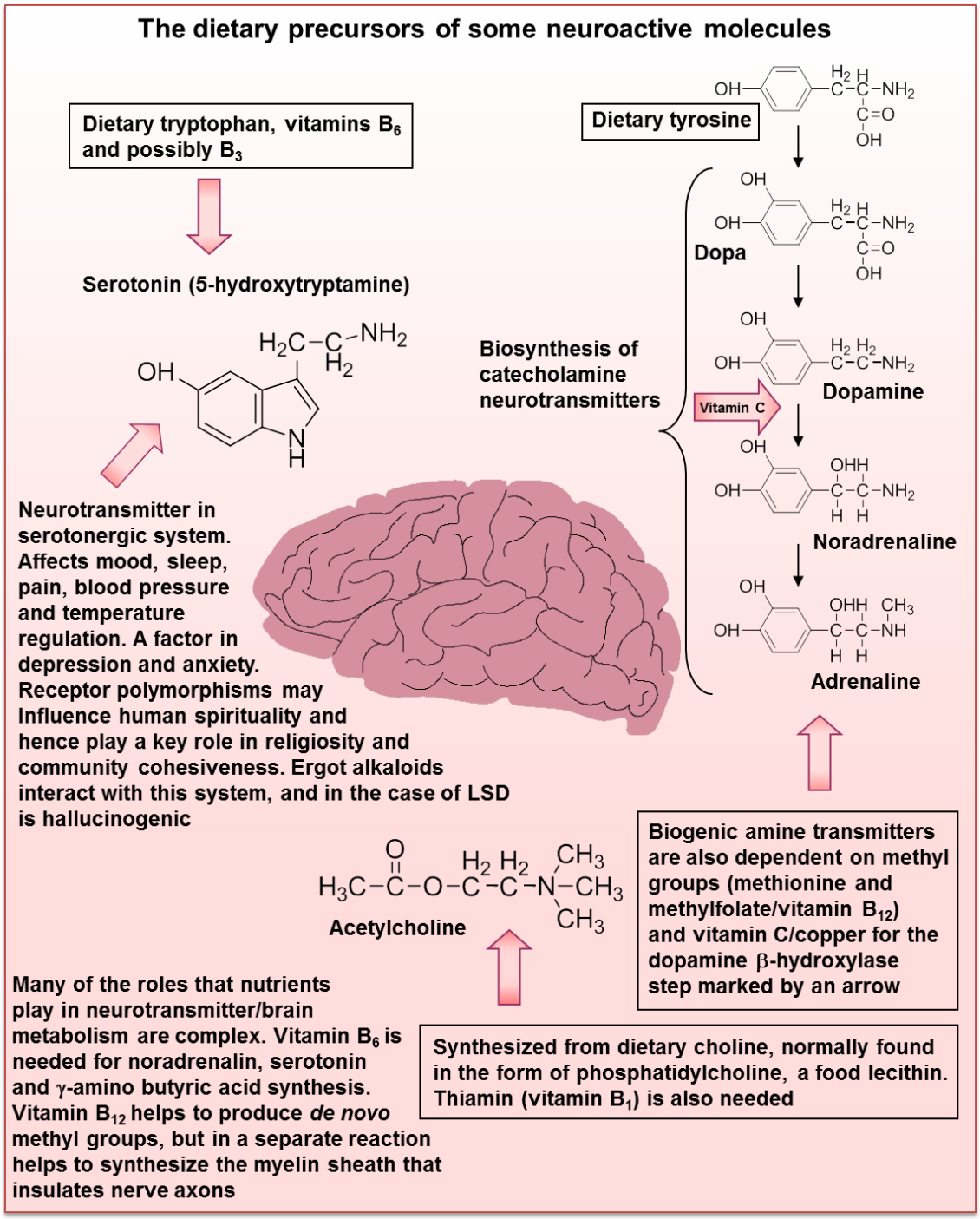

Figure 1-1: The corollary between certain key dietary nutrients and brain neurotransmitter metabolism (courtesy of A/Prof Mark Lucock published in Molecular Nutrition and Genomics – Nutrition and the Ascent of Humankind [25])

1.2. Folic Acid – A Key B-group Vitamin

The decline in micronutrient density, as previously discussed, is a key nutritional characteristic of the recent change that has occurred in Western diets, and which is linked to increased morbidity and mortality from a range of chronic diseases. A key group of micronutrients that are at the centre of this story are the B-group vitamins; they consist of several vitamins that are grouped together because of similarities in physiological functions, chemical properties, and distribution in foods. All B-group vitamins are water-soluble and are found in a variety of plant and animal food sources, they are recognised as vital coenzymes that are essential in facilitating metabolic processes in humans. B-group vitamins include thiamine (B_1), riboflavin (B_2), niacin/nicotinic acid (B_3), pantothenic acid (B_5), a group of pyridoxine related compounds (B_6), biotin (B_7), folic acid (B_9) and cobalamin (B_{12}). Table 1-3 summaries the main functions of each B-group vitamin.

Table 1-3: B-group vitamins – functions and deficiency symptoms

Vitamin	General Function & classical deficiency symptoms
Vitamin B_1 (thiamine)	Is a co-enzyme in energy metabolism and plays a role in nerve impulse conduction and muscle action. Deficiency affects the functioning of gastrointestinal, cardiovascular, and peripheral nervous systems. The two principal deficiency diseases are "beriberi" and "Wernicke Korsakoff syndrome"[196, 197].
Vitamin B_2 (riboflavin)	Is required to complete several reactions in the energy cycle, it functions in the conversion of pyridoxine and folic acid into their active coenzymes forms, and it has antioxidant functions. Deficiency alone is uncommon, and usually occurs in combination with deficiency in other B-group vitamins. Some symptoms may include; sore throat, seborrheic dermatitis, cheilosis, and angular stomatitis [197, 198].
Vitamin B_3 (niacin)	Has a role in energy production in cells, specifically coenzymes NAD & NADP in redox reactions. Prolonged deprivation leads to pellegra, a disease characterized by dermatitis, diarrhoea and dementia [197, 199].
Vitamin B_5 (pantothenic acid)	Is required for the metabolism of carbohydrates, proteins and fats, which supply energy from foods. It aids the synthesis of essential lipids, sterols, hormones and neurotransmitters. Pantothenic acid plays a role in detoxification of alcohol and drugs and hence alcoholics are at risk of deficiency, but generally deficiency is rare [197, 200].
Vitamin B_6 (pyridoxine)	Helps the nervous system through neurotransmitter synthesis and the immune system through antibody production. Crucially aids in the formation of red cell production and homocysteine trans-sulphuration. Complications in the peripheral nervous system, elevated homocysteine levels and hypochromic anaemia have been associated with a pyridoxine depleted diet [197, 201].
Vitamin B_7 (biotin)	Functions in the synthesis of fatty acids, amino acids and glucose. Biotin helps the body make hormones and plays a role in the maintenance of healthy skin and hair. Biotin deficiency results in anorexia, nausea, vomiting, depression, and dermatitis [197, 202].
Vitamin B_9 (folate/folic acid)	Primary function is as a carrier of single-carbon units, which are used in multiple important biochemical reactions, including the biosynthesis of methionine, thymidine, purines, and glycine, and in the metabolism of serine, formate and histidine. Folate helps the cells in the body make and maintain DNA, and is important in the production of red blood cells. Severe folate deficiency leads to megaloblastic anaemia; however it is also associated with many types of cancer, increased homocysteine levels which are implicated in CVD and many other diseases. Pregnancy complications such as NTD's are well recognised as being associated with folate deficiency [197, 203].
Vitamin B_{12} (cobalamin)	Plays an important role in growth and development. It also has a role in the formation of blood cells and the myelin sheath of nerve axons. Critically, cobalamin is involved in the regeneration of folic acid and specifically as a coenzyme in two metabolic reactions i) the methionine synthase reaction with methylcobalamin, ii) the methylmalonyl CoA mutase reaction with adenosylcobalamin. Deficiency most commonly results in pernicious anaemia [197, 204].

As highlighted in the table above, folic acid (or its anion, folate), is a key B-vitamin, which has garnered considerable interest over the past 20 years in relation to health benefits, and lately due to potential negative effects. Early interest was greatly accelerated in the 1990's, when it was shown beyond any doubt that this vitamin had a protective role against neural tube defects (NTD) [183]. It was this relationship with NTD that initiated a worldwide interest in folate related metabolic loci. However, it is the specific relationship between folate, homocysteine and a range of other age related chronic diseases such as CVD that has helped to maintain this interest to the present day. We now know that vitamins B_2, B_6 and B_{12} also play an important role in the equation, as indeed do the many genetic variants that encode critical folate dependent proteins.

1.2.1. Discovery

The story of folate is an interesting one. Folate is a generic term for a large group of similar water-soluble B-vitamins and is named after the Latin term *folium*, meaning leaf. Benefits of this vitamin were initially reported in 1931, when Lucy Wills used a yeast extract to effectively treat tropical macrocytic anaemia, a common problem during late pregnancy in India [205]. The active compound was later found to be a haemopoietic factor, with several researchers contributing to its isolation and identification. Its correct nomenclature is N-(4-(((2-amino-4-hydroxy-6-pteridinyl)methyl)amino)benzoyl) glutamic acid [206]. Fortunately, the name "folic acid" was proposed in 1941 by Herschel Mitchell [206]. Today we recognise that the term folate/folic acid refers to a wide range of natural, closely related compounds. Pteroylmonoglutamic acid is a synthetic form of the vitamin and is the form given as a supplement or taken as a food additive where mandatory fortification is in place. Confusion can arise because while folate/folic acid is a generic term for all folyl vitamers, it is also used specifically to describe the synthetic fully oxidised form pteroylmonoglutamic acid which is the form used as a supplement and/or fortificant. For these reasons, it is necessary to be explicit when using this term to avoid any ambiguity arising from its two meanings. Throughout this thesis, the term pteroylmonoglutamic acid is used when describing the synthetic form of the vitamin, and folate/folic acid is used when speaking generically.

Wills and Mitchell played a major role in the discovery of folate. Their work and other historical developments between the 1930's and 1980's are summarised in figure 1-2 which highlights many of the key scientists associated with the history of folate.

Date	Researcher [Ref]	A timeline for the discovery of folic acid
1931	Wills *et al.* [205]	In India, observed the effect of liver and yeast extracts on tropical macrocytic anaemia, particularly common in late pregnancy, and concludes that this disorder must be due to a dietary deficiency.
1932	Vaughan *et al.* [208]	Found that marmite corrects anaemia of coeliac disease.
1938	Day *et al.* [209]	Finds an anti-anaemia factor for monkeys in yeast and designates it "vitamin M."
1940	Hogan & Parrott [210]	Discover an anti-anaemia factor for chicks in liver extracts, which they name "Vitamin BC".
1940	Snell & Peterson [211]	Identifies growth factors for *Lactobacillus casei* and *Streptococcus lactis*, & coins the term "norite-eluate factor".
1941	Mitchell *et al.* [206]	Suggested the name "folic acid" (folium, Latin for leaf) for the factor responsible for growth stimulation of *Streptococcus lactis*, which was isolated from spinach and suspected of having vitamin-like properties for animals.
1943	Stokstad *et al.* [212]	Synthesized the "folic acid" compound in pure crystalline form.
1945	Angier *et al.* [213]	Working in Lederle Laboratories reported the synthesis of the compound identical to the L. casei factor isolated from liver. They describe folic acid's basic chemical structures (pteridine ring, *para*-aminobenzoic acid and glutamic acid) and name it "pteroylglutamic acid".
1945	Moore *et al.* [214] Vilter *et al.* [215]	Demonstrate that folic acid cures megaloblastic anaemia of all types and also coeliac disease, and pregnancy malnutrition.
1946	Amill & Wright [217]	Found that folic acid was only temporarily effective. It became clear that the anaemia relapsed and neurological damage was not improved and was even made worse.
1948	Farber *et al.* [218]	Demonstrates that antifolates (i.e. methotrexate) are effective in childhood acute lymphoblastic leukaemia which led to a wide variety of anti-cancer agents that inhibits the enzyme DHFR, which is required to return folate oxidized by the thymidylate synthase reaction.
1962	Noronha & Silverman [219]	Hypothesised that there was failure of conversion of 5-methyl-H_4PteGlu to H_4PteGlu in vitamin B_{12} deficiency with a consequent increase in 5-methyl-H_4PteGlu in plasma but a shortage of other folate forms in cells, the 'methyl folate trap' hypothesis.
1962	Herbert *et al.* [220]	Consumes a folate-deficient diet for several months and records his development of deficiency symptoms. His findings set the criteria for the diagnosis of folate deficiency and he estimates the folic acid requirements for adults, which still serve as a basis for many RDIs.
1962	Carson *et al.* [222]	Described the clinical syndrome associated with homocysteinuria, namely mental retardation, skeletal malformation and premature thromboembolic disease.
1964	Mudd *et al.* [223]	Demonstrated that the cause of the homocysteinuria was deficiency of cystathionine synthetase, which controls the trans-sulphuration pathway for homocysteine metabolism. Also showed homocysteine was being remethylated to methionine, which produced the high levels of methionine in humans.
1976	Smithells *et al.* [224]	First suggested that folate deficiency might lead to complications of pregnancy. These included abruption placentae, antenatal haemorrhage and prematurity, plus NTD (anencephaly, encephalocoele and spina bifida) in the foetus.
1979	Matthews & Haywood [226]	Describe the structure and function of 5-methyl-H_4PteGlu in the remethylation of homocysteine. It is synthesized by MTHFR which, under physiological conditions, is a one-way reaction.
1988	Kang *et al.* [227]	Initially defined folate protein binding and then went on to describe the thermolabile form of MTHFR which occurs in around 10% of the Caucasian population and which is now recognised as being caused by the 677C>T MTHFR variant.
1988	Brattstrom [228]	Showed that taking folic acid was associated with significant falls in serum total homocysteine


Dr Lucy Wills (1888-1964) [207]


E.L. Robert Stokstad (1913-1995) [212]


Esmond Emerson Snell (1914-2003) [216]


Victor Herbert (1927-2002) [221]


Richard W Smithells (1924-2002) [225]

Figure 1-2: The historical timeline showing how our understanding of folic acid developed

1.2.2. A paradigm shift in understanding the role of folate in health and disease (1990-2011)

During the past 20 years, the story of folate has exploded in terms of researchers examining its role and relationship to many chronic diseases. Although key events precede this, (see figure 1-2) in 1991 the British Medical Research Council Vitamin Study established that folic acid therapy at 4mg daily, given periconceptually, afforded around 75% protection against NTD affected pregnancy recurrence [60]. This was the ultimate proof that established that folate had a protective role against NTD affected pregnancy.

The following year, Czeizel *et al.* [229] demonstrated that first-time occurrence of NTD may be largely eliminated with a multivitamin containing folic acid taken during the periconceptional period. Later developments in 1998 by Daly *et al.* [230] showed that the addition of 400µg of folic acid in the diet raises red cell folate levels to those associated with a 75% reduction in NTD incidence in at-risk mothers. In addition to these events, Van der Put *et al.* [231] examined the genetics involved in folate metabolism and found that a common mutation of the enzyme MTHFR (677C>T) occurs more frequently in the affected NTD foetus as well as in their parents than it does in controls.

Global interest in the health benefits of folic acid were established through the role this vitamin has in preventing NTD. Despite this, concurrent work was also being conducted during the 1990's, particularly looking at folate and its relationship to vitamin B_{12}, methionine and homocysteine. A meta-analysis published in 1995 presented data from twenty seven studies, showing that homocysteine was an independent risk factor for atherosclerotic disease in the coronary, cerebral and peripheral vessels [232]. This was followed by a recognition that dietary folate lowers homocysteine through *de novo* biosynthesis of methionine, and that elevated homocysteine was observed in vitamin B_{12} depleted vegans [233, 234]. These findings opened a new avenue for intervention with B-vitamins to prevent a range of diseases [235].

Other key developments during the mid to late 1990's included findings by Butterworth *et al.* [236] that higher than normal serum levels of folate are associated with decreased risk of cervical cancer in women infected with the human papilloma virus. Also, Giovannucci *et al.* [237] showed convincingly that nurses who took multivitamin tablets containing folic acid had a progressive decline in the incidence of colon cancer over a period in excess of 15 years. In that same year (1998) Clarke *et al.* [130]

suggested that hyperhomocysteinemia may be associated with disease of the microvasculature supplying the hippocampal areas of the brain, resulting in vascular dementia, AD and Parkinson's disease. From this point on, 677C>T MTHFR and other folate associated single nucleotide polymorphisms (SNP) went on to be investigated in areas other than NTD, but particularly in the areas of cancer and CVD.

By the turn of the century, folic acid was associated with a vast array of health related diseases including developmental problems such as spina bifida [238], Down's syndrome [239], oral cleft [240, 241] and several complications of pregnancy (spontaneous abortion [242], preeclampsia [243, 244], foetal growth restriction [245]). It has also been implicated in many adult diseases such as, a wide gamut of cancers [246] (breast [247], colon [168, 248], lymphocytic leukaemia [249, 250]), infertility [251, 252], CVD [253, 254] (hypertension [255], occlusive vascular disease [256-258]), and with mental health issues such as depression [259], schizophrenia [195, 260] as well as with AD and dementia [138, 184].

After all these years of supportive studies and research work into the benefits of folate, governments around the world began to voluntarily, and later, mandatorily fortify food products with folic acid, specifically via flours and grains. Not long after fortification began in some countries, the tide began to change; one of the first papers discussing possible problems with fortification was by Choumenkovitch *et al.* [261] who reported that in the US, more people than expected were being subjected to the established tolerable upper limit of exposure to synthetic pteroylmonoglutamic acid following fortification. In addition to this, research conducted by Kadir *et al.* [262] found that despite an increase in folate consumption between 1991 and 1996, there was no fall in the incidence of NTD. However, at this point there still was a consensus that fortification was important to ensure an increase in daily consumption from 200µg to 400µg per day but, despite this, concerns were still raised, and continue to be raised [263, 264].

Over the following years, more information has come to light concerning the dangers of fortification. Of paramount importance is the association with cancer. Although in the past, folate status appeared to be beneficial towards a range of cancers and cancer risk, it has now become apparent that this association has a temporal dimension [265]. It was discovered that folate acts to prevent tumour initiation, but despite this, it seems to actually facilitate progression of precancerous lesions [266, 267]. This dichotomy was first shown in colorectal adenomas [170, 265], but is also associated with breast

and prostate cancers [268-270], which have both seen increased rates since the introduction of fortification.

Other areas of concern include potential risks to older people, particularly in the context of masking the diagnosis of vitamin B_{12} deficiency [271, 272]. There is also concerns over potential adverse effects of folate fortification on drug control of epilepsy [273-275]. Additional issues raised have included the suggestion that folate fortification increases the rate of multiple births and can play a role in embryo selection [276-280]. Fortification may also impact the effectiveness of antifolate chemotherapy drugs [281], and generalised concerns exist regarding the body's ability to deal with unmetabolised folic acid in the systemic circulation [282]. These concerns are discussed in greater detail in section 1.3.4 on page 88. In the 80 years since Lucy Wills' originally discovered yeast extract contained a factor which corrected macrocytic anaemia in pregnancy, many aspects of folate metabolism have been studied, although despite this, the exact mechanistic role of folate in NTD in particular remains unclear. It is therefore perhaps, not surprising that folate deficiency, abnormal metabolism, and folate excess in the pathogenesis of neurological and vascular diseases, and certain types of cancer remain important areas of endeavour for contemporary research scientists.

1.2.3. Folate biochemistry

Folic acid is made up of three distinct moieties, all of which must be present for vitamin activity; figure 1-3 below shows the structure of tetrahydrofolic acid (H_4PteGlu) which includes a pteridine ring, a *para*-aminobenzoic acid residue and a glutamic acid which may exist on its own, or conjugated to form a polyglutamic acid tail [283]. One-carbon units, such as methyl, methylene, methenyl, formyl and formimino groups, are added at either positions N5 and/or N10 of the pteridine ring, as shown in black. Synthetic folic acid/ pteroylmonoglutamic acid is considered to be the parent structure and consists of the same three moieties. There are a large number of different folate structures due to variation at three sites on the parent molecule which involve:

1. Oxidation status of the pteridine ring
2. Nature of the one-carbon substituent at the N5 and/or N10 positions
3. The number of γ-glutamyl linked glutamic acid residues conjugated to form an oligo-γ-glutamyl tail

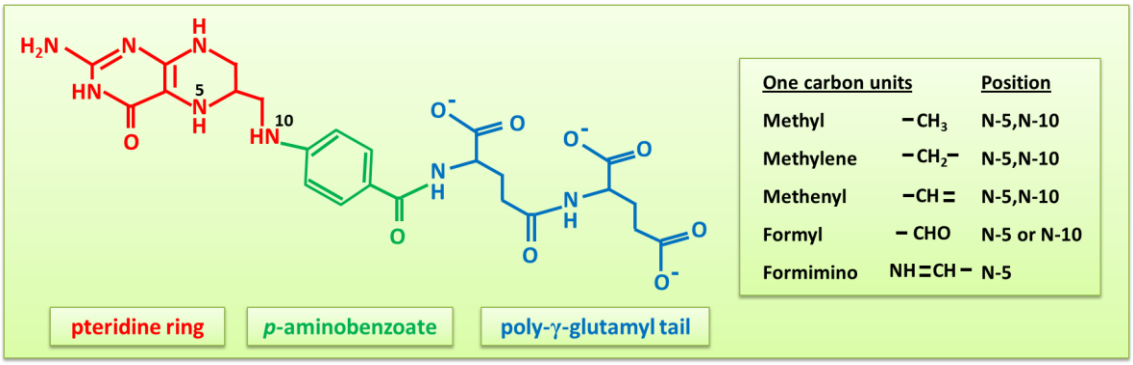

Figure 1-3: The structure of tetrahydrofolate and its role as a carrier of one-carbon units

In the body, metabolically active cellular folate has multiple glutamic acid residues attached, although humans can synthesise all the component parts of the vitamin, they do not have the enzyme necessary for the coupling of the pterin to *para*-aminobenzoic acid to form pteroic acid [284]. Given that there are multiple structural forms of folate, that it occurs in small amounts and it is extremely labile, there has been tremendous difficulty in characterising its biological occurrence, though modern analytical methods such as high performance liquid chromatography (HPLC) now offer a precise and specific way for analysis in foods and biological samples [283].

1.2.4. Dietary sources

Dietary sources of folate are of two distinct types; reduced mono and polyglutamyl forms (natural food folate), or the monoglutamyl oxidised form (synthetic pteroylmonoglutamic acid used in supplements and fortification). Natural polyglutamyl folate is an essential biochemical constituent of living cells, reduced folates generally exist in nature bound to proteins but are also bound to storage polysaccharides in foods [285]. Green leafy vegetables are rich sources of folate - hence the origin of the name (discussed earlier). Additionally, folate can be found widely distributed in many foods such as legumes, dairy products, poultry and meat, but particularly liver and kidney, as well as seafood, fruits, citrus juices, nuts and grains [283].

The highest concentrations of polyglutamyl folates exist in yeast, spinach, liver, and dried beans, but table 1-4 below gives the folate content of a broad range of foods. Raw foods typically are higher in folate than cooked foods because of folate losses incurred as a result of cooking [284]. The broad distribution of folate helps explain why deficiency usually follows from a poor diet rather than from poor intake of any single food group [203]. In supplements and fortified foods, pteroylmonoglutamic acid is used as it is fully oxidised at the pteridine ring level and hence is a very stable form of the

vitamin [284]. As a supplement, pteroylmonoglutamic acid is almost completely bioavailable (especially if consumed on an empty stomach) [284]. When fortified foods are consumed with natural sources of folate the vitamin is about 85% bioavailable [284].

Table 1-4: Folate content of various food products

Food	Content (µg per 100grams/100mls)	
	Natural (5-methyl-H_4folic acid)	Synthetic (pteroylmonoglutamic acid)
Chicken liver cooked	1530	Nil
Spread, yeast, Vegemite ™	793	2993
Red kidney beans dried	394	Nil
Spinach, raw	225	Nil
Beetroot, fresh, peeled, raw	120	Nil
Asparagus, raw	114	Nil
Breakfast cereal, whole wheat biscuit	90	170
Egg, whole hard-boiled	83	Nil
Cashew, roasted, salted	64	Nil
Lettuce, cos, raw	57	Nil
Juice, orange with added vitamins	53	31
Broccoli, raw	49	Nil
Bread, mixed grain (pre fortification)	49	Nil
Bread, white (post fortification)	49	123
Milk fluid, reduced fat 1%	27	Nil
Potato, new, peeled, baked	15	Nil
Centrum ® multivitamin *	0	300

Sourced from Food Standards Australian New Zealand NUTTAB 2010
* Pfizer consumer healthcare

In the Australian diet, cereal products, vegetables and milk products provide approximately 55% of folate intake for all ages [286]. In addition to this, tea makes a moderate contribution to women's folate intake as does beer to men's intake [286]. Across all ages, potatoes, dairy milk/vegetable juices and drinks and yeast extracts also moderately contributed to total folate intake [286]. Other contributions of folate in the diet include vitamin supplements, use of which is on the rise. In general, supplement use is greatest amongst older individuals and more common among women. A 2005 review of dietary supplement users found a prevalence of between 16-60% usage in individuals over 60 years of age [287]. Of the Australian studies reviewed, the range was between 20-43% [287].

Although it is no longer recent data, the National Nutritional Survey (Aust.) conducted in 1995 found that 27% of adult women and 15% of adult men reported supplement use [48]. Of the supplements used B-group vitamins were reported for 8% of women and 4% of men [48]. A smaller study conducted in 2001 in a New South Wales (NSW) rural community reported the use of multivitamins at 45.6%, and B-group vitamins at 30.1% of all people surveyed [288]. Both types of supplement contribute sources of folic acid to the diet. However, in the US, the National Health and Nutrition Examination

Survey reports that 63% of individuals over 60 years of age take a dietary supplement; 40% take folic acid containing multivitamins, 7% B-complex vitamins and 2% folic acid supplements [171, 289]. This survey also found that 55% of women were taking multiple supplements with 17% consuming four or more [171, 289, 290]. These US statistics may act as a surrogate for current supplement use in Australia.

1.2.5. Folate bioavailability

Folate bioavailability from foods varies from about 10% to 98% due to a variety of factors [284, 291]. Variations in intestinal conditions such as pH, genetic variations in enzymatic activity needed for folate digestion, dietary constituents such as inhibitors and the nature of the food matrix are all examples of factors that influence bioavailability [284]. The greatest effect on bioavailability seems to relate to the form in which the vitamin is present, natural polyglutamyl form, or the synthetic monoglutamyl folic acid used in supplements and fortification.

Folate in food is usually reduced, often methylated, and sometimes protein-bound, it can have a variable availability estimated to be no more than 50%.[203, 292] This is because natural polyglutamyl folates unlike synthetic folic acid must undergo enzymatic deconjugation in the small intestine before they can be absorbed [203]. Also, organic acids present in many foods can inhibit deconjugase activity and this may explain why the natural polyglutamyl folate present in food sources is less bioavailable than synthetic folic acid [203, 293]. Deconjugase or γ-glutamyl-hydrolase is an enzyme found in the intestinal brush border that converts natural polyglutamyl folates into monoglutamyl vitamers [284, 292]. The folate in milk for example may have altered absorption because it is bound to a high-affinity folate-binding-protein, which appears to enhance its bioavailability [284].

Synthetic folic acid taken as a supplement exists in oxidised form as a monoglutamate and has an availability approaching 100% of the content of a usual dose [203, 294]. Another factor that can affect folate bioavailability is the nutritional status of a person. Research has found that deficiencies in key micronutrients such as iron and vitamin C are associations with impaired absorption and metabolic function [285]. Generally, folate bioavailability from a mixed diet is thought to be about 50% efficient [284, 295].

1.2.6. Dietary requirements and assessment of nutriture

In Australia, the recommended dietary intake (RDI) of folate is 400μg for adults; this level is the same across many developed countries including the US, United Kingdom

(UK) and Canada [263]. This level was set in 2005 when the National Health and Medical Research Council (NHMRC) reviewed forty different nutrients, as a requirement for all age groups, as well as upper levels of intake and estimated average requirements [263]. The outcome of the 2005 revision resulted in most B-group vitamins increasing 50% and folate increasing 100%. Prior to the NHMRC review, the RDI for folate was 200µg, a level set in the 1981-1989 review, which was recommended jointly by the Food and Agriculture Organisation of the United Nations and the WHO [263]. At this time, the folate value along with all other B-vitamins was set in relation to energy or protein needs and it was lowered based on the amount of absorbed folate required to treat or fully prevent deficiency disease. Prior to this, (pre 1980's) it was 400µg [263].

In the 2005 revision, the NHMRC doubled the RDI for folate based on emerging evidence of the connection between diet and health, and recent recommendations from other countries. Specifically, they examined data looking at dietary intake in relation to maintenance of plasma and red cell folate, and homocysteine level. Whilst the recommendations were increased, the Australian government via Food Standards Australia New Zealand (FSANZ) was also considering mandatory fortification, Food Standard 2.1.1 was accepted by ministers in June 2007 which provided a two year window for implementation [52]. By September 2009, it was mandatory for all Australian millers to add synthetic folic acid to wheat flour which is used for bread making purposes. The level set by FSANZ was 120µg of folic acid per 100g of flour [52]. Although FSANZ recommended mandatory fortification, the New Zealand government decided to delay the decision until 2012 at which point they opted for a voluntary program [296]. This decision was due to a range of issues; from both industry costs and production, to a preference for applying the standard to bread instead of flour. However, it was also in relation to potential adverse health effects and the overall effectiveness of fortification in preventing NTD in pregnancies. Despite this, voluntarily fortification does continue to occur [52].

The move to fortify in Australia has been one repeated around the world, beginning with the US in 1998, which mandated supplementing cereal and grain products with folic acid at 140µg per 100g [297]. Fortification levels vary around the world, at the highest level Chile fortified at 220µg per 100g of flour [298]. Other South American nations such as Brazil, Argentina, Mexico to name a few all mandatorily fortify at 150µg per 100g of flour [299]. Fortification levels are set by individual nations, and in many countries have extended beyond wheat flour to included maize flour/meal, rice flour

and even milk [299]. The implications of folic acid fortification are not fully understood as new information continually emerges. However, the consensus view for mandating was an effort to prevent NTD. Further details regarding dietary folate requirements, specifically dietary insufficiency, excess, and adverse effects of fortification are discussed later (section 1.3 beginning on page 73).

Folate status is generally assessed by measuring folate concentrations in plasma, serum, or red blood cells; most often both measurements are assessed. Serum or plasma folate levels reflect recent dietary intake, thus true deficiency must be interpreted through repeated measures, whereas red cell folate concentrations are more reflective of folate tissue status and represent vitamin status at the time of red blood cell synthesis [284]. Red cell folate concentration is known as the most robust parameter in defining folate status, with concentrations below 370nmo/L being considered deficient in a normal population [300]. Analytical methods used to assess folate status are usually based on microbiological (bioassay) or ligand binding assay (radio immunoassay or chemiluminescence immunoassay) [301]. Other methods include chromatography separation followed by ultraviolet (UV), electrochemical or fluorescence detection or hyphenation with ion capture, tandem mass spectrometry [301].

1.2.7. Absorption and transport of dietary folates

As described earlier, dietary folate exists predominantly in the polyglutamyl form containing up to seven or more glutamate residues. Most of the total folate intake from a mixed unfortified diet is estimated to be in the polyglutamyl form, derived mainly from vegetables (see table 1-4 above for folate content of foods). The intestinal absorption of the polyglutamyl form is a two-step process that involves the hydrolysis of folate polyglutamates to the corresponding monoglutamyl derivatives followed by their transport through the intestinal membranes into the enterocyte [302]. Hydrolysis of the polyglutamyl chain is an essential step in folate absorption because only monoglutamyl forms are able to cross cell membranes. The hydrolysis of polyglutamyl forms requires an important brush-border enzyme located in the proximal part of the jejunum called glutamate carboxypeptidase II (GCPII) (see Figure 1-4 below) [302]. This pH sensitive enzyme acts as an exopeptidase, cleaving terminal γ–linked glutamate residues from the polyglutamyl folates in a stepwise manner to release folates with different glutamate chain lengths, but ultimately rendering a monoglutamyl vitamer [302-304].

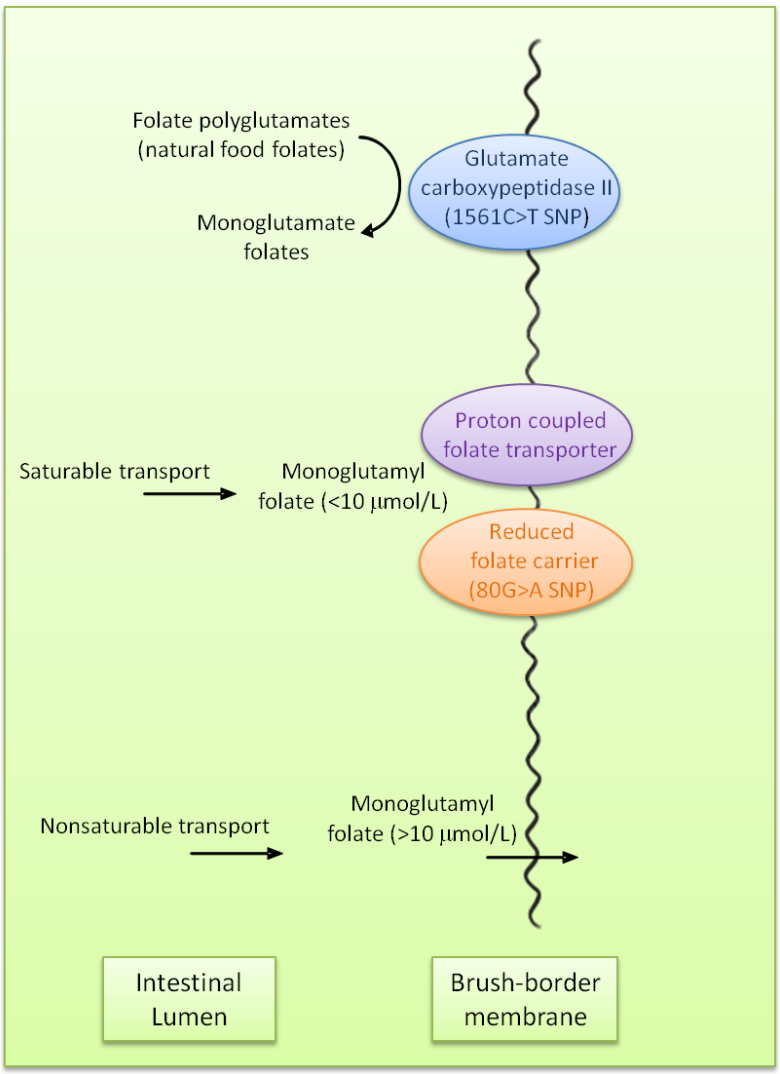

Figure 1-4: Simple schematic representation of intestinal absorption of folate (adapted from McNulty, H. and K. Pentieva, Folate Bioavailability, Folate in health and disease, L.B. Bailey, p. 28 [302])

The cellular uptake of folate may involve two specific processes, transmembrane carriers and folate-binding protein mediated systems, which are expressed in some epithelial cells. Folate-binding proteins are also known as folate receptors. Three high-affinity, closely related α, β and γ, folate receptors are known to exist [305, 306]. Transport via folate receptors is a relatively slow process compared with transport via the transmembrane transporters. Tissues rich in this receptor include; choroid plexus, vas deferens, ovary and testes [307].

The best examined process involves the transport of folate into the enterocyte which requires transmembrane proteins, both reduced folate carrier (RFC) and proton coupled folate transporter (PCFT) [308]. However, the transport of folate by this absorbing cell is not fully understood [302]. It has been suggested that PCFT is principally responsible because it is expressed in the brush-board of the jejunum, it has

an optimum acidic pH and affinity for folic acid and reduced natural folates; these characteristics are consistent with the requirements for folate transport [309]. Additionally, a rare autosomal recessive disorder which has been identified through severe folate deficiency is linked to mutations in the gene that encodes the PCFT [310]. Although it may not be responsible for the bulk of folate transport in all tissues, RFC is thought to be a facilitative carrier; it has been more extensively studied than the PCFT. RFC is expressed in tissue culture cells, tumour cells, foetal tissues and is found universally in normal adult tissues; it preferentially transports reduced folate such as 5-methyl-H_4PteGlu and 10-formyl-H_4PteGlu across cell membranes [306]. RFC has a relatively low affinity for oxidised folates such as pteroylmonoglutamic acid and decreased activity may reduce the uptake of circulating 5-methyl-H_4PteGlu into cells, and disturb folate metabolism by reducing the cellular pool of one-carbon donors [306]. Thus its role is very significant and hence it's a protein that has been extensively researched.

After folate absorption into the portal circulation occurs, much of the folate can be taken up by the liver where it is either; metabolised to polyglutamate derivatives and retained or it is released into blood or bile [306]. During metabolism, the monoglutamyl forms are converted in the intestinal mucosal cells (and liver) to 5-methyl-H_4PteGlu the major circulating vitamer. This form is taken up by cells but cannot be retained intracellularly unless it is first metabolised to H_4PteGlu [264]. Once H_4PteGlu is formed it can be conjugated into polyglutamyl forms, which prevents it leaving the cell (metabolically traps it) [264]. Polyglutamyl H_4PteGlu acts as an acceptor of one-carbon units, producing a range of other folates, which in turn, are specific coenzymes in several intracellular reactions [311]. In the case of synthetic folic acid, because it is not conjugated, it is more bioavailable than natural vitamers. However, before it can become 'metabolically active', it has to first be reduced to H_4PteGlu by dihydrofolate reductase (DHFR), then it is methylated during transit through the intestinal enterocyte and also in the liver [233, 312]. Importantly, the capacity for this conversion by the intestinal enterocyte and liver is limited, and both Lucock *et al*. [313] and Kelly *et al.* [314] found that single doses of oral synthetic folic acid in excess of about 260-400µg are not completely methylated, with unmetabolised folic acid appearing in the systemic circulation. The long-term effects of exposure to unmetabolised folic acid in humans are unknown (considered further below in section 1.3.4).

1.2.8. Folate-mediated one-carbon metabolism

As described, in the cell, 5-methyl-H_4PteGlu serves as a methyl donor and as a source of H_4PteGlu. H_4PteGlu accepts and transfers one-carbons in a network of biosynthetic and catabolic reactions known as folate-mediated one-carbon transfer metabolism (see figure 1-5 below). One-carbon metabolism and the supportive folate coenzymes function in three compartments of the cell; the cytoplasm, mitochondria, and nucleus, each compartment carries out specialised metabolic functions and has specific folate coenzymes that support its function [315]. These coenzymes rarely exchange between cellular compartments. One-carbon metabolism in mitochondria generates one-carbon units in the form of formate through the catabolism of serine, glycine and choline [315]. One-carbon metabolism in the cytoplasm uses the derived formate for nucleotide biosynthesis and for the remethylation of homocysteine to methionine [315]. Folate dependent one-carbon metabolism in the nucleus generates thymidylate during DNA replication and repair [315].

Even though each cellular compartment appears to have a specific function, all folate pathways are interdependent; each compartment is competing for a limiting pool of folate coenzymes, the interdependence results from the shuttling of intermediates such as serine, glycine and formate [315]. It has been shown that in the presence of excess folate, the concentration of folate-binding proteins exceeds that of folate coenzymes, thus the volume of free folate is small [315-317]. Folate-dependent one-carbon metabolism relies on intermediaries that bind H_4PteGlu cofactors; functionally these intermediaries must generate H_4PteGlu activated one-carbons, interconvert one-carbon-activated H_4PteGlu cofactors, catalyse one-carbon transfer reactions, and bind or seize H_4PteGlu cofactors without metabolising them [315].

Folate-dependent one-carbon metabolism is central to this thesis because disruptions in this complex pathway are associated with development anomalies and many pathologies. The biochemical mechanisms involved may be responsible for the initiation and/or progression of many common chronic diseases such as cancer and CVD. The central focus for associating folate-mediated one-carbon metabolism and disease is the nexus between two key pathways; one primarily concerned with nucleotide synthesis and the other with the methylation cycle (see figure 1-5), these two segments intersect at MTHFR, which catalyses the reduction of 5,10-methylene-H_4PteGlu to 5-methyl-H_4PteGlu [318]. The 5-methyl-H_4PteGlu generated by MTHFR is the primary methyl donor for the remethylation of homocysteine to methionine by methionine synthase (MTR), the proper function of MTR is maintained by a redox

partner; methionine synthase reductase (MTRR) [318]. Both MTR and MTRR enzymes rely on a cobalamin (vitamin B_{12}) cofactor, and the reaction proceeds via a methylcobalamin intermediate [319, 320].

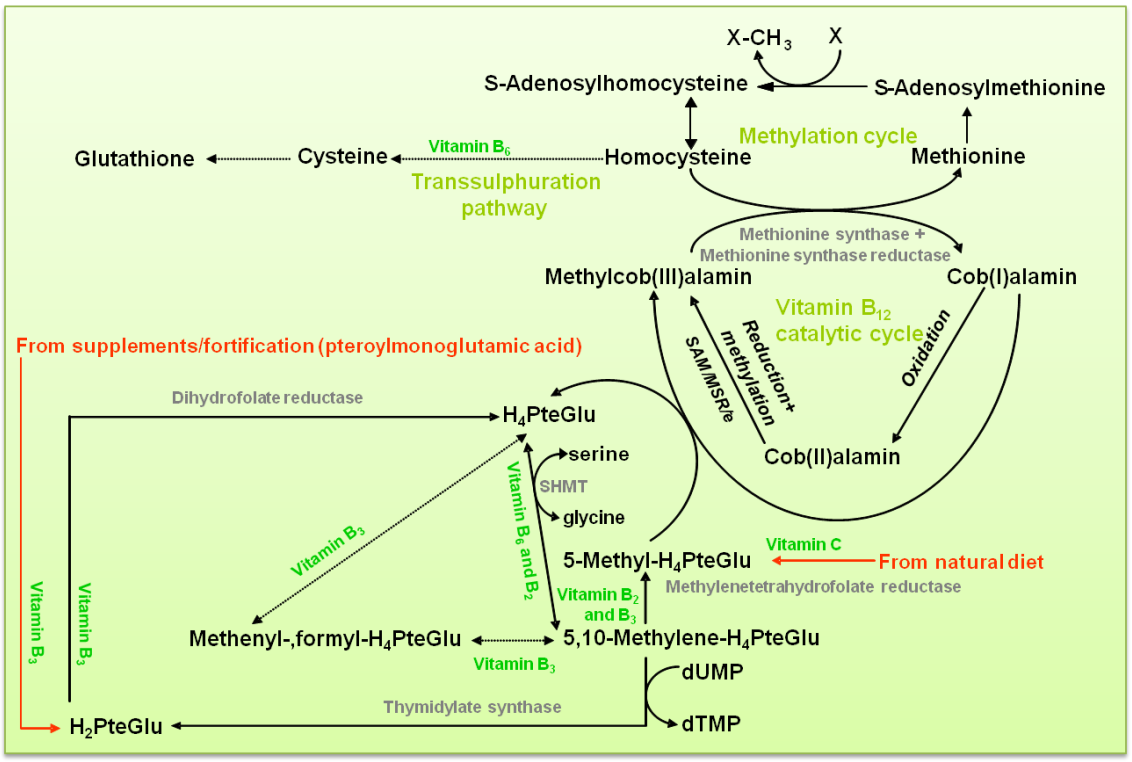

Figure 1-5: Folate-mediated one-carbon metabolism (courtesy of A/Prof Mark Lucock article Folic acid: an essential nutrient with added health benefits [321])

This reaction is key in methyl group metabolism as it allows for the re-utilisation of homocysteine which is used as a carrier of methyl groups that are derived primarily from serine (transmethylation pathway). During this reaction methionine is regenerated from homocysteine, and then converted to *S*-adenosylmethionine (SAM). SAM can then donate labile methyl groups it obtained from 5-methyl-H_4PteGlu to biological methylation reactions [319]. These reactions may include the methylation of DNA, ribonucleic acid (RNA), protein, lipids and lipoproteins. In addition, homocysteine can also condense with serine via an irreversible reaction which forms cystathionine. The reaction is dependent on the vitamin-B_6-containing enzyme, cystathionine-β-synthase (CβS), which also makes up part of the transsulphuration pathway [319]. Most tissues depend on folate-dependent enzymes for homocysteine remethylation, however in some areas betaine can be used as a carbon donor for homocysteine remethylation via betaine-homocysteine methyltransferase [318, 322].

Further to the methylation segment of the one-carbon transfer pathway, folate-mediated one-carbon metabolism is also essential for the synthesis of

deoxynucleotides. The MTHFR substrate 5,10-methylene-H_4PteGlu is generated in the cytoplasm by either 5,10-methylene-H_4PteGlu dehydrogenase or by serine hydroxymethyltransferase (SHMT) [318]. SHMT is a pyridoxal-5'-phosphate containing enzyme - (the active form of vitamin B_6) which catalyses the reversible transfer of formaldehyde from serine to H_4PteGlu to generate glycine and 5,10-methylene-H_4PteGlu [319, 323]. The generation of 5,10-methylene-H_4PteGlu can then be used as a one-carbon donor by thymidylate synthase (TS). This reaction catalyses the transfer of formaldehyde from 5,10-methylene-H_4PteGlu to the 5' position of deoxyurdine monophosphate (dUMP), producing deoxythymidine monophosphate (dTMP) [318] (see figure 1-5 above). During this TS reaction, H_4PteGlu is oxidised to dihydrofolate (H_2PteGlu), which requires reduction by a further enzyme called DHFR [318]. In addition, the one-carbon moiety 5,10-methylene-H_4PteGlu can also be oxidised to 10-formyl-H_4PteGlu by 5,10-methylene-H_4PteGlu dehydrogenase for *de novo* purine synthesis [319].

Given the importance of deoxynucleotides as the immediate substrates for the polymerases involved in replication and repair of DNA, the fidelity of DNA synthesis is crucially reliant on the availability and precise balance of deoxynucleotides [246]. Hence, folate-mediated one-carbon metabolism is essential in maintaining the integrity of DNA. In cells, the *de novo* synthesis of deoxythymidylate from deoxyuridylate is a rate-limiting step required for DNA synthesis, inhibition of healthy folate metabolism can result in uracil misincorporation; this is supported by findings that in human's folate deficiency results in excess uracil incorporation into DNA [319, 324]. Repair of uracil residues that are narrowly spaced on the opposite strands of DNA can result in double-strand breaks and deletions [325], chromosomal breaks [326], micronuclei formation [327] and loss of heterozygosity [328], all of which are linked to many cancers and other developmental abnormalities [319].

Clearly, folate-mediated one-carbon metabolism is a highly complex area, with many of the intermediaries and enzymes playing key roles in normal homeostasis. Following on from this, is a focused look at the transsulphuration pathway with specific consideration of homocysteine and its relevant thiol metabolites.

1.2.9. The homocysteine transsulphuration pathway and the relevance of its metabolites

In folate-mediated one-carbon metabolism, homocysteine, a natural sulphur containing amino acid, is formed as an intermediate during the conversion of methionine to

cysteine. It is regulated by two metabolic pathways; transmethylation and transsulphuration, and its metabolism depends primarily on several B vitamin cofactors and several key enzymes (see figure 1-5 above for diagram). A deficiency in these vitamins and genetic mutations in the enzymes can lead to homocysteine accumulating, resulting in the condition known as hyperhomocysteinemia (see section 1.3.1 the impact of folate deprivation, page 74 for more detail). Abnormal plasma levels of this toxic amino acid have been implicated in many pathological conditions, including CVD, AD and several pregnancy complications such as NTDs [24, 133, 138, 329-333].

As indicated on page 42, the transmethylation and transsulphuration pathways are the two major metabolic mechanisms that regulate and expedite the removal of homocysteine in cells. During transsulphuration, homocysteine is degraded into cystathionine by the action of the regulatory enzyme CβS. Cystathionine is then converted to cysteine by the action of cystathionine-γ-lyase (CγL). These reactions require the presence of a pyridoxine (vitamin B_6) cofactor. Cysteine subsequently forms the cellular antioxidant glutathione [334]. Alternatively, the remethylation of homocysteine into methionine occurs via the aid of the catalysing enzyme MTR, which requires a cobalamin (vitamin B_{12}) cofactor. In order for remethylation to occur, the presence of methyl groups donated entirely from the H_4PteGlu dependent methionine cycle is essential, and is a process that also requires the action of the MTHFR enzyme. Alternatively, a second reaction which catabolises betaine can also be utilised in the remethylation of homocysteine [319]. These two pathways synergistically work to ensure that homocysteine is metabolised and kept at a moderate level in the cell. Obviously, a micronutrient deficiency of folate, vitamin B_6 and/or B_{12} could adversely affect homocysteine concentrations, as could genetic mutations and disruptions in the enzymatic actions of MTR, CβS, CγL, and MTHFR (regarding genetic mutations see section 1.2.11 below on page 52). Combined, these insufficiencies can lead to homocysteine toxicity and ultimately to increased disease risk.

Whilst considerable research into the role of folate-mediated one-carbon metabolism and the role of homocysteine in chronic disease has been conducted, there is very little attention paid to other key amino-thiols that are formed during the transsulphuration pathway. Within this metabolic pathway, homocysteine is irreversibly condensed with serine to form cystathionine, this occurs via the action of CβS [335]. Cystathionine is then converted to cysteine which is a limiting amino acid used for the synthesis of the antioxidative tripeptide, glutathione (γ-glutamyl-cysteinyl-glycine) [336, 337]. This reaction involves γ-glutamylcysteine synthetase and glutathione synthetase [336]. The

catabolism of glutathione leads to formation of cysteinyl-glycine through the action of γ-glutamyl transpeptidase [338]. Cysteinyl-glycine can be catabolized through the action of a dipeptidase to form cysteine and glycine, which can then be reused to form glutathione or other proteins and peptides [339].

A disruption to this transsulphuration pathway can lead to excessive homocysteine production, resulting in loss of cellular redox homeostasis, and reduced production of glutathione [340]. These changes can also impair DNA methylation and cause DNA damage due to loss of antioxidant protection [340]. Despite not having received as much attention as homocysteine in terms of any association to health and disease, each of these amino-thiols; cysteine, cysteinyl-glycine, and glutathione are all metabolically linked to homocysteine and hence have important roles in one-carbon metabolism.

Cysteine is one of the most abundant thiols in plasma and is structurally related to homocysteine, it is a key extracellular reducing agent, and also a critical substrate for protein synthesis [341]. Cysteine is also a rate-limiting precursor for taurine and glutathione synthesis [342]. Like homocysteine, there is a significant correlation between cysteine levels and a person's age, total cholesterol concentration, diastolic blood pressure [343, 344], and there is also evidence of high levels of cysteine in patients with peripheral and cerebral vascular disease [345-348]. In fact, Ozkan *et al.* suggested plasma cysteine concentrations may be as significant as high homocysteine levels after finding a negative association between cysteine and coronary heart disease [349]. In addition to this, cysteine levels were found to be higher in patients suffering from AD, Parkinson's disease and motor neuron disease [350].

Conversely, cysteine may have a protective effect in relation to cancer. One study found women in the highest quintile of total plasma cysteine had an approximately 50% lower risk of breast cancer [351], although other reports have not been consistent in this respect [352]. Another study on cervical dysplasia also associated increased plasma cysteine levels with a possible reduction in risk [353].

It has been estimated that around 70% of cysteine is used for the synthesis glutathione [354], a powerful intracellular antioxidant. Glutathione participates in many cellular reactions, and is important in the defence against reactive oxygen species and free radicals where it serves as a non-enzymatic electron donor [355, 356]. Glutathione also has a role in nitric oxide metabolism [357]. A deficiency of glutathione and therefore increased oxidative damage has been shown to lead to apoptotic cell death. Given

these roles, an imbalance of glutathione is observed in a wide range of pathologies, including, CVD, cancer, neurodegenerative disorders, HIV and even ageing [357-360]. The loss of glutathione resulting in an increased neuronal loss has been associated with the development of AD, dementia and Parkinson's disease [336, 361, 362]. Glutathione along with homocysteine are recognised ligands for the *N*-methyl-d-aspartate (NMDA) subclass of glutamate receptor [363]. Disturbance to these receptors and to glutathione metabolism itself has been associated with the pathogenesis of psychiatric disorders such as depression and severe suicidal tendencies [364].

During the catabolism of glutathione, cysteinyl-glycine is generated. It is a pro-oxidant thiol, which is inversely correlated with both cysteine and homocysteine [339, 365]. Increased levels of plasma cysteinyl-glycine are associated with increased oxidative stress [366], although it is possible that this may be due to an increase in the degradation of glutathione. Elevated γ-glutamyl-transferase which assists in the formation of cysteinyl-glycine is a recognised predictor of CVD, partly because it catalyses the cleavage of glutathione [367-369]. Cysteinyl-glycine has also been positively associated with ischemic heart disease [370], myocardial infarction [371], and it also has a role in the iron-dependent oxidation of LDL within atherosclerotic plaques [372]. A very small study has also found reduced cysteinyl-glycine and glutathione levels in AD patients [373]. In another recent study using an animal model, it was demonstrated that there was decreasing spontaneous activity and increasing sedation with the administration of cysteinyl-glycine [374]. It has also been suggested that it may be associated with depression [375].

In terms of cancer risk, higher cysteinyl-glycine levels were marginally associated with an increased risk of breast cancer in subjects who had high oxidative stress profiles such as alcohol consumption, obesity, and postmenopausal hormone use [365]. However, this study and the others reviewed contain small sample sizes. To date, there is inadequate research into the impact of cysteinyl-glycine and the other key amino-thiols in terms of investigating their role in contributing towards risk for certain chronic diseases. Whilst homocysteine dominates research due to the central role it plays in bridging the transmethylation and transsulphuration pathways, it is the availability of folate for donating one-carbon units and particularly methyl groups, which ultimately maintains the proper functioning of the entire pathway. The molecular mechanisms and the impact of folate deficiency is discussion in section 1.3.1 on page 74 below, this includes more specific detail about the role homocysteine has in health and disease.

Next is a brief description of some of the other key micronutrients that share a functional relationship with folate and which therefore have an impact on one-carbon metabolism.

1.2.10. Nutrient-nutrient interactions related to folate dependent one-carbon metabolism

While the highly complex nature of one-carbon metabolism is centred on folate, many other nutrients interact with folate at this metabolic nexus. Such interactions can affect the metabolism and bioavailability of folate. For example, vitamin C, like folate is an essential plant-based vitamin, and can affect folate absorption because as an antioxidant it is known to interact with dietary 5-methyl-H_4PteGlu in the stomach lumen helping to maintain the reduced status of 5-methyl-H_4PteGlu [376]. Vitamin C salvages labile 5-methyl-H_2PteGlu by reducing it back to acid-stable 5-methyl-H_4PteGlu [377]. This makes vitamin C critical for optimising the bioavailability of food folate since at the oxidation level of 5-methyl-H_2PteGlu it cannot enter the body's active folate pool [378]. The reducing properties of vitamin C are subject to specific intestinal pH conditions, and it is believed that a deficiency of this vitamin will influence natural folate stability and hence absorption. However, it is important to note that vitamin C does not affect synthetic folic acid absorption because this vitamer is stable across all likely gastric pH values [378].

Other important nutrients that impact folate homeostasis include other B-group vitamins; specifically B_2, B_6 and B_{12} which are all directly involved in one-carbon metabolism. To expound on the information in table 1-3 above, vitamin B_2 or riboflavin consists of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) two biologically active forms which are required by all flavoproteins [379]. These two coenzyme derivatives play an integral role in a range of redox reactions; in particular, folate dependent one-carbon metabolism requires FAD because it is a cofactor for MTHFR activity [315]. It has been reported that one-carbon metabolism is interrupted by a diminished riboflavin status which alters MTHFR activity [380]. Other findings have also established interactions, including between riboflavin status, folate status and homocysteine concentrations [381].

Given riboflavin's role in flavin coenzyme activity, deficiency closely resembles that of folate, exhibiting similar foetal development abnormalities [382]. Hepatic MTHFR concentration is lower in rats fed a riboflavin-deficient diet compared to controls [383] and in humans, both low dietary and plasma riboflavin concentrations are associated

with higher plasma total homocysteine concentrations [380, 381, 384]. In addition, riboflavin may also have an effect on blood pressure regulation similar to that of folate. A study by Wilson *et al.* found that in 677TT MTHFR individuals, riboflavin supplementation lowered blood pressure more effectively than antihypertensive drugs [385]. It was hypothesised that riboflavin may restore 5-methyl-H_4PteGlu concentrations in vascular cells, therefore improve nitric oxide bioavailability, and in turn lower blood pressure [385]. Given riboflavin's role in folate metabolism and regulation of homocysteine, it's not surprising it is gaining attention, particularly as a potential protective agent against both CVD and cancer.

Recent research has also found that riboflavin is a strong photosensitiser that can affect folate stability. Following UV radiation exposure, flavins produce reactive oxygen species; this has implications for folate because it is very light sensitive and it oxidises when exposed to reactive oxygen species [377, 386]. Evidence has demonstrated that 5-methyl-H_4folic acid and pteroylmonoglutamic acid are not sensitive to solar radiation *in vitro*, but do degrade in the presence of photosensitisers such as riboflavin [387]. Further research in this area is needed but this does further demonstrate the close relationship between these two water soluble B-vitamins.

Vitamin B_6 like B_2 is also a critical factor in one-carbon metabolism, and shares a nutrient interaction with folate. Vitamin B_6 is a generic descriptor for a group of similarly related compounds which have 5'-phosphate esters, the main three being pyridoxine which is an alcohol derivative; pyridoxal, an aldehyde compound and an amino containing vitamer, pyridoxamine [388]. The pyridoxal 5' phosphate (PLP) and pyridoxamine 5' phosphate are the active coenzyme forms of vitamin B_6, they are both involved in amino acid metabolism, PLP however, is the predominate coenzyme involved in metabolism of carbohydrates, lipids and one-carbon units [201].

The roles of PLP in one-carbon metabolism are critical, thus making vitamin B_6's relationship to folate an obligatory one. PLP is responsible for SHMT and glycine decarboxylase, both of which are involved in the transfer of one-carbon units to H_4PteGlu from serine and glycine [318]. This in turn is important for purine and thymidine synthesis which is critical in DNA formation (refer to previous section for more detailed information). PLP is also involved in the transsulphuration and methylation components of one-carbon metabolism; CβS and CγL are PLP-dependent enzymes that catabolise homocysteine a process that involves condensation with serine to produce cystathionine, and subsequent cleavage to create cysteine and α-ketobutyrate [389].

Folate-mediated one-carbon metabolism has been shown to be severely impaired in an animal model of B_6 deficient rats [390] and a recent meta-analysis has established an inverse association with the risk of colorectal cancer between blood PLP levels and vitamin B_6 intake [391]. It is likely that low vitamin B_6 levels may increase risk for all diseases associated with folate and its metabolism, which covers an array of cancers and vascular complications. Not only does vitamin B_6 share this relationship with folate, its metabolism and functions rely on riboflavin, niacin and zinc [201]. Deficiency in any of these essential micronutrients may affect the metabolic utilisation of vitamin B_6 and hence impact folate metabolism. Examples of this include; the enzyme kynureninase which is PLP-dependent and is required for the *de novo* biosynthesis of niacin from tryptophan [201]. Also, zinc is a cofactor for pyridoxal kinase which catalyses PLP and finally, riboflavin in the form of FMN and FAD is required to serve as a coenzyme for aldehyde dehydrogenase which is required by pyridoxine phosphate, a B_6 related compound [201].

Vitamin B_{12} also shares a synergistic relationship with folate and a deficiency or excess can drastically affect the utilisation and status of folate. Vitamin B_{12} or cobalamin as alluded to previously, plays an essential role in folate-mediated one-carbon metabolism. Key intermediaries MTR and MTRR require vitamin B_{12} as a cofactor, additionally; methylmalonyl CoA also requires vitamin B_{12} although this step does not directly impact the folate pathway [392]. Cobalamin-dependent MTR is central to the effective utilization of 5-methyl-H_4PteGlu, the major circulating form of the vitamin. Because, 5-methyl-H_4PteGlu is a poor substrate for polyglutamylation, the folate moiety must be converted to H_4PteGlu through the MTR reaction in order for it to be incorporated into the useable intracellular polyglutamate folate pool so it can be utilised for one-carbon metabolism [392].

The absence of vitamin B_{12} and thus MTR activity will result in normal circulating levels of 5-methyl-H_4PteGlu, although the vitamin will be unusable by the cell. This unusable form of the vitamin leaves folate ‘trapped’ and the problem is further compounded by the irreversible reaction involving MTHFR (5,10-methylene-H_4PteGlu is reduced to 5-methyl-H_4PteGlu and is unidirectional see figure 1-5 page 42) [392]. This phenomena is more commonly referred to as the ‘methyl trap hypothesis’ and is supported by clinical, animal and cell culture studies [393-395]. The trapped circulating 5-methyl-H_4PteGlu cannot partake in either of the two folate cycles - methionine or nucleotide synthesis, and as a consequence, this leads to folate deficiency [234]. Therefore, long term ‘trapped’ folate can result in symptoms of megaloblastic anaemia, and if untreated,

long-term individuals can go on to develop irreversible and potentially deadly pernicious anaemia [392]. This anaemia occurrence is of particular concern for elderly people as vitamin B_{12} deficiency is usually a result of either atrophic gastritis or due to a lack of intrinsic factor – which is associated with an impaired immune system [396]. Elderly people are more susceptible due to decreasing intrinsic factor with age [397]; this is expanded on in greater detail below in section 1.3.4 because folate fortification programs may result in the masking of symptoms due to vitamin B_{12} deficiency. In addition to elderly people who may lack intrinsic factor, individuals who practice strict vegetarianism are also likely to suffer from vitamin B_{12} deficiency, as dietary sources are predominately found in animal products such as liver, shellfish, and red meats.

Finally, vitamin B_{12} deficiency obviously causes disruptions to homocysteine remethylation which in turn leads to elevation of homocysteine in tissues [392]. It has been reported that in populations with optimal folate status such as post fortification populations', vitamin B_{12} status can be used as the major predictor of total homocysteine concentration [398, 399]. Vitamin B_{12} and folate are inextricably required for optimal health, and prevention of common chronic conditions associated with folate metabolism. An important cation also plays a role in this relationship; human, animal and cell culture models have all demonstrated that iron deficiency impairs folate metabolism, although the underlying mechanisms for this has received little attention.

Iron is an essential micronutrient responsible for carrying oxygen around the body; an intracellular protein that stores and releases iron as well as playing a key role in metabolism is called ferritin [400]. Ferritin is a heterogeneous three-dimensional structure composed of 24 heavy and light chain subunits. Recently heavy chain ferritin was identified and isolated from rat liver extracts, and was found to be capable of catalysing the formation of *para*-aminobenzoylglutamate from 5-formylH_4PteGlu [401]. This may have implications for folate catabolism and be an important component in the regulation of folate status. Other research has also demonstrated that increased heavy chain ferritin synthesis decreases intracellular folate concentrations in cell culture independent of exogenous folate levels [402]. Both iron and folate deficiency-states result in anaemia's which are shared by similar morphological alterations in granulocytes and neutrophil hyper-segmentation [400, 403]. Clearly, iron adversely affects folate by decreasing its utilisation as a cofactor, its bioavailability, and ultimately its status. However, the underlying biochemical mechanism shared between these two key micronutrient remains poorly defined.

The final micronutrient to be explored for its close relationship with folate is another cation and essential trace mineral, zinc. Zinc, like folate plays a role in a number of biochemical processes related to human growth and development. They are both required for nucleic acid synthesis, but yet there has been little research on the influences they may have on each other [404]. It was first established that the intestinal brush-border, pH sensitive enzyme, GCPII requires zinc for its activity; this was one of the first clues of a metabolic interrelationship [405]. Another observation in patients with megaloblastic anaemia with secondary folate deficiency was elevated red cell zinc levels [406]. Through supplemental folic acid treatment, these elevated levels decreased [406].

Many reports have further established an inverse relationship between folate and zinc nutriture; zinc deficient rats have shown reduced hepatic folate [407] and suppressed liver and intestinal folate conjugase activity [408]. A study by Tamura *et al*. [409] showed zinc-depleted men had reduced intestinal absorption of polyglutamyl folates, and another study showed when males supplemented with zinc were fed 400µg of folic acid every other day they had significantly greater faecal zinc excretion while urinary zinc excretion was reduced by 50% when compared to controls [404]. Several *in vivo* transport studies have suggested that the effect of folate on zinc was related to the presence of folate and zinc together in the intestinal lumen [410]. Some studies have suggested that in the stomach, zinc may form insoluble complexes at an acidic pH, however once the complexes reach the higher pH of the small intestine these complexes most likely dissolve [410-412]. So, under normal physiological conditions zinc and folate do not form complexes; but rather, the interaction between zinc and folate occurs at the intestinal membrane level [410]. In conditions such as pancreatic insufficiency where the pH of the intestine is below 6.0, these zinc-folate complexes may not dissolve and therefore result in less absorption of both key micronutrients [410].

These studies demonstrate that at the intestinal level there is reciprocal inhibitory effect for zinc and folate transport. This may explain the clinical observations that have been reported for dietary supplement forms of both nutrients. However, more recently, other studies into high dose folic acid supplementation found no effect on zinc status or absorption in humans, and the level of zinc intake did not impair folate utilization [413-415]. Despite this, there is clearly an interaction between zinc and folic acid at the intestinal level, but like iron, further research on the effect of each micronutrient is required.

Folate is clearly a key component in a broad range of nutrient-nutrient interactions that involve vitamin C, B_2, B_6, B_{12} and trace elements like iron and zinc. Many other intermediaries not described in detail in this thesis also play a role in maintaining folate-mediated one-carbon metabolism. To add another layer of complexity to the understanding of folate-related biological process, there has been considerable research in the past 15 years on understanding the role of cognate genes. It is now known that folate status is partially under genetic control. The recent cloning of genes encoding proteins required for intestinal absorption of folates, delivery of folates to the cell, as well as the folate cycle provides the basis to identify rare mutations associated with severe enzyme deficiencies as well as common genetic polymorphisms that affect folate status [416-425].

1.2.11. Genetic variation within folate metabolism

One of the biggest discoveries in the 20th century was DNA; a double helix structure consisting of two single strands containing millions of nucleotides. In the human genome, the primary nucleotide sequence is approximately 99.9% identical, the remaining 0.1% contains variation that is referred to as polymorphic [426]. Within this polymorphic classification there are variations that are called either micro/macro-satellite repeat sequences, viral insertions or the highly common SNP [427, 428]. SNPs are defined as nucleotide base pair (bp) differences which could be insertions, deletions or substitutions of one bp for another [429]. SNPs influence human phenotypic variation, which may include alterations in human behaviours [430], susceptibility to diseases [431], physiologic responses to environment such as diet [432], pharmaceuticals [433], pathogens and toxins [434]. Often a SNPs impact on health and disease is influenced by an individual's nutritional status, with the dynamic relationship between genes, diet and health being the basis of the concept known as "nutrigenomics" [435].

Over the past 20 years, many genetic polymorphisms in folate metabolism have been identified. In general, these mutations are subtle, especially when compared to rarer mutations that result in inborn errors of metabolism. Although subtle, these mutations have a much greater impact on populations because they have been linked to a wide variety of common diseases, such as CVD and cancers. Displayed below (figure 1-6) is a simplified figure which demonstrates how genetic variability and selected nutrient-nutrient interactions might influence cell biology and, hence phenotype.

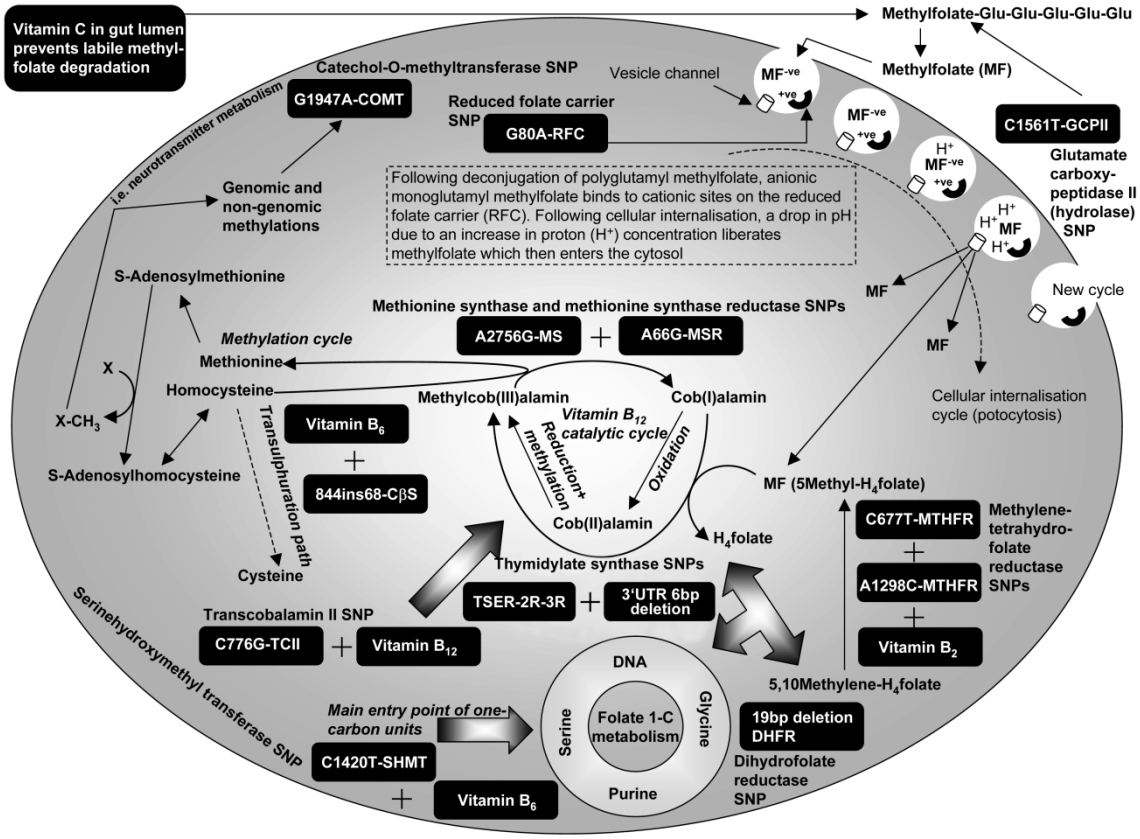

Figure 1-6: Schematic representation of genetic variation within folate metabolism (courtesy of A/Prof Mark Lucock published in Molecular Nutrition and Genomics – Nutrition and the Ascent of Humankind [25])

(Note: transcobalamin II and CβS SNPs were not examined in this thesis but are central to metabolic integrity).

Gene variants in folate metabolism have been the subject of intense investigation as risk factors for many disorders, the most extensively investigated polymorphism is the 677C>T MTHFR. This genetic variant was first identified in 1995 [416], and since then there has been a proliferation of research linked to this gene. It is estimated that between 250 and 300 articles are published per year about it [436]. More recently, interest in other genetic variants has increased but in some cases overall impact remains to be established. For some, they may result in no effect on human health and disease risk, but for others there is potential for phenotypic effects given their ability to alter enzyme activity and regulation, as well as influence gene expression. This ultimately impacts blood metabolite concentration, and given folate is crucial in one-carbon metabolism, it's not surprising that genetic variation is linked to a wide variety of health problems.

In order to better understand many of the common folate SNPs that either do, or might, influence clinical phenotype, eleven are described in detail with an examination of the

biochemical and metabolic impact of each mutation, as well as the prevalence within populations and the impact on human health and disease risk. All of these have been examined in blood samples, in the context of this thesis.

I. 677C>T MTHFR

As previously discussed, MTHFR catalyses the reduction of 5,10-methylene-H_4PteGlu into 5-methyl-H_4PteGlu, which is the major circulating form of folate. MTHFR is a crucial enzyme within the folate pathway, it acts as a methyl donor in the remethylation of homocysteine to methionine and it influences both bioavailability of folate for dTMP synthesis and maintains methylation patterns at CpG islands known to regulate gene expression [436-439]. As described above, this MTHFR gene polymorphism (*rs1801133*) was discovered in the mid 1990's. The gene is approximately 20.3 kb long and consists of 11 exons ranging in size from 102 to 432 bp [416, 440]. The major gene product has been mapped to the short arm of chromosome 1 at 1p36.3. It is a catalytically active 77 kDa protein and it consists of 656 amino acids [438]. The most extensively investigated polymorphism within MTHFR is the 677C>T transition; its role in folate metabolism has made it a popular locus for study and is widely referred to as a classic gene-nutrient interaction with ramifications for disease susceptibility. This polymorphic variant is a cytosine (C) to thymine (T) transition at position 677 within exon 4, that occurs at the folate-binding site and results in an alanine (A) to valine (V) substitution (A222V) [416].

The prevalence of the TT genotype varies widely based upon geographical locations and ethnic backgrounds, a large study of 7000 newborns from 16 locations around the world found the TT genotype was particularly common in Northern China (20%), Southern Italy (26%) and Mexico (32%), low frequencies were observed in African-American populations (2-3%) [441]. Wilcken *et al.* [441] further describes a geographic gradient in which the TT changes within a continent; In Europe for example, low frequency (4-7%) was observed in Finland, Russia and the Netherlands. An intermediate frequency (8-10%) was observed in France and Hungary and the frequencies continued to increase in Southern Europe (12-15%) in Spain and Northern Italy, peaking in very southern locations of Italy; Sicily and Campania (20-26%).

This geographical gradient was also observed on the American continent; the TT homozygote's increased from Western Canada, to Atlanta Georgia (South East, USA) and frequencies peaked in Mexico. The same study also reports newborns in metropolitan Atlanta had TT genotypes that corresponded to ethnic background; newborns of Hispanic origin was 15%, of European origin was 11% and of African-

American origin was 3% [441]. In addition to the global prevalence of the T allele, preliminary work has uncovered a related effect of dietary folate: It is understood that if high maternal folate exists, then TT homozygote's in utero may have increased survival potential [278, 442]. This phenomenon likely stems from preferential one-carbon flux into nucleotide biosynthesis and may also help explain the north-south gradients seen; because there is a higher folate content of the Mediterranean diet (Southern Europe) [443-445]. Also Lucock *et al.* [446] observed a high TT frequencies in people with a UK 'spring time' conception; a time when there is a higher abundance of folate rich foods.

The biochemical impact of this variant is significant given MTHFR is the sole producer of 5-methyl-H_4PteGlu. The C to T transition results in a thermolabile enzyme with reduced enzyme activity at 37^0C *in vivo*. Because of this, TT carriers have only about 30% of activity in lymphocyte extracts as compared to CC individuals [416]. One reason to explain the reduced activity is the mutant valine residue loses the ability to retain its essential flavin cofactor [447]. Primarily, reduced MTHFR activity has been shown to increase homocysteine concentrations by decreasing the 5-methyl-H_4PteGlu available for homocysteine methylation. Furthermore, multiple studies have reported elevations in homocysteine by as much as 70% in TT individuals when compared with CC individuals [416, 448-452]. Evidence suggests that the homocysteine raising effect of the gene variant is modified by folate status, with greater effects observed in individuals who have poor folate status [447, 453, 454], and diminished effects are seen after consuming a folic acid rich diet or taking supplements [455]. Low riboflavin and vitamin B_{12} have also been linked to elevated homocysteine, particularly when low folate also exists [381, 449, 456]. The TT genotype is also linked to reduced plasma betaine, which is also required for the remethylation of homocysteine [452].

Secondly, MTHFR activity impacts the distribution of folate derivatives; reduced MTHFR results in increased 5,10-methylene-H_4PteGlu, which can then be used for dTMP synthesis or it can be converted to formyl-H_4PteGlu; a folate derivate not normally found in red blood cells [457]. Observations of TT individuals have found increased dTMP synthesis and altered folate distribution in red blood cells [458-460]. Given this increase in 5,10-methylene-H_4PteGlu, the major transport form of folate, methyl-H_4PteGlu is decreased. The TT genotype is frequently associated with a 10% to 35% reduction in circulating folate [448, 449, 452, 461]. Finally, reduced MTHFR activity may decrease methylation activity because of an increased level of the methyltransferase inhibitor, *S*-adenosylhomocysteine (SAH), which results in decreased SAM levels. Changes in methylation are particularly critical to DNA

expression; reports have associated the TT genotype with decreased DNA methylation and increased genetic instability [457, 462]. Also, altered chromatin conformation and gene expression has been seen in cancer which is attributed to altered DNA methylation [463].

This gene variant was first studied in developmental abnormalities such as NTD and other birth defects, particularly because hyperhomocysteinemia and/or low folate were established as risk factors for such disorders. In 1995; van der Put *et al.* [231] demonstrated this 677C>T MTHFR variant was a genetic risk factor for spina bifida. Today, many meta-analyses have established that carriage of the T allele (either TT or CT genotypes) in either mother or offspring increases risk for NTD [464, 465]. One meta-analysis indicated that TT individuals had a doubling in risk of having an affected child [444]. Other work has found that low maternal blood folate combined with no periconceptional folate supplementation is associated with increased risk for the T allele [238, 466]. A second common mutation within the MTHFR has been uncovered and is discussed in detail below; individuals who have compound heterozygosity for both mutations may have an increased spina bifida risk compared to individuals who exhibit wild-type combinations [444, 467-469].

The 677C>T variant has also been investigated for its links with congenital heart defects; van Beynum *et al.* [470] established a weak association, other inconclusive research involving this variant and cleft lip, cleft palate and Down syndrome exist [333, 465]. Given the recent impact of mandatory folate intake on this variant, it may make it difficult to establish meaningful associations between homocysteine and specific clinical phenotypes. Despite this, the TT genotype alone or coupled with hyperhomocysteinemia has been associated with an increased risk of spontaneous abortion [471], recurrent pregnancy loss [472], placental abruption, severe hypertension and preeclampsia [473, 474]. Similarly, this variant has also been investigated extensively as a risk factor for CVD. Just as birth defects are associated with hyperhomocysteinemia, studies involving coronary artery disease, stroke, hypertension, and venous thrombosis have utilised the same link which involves this genetic mutation. Today we know hyperhomocysteinemia is a recognised independent risk factor for atherosclerosis.

Several meta-analyses have established a 20% increase in risk for coronary artery disease [24, 475, 476], and a 30% increase in risk for stroke [257, 477, 478] for individual's carrying the TT genotype. Also reported, is a 1.5 fold increase in risk for venous thrombosis with the TT genotype [24, 479-481]. This is likely a result of

reduced MTHFR activity resulting in increased homocysteine, but other risk factors and folate intake may play a role in risk. There are however, mixed results, principally among the meta-analyses. This is possibly due to limitations of the Mendelian randomization approach; the need for very large sample sizes to detect associations when the genotype influence on phenotype is small and when the association of the phenotype to outcome is modest [476, 482]. The Klerk *et al.* [475] meta-analysis involved over 11,162 cases and the more recent study by Lewis *et al.* [476] involved over 26,000 cases, but both found that after stratification by region, heterogeneity was reduced. Lewis *et al.* [476] then only found increased odds of coronary heart disease in the Middle East and Asia. Whereas in the Klerk *et al.* [475] study, the associated risk of the TT after stratification was confined to European studies and risk was absent in the North American populations. It was hypothesised from this study that continent-of-origin differences could be the result of effect modification by folate status, given at the time of assessment that vitamin use and breakfast cereal fortification were more common in North America than in Europe [475].

This variant has been investigated as a risk factor for most folate-related disorders; it is reported that the TT genotype may increase the risk for schizophrenia [483] and depression [484] by as much as 40%. The mechanisms may involve either hyperhomocysteinemia and/or reduced neurotransmitter metabolism. Elevated homocysteine levels have also been strongly linked with AD, with Alzheimer's patients also having been found to be deficient in certain key micronutrients such as folate and vitamin B_{12} [130, 132, 133, 331]. No association or increased susceptibility has been established between this variant and AD [485-487]. However, one study found that female TT homozygotes have significant cognitive decline compared to wild-type and heterozygotes [488], and another study in a Japanese population found that the combined MTHFR haplotype (677CC, 1298CC, 1793GG MTHFR) was protective against late onset AD [489].

Finally, low folate intake has been shown to be associated with increased cancer risk, and thus there is a potential role for the 677C>T in carcinogenesis and/or cancer progression [165, 490]. Overall, results for examination of this SNP and cancer risk appear to be somewhat inconsistent, as the influence of this variant may depend on many site and cancer specific molecular mechanism which mediate pathogenesis. Quite often individual studies report significant findings however, pooled and meta-analysis for a range of site-specific cancer often reveal no association [491]. Examples of negative associations include; a reported 2.8-fold increased risk for endometrial

cancer in individuals who are homozygous recessive [492]. Also a 50% increased risk of gastric cancer has been associated with the TT genotype [493, 494]. These findings are in marked contrast to the relationship between colorectal cancer and the 677C>T MTHFR variant; initial reports suggested a possible protective effect of the TT genotype. There now has been confirmation that indeed the TT genotype correlates to a 1.2 to 3.0-fold reduced risk for colorectal cancer [495-497].

There is, however, a large 'but'. The reduction in cancer risk attributed to the T allele exists only in the presence of a high folate concentration; protection is lost or the TT genotype may become a risk factor if folate concentration is low [495, 498, 499]. The protective effect of this variant may be due to the diversion of folate to purine and thymidylate synthesis. This leads to an increase in 5,10-methylene-H_4PteGlu for conversion of dUMP to dTMP, reducing DNA damage resulting from uracil misincorporation into DNA [163, 498, 500]. Alternatively, increased homocysteine or its metabolites could increase apoptosis in transformed cells in the intestine [501]. So far, the variant does not appear to affect overall risk for colorectal adenoma [498, 502-505], which adds to the suggestion that it may help prevent adenomatous polyps from developing into carcinomas. Ulvik and colleagues investigated 677C>T MTHFR and adenoma risk, they divided their data into above and below mean red cell folate value (263 nmol/L), and found that in the low folate group, the CT and TT genotypes had a 3.53 and 6.06 fold elevated risk of adenomas respectively compared to CCs, while in the high folate group the risk for all three genotypes was similar [506]. In addition to the role of folate concentration, several other factors such as tumour location [507], alcohol intake [496, 508, 509], smoking [506] and ethnicity [510, 511] impact this variant's relationship to cancer risk.

In addition to these findings, the TT genotype has been associated with a 4.3-fold reduced risk for acute lymphocytic leukaemia [249], and conflicting results for breast cancer exist; a 40% decreased breast cancer risk in postmenopausal women who used hormone replacement therapies has been reported in TT individual's [512]. However, other studies have indicated the TT genotype confers an increased risk of breast cancer [513-515], while others have found no association [516, 517]. Also, the TT genotype has been associated with the increased expression of the tumour suppressor gene p16 in lung cancer patients [518]. Thus, reduced MTHFR activity may prove to be protective by inhibiting hyper-methylation that could lead to CpG Island silencing of certain tumour suppressor genes, but further work needs to be undertaken to support this. Finally, this genetic variant has also had pharamacogenetic effects reported; the

mutation may influence the response to treatment with antifolate chemotherapeutics (such as methotrexate and 5-fluorouracil), and anticonvulsants drugs [519]. This area of research is relatively new, but given this mutation's role in MTHFR regulation, promising results may help determine the effectiveness of such drug treatments on individuals with varying genetic profiles (pharmacogenomics).

The total biochemical impact of the 677C>T MTHFR variant is extensive. Reduced enzyme activity results in increased homocysteine and a decreased folate, also a decreased capacity for methylation reactions, altered folate distribution and abnormal nucleotide synthesis. All of these mechanisms may contribute to clinical outcomes and thus influence disease risk.

II. 1298A>C MTHFR

This is the second common variant occurring in the MTHFR gene-coding sequence; it involves an adenine (A) to cytosine (C) transition at position 1298 within exon 7 that results in a change from a glutamate (E) to an alanine (A) residue (E429A) (*rs1801131*) [417, 469]. The frequency of the CC genotype varies among ethnic groups. In white Caucasian populations such as in North America and Europe, the frequency ranges from approximately 6-11% [444, 449]. The prevalence is lower in African, Hispanic and Mexican populations (2-4%), and lower again in Asian populations (1.4-3.7%) [448, 494, 520]. Unusually, a small study found the frequency to be 24% in a healthy Lebanese population [521]. A meta-analysis has reported the mutant 677T and 1298C MTHFR alleles are rarely found in the *cis* configuration, and therefore the compound homozygous genotype is rarely reported [522]. This same meta-analysis reports that the two variants are in linkage disequilibrium, meaning the 677T and 1298C alleles are coupled with 1298A and 677C alleles respectively [522]. The biochemical impact of this variant is much more subtle than the previous 677C>T variant given this variant does not result in a thermolabile enzyme. However, this 1298A>C MTHFR variant resembles the 677C>T variant by showing decreased MTHFR enzymatic activity, a phenomenon most prominent in individuals with the CC genotype [447, 469, 523]. It has been reported that reduced MTHFR activity in lymphocyte extracts is 60 to 70 % in CC individuals compared to AA individuals and this is smaller than the effect observed in 677TT individuals at 30% [417, 469].

This variant has not been studied as extensively as its better known 677C>T counterpart. Observations between this variant and homocysteine levels have been highly inconsistent [417, 469, 524-527], with some studies having reported no associations [525, 527] whilst others report the CC genotype having lower or higher

homocysteine concentrations [524, 526]. Some research reports that compound heterozygosity for both 677C>T and 1298A>C is associated with both higher plasma homocysteine and lower plasma folate concentrations [417, 462, 524]. Despite this, strong evidence has been reported in a large-scale study involving over ten thousand participants; it established that the *C* allele does have a functional impact, which is independent of the 677C>T polymorphism. Ulvik *et al.* [528] reports that with each additional C allele, homocysteine increased statistically significantly and serum folate levels decreased. In terms of CVD, there has been limited evidence to suggest risk is increased with this variant [529], although one study found the *C* allele was associated with early-onset coronary artery disease, even when total homocysteine levels were not elevated [530]. In relation to NTD, the majority of publications have found this variant is not an independent risk factor [417, 467, 469, 531, 532], a select few have suggested that the compound heterozygosity of these two variants increases NTD risk [444, 467, 469], but this has not been consistently observed [531]. Additionally, this variant has been investigated for risk of other birth defects, cleft lip and palate and congenital heart defects and no associations have been found [333].

Studies that have investigated the association between the 1298A>C MTHFR polymorphism and colorectal cancer risk have yielded mixed results, with no effect or a non-significant reduction in risk association with the CC genotype [497, 499, 533, 534]. A meta-analysis reported a protective effect of the CC genotype [510] and the 'Physicians' Health Study' reported a weak association which was unaltered by folate status [533]. Wang *et al.* [535] published work supporting the 'Physicians' Health Study', however, Keku *et al.* [534] described the same relationship among Caucasians, but not among African Americans. In the 'Physicians' Health Study' findings, it is noted that this weak association was not due to a confounding 677C>T MTHFR effect [533]. It is, in fact, thought that a greater protective effect occurred in individuals who carried both 677T and 1298C alleles compared to wild-type subjects [497].

In addition to this, Martinez *et al.* [536] observed that individuals with both the recessive homozygous variant 1298CC and 677TT genotypes were at an increased risk for colorectal adenoma recurrence, with a higher risk in the presence of low folate. In relation to DNA methylation, some research has shown that individuals with the 1298AA genotype had reduced genomic DNA methylation in the presence of low folate compared to the other genotypes [457, 537, 538]. Finally, several studies have reported that 1298CC genotype and compound MTHFR heterozygosity (CT and AC) were associated with a reduced risk of developing breast cancer [515, 539, 540].

However other studies have not be able to replicate these findings [541-544], and the same applies to research into gastric cancer, where a recent meta-analysis found no associations between this SNP and risk for gastric cancer [493, 494].

Many other MTHFR polymorphic variants exist; particularly the relatively common 1793G>A and 1317T>C MTHFR, but these and others have not been reviewed here and are not examined in this thesis.

III. 19-bp deletion DHFR

As described earlier (see page 41), the enzyme DHFR reduces pteroylmonoglutamic acid and H_2PteGlu to H_4PteGlu, using nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor, which can then be converted to H_4PteGlu, the primary cofactor necessary for one-carbon transfer chemistry. In humans, the DHFR enzyme is encoded by the DHFR gene, it is found in the q11-q22 region of chromosome 5 [545]. In 2004, the polymorphic 19 bp deletion within the highly conserved intron-1 of the DHFR enzyme was discovered (*rs70991108*) [418]. The homozygosity frequency of the 19bp deletion alleles in white populations is 17% to 22% [418, 546-548]. The 19bp deletion variant removes a putative binding site for the transcription factor Sp1, which is required for transcriptional activation/repression involved in gene expression, and may affect the splicing process and therefore may affect DHFR expression and activity [418]. Two studies have observed an increase in DHFR expression as a result of the 19bp deletion allele, whereas a third study reported no changes in DHFR expression levels among genotypes [547-549]. In addition to these studies, other evidence suggests that the deletion is a functional mutation because it occurs in a noncoding part of the DNA sequence, and in other genes this is an important site for transcription, translation and other regulatory functions [550-552]. The 19 bp del/del genotype has been associated with up to a 4.8-fold increase in messenger ribonucleic acid (mRNA) levels compared with the wild-type genotype [548], but similar studies have reported little or no effect [547, 549].

The 19bp deletion DHFR variant has been investigated for functional impact in a range of diseases, although findings in relation to NTD risk have been contradictory [418, 549, 553]. Work carried out by Gellekink *et al*. [546] demonstrated that the 19 bp del/del genotype is associated with a reduced plasma total homocysteine of about 1.5µmol/L (14%) compared to wild-type subjects, suggesting that the polymorphism increases DHFR expression, thereby facilitating homocysteine remethylation. But this was not supported in another study [547]. Other impacts of the 19bp deletion DHFR variant on health include a large cohort study which found the combination of del/del

genotype and multivitamin use was associated with a 50% increase in breast cancer [548]. But overall risk of breast cancer was not contingent upon genotype alone and other studies support this finding [548, 554].

Finally, the 19bp deletion DHFR polymorphic variant was examined in terms of having a gene-nutrient interaction. Kalmbach *et al.* [555] examined the genotypes effect on dietary folic acid intake. They found that the genotype alters the capacity to reduce dietary folic acid, thereby limiting the assimilation of synthetic folic acid into endogenous forms of folate. Clinically, their findings are significant, particularly in individuals with either low folic acid intake, diminished tissue folate stores, or very high intake which increases folic acid in the circulation [555]. This research has also led to questions on the safety of high folic acid intake; the 19bp deletion DHFR could add additional risk.

IV. 2756A>G MTR

MTR catalyses the methylation of homocysteine to methionine with simultaneous conversion of 5-methyl-H_4PteGlu to H_4PteGlu [556] (further details have been described earlier). The MTR gene has been mapped to 1q43 near the telomere of chromosome 1, the entire coding region has a length of 3,795 bp with a molecular mass of 140 kDa, and the gene product consists of 1,256 amino acid residues [556-559]. A polymorphic variant in the MTR gene was first described by Chen *et al.* [556] it is an adenine (A) to guanine (G) transition at nucleotide 2756 in exon 26 where, at the catalytic site an aspartic acid (D) replaces a glycine (G) at codon 919 (D919G) (*rs1805087*). The frequency of the GG genotype is fairly consistent worldwide ranging from 1-5% [419, 499, 560, 561]. For Japanese, Chinese and Korean populations, frequencies are 2-3% [562, 563], and many European studies report a 3% frequency for the GG genotype [452, 564-567]. Two studies report higher frequencies (10%) in white populations; specifically white women and children in Canada and white persons in Hawaii [420, 497].

Given the close biochemical relationship with MTHFR, the 2756A>G MTR has been investigated as a risk factor for many of the same disorders as the two MTHFR variants previously discussed. A number of studies have demonstrated a relationship between this variant and lower homocysteine concentration [452, 499, 568-571], although other studies have shown no effect [419, 567, 572-574]. Also, folate and vitamin B_{12} levels do not appear to be influenced by this variant [452, 574]. In terms of CVD, studies have been mixed; one study by Hyndman *et al.* [572] reported that AG genotypes are 3.4 times less likely to have a recurrent myocardial infarction, heart failure or bypass

surgery than AA genotypes. Klerk *et al.* [573] reported the GG genotype represented a four-fold increased risk of coronary heart disease when compared to the AA genotype. Additionally, Yates *et al.* [571] found that the prevalence of the mutant *G* allele was lower in subjects who had experienced a thrombotic event compared to matched controls. Other studies have reported similar but non-significant relationships between this variant and myocardial infarction risk [568], homocysteine level [573] and coronary artery disease [565].

With respect to NTD, the variant in the mother or child has not been observed to increase risk [419, 575]. However, two studies have reported a substantially elevated NTD risk in individuals who are doubly homozygous for this variant and the 66A>G MTRR (see below) GG/GG compared to the wild-type AA/AA [575, 576]. Other research conducted on this variant has uncovered a relationship to AD. Several studies have reported the AA genotype increases risk for AD by about three-fold in European populations [564, 577, 578]. Other genetic risk factors have been reported to further increase risk, one of these being the ε4 allele of the apolipoprotein-E (Apo-E) which when combined with the AA genotype increased Alzheimer's risk by 3.3 fold [564].

Finally, in relation to cancer risk, this 2756A>G MTR polymorphism shares few associations. It has been investigated in relation to colorectal cancer [497, 561, 574, 579, 580], with the GG genotype reported to protect against colorectal cancer at specific sites [567] and in men who consume less than one alcoholic drink per day [574]. No significant relationship has been reported with colorectal adenoma [581].

V. 66A>G MTRR

MTRR plays an essential role in maintaining the function of MTR, specifically by restoration of its activity for the remethylation of homocysteine [558]. Further details on the function of MTRR are described on page 41. The MTRR gene is located on chromosome 5p15.2-p15.3 [558] and encompasses approximately 34 kb comprising 15 exons. These vary in size from 43 to 1,213 bp. There are 14 introns, with sizes ranging from 108 bp to 5 kb [557]. Wilson *et al.* [420] were the first to describe this mutation on the MTRR gene, located at nucleotide position 66 in exon 2. The 66A>G MTRR (*rs1801394*) transition results in an isolecine (I) to methionine (M) substitution (I22M). The allele frequencies of this variant when originally described by Wilson *et al.* [420] were almost identical (51% *G* allele). The *G* allele was designated to be the "mutant" allele because of the conservation of isolecine at this position in other species [420]. Other studies have reported that the GG genotype frequency in white populations is

20-30% and considerably lower (7-10%) in Asian and African populations [449, 452, 499, 561, 582].

The functional impact of the 66A>G MTRR polymorphism is not well defined, Gaughan *et al.* [583] reported that the GG genotype was associated with a higher total homocysteine concentration in men when compared with the AA and AG genotypes. Kluijtmans *et al.* [584] also detected a trend toward higher homocysteine with the presence of the *G* allele. These findings have not been consistently observed [420, 585-589], and current thoughts are that this change in homocysteine concentration may depend on low vitamin B_{12} concentrations [565]. These findings are considerably different to findings from the third National Health and Nutrition examination Survey (NHANES III) DNA bank study. Yang *et al.* [449] reported a statistically significant interaction involving the 66A>G MTRR and 677C>T MTHFR polymorphisms. Contrary to other findings they found individuals who were doubly homozygous for the GG (MTRR) and TT (MTHFR) genotypes had a 25% lower total homocysteine concentration than did individuals who had AA (MTRR) and TT (MTHFR) genotypes [449]. The differences between the Yang *et al.* [449] and Gaughan *et al.* [583] studies may be due to some confusion surrounding the identification of the mutant allele, for the purposes of this thesis the *G* allele has been assigned as the mutant allele.

The research conducted on this variant has shown little significance in relation to the disposition of folate, vitamin B_{12} levels, and to methylmalonic acid concentrations [449, 452, 560, 565, 585] and no impacts on serum SAM/SAH ratios have been observed [585]. The results of this variant and CVD appears to be mixed [560, 565, 586] and the impact on cancer also requires additional studies. This variant has been linked to colorectal cancer [562], but again this has not been consistently observed [497, 561, 579]. Vitamin B_{12} and folate concentrations in combination with this variant may play a role in risk for developing colorectal adenoma [581] and lung cancer [590] .

In developmental disorders, this variant has shown some strong relationships; a meta-analysis found maternal GG carriage increased spina bifida risk by 55%, increasing further when 677TT MTHFR was combined, but the child's genotype had no impact [576]. This same study also reports a possible interaction between the GG genotype and high plasma methylmalonic acid and risk for NTD [576], and in the original paper by Wilson *et al.* [420] maternal GG genotype in combination with low vitamin B_{12} increased NTD risk about five-fold. Surprisingly, in both studies folate did not impact risk. Given the close relationship between MTR and MTRR, both polymorphic variants have been examined together and have been shown to increase NTD risk, but data is

limited [575, 576]. Another developmental disorder examined in relation to this variant relates to congenital heart defects, with the 66A>G MTRR variant in combination with elevated methylmalonic acid increasing the risk more than six-fold [591]. By contrast, another study found no relationship between the variant in combination with low vitamin B_{12} [592].

Finally, a mouse model for MTRR deficiency has been established using a gene trap approach to greatly reduce MTRR; the complete lack of MTRR may be lethal to the embryo [593]. Although not specific to this 66A>G MTRR genetic mutation, mice have been used to investigate the impact of MTRR deficiency on birth defects and pregnancy complications; research concluded that reduced maternal MTRR activity increases pregnancy loss and embryonic delays [594].

VI. 1420C>T SHMT

SHMT catalyses the reversible transfer on one-carbon units from 5,10-methylene-H_4PteGlu to glycine to form serine and H_4PteGlu. This reaction requires the cofactor pyridoxal-5-phosphate (active vitamin B_6); further details on the role of SHMT are described above. There are two iso-enzymes of SHMT; the cytoplasm form located on chromosome 17p11.2, which consists of 12 exons and 12 introns [595], and a second mitochondrial form localised on chromosome 12q13.2, which consists of 10 exons and 10 introns [596]. Both forms of SHMT have polymorphic variants, but the most commonly researched, and the focus in this thesis, is the cytoplasmic 1420C>T. This variant is represented by a cytosine (C) to thymine (T) transition in exon 13 resulting in a leucine (L) to phenylalanine (F) change (L474F) (*rs1979277*) [423]. The frequency of the TT genotype is 7-13% in white populations [423, 532, 595, 597, 598], 6.7% in African Americans [598] and 0.2% to 1.7% in Asians [599].

In terms of a functional impact on health, there have been several studies conducted on this variant. While the relationship with NTD risk is unclear [423, 532], a study found that the maternal *T* allele confers a doubling in risk of preterm birth, although this was only shown in white American women. The same study did not observe this risk in African American women, except in women who had a combination of low folate intake and the *T* allele [598]. Other research focused on homocysteine concentrations has found an inconsistent effect in relation to this genetic variant [423, 467, 532]. Further work has confirmed that this maybe the result of other folate variants influencing the 1420C>T SHMT polymorphism. Lim *et al.* [600] found that the variant does not appear to influence CVD risk on its own, but in combination with 677C>T MTHFR variant, the 1420TT genotype increased CVD risk five to ten fold. They report that the 1420TT

genotype acts synergistically with the 677TT MTHFR genotype to increase homocysteine concentrations. Just like CVD, current research to date suggests this variant alone has no impact on cancer risk including colorectal/ adenoma cancer [581, 597]. However, two publications report that the *T* allele combined with a polymorphism in the thymidylate synthase enhancer region (TSER), a triple tandem repeat variant may protect against cancer (breast [520] & malignant lymphoma [599]) given the close relationship between SHMT and TS in the prevention of DNA damage via the limiting of dUMP accumulation.

VII. 2R>3R repeat/insertion TSER

TS is a key enzyme in folate metabolism and the primary target of the anticancer drug 5-fluorouracil. TS catalyses the intracellular conversion of dUMP to dTMP using 5,10-methylene-H_4PteGlu as the carbon donor [601]. The TS gene is located on chromosome 18 p11.32 [602] and is about 16kb in length [603], it has a polymorphic tandem repeated sequence in its promoter enhancer region [425]. The 5'-untranslated region (5'UTR) contains a repeated 28bp sequence, it is required for efficient gene expression, and is usually found in triplicate 8bp's upstream of the start codon. This sequence is referred to as the triple tandem repeat (3R) (*rs45445694*) [424]. Protein expression from the 2R/2R TSER is 3 to 4 fold less efficient than the 3R/3R [425, 604]. The frequency of the 2R/2R genotype varies by ethnicity; highest frequencies have been recorded in white populations (19-23%) [605-607], a lower prevalence has been recorded in Africans (14-19%) [608] and a very low prevalence of only 2-10% has been observed in Asian populations [499, 599, 609, 610]. Studies into this genotype have generally focused on cancer, frequently colorectal cancer, but also publications have examined the effectiveness of chemotherapeutics such as 5-flurouracil in relation to this variant [602].

Two studies have investigated this variant in relation to birth defects, Volcik *et al.* [611] demonstrated that the double repeat is associated with NTD risk in infants, especially in non-Hispanic US whites, whereas Wilding *et al.* [612] were unable to demonstrate an association between this variant and NTD risk in subjects with NTD and their parents. In terms of folate, the 3R/3R genotype among Singaporean individuals was found to be associated with reduced plasma folate and increased plasma homocysteine concentrations in individuals with low intake [613]. The authors suggest that this variant and MTHFR compete for limiting supplies of folate required for the remethylation of homocysteine and this may impact risk for CVD, however other studies has reported homocysteine concentrations being unaffected by this variant [614-616].

Ulrich *et al.* [580, 607, 617] have studied this variant extensively, particularly in relation to colorectal adenomas and cancer. In 2002, Ulrich *et al.* [607] initially reported little association between this TSER variant and a second variant, the 3'UTR 6bp deletion described below. However, they did report a statistically significant interaction. Among individuals with the 3R/3R genotype, persons taking >440µg per day of folate were at a 2-fold decreased risk colorectal adenomas compared to person taking <440µg per day [607]. Conversely, among individuals with 2R/2R genotype, high folate intake was associated with a 1.5-fold increased risk of colorectal adenomas, a similar trend was also established for vitamin B_{12} [607]. In 2005, Ulrich *et al.* [580] observed this TSER variant was associated with a statistically significant decreased risk of colorectal neoplasia among men (but not women), also individuals with both TS polymorphisms (combined) were at a reduced risk, with statistically significant results for women. Additionally, when dietary intakes were taken into account, individuals with at least one variant TSER allele (3R/2R or 2R/2R) were at reduced risk of colon cancer in the presence of both low folate and methionine intakes [580]. This work supports findings from adenoma studies indicating that purine and pyrimidine synthesis may be a relevant biological mechanism linking folate metabolism to colon cancer risk [460].

Other research findings on this TSER variant have reported no overall association between the TSER promoter polymorphism and adenoma risk [605]. However, Chen *et al.* [605] observed a significant TSER-alcohol interaction, although individuals with the 2R/2R genotype were not at an increased risk if they had high alcohol consumption (>30 g/d). Heterozygotes and those with the 3R/3R genotype showed an elevated risk of colorectal cancer [605]. Given the enzymatic role of TS, research into 2R>3R TSER has focused on its impact and response to fluropyrimidines, since polymorphisms in this gene have been suggested to influence 5-fluorouracil sensitivity [618]. In gastric cancer, a non-significant association was observed between the *2R* allele and longer survival in patients who had oral fluropyrimidine therapy [619]. Additionally, Ott *et al.* [620] reported the 3R/3R is a risk factor for tumour related survival in advanced gastric cancer, however, several other studies did not observe any significant difference in the outcome of patients according to 2R>3R TSER genotype [621-623]. Similarly, several studies on colorectal cancer have found that patients with the 3R/3R genotype may benefit less from 5-fluorouracil than those with the *2R* allele in terms of survival [624, 625] - again these findings have not been consistently observed [626].

VIII. 3'UTR 6bp deletion TS

A second functionally relevant polymorphic variant has been described in the TSER; it occurs in the 3'UTR, and is a 6 bp deletion at position 1494 (*rs34489327*) [424]. This variant is in linkage disequilibrium with the previously reported 5'UTR variant [627, 628]. It also varies greatly amongst ethnic populations - the frequency of the 6bp deletion allele is reported to be 30-40% in white Caucasians, approximately 26% in Hispanics, 52% in African Americans and 76% in Singaporean Chinese [580, 629, 630]. This TS variant has not been as extensively studied compare to the previous variant, but most significantly it has been associated with reduced mRNA stability and translation *in vitro* and lower intratumoural TS expression *in vivo* [424, 629].

This 6bp deletion genotype confers possible protection against diseases for which a low folate/high homocysteine phenotype appears to be an etiologic component. Kealey *et al.* [631] found that in non-smoking individuals, red cell folate concentration was much higher for del6/del6 subjects compared with ins6/ins6 or ins6/del6. Equally, homocysteine concentration for del6/del6 was considerably lower compared with the other genotypes [631]. However, other studies have reported null findings [615, 632], and one study has reported a significant difference, but only when examined in combination with other TS genetic variants [628]. The non-deleted genotype has been associated with increased NTD risk only in non-Hispanic US white subjects [611], but limited numbers of studies exist to support these findings.

Most of the research conducted on this polymorphism, like the previous one, is in relation to cancer. A significant risk for gastric cancer susceptibility was observed in carriers of the del6 allele [633]. However, inconsistency exists, with one study finding no association [621], and another study finding the opposite outcome. Huang *et al.* [618] found that overall, gastric cancer survival in patients with the ins6/ins6 genotype was significantly shorter than those in patients with the del6/del6 and ins6/del6 genotypes. Non-Hodgkin lymphoma has also been investigated in relation to this variant; Skibola *et al.* [606] found an inverse association in the presence of a single copy of the *del6* allele. They also examined the combined 2R>3R TSER and 3'UTR 6bp del genotypes; individuals with the 2R/2R TSER and ins6/ins6 had a 1.5-fold increased risk of non-Hodgkin lymphoma compared with the 3R/3R + ins6/ins6 [606].

A case-control analysis of the combined TS polymorphisms and risk of lung cancer was conducted by Shi *et al.* [634] they established the 2R>3R TSER was not associated with increased risk of lung cancer. But the 3'UTR 6bp deletion, ins6/ins6 and ins6/del6

genotypes were associated with a significant increased risk of lung cancer compared to the del6/del6 genotype [634]. This significant association increased for individuals who were; over 55 years of age, male, heavy smokers and current alcoholic drinkers [634]. TS SNPs have also been reported as risk factors for squamous cell carcinoma of the head and neck; the del6/del6 genotype being associated with a significantly decreased risk compared to the ins6/ins6 genotype [635]. This research also examined the combined TS genotypes and found carriage of four protective alleles was associated with a significantly decreased risk; the four alleles being 3R/3R + del6/del6. In addition to these findings, the del6 genotypes were linked in an allele dose-dependent manner with a decreased risk of oral cancer progression [635].

Finally, some reports have investigated this variant in relation to colorectal adenoma and other cancers, however; so far, reports have established no clear pattern. Chen *et al.* [615] and Ulrich *et al.* [607, 615] both found no influence on colorectal cancer risk and survival. Additionally, Chen *et al.* [615] found the variant did not modify plasma folate and total homocysteine levels. One small and limited studied reported that tumour mRNA levels in patients with ins6/ins6 were 4.2-fold higher compared with those patients carrying the del6/del6 genotype, but these results have not been replicated [636].

IX. 80G>A RFC

The RFC is responsible for the uptake of dietary 5-methyl-H_4PteGlu from the plasma to the cytosol [637]. Decreased RFC activity may result in a lower uptake of circulating 5-methyl-H_4PteGlu into cells, thus disturbing folate metabolism by reducing the cellular pool of one-carbon donors [637]. The RFC gene is located on chromosome 21q22,2-22.3, and consists of 5 exons, the entire coding region includes 1,776 bp which consist of 591 amino acid residues [638-641]. Additionally, this gene is sometimes referred to as solute carrier family 19 member 1 (SLC19A1). Given the role of RFC, any mutations occurring in the gene may result in low folate levels. A common polymorphism was located at nucleotide position 80, where a guanine (G) is changed into an adenine (A) in exon 2, this translates to an arginine (R) replacing a histidine (H) at position 27 in the functional carrier protein (R27H) *(rs1051266)* [637]. Prevalence of this mutation varies across the world for reasons that appear unknown. Frequencies of the GG genotype are said to be 24-33% in whites, about 20% in African Americans, and 26-32% in Hispanics [580, 637, 642-644]. The GG frequencies in Asia range from 19-33% [645, 646] and in Northern Europe vary from 26-41% [451, 452, 532].

Several publications involving a RFC deficient mouse model have determined that RFC is not solely responsible for the uptake of folate from the diet. However, further work has confirmed the other folate transporters cannot completely compensate for the absence of the RFC [647-649]. In relation to folate metabolism, this variant alone does not appear to affect plasma folate, red cell folate, homocysteine, SAM or SAH concentrations [451, 452, 467, 585, 637, 642]. When this variant is considered along with 677C>T MTHFR, researchers have detected an increase in homocysteine concentration in individuals who were doubly homozygous i.e. those carrying both 80GG RFC and 677TT MTHFR genotypes compared with individuals who were 80GG RFC and 677CC MTHFR or 677CT MTHFR [637]. In addition to these findings, higher plasma folate levels were seen in the compound 80AA RFC and 677CT MTHFR genotype compared to 80GG RFC and 677CT MTHFR individuals [637].

Other impacts on health attributed to this polymorphism include NTD. The GG RFC genotype has been linked to a 2.5 fold increase in NTD risk [645, 650], but this has not been consistently observed [467, 643, 651]. Pei *et al.* [645, 651] and Shaw *et al.* [645, 651] found that the combination of low folate intake (did not consume folic acid supplements during pregnancy) and maternal GG genotype increases risk for NTD significantly[645, 651]. Additionally, Relton *et al.* [532] describe a combination of 80G>A RFC and the *CβS* 844ins68 SNP as having an increase in risk for NTD pregnancy. Other investigative studies have demonstrated a link between this variant and congenital heart defects and Down syndrome [644, 646, 652].

To date, this genetic variant has not been associated with colorectal cancer [580, 653], though it has been shown to have an impact on chemotherapy treatment outcomes. The A allele is associated with shorter survival estimates [654] and increased hepatotoxicity after methotrexate treatment [655] in patients with acute lymphoblastic leukaemia. A further study supports this and found that GG individuals have a reduction in methotrexate uptake in cultured cells [656].

X. 1561C>T GCPII

The GCPII enzyme (see page 38) influences folate bioavailability by catalysing the reaction that hydrolyses the glutamate residues of the folate polyglutamate chain to yield a monoglutamyl folate [657]. This process regulates the bioavailability of folates, and allows efficient transport of dietary folate as well as helping maintain the level of circulating folate. Because of this, reduced GCPII activity could lead to a generalised folate deficiency [422]. The GCPII gene is located on chromosome 11p11-p12, surrounding about 60kb and 19 exons, and encoding a 750 amino acid expression

products [658-660]. In 2000, Devlin *et al.* [422] reported a polymorphic variant resulting in a cytosine (C) to thymine (T) substitution at nucleotide 1561 (*rs61886492*). This non-synonymous mutation is located in exon 13 and results in a substitution of a histidine (H) to a tyrosine (Y) residue in the putative catalytic region (H475Y) [422]. The variant is not common worldwide, since the reported frequency of the TT genotype ranges from less than 0.5% to 2% [451, 532, 581, 582, 597, 642, 653, 661, 662]. The present research bears this out since in the work described in this thesis, the TT genotype was not seen.

When examined *in vitro*, the GCPII function was 53% less in the variant form compared to the wild-type [422]. Although a second study found that bioavailability of folate was not affected in individuals who are CT as compared to CC [663]. Many studies conducted on this variant have found conflicting results; the *T* allele either increases or has no effect on plasma and red cell folate concentrations [451, 597, 642, 661, 663]. Additionally, the variant decreases or has no effect on homocysteine concentrations [451, 597, 642, 653, 661, 662]. The impact of the 1561C>T GCPII SNP on health, thus far appears limited. No associations with NTD occurrence have been observed [532, 642] and it does not appear to have an influence on CVD or colorectal cancer [581, 597, 653, 661].

XI. 1947G>A COMT

This polymorphic variant is not normally associated with folate one-carbon metabolism, but in recent years with research linking folate to neurological function and disease there has been interest in the expression product because it relies on methyl groups derived from folate. This may be relevant in cognitive function/AD and depression which are phenotypic traits that are examined in this thesis. Catechol-*O*-methyltransferase (COMT) is a methylation enzyme which catalyses methylation of endogenous catecholamines (e.g. dopamine, epinephrine and norepinephrine) [664]. O-methylation occurs in the presence of SAM and magnesium ions. Given this role, COMT has been closely associated with the underlying pathogenesis of many mental disorders [665, 666].

COMT exists as soluble and membrane-bound isoforms encoded by a single gene located at chromosome 22q11 using alternative promoters [667]. COMT activity is governed by a common SNP located in exon 4 that is associated with 3 to 4-fold differences in COMT activity among homozygous subjects, whereas an intermediate biochemical phenotype is observed in heterozygous subjects [666, 668, 669]. This SNP

is a guanine (G) to adenine (A) transition at position 1947(*rs4680*); it results in a valine (V) to methionine (M) substitution at amino acid 158 in the soluble form and at amino acid 108 in the transmembrane form [665, 666, 670]. This SNP is more commonly referred to as Val^{158}Met COMT and sometimes the alleles are assigned according to activity High (H) and Low (L) designations. The frequency of the AA (Met/Met) genotype is approximately 18-27% in Europeans and American Caucasians [671]. Significantly lower frequencies are seen in Asians (1.5%) [672] and Africans (~7%) [673].

COMT has been investigated for its role in modifying homocysteine concentration because SAM is the necessary methyl donor for COMT catalysed reactions. Upon the methylation of a COMT substrate, SAM is converted to SAH, a known COMT inhibitor repressing a negative feedback loop. A report in Finnish men found no association between this polymorphic variant and total homocysteine [674]. However, Tunbridge *et al.* [675] hypothesised and established that A allele carriers (individuals with low activity) display lower total homocysteine levels than G carriers since they have lower SAM usage, and therefore lower SAH and homocysteine levels.

In terms of cancer, the low activity COMT A allele has been found to be associated with oestrogenic cancers in multiple published studies [676-679]. Goodman *et al.* [679] found that high homocysteine and/or low folate levels in combination with an increasing number of A alleles is associated with an increased breast cancer risk. Additionally, Lavigne *et al.* [680] found that the association between AA genotype and postmenopausal breast cancer was statistically strong in women with a BMI >24.47. By contrast, one study reported no such association between COMT and breast cancer risk [681].

Catecholaminergic neurotransmission has been suggested to be involved in a range of mental disorders; the COMT gene is proposed to impact risk for such disorders given its role in encoding a key dopamine catabolic enzyme [682]. This COMT candidate polymorphism has been very well studied in schizophrenia patients. A 2002 meta-analysis found that only two [683, 684] out of fourteen case-controlled studies yielded significant evidence for association (A allele carriers) [685]. But this is contrasted by a 2003 meta-analysis that found significant evidence for associations of the *G* allele and schizophrenia, although when the Hardy-Weinberg equilibrium was accounted for, these effects where no longer significant [686]. Overall, extensive research has failed to find a consistent relationship [672, 687-693]. One Australian study showed significance for the COMT SNP and schizophrenia, however, this association was only

seen in a haplotype analyses which included other COMT related mutations. The authors suggest inconsistent results attributed to this variant and schizophrenia may be because of separate and interacting effects within this haplotype [694]. It is also worthwhile noting that there is robust evidence that the COMT mutation influences frontal lobe n which neuroprocessing impacts cognitive function - a trait marker for schizophrenia [695].

COMT has also been associated with attention deficit hyperactivity disorder (ADHD) [696-700], substance dependence [701] (alcohol [702, 703], cannabis [704, 705], amphetamine [706, 707], cocaine/heroin [708, 709] usage), bipolar disorder [710-714], depression [666, 715-717], obsessive-compulsive disorder [718-721], anorexia nervosa [722, 723], suicidal [724, 725] and aggressive personality traits [693, 726] to name a few [682]. The vast majority of these studies have resulted in only weak associations, inconclusive or negative effect outcomes. However, the COMT mutation may increase overall susceptibility to psychiatric disorders in general, but other mechanisms such as other genetic or environmental factors may act in a disorder-specific manner [718].

These introductory sections cover nutritional and clinical aspects germane to this thesis. However, many of the associations that have been found, and which have been discussed in this section are based on a limited number of molecular mechanism that are, in all probability, directly responsible for defining several important clinical phenotypes. The next section will address these in greater detail.

1.3. B-Vitamin Related Molecular Mechanisms That Underpin Disease

It is the scientific endeavour of early researchers like Lucy Wills, Victor Hebert, and Richard Smithells who have initiated our current ideas on the functions of folate in the body, and the many consequences of folate deficiency. Wills first discovered the critical need for folate in pregnancy, and many years later, Herbert used himself to experiment with a folate deficient diet, observing an array of blood anaemia's and psychiatric symptoms. Richard Smithells was the first to suggest that folate deficiency led to NTD in pregnancy (see historical timeline figure on page 30). Today, we know that folates are required to provide one-carbon units and methyl groups; they are needed in the synthesis of DNA and RNA precursors, and are important in amino acid metabolism. Given the central role folate plays in human biology, it's not surprising that deficiency can affect many areas of the body. Rapidly proliferating and/or sensitive cells and tissues, such as those in the gastrointestinal tract, cardiovascular and reproductive

systems are key areas in which a lack of folate and potential disease development interconnect.

In the past, classic folate deficiency was known to cause megaloblastic changes in the bone marrow, peripheral blood, and intestinal epithelium. In terms of anaemia, this results in increased mean red blood cell volume, red cells that retain their nucleus, and neutrophil hyper-segmentation [403, 727]. Diminished folate status of a magnitude insufficient to cause megaloblastic anaemia still causes homocysteine levels to increase; this hyperhomocysteinemia is associated with a range of cardiovascular health complications, AD, mental health problems, and putative NTD related effects. Also, as we know today, folate deficiency is associated with various forms of cancer, given its role in DNA elaboration and stability. This final section explores the impact of folate deprivation through the major metabolic mechanisms that have a role in the development of many common chronic diseases. Furthermore, it evaluates current research on the potential harm of consuming too much synthetic folic acid as a result of fortification programs.

1.3.1. The impact of folate deprivation

Clinically, there are several biomarkers of human folate deficiency; the most common and hence robust assessment is red cell folate concentration (levels <370nmol/L) [728, 729]. However, this alone does not provide an adequate diagnosis; serum folate, vitamin B_{12}, and plasma homocysteine concentrations provide a wider and equally effective gamut of biomarkers for determining a person's nutritional folate status. As described in section 1.1, progress in nutritional research during the 20^{th} century resulted in altered patterns of food consumption and therefore altered health outcomes. Early in this century, under nutrition was linked to many deficiency diseases including classic megaloblastic anaemia resulting from folate deficiency. Although under nutrition is now rare in Western countries, we do see more over consumptive malnutrition where deficiency arises through the consumption of energy-dense, nutrient-poor foods that may contain few key nutrients, including B-group vitamins. The deprivation of folate in the human body can generally occur through three different mechanisms; reduced folate intake, impaired absorption and retention, and of most significance to this dissertation, alterations in folate dependent metabolism.

Inadequate folate intake may arise through an inability to access foodstuffs that contain this micronutrient such as green leafy vegetables, or through poor dietary choices that contain limited folate content. Deficiency in infants may occur when exclusively fed a

low folate milk such as goats milk [730]. Malabsorption of folate can occur in individuals with chronic inflammation of the gastrointestinal tract, such as in conditions like atrophic gastritis, celiac or Crohn's disease and there are rare cases of congenital syndromes in which folate absorption is impaired [731-733]. During pregnancy, there is an increased demand for folate due to the rapid cell division and growth of the embryo and foetus; if intake is not adjusted, this increased need may also induce folate deficiency. Likewise, as is well documented, a shortage of folate during pregnancy can lead to an array of birth defects [60, 224, 734-736].

Outside these aberrant physiological conditions, folate absorption may also be impaired by long term use of medications such as aspirin, antacids and by consumption of alcohol [737]. These drug types alter the gastrointestinal pH; this would affect both the absorption process and bioavailability of folate resulting in a potential deficiency state [737]. Additionally, antifolate drugs such as methotrexate and 5-fluorouracil used in chemotherapy and anticonvulsants such as phenytoin, carbamazepine and valproic acid can induce secondary folate deficiency as these drugs unambiguously impair folate metabolism [738].

At a molecular level, deprivation of folate in the human body results in altered one-carbon metabolism and it is this that links folate to a range of diseases, which includes common chronic degenerative disorders such as cancer and CVD. As described in section 1.2.8 (page 41), folate-mediated one-carbon metabolism is highly complex, involving many intermediaries and enzymes which play a key role in normal homeostasis. It can be impaired by insufficient folate or a shortage of other B-group vitamins, and by genetic polymorphisms. As previously discussed, one-carbon metabolism contains several key pathways, although the two of greatest interest are arguably the methylation cycle and nucleotide synthesis. Centred close to the nexus between these two pathways is MTHFR, whose role is to reduce 5,10-methylene-H_4PteGlu to yield 5-methyl-H_4PteGlu. The methyl group on this vitamer is required for the remethylation of homocysteine to methionine.

During folate deficiency, lack of 5-methyl-H_4PteGlu in one-carbon metabolism slows the conversion of homocysteine to methionine, resulting in elevated levels of homocysteine (hyperhomocysteinemia) [739]. An insufficiency in methyl groups also increases cellular SAH, as the equilibrium favours it and therefore results in reduced intracellular SAM concentrations. As SAM is critical for methylation reactions, which involve DNA and protein methyltransferases, a reduced SAM and an increased SAH is associated with *hypo*methylated DNA [739]. In addition to this *hypo*methylated DNA, folate

deficiency also causes an imbalance in nucleotide pools, which results in an accumulation of dUMP and depletion of dTMP. This accumulation leads to uracil misincorporation in DNA, a process eventually causing DNA instability and chromosome breakage [163].

Therefore, there are three important metabolic consequences of a low folate status; hyperhomocysteinemia, decreased methylation potential, and misincorporation of uracil into DNA. Each of these metabolic aberrations that stem from folate insufficiency have been associated with disease development. Examples of this are elaborated on below.

Hyperhomocysteinemia: Hyperhomocysteinemia is biologically defined as a fasting plasma homocysteine value of more than 15µmol/L [740]. Importantly, homocysteine concentration is much lower in children, but increases with age [741] and is significantly higher in men than in women, particularly pre-menopausal women [742]. The first clue that homocysteine was associated with chronic disease was discovered over 40 years ago (see timeline figure on page 30), through the observation of two children with inborn errors of metabolism which resulted in extremely high levels of homocysteine that became fatal [743]. The first child had a defect in methionine metabolism and the other had a deficiency of the CβS enzyme, but they both shared widespread arteriosclerotic plaques and severe vascular damage [743, 744]. After examining those children, McCully *et al.* [744] questioned whether mildly elevated homocysteine levels with no apparent defects in functioning enzymes could cause vascular changes and lead to CVD. His theory has led to decades of research examining this possibility. Today, it is widely accepted that mildly elevated homocysteine is associated with atherogenic, thrombogenic and hypertensive vascular disease [258, 740, 742, 745, 746]. Hyperhomocysteinemia is now recognised as an independent risk factor for CVD [125]. These findings have also generated extensive research into whether folate might play a role in vascular disease prevention via its influence on homocysteine levels or via other mechanisms related to genetic mutations in folate-mediated one-carbon metabolism.

To date, there have been many key pieces of research in this area; in 1995 an influential meta-analysis conducted by Boushey *et al*. [232] showed that an increment of 5µmol/l plasma homocysteine was associated with an increased risk of coronary heart disease of 80% for women and 60% for men. A 2002 meta-analysis found that after controlling for blood pressure and other CVD risk factors, decreasing homocysteine concentration by about 3umol/L was associated with a 11% lower risk of ischemic heart disease [747]. Although there is convincing evidence that even mild

hyperhomocysteinemia is a prevalent risk factor for CVD, stroke and venous thromboembolism there has been relatively little success of research into vascular protection via homocysteine-lowering B-group vitamins. Many prospective and observational studies found evidence for an inverse relationship between folate status and stroke and coronary heart disease, although such findings have not generated consensus [748-753]. Many randomised controlled trials examining the use of high dose folic acid, vitamin B_6 and vitamin B_{12} supplementation for secondary prevention of CVD have largely failed to find any benefit [256, 754-758], this is despite very recent reports that following folic acid fortification, the average homocysteine concentration in the US has decreased by about 10% [759].

There is, to a certain extent, a mismatch between results from observational and interventional studies. Reasons for this include the possibility that hyperhomocysteinemia is not causal but in fact is only a predictor of CVD (i.e. a biomarker) [760]. If correct, treatment via B-vitamin supplementation may not reduce clinical disease. In addition, many interventional studies to date have had short follow-up periods (i.e. <=5 years), which is a small time frame relative to the development of atherosclerotic disease [760]. Potential long-term benefits are yet to be seen. As folate is critical for cellular proliferation, another reason for the disparity could be that folic acid supplementation may actually promote inflammation and the spread of atherosclerotic lesions seen in CVD [760]. Therefore, the disparity in the research may have begun to break down from the moment that discretionary folic acid intakes increased, making interpretation of the relationship between homocysteine and CVD difficult among populations [761]. This has certainly been further hampered by mandatory fortification programs.

As previously described, MTHFR plays a critical role in homocysteine metabolism, and as discussed extensively in section 1.2.11, the 677C>T genetic mutation results in homozygous carriers having homocysteine concentrations that are higher than individuals who are wild type [416, 448-452]. This single genetic mutation alone can in fact mimic conditions of a lifelong low folate intake. Several meta-analyses have examined the relationship between this gene mutation, hyperhomocysteinemia, risk of CVD and the vascular protective role of folate. Results have been mixed in part due to the need for very large sample sizes to detect gene based associations. A large 2002 meta-analyses by Klerk *et al.* [475] examined 40 studies and found that compared to CC individuals, TT carriers have a 16% greater risk of coronary heart disease. Wald *et al.* [24] supported this with an even larger number of studies within the analysis, and

also found the TT genotype carried a 21% greater risk of coronary heart disease when compared to the CC genotype. Klerk *et al.* [475] found that the effect was more evident when folate status was low, which suggests that impaired folate metabolism resulting in hyperhomocysteinemia is causally related to risk of CVD [475].

The molecular mechanisms that involve folate, its metabolism and the phenomenon of hyperhomocysteinemia along with the associated effect on cardiovascular cells and tissues still lacks clarity; one mechanism in which hyperhomocysteinemia promotes endothelial dysfunction has been extensively researched, particularly using animal models [762-764]. The cardiovascular system is lined with endothelial cells, these cells produce nitric oxide which has a critical role in modulating vasodilation and hence blood flow and pressure. Increased homocysteine increases oxidative stress, leading to injured endothelium and altered elasticity of the vascular wall [761]. Additionally, asymmetric dimethylarginine, which is elevated in hyperhomocysteinemia, has been found to inhibit the production of nitric oxide, which promotes the uncoupling of *eNOS*, directly contributing to increased oxidative stress [765, 766]. Oxidative stress induced by homocysteine may also result in the decreased activity of the antioxidant enzyme glutathione peroxidase, and may result in activation of pro-oxidant enzymes such as NADPH oxidases [745, 767-769].

Tetrahydrobiopterin is an essential cofactor for nitric oxide biosynthesis, and its activity may be modulated under conditions of hyperhomocysteinemia. Homocysteine affects endothelium-derived nitric oxide production by increasing the production of reactive oxygen species, which in turn could lead to oxidation and depletion of this biopterin cofactor [761]. Like asymmetric dimethylarginine, oxidative stress is further induced by the uncoupling of *eNOS*. This creates a superoxide radical producing generator, depleting 5-methyl-H_4PteGlu which can lead to uncoupling of *eNOS* from its cofactor, tetrahydrobiopterin [761, 770, 771]. Other examples of the molecular mechanisms involving homocysteine and its effect on cardiovascular function have been reviewed by Undas *et al*. [740]. In relation to thrombosis and endothelial dysfunction, it was suggested that homocysteine is involved in several mechanisms which include: elevated tissue factor expression, enhanced platelet reactivity, increased thrombin generation, reduced anticoagulant processes and fibrinolytic potential, and also additional factor V activity [740]. Ultimately, homocysteine is a potentially toxic thiol that has been found to be a pro-thrombotic and pro-inflammatory factor; a vasodilation impairing agent and an endoplasmic reticulum stress inducer. Combined, these factors

contribute to the prevailing view that elevated homocysteine is an independent risk factor for CVD.

Given the evidence for homocysteine's negative role in vascular health, it is not surprising that elevated concentrations are also considered to be embyrotoxic. Indeed, the effect of homocysteine levels on pregnancy outcomes has also been thoroughly researched. Evidence has linked increased concentrations with repeated miscarriages [772-775], NTDs [776, 777], pre-eclampsia [243, 778-780], and intra-uterine growth retardation [245, 781]. In the early phase of pregnancy, MTHFR actively converts homocysteine into methionine in the amniotic fluid, a deficiency of available methyl groups from a low folate status and/or a mutation in MTHFR such as the 677C>T polymorphism can result in elevated homocysteine. It is suggested that this leads to intrinsic toxicity within the embryonic fluid that can then result in miscarriage [774]. NTDs on the other hand may be caused directly by a functional abnormality of the enzyme MTR. As described earlier, MTR is key in remethylating homocysteine into methionine, but it also has a role in the production of myelin's core protein [334]. Abnormities in MTR function like MTHFR, can led to hyperhomocysteinemia and ultimately to NTD [334]. Other enzyme deficiencies and gene mutations of folate metabolism could also account for NTD and pregnancy complications, but overarching all causes is the central role that low folate status results in hyperhomocysteinemia.

Finally, the same mechanisms that underpin the role of homocysteine in vascular function and which promote CVD, extend into the pathoaetiology of cerebral disorders. Hyperhomocysteinemia is associated with an increased risk of vascular dementia, mild cognitive impairment, age-related memory loss, and has also been confirmed in patients with AD [133, 135, 138, 184, 782-784]. Similarly, some evidence exists for a role of elevated homocysteine in mood disorders such as depression [189, 259, 785, 786], as well as in schizophrenia [195, 787, 788]. Ravaglia *et al.* [136] found that elevated plasma homocysteine concentrations and low serum folate are independent predictors of the development of both dementia and AD, and Seshadri *et al.* [133] found hyperhomocysteinemia at baseline was significantly associated with a subsequent diagnosis of AD.

As in vascular disease, it is thought that oxidative stress is an underlying cause of neurodegeneration and possibly even psychiatric disorders, and that homocysteine may promote neurotoxicity through the formation of reactive oxygen species [769, 789]. Hyperhomocysteinemia may also promote excitotoxicity, and lead to increased neuronal injury and cell death; it is thought that in the brain, homocysteine may partially

block the glycine site of the NMDA receptor leading to excitotoxicity [192, 790]. Other suggested mechanisms include that homocysteine increases cytosolic calcium, causes phospho-tau immunoreactivity, and possibility even apoptosis [789, 791]. Each of these may affect neuronal homeostasis leading to AD. Lastly, there has been consistent epidemiological evidence for a connection between folate status and depression, although it has not been as extensively studied as AD. There have also been several studies, which have identified hyperhomocysteinemia as a risk factor for depression [189, 190, 786].

Elevated homocysteine resulting from a shortage of folate highlights a key metabolic mechanism that links this B-vitamin to the potential development of various diseases. Folate dependent one-carbon metabolism also plays a critical role in DNA methylation another mechanism by which folate deficiency impacts disease risk.

DNA hypomethylation: DNA methylation in mammalian cells is an important factor in gene expression. The process involves a methyl group addition at carbon 5 of the pyrimidine ring of cytosine residues that precede a guanosine in the DNA sequence [792]. This is referred to as a CpG site, and when concentrated together these sites are called CpG islands [793]. These methylation sites are generally located in the 5' untranslated region of genes [793]. Nearly half of all CpG sites are methylated, particularly sites that are within coding regions of genes, but generally CpG islands or clusters are found in an unmethylated state [792]. In order for DNA methylation to occur (cytosine is converted to 5-methylcytosine), activation by DNA methyltransferases is required. These enzymes are necessary for the maintenance of methylation profiles, and without them DNA replication would be impaired [792].

As alluded to earlier, biological methylation requires SAM as a donator of methyl groups. 5-methyl-H_4PteGlu is the only nutrient capable of achieving *de novo* synthesis of methionine and hence SAM. Methylation of DNA, RNA and proteins are SAM dependent, and experimental observations across each of these compounds have found that an inadequate supply of one-carbon nutrients results in low levels of methylation [794-797]. Abnormalities in DNA methylation are very common in cancer, and are most likely often causal in its development. Quite often in epithelial cancer cells, the main abnormality in DNA methylation is the overall *hypo*methylation of the genome. However, this is coupled with the opposing dynamic of *hyper*methylation within CpG islands [792]. It is thought that hypermethylation of CpG islands leads to repression of tumour suppressor genes via gene transcriptional silencing [798].

However, there are several other pathways that could lead to cancer that hypermethylation is associated with including DNA repair and apoptosis.

Overall, genomic *hypo*methylation is commonly observed in dysplastic cells and is the earliest of all molecular anomalies observed in cancer. Incremental increases in grades of dysplasia are associated with decreased degrees of genomic methylation [799]. Under experimental conditions, folate depletion in humans, animals and cells has been shown to produce genomic DNA *hypo*methylation [794, 797, 800, 801]. The likely mechanism by which a lack of folate causes *hypo*methylation is through an increase in SAH concentrations and the resultant decreases in SAM. Methylation is dependent on both normal levels of SAM and on DNA methyltransferases enzymes, whose actions are inhibited by SAH [463]. Also, as one-carbon metabolism is regulated at the level of the enzyme MTHFR, which is an allosteric enzyme controlling the remethylation of homocysteine to methionine, it is therefore not surprising that genetic variants of this enzyme are associated with aberrant methyl group metabolism and hence cancer development.

Although folate depletion is associated with DNA *hypo*methylation, results have not always been consistent. Other factors such as age and the status of other B-vitamin related nutrients may impact of DNA methylation. Indeed, systemic fluctuation in one-carbon related nutrients and timing of sampling may account for observed inconsistencies. Pogribny *et al.* [801] used rodents on a methyl-deficient diet and observed an early occurrence of genomic *hypo*methylation, followed by small increases in methylation[801]. This was followed by increases in the enzymatic activity of the DNA methyltransferases, causing increases in methylation at specific foci [792, 801].

Furthermore, there is evidence that *hypo*methylation may be dependent on the magnitude and duration of the induced depletion of folate. Two rodent based studies that shared similar protocols, but differed in the level of folate depletion, observed induced genomic *hypo*methylation under severe folate deficiency [794, 802]. The second study achieved a moderate level of depletion and produced little evidence of *hypo*methylation [802]. As demonstrated in classic folate deficiency, bone marrow is extremely sensitive to limited availability of folate, this is in part due to the bone marrow undergoing rapid proliferation of cells requiring high rates of DNA synthesis. This phenomenon underpins the pathoaetiology of the deficiency disease – megaloblastic anaemia. Another tissue which exhibits evidence of increased vulnerability is colonic mucosa, a tissue area with a high cellular turnover rate. This may help explain why the vast majority of data linking increased cancer risk and diminished folate status is most

compelling for the colon. Colonic mucosa has been found to be susceptible to the induction of *hypo*methylation; one study found a synergistic effect when multiple one-carbon related nutrients (folate B_{12}, B_6, B_2) were depleted. Following this treatment, a 50% reduction in genomic DNA methylation was observed in mice colonic mucosa [803]. This occurred even though folate alone produced no effects [803]. In considering nutrient-gene interactions, several studies have shown that a reduced folate status in combination with the TT genotype of the MTHFR 677C>T polymorphism affords diminished genomic DNA methylation potential, although there are many confounders [457, 804]. Indeed, the measurement of DNA methylation itself is a confounding variable.

There have been a limited number of human intervention trials that examined whether supplementation with folic acid at extremely high doses might increase genomic DNA methylation. Some have indicated possible benefits, others have reported null effects [805, 806]. Kim *et al.* [807] found increased genomic DNA methylation in the colon of subjects who were previously diagnosed with colorectal adenomas after they were treated with 5mg/day of folic acid compared to placebo controls. However, the replication of neoplastic cells when compared to normal cells is generally much faster; this increased rate of replication and hence DNA synthesis increases the need for folate. In the face of an abundant supply of folate – particularly through the use of synthetic folic acid, it could be expected that this might optimise the proliferative capacity of neoplastic cells. In fact, the administration of high quantities of folic acid has been shown to enhance carcinogenesis in rodent models of colon cancer [266, 267]. This was only achieved in the presence of pre-existing neoplastic foci, creating a strong disposition towards increasing carcinogenesis development. By contrast, restricted access to folate derived methyl groups has been shown to diminish growth of neoplastic foci, i.e. the basis of antifolate chemotherapy drugs. Such work, including very recent research is controversial as it demonstrates the potential harm of too much folic acid, particularly given recent government mandated fortification programs. This public policy is elaborated on in the next section.

Finally, it is important to note that DNA methylation changes are not exclusively associated with cancer. It is believed that disrupted methylation also plays a role in arthrosclerosis development through immune and inflammatory responses [808, 809], in addition to the already discussed role of homocysteine. Furthermore, disorders of the central nervous system, such as dementia and depression have been associated both with folate deficiency and aberrant DNA methylation; this is likely through SAM

deficiency and possibly through altered neural plasticity in the central nervous system [810]. It is also important to note that although DNA methylation is dependent on normal one-carbon metabolism, other nutrients outside the B-group vitamins have been shown to be involved in DNA methylation and include: zinc, selenium, vitamin A, and even polyphenols and phytoestrogens [811].

DNA methylation is an important epigenetic feature of DNA that modulates gene expression and genomic integrity; damage to the genome resulting in aberrant gene expression is the fundamental cause of all cancer. Folate deprivation is a central factor in genome damage as it influences DNA methylation and stability; inadequate levels of folate may also lead to chromosomal breaks through uracil misincorporation. The next section discusses what impact this has upon DNA.

DNA Instability: As alluded to earlier, folate is absolutely essential for DNA replication and repair processes. The well-recognised deficiency state megaloblastic anaemia exhibits impairment in DNA replication as the underlying mechanism that precipitates this condition. Folate deficiency has been hypothesised to increase cancer risk either by disturbing DNA synthesis and repair [324, 812] or by inducing DNA *hypo*methylation and negatively affecting gene expression [794]. Folate in the form of 5,10-methylene-H_4PteGlu is required for the biosynthesis of thymidylate. Therefore, a limited availability of dietary folate and consequently cellular 5,10-methylene-H_4PteGlu means that a methyl group cannot be donated to uracil to form thymine. This causes an increase in the ratio between intracellular deoxyuridylate and deoxythymidylate; this in turn can promote the misincorporation of uracil into DNA [324]. The presence of uracil has been found to promote genomic instability leading to chromosomal fragile sites, chromosome breakage and micronucleus formation [327]. It is worthwhile noting that uracil misincorporation may not be solely due to folate status, as animal models have shown older age can enhance colonic susceptibility to uracil incorporation [813], as can deficiency of vitamin B_{12} [814].

Most of the evidence for the role of folate in uracil incorporation has come from a laboratory setting or individuals with a frank folate deficiency [163, 437, 813, 815, 816]. An animal model (mouse colon) found that very mild depletion of all four one-carbon related vitamins (B_2, B_6, B_{12} & folate) did not enhance uracil misincorporation, suggesting that significant deprivation is required to produce substantial elevations of DNA uracil content [803]. A low level of uracil in DNA has been observed even under normal conditions, and it is only when it becomes excessive that it can result in various types of genetic instability [792]. Fortuitously, human cells have several excision repair

systems that are designed to eliminate uracil residues and insert the intended nucleotide [817]. The process of removal begins with the creation of an abasic site, where DNA glycosylase recognises the damaged base and cleaves the N-glycosidic bond which releases the impaired base; this is then followed by a break in the phosphodiester backbone of DNA, creating a gap which is filled by the missing base with the help of DNA polymerase [792].

When folate insufficiency continues to occur, and uracil incorporation reaches a much higher than normal level, it can lead to single and double stranded DNA breaks [792]. Double stranded DNA breaks are much harder to repair and are considered to be highly mutagenic, as they can lead to gene deletions, chromosomal translocations and gene amplification of oncogenes [325, 818-820]. A vast amount of epidemiological evidence supports a role for low folate status in cancer risk. Excessive uracil incorporated into DNA is one of central paradigms of how inadequate folate might initiate and promote carcinogenesis.

Several human trials have attempted to find out whether supplementing with one-carbon metabolism related vitamins might change the level of uracil incorporation, Basten *et al.* [821] found that prior to intervention, a significant association existed between red cell folate and uracil misincorporation. After supplementation, a significant reduction in misincorporation was observed [821]. They also found that increasing folate status decreased the base excision repair capacity significantly in individuals with the lowest folate status prior to intervention [821]. However, there were no associated changes for increased supplemental folate status and a reduced level of DNA strand breakage or DNA methylation [821]. This association has not been consistently observed as van den Donk *et al.* [822] found the uracil content of colonic DNA did not diminish after a 6 month intervention of high dose folic acid and vitamin B_{12}.

Whether supplement intervention in humans can reduce uracil misincorporation remains ambiguous. Two rodent based studies have found diets deficient in several one-carbon related nutrients or in folate alone induce DNA strand breaks at a genomic level [823, 824]. Interestingly, they found that as a result of folate depletion, single genes in specific regions are far more susceptible to strand breakage then other regions of the genome [825]. The highly conserved regions (mutation cluster regions) in particular are more vulnerable, which is the site most frequently associated with mutations in human carcinogenesis [792]. The impact of folate deprivation results in uracil being inserted into a site where thymidine is intended resulting in a U:A mispair [792]. The consequences of this mispair are distortions in the structure of DNA which

can interfere with the genetic regulatory mechanism, leading to chromosome breakage and fragility, ultimately resulting in malignant transformation [792].

Out of all the vitamins, folate has been the most extensively studied because of its significant role in health and disease. The past 30 years of research has established links with low folate intakes and many common chronic diseases; prominent examples include classic megaloblastic anaemia and complications of pregnancy which include birth defects. But of most significance to this research is the role in CVD, cancer, cognitive health and depression. Clearly, the impact of folate deprivation in humans causes disruptions to folate-mediated one-carbon metabolism, which can result in molecular changes that impact DNA synthesis and stability, and create a toxic homocysteine environment. It is important to note that with all the knowledge we have on the role of folate in human health and disease, utilising synthetic folic acid as a disease preventive agent has had conflicting and limited success. There is no doubt that synthetic folic acid reduces homocysteine levels and of course there is the successful use in prevention of NTD. But, limited success has been found in relation to CVD and in the case of cancer, leading to many concerns being raised. The next section examines the issues related to too much folic acid and the potential negative impact this might have on disease prevention.

1.3.2. Folate excess

Upon examining folate in the context of insufficiency, it is necessary to also examine the concept of too much folate. Prior to 1943 when folic acid was first synthesised by Stokstad *et al*. [212], the concept of too much folic acid was never considered. The form of folate used to fortify foods is synthetic pteroylmonoglutamic acid, a pure crystalline substance. The synthetic form of folic acid is different from the natural forms of folate because it is in an oxidised state and contains only one conjugated glutamate residue [826]. One of the benefits of using synthetic folic acid is that it has a higher bioavailability and hence is easily absorbed, unlike natural polyglutamyl forms it isn't required to undergo enzymatic deconjuagtion in the small intestine before it can be absorbed [826]. A downside of using this form is it's not a normal metabolite and must be reduced to H_2PteGlu by the enzyme DHFR before it can be utilised in the folate pathway [312]. Recent research has found that DHFR activity can vary considerably between individuals and is lower in humans than in other animals that had previously been studied [314, 827-829]. An inability of this enzyme to reduce synthetic folic acid can lead to accumulation of this unmetabolised form in the systemic circulation, (elaborated on in detail below). An additional problem with synthetic folic acid is that it

is likely to compete with natural reduced folates for binding enzymes and proteins, and carrier proteins, resulting in interference in the normal cellular transport, metabolism, and regulatory functions of natural folates [827].

Past history has taught us a lot about under-nutrition and the consequences of diseases attributed to deficiency of micronutrients, however, access too much synthetic folic acid is a new development. No adverse effects have been reported with consumption of high amounts of natural folates in food [830] and concerns regarding the safety of high intakes are restricted to the synthetic folic acid form [264]. Most of the developed world, US, UK, Europe and Australia have set an upper tolerable intake limit (UL) at 1000µg per day exclusive of food folate, which is a guideline for the maximum amount individual adults should be exposed to [264, 831, 832]. The following section details the folic acid fortification programs that have been implemented, with the view that serious concerns exist surrounding these decisions.

1.3.3. Folic acid fortification

Just over two decades ago, the scientific community received conclusive clinical evidence that folic acid could prevent up to 70% of NTDs, which are known to be among the most severe of all birth defects [60]. This evidence resulted in many countries around the world adopting government-sponsored public health campaigns encouraging women of childbearing age to increase their daily folate intake. On top of this, organisations related to food standards permitted food industries to voluntarily add folic acid to cereals and other foods, and allowed advertising of the health benefit claims relating to prevention of birth defects [833, 834]. In addition to this, further research conducted during the mid-1990's also showed folate plays a major role in cardiovascular health and cancer prevention via mechanisms which include the reduction of homocysteine and altered methyl group metabolism [184, 230, 237, 835, 836].

So, with this body of evidence, the US quickly followed by Canada decided to enrich cereal grains with folic acid. As described above in section 1.2.6 (page 36) the US fortified all grain with folic acid at 140µg per 100g of flour [297]. During the past 13 years many countries have adopted mandatory programs, these programs vary in the level of fortification, with Chile at the highest level of 220µg per 100g and various food products being targeted from wheat flours and cereals to rice flour and milk [298, 299]. Just over 10 years after the US began its mandatory program, Australia adopted the same policy which required fortification of wheat flour at 120µg per 100g [52].

Proponents of mandatory fortification argued that although voluntary fortification had reduced preventable NTD cases to approximately 500 cases per year in Australia, it was not equitable across all of society resulting in inequality in some sub-population groups [832, 837, 838]. It was also noted that adding folic acid was very cheap, with a cost of less than 20 cents per ton of flour [838].

Other reports taken into consideration were that the US median serum folate levels in non-pregnant women of reproductive age had doubled since the mandatory program began [839]. Also, in countries where programs were implemented, significant reductions in NTD rates had been reported; US 30% [839], Canada 50% [840, 841] and Chile 70% [842]. At the time it was thought that mandatory fortification would not only help prevent NTD but also benefit the wider population, given the beneficial and central effect of folate in disease prevention (i.e. lowering of vasculo-toxic homocysteine) [843]. Some concerns had been discussed such as the perceived risks of masking pernicious anaemia in the elderly and the neurological manifestations that could be created as a consequence, and also the perceived risk of increased cancers. However, the wider benefit to the community was accepted and mandatory fortification was implemented.

At the time of preparing this thesis, limited analyses exist on how effective the fortification program has been in Australia. Brown *et al.* [729] published a retrospective analysis on the prevalence of low serum and red cell folate levels in a large public hospital diagnostic pathology laboratory. Although not necessarily a representation of the whole Australian population, they reported that between April 2009 and April 2010 there has been a 77% and 85% reduction in the prevalence of low serum folate and red cell folate levels respectively [729]. During that same time period they found a 31% increase in mean serum folate level (from 17.7nmol/L to 23.1nmol/L) and a 22% increase in mean red cell folate level (from 881nmol/L to 1071nmol/L) [729]. This indicates that the program is working effectively by reducing the prevalence of folate deficiency in Australia, including in women of childbearing age. At this point, limited information exists on the changes to NTD rates in Australia, and as discussed below there is concern that we may not see any dramatic change in NTD prevalence with folic acid fortification.

In the 13 years since the US led the way with mandatory fortification, increasing levels of concern are being raised. Early on, data reported that the fortification target is often exceeded in the US, reaching concentrations of 190µg/100g [261], and the daily intake of folate may greatly exceed predictions. This may be a concern for Australia as the

previous paper (Brown *et al*. [729]) also reported that in the same period the prevalence of serum folate levels >45 nmol/L (upper limit of assay detection in serum) increased 6.6% to 12.8%[729]. Additionally, the prevalence of red cell folate levels >2000nmol/L (upper limited of assay detection) increased from 6.9% to 7.9% [729].

Clinically relevant folate deficiency with megaloblastic anaemia has become very uncommon, although milder, usually subclinical folate insufficiency still exists, especially within some subsets of the general population [203]. This milder deficiency may be increasing in part due to the decreasing micronutrient density of contemporary western diets and food groups. In terms of fortification, there will always be an argument for a more considered and focused approach that takes subgroup variations, genetic influences, and individuals into account. Also, the effects of fortification may be impacted by the growing use of vitamin supplements in many Western countries such as Australia; this again adds concern that certain population sub-groups may be exposed to too much folic acid. The following section examines the potential harm that excess folic acid may cause.

1.3.4. Possible adverse effects of mandatory fortification of flour with folic acid

The ongoing controversy regarding the possible harmful effects of folic acid intake from fortified products has many scientists worried. Most are not challenging the established benefits of fortification in the prevention of NTD but are concerned that it may not be good for everyone and that some people may get too much. David Smith's commentary on the topic asked the correct question that – "is the benefit to the relatively few mothers and children sufficient justification for exposing the entire population to an increased intake of folic acid?" [169]. He asks this question because a UK advisory committee estimated that between ~77 and 162 NTD affected pregnancies would be prevented each year by fortification [264]. The same advisory group also estimated that for each infant saved through this program between 370,000 and 780,000 people will be exposed to extra folic acid [169, 264]. Although Australia's population is considerably smaller than the UK, extrapolating the data could potentially see a possible quarter of a million Australians being exposed to too much folic acid; within this population, it is possible that hundreds could be adversely affected.

What is also extremely troubling is US data published by Pfeiffer *et al.* [844] highlighted in David Smith's commentary [169] that since fortification, the population with the highest blood concentrations of folate is children under 5 years of age. Within this group 10% exceed the adult upper limit, the next cohort was children aged 6-11 years

and the third was people over 60 years and of those, 38% had exceeded the upper measurable limit of the assay at >45.3nmol/L [844]. Young children and elderly populations are not the intended group for the fortification programs but yet these groups are receiving much more than normal, this is particularly troubling in children who are undergoing rapid growth and development.

It is important to explore the potential harm and key evidence for concerns surrounding mandatory programs. There has been extensive research into the role of folate in cancer and it is becoming clear that this association has a temporal dimension to it. Also important are the effects on vitamin B_{12} deficiency, antifolate drugs, reproductive health, immune function and the unknowns associated with unmetabolised folic acid in the systemic circulation.

Dual modulatory role of folate in cancer: Despite the indisputable success of folic acid fortification in reducing NTD, the debate has not ceased; during the past 5 years an increase in evidence of folic acid's negative role in cancer has been uncovered. Folate is critically required for cell growth and division (described in the metabolism section on page 41), it acts as a cofactor in the *de novo* synthesis of thymidylate and purines which are central to nucleic acid synthesis and DNA repair [792]. Epidemiologic evidence has consistently suggested an inverse, dose-dependent relationship between folate status (measured by dietary and supplemental intake or blood biochemistry) and the risk of many forms of cancer exists. Breast, oesophagus, stomach, pancreas, lung, leukaemia are some of the many forms of cancer with this negative folate association, some of the best evidence however, exists for colorectal cancer [168, 247, 516, 845-848].

Many large prospective studies have suggested as much as a 40% reduction in the risk of colorectal cancer and its precursor adenomatous polyps in individuals with the highest intake of folate compared to individuals with the lowest intakes [168, 849]. This data is supported by several meta-analyses in which subjects with the highest folate intake compared with those with the lowest intakes confer a 20-25% reduction in risk of colorectal cancer [166]. A closer examination of research focused on folic acids possible beneficial effects on colorectal cancer risk and mortality in subjects that took a multivitamin supplement containing more than 400µg folic acid per day [237, 850]. Although, data was not consistent with the very recent Iowa Women's Health Study that reported in older women multivitamin and mineral supplements were associated with an increased risk of mortality, this did include a slight increased risk with folic acid use [851].

Cancer cells exhibit rapid cell division and DNA replication. Blocking folate metabolism inhibits tumour growth and development; this premise is the basis for antifolate drugs [169]. Given this, it seems paradoxical that epidemiological evidence supports high folate intake in protection against several cancer types. Moreover, other evidence from *in vitro*, animal and human studies has associated low folate status with altered DNA methylation, uracil induced genomic instability, DNA strand breaks and increased mutation; all precursors to carcinogenesis [163, 167, 437, 813]. Many studies have found negative results for the combined effects of folate on cancer; as far back as the late 1940's observations found folic acid administration in children with acute leukaemia resulted in an acceleration of the disease process [852]. But only recently have questions surrounding the type of folate been raised. There is some evidence now that utilising the synthetic folic acid vitamer to treat or prevent cancer may in fact promote cancer [162, 167, 170, 266, 267, 853].

A large study conducted by Mason *et al.* [265] examined colorectal cancer rates in the US and Canada. They demonstrated that concurrent with folic acid fortification these two countries experienced immediate reversals of the previous downward trend in colorectal cancer incidence that had been seen in previous decades [265]. Rates of colorectal cancer began to increase in the US in 1996 and in Canada in 1998 and reached a maximum in 1998 and 2000 respectively. The incidence has continued to exceed pre-fortification levels by as much as 15,000 extra cases per year [265]. Changes in endoscopic screening procedures do not seem to account for the increased rates [265]. Similar data is also present in Chile, where evidence exists for an increase in colon cancer rates since the introduction of folic acid fortification [854]. Importantly, Chile implemented one of the world's highest fortification levels at 220µg per 100g of maize flour making it valuable to examine the effect of fortification in follow up studies. The observational form of these studies does not prove causality but is consistent with other animal based studies on colorectal neoplastic cells. Several studies have found that if folic acid supplementation is started before the establishment of neoplastic foci, the development and progression of the tumour is suppressed [167, 266, 267]. Unfortunately, if the supplementation commences after the established presence of neoplastic foci, then folic acid was found to enhance growth and progression of the mutated cells [167, 266, 267]. This work has led many scientists to describe folate as having a dual role in cancer, or describing fortification as a double-edged sword, in which folate exposure including form and timing are critical in cancer formation [169, 849, 855, 856].

Other recent clinical findings include results from the large Aspirin-Folate Polyp Prevention Study, in which patients with a recent history of colorectal adenomas were involved in a double-blind, placebo-control study. The study found that using folic acid supplementation at 1mg per day for up to 6 years did not prevent the recurrence of colorectal adenomas [170]. During the 3 to 5 year follow-up period it was found that folic acid conferred a 67% increased risk of advanced lesions; lesions that had a high malignancy potential and a reported greater than 2-fold increased risk of having three or more adenomas [170]. This study raises serious concerns about the lack of efficacy of folic acid in colorectal cancer prevention and as previously suggested in an animal model, the likely cause of these results is that folic acid might have promoted the progression of already existing, undetected pre-neoplastic lesions.

Unexpected secondary findings were also found in the folic acid supplemented group of this study. The risk of cancers other than colorectal cancer was also significantly increased, specifically large numbers of prostate cancer [170]. In explaining these findings, 64% of the population were males and the mean aged was 57. It is therefore possible that some of the male patients harboured precursor lesions in the prostate, which developed more rapidly within a folic acid rich environment [170]. On commenting on this large study, Ulrich *et al.* [856] note that there was also no evidence for reduced cardiovascular effects through lowered homocysteine which is a central argument for supporting mandatory fortification.

Obviously, this study contradicts the vast majority of epidemiological studies that normally show an inverse relationship for folate status and cancer. Additionally, future research and studies of this type will become increasingly difficult to conduct in Australia due to the mandatory fortification program. Another recent research publication which used model-based predictions, suggested that folic acid fortification may reduce colorectal cancer rates if commenced early in life, however it can increase rates if begun after age 20 [857]. If this modelling study approximates reality, then mandatory folic acid fortification would be highly detrimental to a significant proportion of the Australian population.

More well designed studies are required to clarify the evidence for this dual role of folate in cancer formation, particularly in relation to dose of folate and timing. It is also worth considering that some of the contradictions in the research may be due to other biological factors. Indeed, folates effect on carcinogenesis could be due to a significant number of other factors, and is likely that patterns of these risk factors could differ between individuals. Some examples include observational studies in which smoking

and alcohol intake affect folate functioning in terms of cancer risk [268, 858]. But more importantly, folate has a requirement for other B-vitamins, particularly vitamin B_{12}, and is affected by genetic polymorphisms in one-carbon metabolism [499, 607, 859, 860].

Importantly, the effect of folate on cancer risk can be changed from a protective effect into a harmful effect simply based on which genotype a person carries. Discussed in an earlier section (1.2.11 Genetic variation within folate metabolism page 52) was a study that found a reduced risk of colorectal adenoma with a folate intake of less than 400µg per day in individuals that are homozygous for the 3-repeat TSER polymorphism [607]. Conversely, at the same level of folate intake, individuals homozygous for the 2-repeat TSER polymorphism were associated with an increased risk of colorectal adenomas [607]. Another study which examined the interaction of risk factors (nutrient-gene) found two high risk groups. The analysis spilt the cohort into folate intakes above and below the median and smokers and non-smokers, and examined the very common 677C>T MTHFR polymorphism. The results concluded that individuals that carry the CC genotype, and who smoked and had high folate levels have a greatly increased risk of either pre-neoplastic adenomas and/or hyperplastic polyps [506]. The other high risk group was found to be in carriers of the T allele. Interestingly, individuals with low folate that smoked had an increased risk but in smokers with high folate the risk for colorectal polyps no longer existed [506].

These two examples (there are many others), clearly demonstrate that it is not justifiable to assume that high folate in a whole population is the best for all people within that population. Despite undisputable epidemiological evidence of folate being protective in cancer risk, this appears not to translate into using folic acid as a “blanket” preventive measure. It is worthwhile mentioning a final study, which has also challenged previous cancer epidemiological studies. The recent Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial which included over 25,000 postmenopausal women found that when compared to non-supplemental users, subjects who took folic acid of more than 400µg per day had a 20% increased risk of developing breast cancer [268]. Interestingly, when analysed separately, food folate was not significantly linked to breast cancer risk but total folate mainly from supplementation significantly increased risk by 32% [268, 861].

The role of folate in cancer treatment and prevention so far consists of evidence that is inconsistent and often conflicting. What is clear is that folate has a dual modulatory effect and timing and dose are critical. The bulk of research has focused on colorectal cancer and more research into other cancer types may help understand the

relationship better. What is needed is better quantitative experimental data, which spans cancer aetiology and examines folate status in relation to epigenetics. Even prior to mandatory fortification, population exposure to synthetic folic acid was high when delivered through supplements and fortified foods. Unfortunately, mandatory fortification will make it increasingly difficult to research and examine this folate cancer relationship within a population such as in Australia. Either way, the question of what harm higher folate concentrations could have on the majority of the population requires immediate further attention.

Risks to older people: The strong interrelationship between folate and vitamin B_{12} was discussed earlier and is often the most commonly raised issue in relation to too much folic acid. That is excess folic acid could mask vitamin B_{12} status. The elderly population is at greatest potential risk of harm because of the age-related decline in vitamin B_{12} absorption. In particular, release of B_{12} from protein food sources, and increased autoimmunity against intrinsic factor are problematic in older people [862]. Knowledge that treating anaemia with high folate concentrations masks the hematologic signs of B_{12} deficiency was discovered a long time ago [863]. This masking can delay diagnosis and potentially lead to irreversible neurological damage [864]. Although clinically it is possible for this masking to occur, it is rarely seen. In fact, several studies into this phenomenon have shown little evidence that it actually occurs. Mills *et al.* [271] assessed the incidence of low serum B_{12} in megaloblastic anaemia patients before, during and post implementation of folic acid fortification and found no differences in serum B_{12} status. Also, the numbers of cases of pernicious anaemia or degeneration of the spinal cord have not increased post-fortification in the US [264, 865]. However, a Canadian study into women aged over 65 reported a 64% increase in serum folate concentration post fortification and a small increase in serum B_{12} concentrations [272]. However, unfortunately the prevalence of low B_{12} concentrations (<150pmol/L) in combination with supraphysiological concentration of folate (>45nmol/L) increased significantly post fortification [272], and it is this combination that is of greatest concern.

A 2005 study (US post fortification) reported an increased risk of cognitive decline in elderly persons who took folic acid supplements of 400µg per day or more [866]. Importantly, the cognitive decline was slower in individuals who also took high dose vitamin B_{12} supplements [866]. Two years later the same authors published a larger study and found people with normal folate status and low vitamin B_{12} concentrations had a massive 70% increased risk of cognitive impairment [784]. Unfortunately,

individuals with high folate (>59 nmol/l) and low B_{12} (<148pmol/L) have even greater risk, particularly when compared to individuals with normal levels of both vitamins. Therefore cognitive impairment associated with low vitamin B_{12} is made much worse by a high folate status; these findings are also supported by reports of low dose folic acid aggravating neurological symptoms in individuals with vitamin B_{12} deficiency, with the severity of impairment increasing with rising serum folate concentrations [867-869].

A possible explanation for this is that under high concentrations of synthetic folic acid, pteroylmonoglutamate may act as an antagonist at the enzyme DHFR, which is responsible for its conversion to H_2PteGlu. As H_2PteGlu can act as an inhibitor of MTHFR reducing formation of 5-methyl-H_4PteGlu, this may impair methionine synthesis. In people with low vitamin B_{12} levels, methionine synthesis is already compromised, and so high folate levels could worsen symptoms, consistent with data described in the above studies on cognitive health. These studies have greatly highlighted the concerns surrounding the US fortification policy and others implemented in different countries around the world, including Australia. The metabolic consequences of imbalances in folate and vitamin B_{12} status may well be a contributing factor in determining/modulating disease risk. This has led to increasing calls for fortification of flour to not be limited to just folic acid but to also include Vitamin B_{12} [870-873].

In the previously discussed publication on cognitive impairment (Morris *et al.* [784]) it was noted that in subjects with a normal vitamin B_{12} status, and high serum folate (>59nmol/L) there was a protective effect [784]. Consequently, if vitamin B_{12} status is normal, then fortification does greatly benefit you, but the same cannot be said if you have inadequate levels. Another publication using the NHANES data, pre and post-fortification examined vitamin B_{12} biomarkers; total homocysteine and methylmalonic acid [874]. These were examined in relation to a combined low B_{12}/high folate status. In the pre-fortification data, participants with low vitamin B_{12} had higher homocysteine, and as expected there was a trend towards reduction of homocysteine with high folate irrespective of vitamin B_{12} status [874]. In the post-fortification cohort a much higher blood folate concentration was reported and total homocysteine was overall lower (evidence of fortification program working). However, trends in subjects deficient in vitamin B_{12} suggested higher total homocysteine and high methylmalonic acid, which was seen even as folate levels increased [874]. These findings add weight to the idea that vitamin B_{12} should be added to the fortification policy, or at least there should be a rethink of the folate program.

Another area of concern relating to too much folic acid is in the context of CVD. Although not specifically confined to older people, the prevalence of CVD does increase with age and in general is much higher in older populations. The relationship between CVD and folate remains inconsistent despite the large number of observational studies/epidemiology that has shown low folate is a risk factor for this disease [748-753]. Fortification programs were generally implemented based on the evidence for NTD, but also often quoted was the potential benefit for lowering homocysteine levels and thus reducing risk of CVD. A cross-sectional study that examined mortality rates from stroke before and after fortification in the US and Canada observed a significant reduction [875]. However, the authors note that the decline in stroke mortality may be largely attributable to a reduced case-fatality rate [875]. Other studies have suggested that folic acid supplementation can effectively reduce the risk of stroke [755, 757, 876].

In relation to homocysteine related CVD risk, clinical trials using folic acid have found limited or no effect on CVD risk [256, 754, 755, 757], one meta-analysis of 37,000 people found no effect of folic acid supplementation after 5 years [756]. In terms of too much folic acid, one study found increased risk of myocardial infarction in patients who were treated with a combination of folic acid (800µg/day), vitamin B_6 (40mg/day) and vitamin B_{12} (400µg/day) [754]. Another study in patients who had balloon angioplasty found folic acid together with B_6 and B_{12} reduced the need for revascularisation interventions [877]. However, the more common treatment today is for coronary stents and one study found folic acid at 1.2mg/day increased the degree of re-stenosis, particularly in men [878]. To date no trial has convincingly shown that folic acid and other B-vitamins are beneficial in reducing heart disease risk, although no research has found largely damaging evidence for too much folic acid through fortification or supplementation. More targeted research is really required to examine the effect of natural folates as opposed to the synthetic folic acid form on CVD.

Impact on the effectiveness of antifolate drugs: Antifolate drugs are wildly used in the treatment of a vast array of diseases such as cancer, rheumatoid arthritis, malaria, psoriasis and epilepsy to name a few. These drugs aim to interfere with the folate metabolic pathway often by targeting enzyme actions within this complicated nexus. Most are in fact designed to inhibit the enzyme DHFR thus preventing the formation of H_4PteGlu, although, there are some other forms that aim to inhibit TS instead (e.g. 5-fluorouracil) [879, 880]. Because antifolates are generally designed to inhibit DHFR these types of drugs actively compete with folic acid for the active site of the enzyme.

Given this, it is possible that increasing folate concentrations could lead to an up-regulation of DHFR activity and thus lead to drug resistance. Antifolate resistance could also arise through multidrug resistance proteins – these transporters are up-regulated by exposure to raised concentrations of folates and this could enable transport of antifolates out of the cell [881]. A rise in folate concentrations through fortification could result in detrimental effects on the effectiveness of this class of drug.

A common antifolate is methotrexate; it was originally developed to be used in chemotherapy treatments, and was found to be effective in a range of cancers including childhood leukaemia, breast and lung cancer [882]. More recently, methotrexate has been effectively used in the treatment of autoimmune disorders such as; rheumatoid arthritis, psoriasis, lupus and crohn's disease [880, 883, 884]. In David Smith's commentary [169] he reported that over 10 million methotrexate prescriptions are written each year in the UK, he suggests that with such large number of people prescribed this antifolate it is possible that changes in the consumption of folic acid through fortification may interfere with the effectiveness of this drug [169]. Unfortunately, this area has received little attention – questions therefore remain about the effect of a person's folate status on the efficacy of this type of medication. It has been reported that head and neck cancer patients with higher folate intake experienced less treatment-related toxicities [885]. Despite this, there is concern that folate may interfere with the effectiveness of the antifolate treatment and possibly support tumour growth [281].

Research work conducted on rheumatoid arthritis patients also found negative results; a post hoc analysis of two randomised trials found patients who took 1-2mg folic acid per day had a poorer response to methotrexate [886]. Concerns surrounding antifolates have led to recommendations that supplemental folic acid should be restricted during methotrexate treatment [281, 887]. Unfortunately, where mandatory fortification occurs individuals have less control over their exposure. Research into this area requires a more focused examination, specifically of low dose folic acid equivalent to fortified levels to examine the likelihood of drug resistance forming.

Another antifolate drug is phenytoin, used in the treatment of epilepsy. The drug is known to decrease serum folate concentrations by half [273]. Supplementing with folic acid prevents deficiency, but it also modifies the drugs pharmacokinetics and may result in lower phenytoin concentrations. This could lead to poorer seizure control in patients with epilepsy, although evidence to support this is limited [274]. One population study of post fortification epileptic patients in Canada found no change in

serum phenytoin; fortified at 200µg/day the authors suggest that at this level it is unlikely that fortification would have an adverse impact on anticonvulsant drug metabolism [275]. Another drug family that may have its efficacy reduced in an enhanced folate nutritional environment relates to the antimalarial drugs. There is evidence of high dose 2.5-5mg/day of folic acid reducing the effectiveness of antimalarial drugs [888]. It is not known whether lower doses have the same effect, but either way this should be investigated further, particularly in African and Asian nations where malaria is prevalent and these countries should consider the impact of this before folic acid fortification is introduced.

The effect of too much folic acid through fortification or supplementation has not received enough thorough scientific attention; there are limited studies on folate status and its effect on antifolate drugs, this could have serious consequences for a large number of people. There is potential for people with psoriasis to have more attacks, epileptics to have more seizures and chemotherapy to be less effective in treating cancer - but all this remains uncertain due to a lack of comprehensive scientific investigation.

Reproductive health and neural tube defects: As described earlier, the central purpose for enacting mandatory fortification of folic acid in many countries was due to the overwhelming evidence that it can prevent NTD affected pregnancies. Today, many countries that have fortification programs in place regard it as a tremendous public health achievement; many countries have documented dramatic increases in blood folate concentrations and a concomitant decrease in NTD birth prevalence rates, which equates to a substantial cost savings in medical care [889]. There are still some questions that surround folates role in NTD prevention, particularly the question of what is the minimum effective blood folate concentration for the maximum prevention of NTD? This arises from a published study on an Irish cohort that found NTD risk continued to decrease in women with blood folate concentrations above the classical cut-offs for deficiency [735]. The authors speculate that NTD-affected pregnancies can occur among women who are not necessarily folate deficient [735].

Also, in 2009 Heseker *et al.* [890] argued that not all cases of NTD can be prevented by increasing the intake of folic acid. They demonstrated that in fortified populations, the degree of reduction in NTD prevalence affected by folate is related to the original baseline NTD prevalence [890]. The decline was independent of the amount of folic acid administered and apparently reveals a ‘floor effect’ for folic acid-preventable NTD [890]. This clearly shows that not all cases of NTD are preventable by increasing the

folate intake and that the relative decline depends on the initial NTD rate. They also argue that for the US, setting a target of reducing NTD prevalence by 50% is highly misleading, particularly when the Centre for Disease Control and Prevention report that prevalence has fallen only by 26% since fortification began [891]. This is likely attributed to the relatively low NTD prevalence of the general population in the pre-fortification period, and not to the level of folic acid in fortified foods as suggested by some people [890].

These authors note that countries with NTD prevalence close to the observed floor of approximately 5 cases per 10,000 births (such as Australia [892]), may have already reached the floor of folic acid-preventable NTD. It is too early to tell if this is the case for Australia although our prevalence rates match this theory; there is also evidence that NTD rates have been stable since 1998. Abeywardana *et al.* [892] reported that between 1992 and 2005 there has been a 26% decline in total NTD prevalence, but the main decline occurred prior to 1998. If the 'floor effect' occurs here in Australia and we don't see a further reduction in the prevalence of NTD after fortification, then greater consideration of the benefits of the program will be needed given mandatory fortification results in higher intake of folic acid for the whole population. It may expose a large number of non-target individuals to high levels of folic acid which might have unintended beneficial or harmful effects.

In addition to this 'floor effect', the expected benefits of folic acid fortification often assumes that nearly all causes of NTD are folic acid related and therefore are preventable. However, other environmental risk factors exist, such as alcohol and drug use [893, 894], periconceptional clomiphene use [895], maternal obesity [896] and vitamin B_{12} deficiency [897] , as well as possible genetic variants and disorders that may all play additional roles in NTD development outside of folic acid levels *per se* [898, 899]. It is particularly important to mention the role of vitamin B_{12}, as several studies have indicated low or deficient maternal B_{12} status and a significantly increased risk for NTD [900, 901]. Given the close relationship between folate and B_{12} many argue that adding B_{12} to fortified foods needs to be considered and that in a folate-fortified environment, a further fraction of NTD cases might be prevented by higher periconceptional intake of vitamin B_{12} [871] [873].

Multiple births: A multiple birth is often considered a positive event; however, they do carry greater risk when compared to singleton pregnancies for both mother and child. Greater rates of maternal and perinatal mortality exist with twin pregnancies [902]; also twin pregnancies are more likely to result in preterm delivery, and underweight babies

[903]. Other complications may include higher risk for pregnancy induced hypertension, postpartum haemorrhage and anaemia [903-907]. During the past two decades many Western nations have observed rising rates of twinning. In Australia the prevalence of twin pregnancies has gone from 9.0 per 1000 births in 1977 to 15.1 per 1000 births in 2000 [908]. The rate is highest in women aged 35-39, and a large proportion of the increase can be attributed to increasing maternal age, while some of the increase can be attributed to ovarian stimulation and assisted reproductive technologies [909, 910].

Several studies have reported associations between the consumption of multivitamins which include folic acid or folic acid alone by women during pregnancy and an increase of almost 40% in multiple births [911-913]. Because of this, a number of large studies have investigated whether folic acid fortification or supplement use is associated with an increased prevalence of multiple births. Li *et al.* [914] observed no association between folic acid consumption (400µg/day) and multiple births in a Chinese cohort, and Shaw *et al.* [915] investigated US rates of twinning both pre- and post-fortification (1990 to 1999) of foods with folic acid and also found no association of increased prevalence.

A more thorough study published in 2005 examined US birth records from 1990 to 2000; the analysis was restricted to nulliparous women and excluded any fertility treatments. A total of 25,065 twin and 3,362,245 singleton pregnancies were included, twin gestation rates were found to be stable from 1990-1996 (7.2 per 1000 births) then steadily increased 2.4% per year until 2000 (8.2 per 1000 births) [280]. The authors concluded that although the prevalence of twinning had increased following folic acid fortification the effects were less than forecasted in intervention studies and the rise was difficult to separate from the long-term increase in twinning rates [280].

In addition to this, *In vitro* fertilisation (IVF) has been shown to be a strong confounder in some studies. IVF is associated with both use of folic acid and with twin births [916]. Haggarty *et al.* [279] reviewed data from the US on multiple birth rates arising from IVF treatment following mandatory fortification and found that IVF treatment was associated with an 11-13% increase in the incidence of multiple births. In addition, they also found that the likelihood of twin births following a double embryo transfer was significantly associated with increased plasma/red cell folate concentrations and low age [279]. The authors suggest that the high incidence could be reduced by identifying women at increased risk of multiple embryo transfer based on plasma folate concentrations and age [279]. There is still much debate on whether or not folic acid fortification increases the likelihood of twin pregnancies. For normal pregnancies, the rates don’t appear to be

increasing outside pre-fortification rates, but with more women seeking medical advice for infertility, and therefore the increased use of IVF, it is likely that within this sub-population twinning rates may increase. It is possible that wide-spread fortification/supplementation with folic acid may represent a hazard larger than the postulated beneficial effect on NTD; continued research on this topic is still required.

Genetic/embryonic selection: As described earlier, the polymorphic variant 677C>T MTHFR is associated with altered folate metabolism. T allele carriers have reduced enzyme activity and instability which is associated with elevated homocysteine levels and aberrant epigenetic processes [445]. Because of this, it is thought that T allele carriers have a greater susceptibility to certain diseases. Importantly, T allele carriers in a high folate environment have a reduced DNA instability because MTHFR is protected from loss of its cofactor FAD [381]. The properties of this genetic variant has led to a theory that involves embryonic selection; evidence shows that in pregnancy a T allele infant has a greater risk of spontaneous abortion because of elevated homocysteine concentrations in the mother [442]. However, in theory if there is folic acid supplementation or indeed fortification creating a high folate environment, then the MTHFR enzyme becomes stable and homocysteine levels are lowered. As a result, the risk of miscarriage decreases and there is an increase in the number of infants surviving who carry the T allele [277]. This idea is supported by Spanish data that showed since the introduction of folic acid supplements for pregnant women there has been almost a doubling in the prevalence of the TT genotype [278], although a subsequent publication disagreed with these findings and attributed the results to sampling bias [917].

A more recent publication by Haggerty *et al*. [918] also found that there is no evidence to support the concern that folic acid fortification or supplement use in pregnancy results in selection of deleterious genotypes such as the 677TT MTHFR. The idea of this survival advantage for the 677T MTHFR SNP is concerning given this mutant allele is associated with a broad range of diseases from CVD [475, 476], to many cancers [492, 494, 506, 515], birth defects [444, 464], infertility [471], and with mental health disorders such as depression and schizophrenia [483, 484]. This concern led Lucock *et al.* [276] to suggest that folic acid supplementation could introduce a strong genetic selection pressure or a ‘genetic time bomb’ that results in long term effects that increase chronic degenerative disorders, potentially altering MTHFR gene frequency, and introducing a dilemma for the discipline of evolutionary medicine. More thorough

research is required on this topic and the long-term effect of fortification programs that involve folic acid.

Unmetabolised folic acid in the systemic circulation and immune function: The long term effect of high intakes of folic acid has raised concerns regarding the appearance of unmetabolised synthetic folic acid in the systemic circulation. As described earlier (page 38), in the human liver synthetic folic acid must be converted to H_4PteGlu by the enzyme DHFR in order for it to be fully utilized in one-carbon metabolism. Unfortunately, human liver has low DHFR activity, the reaction is slow and rate limiting and thus results in a poor ability to reduce synthetic folic acid [827]. It has been hypothesised that a modest, regular daily intake of synthetic folic acid could saturate the preliminary liver folate-monoglutamate pool and result in unmetabolised folic acid in the blood system [919]. Several studies have observed this occurrence in humans; most notable was a study that examined the appearance of unmetabolised folic acid in the systemic circulation in response to the consumption of fortified foods. Kelly *et al* [314] reported a threshold intake of 266 µg folic acid per meal at which point unaltered folic acid appeared in the serum samples.

These results are also consistent with the reports of unmetabolised folic acid in plasma and urine [920] and observations of American subjects post fortification displaying the appearance of unmetabolised folic acid in the circulation [921]. This may be further compounded by a 19bp deletion SNP for DHFR, for del/del carriers it has been shown that intakes of more than 500µg per day increased the prevalence of high circulating levels of unmetabolised folic acid compared to wild-type carriers [555]. Overall, there is insufficient data in humans to assess the long-term effects of exposure to unmetabolised folic acid in the systemic circulation; it is likely that this could be detrimental, particularly in individuals with existing, undiagnosed premalignant and malignant lesions, or in individuals with vitamin B_{12} deficiency as described earlier. Other yet unidentified areas of disease development could also be implicated, but further research is required to elucidate such effects.

In association with this saturation of folic acid in the body, other research has also uncovered a role in the correct functioning of the immune system. Natural killer cells are an important part of a healthy immune response; their roles include fighting viral infections and destroying cancer cells [282, 922]. In the same study that observed unmetabolised folic acid in subjects post fortification, an inverse relationship was discovered between natural killer cytotoxicity and the presence of unmetabolised folic acid in plasma, and was most evident in women over 60 years of age [921]. The study

of postmenopausal women also found women whose dietary folic acid intake was less than 233µg per day, and who took a daily supplement containing up to 400µg, displayed better immune function than those who did not take a supplement [921]. However, the women who consumed more than 233µg per day and took a supplement had impaired natural killer cytotoxicity [921]. This clinical evidence suggests another way in which excess folic acid might promote existing malignancies is via the role of natural killer cells in tumour cell destruction which is the first line of defence against cancer. Clearly, excess folic acid from supplements and fortification can impair normal immune function, however like all areas covered in this review research is ongoing.

Overall, there is insufficient data in humans to assess the long-term effects of exposure to unmetabolised folic acid in the systemic circulation. The relationship between folate and health outcomes is extremely complex, synthetic folic acid is unnatural and the human body has a limited ability to convert it into a usable form. Even prior to mandatory fortification, concerns were expressed. These still remain and others are only just emerging. Masking of the haematological symptoms of vitamin B_{12} deficiency was the biggest concern prior to fortification; the 'hottest' topic now is evidence that undiagnosed premalignant lesions could be enhanced in a high folic acid environment, despite vast amount of epidemiological evidence that puts a low folate status and increased cancer risk together.

Mandatory fortification was introduced specifically to prevent NTD and although there is still a continuing need for women of child-bearing age to be exposed to folic acid, the method of fortification of wheat flour exposes the entire population. Non intended population subgroups such as elderly people could be at greatest harm from fortification, as high rates of vitamin B_{12} malabsorption exist and there is now evidence that points to harmful effects of an imbalance between folate and vitamin B_{12}. This has led some people to call for a fortification program to include vitamin B_{12} [871-873]. Additionally, colorectal cancer is associated with an ageing colon and with this, new evidence on folate and colorectal adenomas adds more weight to the dangers older people face if exposed to too much synthetic folic acid. Furthermore, fortification comes on top of the unregulated consumption of over the counter vitamin supplements that often contain folic acid, and the ready availability of voluntary fortified foodstuffs such as breakfast cereals (often at 100µg per serve or more).

Folate-mediated one-carbon metabolism is a very complex and sensitive biological process, current research is insufficient to determine what impact high levels of folic acid has on these processes, and the impact on humans in relation to disease

development. Importantly, variations across populations, lifecycle stages and individual genetic variations all impact on how we determine whether more folate is necessarily beneficial in all circumstances, and whether the benefits of a mandatory fortification program outweigh the possible deleterious effects.

1.4. Thesis Scope

Folate is an essential B-group vitamin that has long been known to play an important role in maintaining human health. It is one of the most intensively researched vitamins, particularly in the past 20 years since the discovery that it can prevent NTD. As explored earlier, folate metabolism involves proteins that are responsible for the uptake and retention of folates, as well as the conversion of one-carbon units that are utilised in an array of biological reactions. As a series of biological cycles, one-carbon metabolism can be conveniently divided into two segments that are heavily interdependent on each other; the methylation cycle in which methionine is generated through the remethylation of homocysteine, in the process synthesising the vital methyl donor SAM [319]. The second segment involves nucleotide synthesis where 5,10-methylene-H_4PteGlu donates a methyl group to TS, enabling the reaction that produces dTMP [318]. Proper functioning of folate metabolism requires several other key B-group nutrients to acts as cofactors for various enzymes, namely vitamin B_{12}, B_6 and B_2. Many polymorphic genes encode these folate related enzymes, several of which can modify gene regulation and expression.

It is important to consider this as a complex metabolic system that contains many regulatory processes and feedback mechanisms, any disturbance to the system causing stress in the pathway can result in phenotypic effects. The premise of this thesis is that the stress of inadequate intake and/or metabolism of folate causes disruption to homeostasis and can result in phenotypic traits that lead to some of our most important chronic degenerative diseases. For example, the genetic polymorphism 677C>T MTHFR is strongly associated with high homocysteine concentrations and low folate status.

As outlined in section 1.2.11 (beginning on page 52), there are many polymorphic genes that encode enzymes involved in the folate-related one-carbon transfer cycles. Tabulated below is a summary of the eleven key polymorphic variants examined in this thesis and the functional role their associated enzymes play in metabolism

Table 1-5: Summary of key polymorphic variants examined

Encoded Protein	Polymorphic Variants	Functional role
Methylenetetrahydrofolate reductase	677C>T MTHFR 1298A>C MTHFR	MTHFR, involved in folate metabolism and maintaining the reduced folate pool used for the synthesis of DNA
Dihydrofolate reductase	19bp deletion DHFR	DHFR, responsible for the oxidisation of H_4PteGlu to H_2PteGlu
Methionine synthase and Methionine synthase reductase	2756A>G MTR 66A>G MTRR	MT and MTRR, are vitamin B_{12}-dependent enzymes that regulate the methionine cycle and modify homocysteine levels
Serine hydroxymethyltransferase	1420C>T SHMT	SHMT, catalyses the reversible transfer on one-carbon units from 5,10-methylene-H_4PteGlu to glycine to form serine and H_4PteGlu
Thymidylate synthase	3'UTR 6bp deletion 2R>3R repeat/insertion	TS, synthesise dTMP from dUMP
Catechol-O-methyltransferase	1947G>A COMT	COMT, catalyses methylation of endogenous catecholamines
Reduced folate carrier and Glutamate carboxypeptidase II	80G>A RFC 1561C>T CGPII	RFC and GCPII, regulate uptake of dietary folate and its subsequent cellular translocation

In addition to these SNPs, this thesis examines the biochemical values of four key amino-thiols. Although, folate is critical in the remethylation of homocysteine to methionine, homocysteine can also be converted to cysteine with cystathionine acting as an intermediate. Beyond transsulphuration, cysteine is a precursor to the important antioxidant glutathione. During the catabolism of glutathione, the pro-oxidant thiol cysteinyl-glycine is generated. Both the determination of these key thiols and the genotypes described are combined with biochemical markers of folate status (serum folate, red cell folate and serum vitamin B_{12}) and the estimated nutrient intakes (natural and synthetic folate). Together these indices paint a clear picture of folate homeostasis. The metabolic profile provided by all these factors has been combined with clinical outcomes to help explain the basis of several important phenotypic traits.

Australian Institute of Health and Welfare (AIHW) estimates that by 2036, the number of people aged 65 years and over is expected to more than double, from 2.7 million to 6.3 million, which will represent 24% of the total population, up from 13% currently [923]. It is therefore pertinent to look at this age group as health costs tend to be most concentrated in this group. Chapter 3 will examine an elderly population (over 65 years) for hypertension and depression. Hypertension is a major risk factor for CVD, which is the single greatest killer of Australians. Hypertension is reported to affect as many as 30% of people over the age of 25 [62]. Additionally, in the 2012 Australia

Health Report [62], anxiety and depression are listed as the second leading cause of disease burden behind coronary heart disease, which is particularly prevalent in the elderly. Chapter 4 will also examine the same folate, thiol and genetic relationships, but the phenotypic trait examined is AD. The final study (chapter 5) will examine a population over 40 years of age who have a diagnosis of adenomatous polyps, which are often precursors for colorectal cancer. This study will again examine nutritional biochemistry and genetic data to establish any relationship to pre-malignant cancer. Additionally, a closer look at the relationship of synthetic folic acid will be conducted given recent data on the harmful effect it may have in colonic mucosa [167, 170, 266, 267, 849]. The next chapter will discuss the methodology employed for each of these studies.

CHAPTER 2

METHODOLOGICAL APPROACH

2. Overview

This chapter describes the basic methodological approaches used to investigate gene-nutrient interactions within defined populations that reflect the common chronic degenerative disease of interest. Analyses include biochemical measurements, determination of gene polymorphisms, estimation of total dietary intake of folates and the applied statistical analysis of the data. For the sake of clarity some specific study and clinical information is presented on a chapter-by-chapter basis.

2.1. Biochemical Measurements

2.1.1. Blood collection and handling

Blood collection varied slightly between each population cohort. Due to logistical arrangements, the participants recruited for the study 'B-vitamin genetics and risk for adenomatous polyps' (chapter 5) donated non-fasting blood. This was due to issues with the clinic location, allocation of staff time and funding. However, in all other study populations, participants fasted for at least 10 hours prior to blood collection, which was conducted first thing in the morning by a trained phlebotomist. Each study was conducted at a different location, varying from hospital clinics, nursing homes, and retirement village offices (for specific clinic details see subsequent chapters). Approximately 30ml of blood was collected from each participant; blood was collected in 2mL Vacuette®-ethylenediaminetetra-acetic acid (EDTA)-tubes for genetic analysis, 4mL Vacuette®-EDTA-tubes for red cell folate measurements, two 4mL anticoagulant free tubes for serum folate and serum vitamin B_{12}, and two 8mL Vacuette®-Heparin-tubes containing a gel separator for thiol determinations. (Vacuette®-tubes supplied by Interpath Services, Heidelberg West, Victoria (VIC)).

The collected blood tubes were stored on ice during clinic, and if required were centrifuged (3000xg for 10 minutes) all within 30 minutes of venepuncture. The whole blood assigned for genetic analysis and the separated plasma and serum samples were return to the Molecular Nutrition PC2 Laboratory, University of Newcastle, Ourimbah, NSW. Once there, the samples were aliquoted into cryo-vials and stored at -80^{o}C until required for DNA extraction and thiol determination. All other tubes were couriered to Westmead Hospital, Westmead, NSW.

2.1.2. Assay of red cell folate, serum folate and vitamin B_{12}

The Vacuette® tubes for the vitamin assays were stored on ice and transferred within an hour of venepuncture to the Institute of Clinical Pathology and Medical Research (ICPMR) at Westmead Hospital. Once there, the blood levels of red cell folate, serum folate and vitamin B_{12}, were measured using an automated Access Immunoassay System.

The instrument used was a Beckman Coulter DXI 800. The Access Folate assay is a competitive-binding receptor assay. For the assay of folate in red blood cells, a whole blood sample is first treated off-line with a lysing agent composed of ascorbic acid and folate free human serum albumin. Serum samples are treated to release folate from endogenous binding proteins. After neutralization of the reaction mixture, folate-binding protein, mouse antifolate-binding protein, folic acid-alkaline phosphatase conjugate, and goat antimouse capture antibody coupled to paramagnetic particles are added to the reaction vessel.

Folate in the sample competes with the folic acid-alkaline phosphatase conjugate for binding sites on a limited amount of folate-binding protein. Resulting complexes bind to the solid phase via mouse antifolate-binding protein. After incubation in a reaction vessel, materials bound to the solid phase are held in a magnetic field, while unbound materials are washed away. The chemiluminescent substrate Lumi-Phos 530 is added to the vessel and light generated by the reaction is measured with an illuminometer. The light production is inversely proportional to the concentration of folate in the sample. The amount of analyte in the sample is determined from a stored, multipoint calibration curve [924]. The normal laboratory reference ranges are recorded in the table below.

Table 2-1: Reference ranges - ICPMR lab guide

	Normal laboratory reference range	Clinical deficiency levels
Red Cell folate	370-1050 nmol/L	< 370nmo/L
Serum folate	5 to 21 nmol/L	< 5nmol/L
Vitamin B12	125-780 pmol/L	< 107pmol/L

The Access Vitamin B_{12} assay is a competitive binding immuno-enzymatic assay. The sample is added to a reaction vessel along with alkaline potassium cyanide and dithiothreitol, this treatment denatures B_{12} binding proteins and converts all forms of the vitamin to the cyanocobalamin form. After neutralization, intrinsic factor-alkaline

phosphatase conjugate and paramagnetic particles coated with goat anti-mouse IgG: mouse monoclonal anti-intrinsic factor are added to the sample.

Vitamin B_{12} in the sample then binds to the intrinsic factor conjugate, preventing the conjugate from binding to the solid phase anti-intrinsic factor. After incubation in a reaction vessel, separation in a magnetic field and washing removes materials not bound to the solid phase. A chemiluminescent substrate, Lumi-Phos 530, is added to the reaction vessel and light generated by the reaction is measured with an illuminometer. The photon production is inversely proportional to the concentration of vitamin B_{12} in the sample. The amount of analyte in the sample is determined by means of a stored, multi-point calibration curve [925]. See table 2-1 above for vitamin B_{12} reference range. It is important to note that other indices may potentially offer improved sensitivity in vitamin B_{12} determination. These include assays for serum holotranscobalamin and serum methylmalonic acid. These were not available in the present study, however if employed they may have offered additional useful information. Future studies might benefit from their adoption.

2.2. Plasma Determination of Thiols

In the past decade, there has been considerable interest in plasma homocysteine determination; this has led to the development of many different methods of analysis. Generally, homocysteine can be measured by either chromatography or enzyme immunoassays. Enzyme immunoassays are highly accurate and are often used in research and clinical laboratories, however this method is only applicable for the determination of homocysteine and is not possible for other thiols. Chromatography was chosen because of its ability to assess not only homocysteine concentration, but also glutathione, cysteinyl-glycine and cysteine concentrations. The only downside of this method is that it requires expensive equipment and reagents as well as being more labour intensive.

The determination of plasma thiol concentration was conducted using HPLC with fluorescence detection, a method evolved from Dudman *et al.* [926] & Krijt, *et al.* [927]. This method uses a fluorogenic 7-Fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid ammonium salt (SBD-F) and also Tris(2-carboxyethyl)phosphine (TCEP) to reduce the disulfide group.

2.2.1. Equipment and chromatographic conditions

The HPLC analysis was performed using a Varian ProStar System™ (Varian, Clayton, VIC) with two pumps (ProStar 210); the system also contained a ProStar 430 auto sampler, which was fitted with a 100 µl sample loop and a tray temperature control set at 8^0C. Separation was performed using an analytical Sphereclone™ 5µODS(2); 250 x 4.60 mm 5 micron column, protected by Security Guard™ C18 4.0 x 3.0mm guard column (Phenomenex, Pennants Hills, NSW), stored in a column oven at 25^oC. Using the Varian ProStar 363 fluorescence detector, the fluorescence intensities were measured with the excitation wavelength set at 385 nm and emission wavelength set at 515 nm. The detector signal was recorded and the peak area was quantified using Star Chromatography Workstation™ version 6.0 software.

The mobile phase consisted of (A) 4% methanol in 0.1M Sodium Acetate pH4 and (B) 15% Methanol in 0.1M Sodium Acetate pH4 (Sigma-Aldrich, Castle Hill, NSW). The HPLC mobile phase was prepared on the day with a Millipore Milli-Q water purification system with resistivity greater than 18 Mµ cm (Millipore Australia, North Ryde, NSW). The solvents were filtered through a 0.45 µm cellulose filter and degassed prior to use. At a flow rate of 1 ml/min, 100% mobile phase A was run for the first 3.6 minutes. The system was then switched to 100% mobile phase B between 3.6 and 7.2 minutes and then switched again to 100% mobile phase A for 10 minutes before the next injection to allow the column to re-equilibrate on solvent A.

2.2.2. Assay reagents and standards

The assay required the following chemicals; 4M potassium hydroxide, 1M sodium hydroxide, 0.5M potassium borate / 5mM disodium EDTA (pH10.5), 0.1M potassium tetraborate / 2mM disodium EDTA (pH 9.5), 0.6 M perchloric acid/1mM EDTA solution, 15% (w/v) TCEP. All chemicals were supplied by Sigma-Aldrich (Castle Hill, NSW) these were made according to standard operating procedure using deionised water and stored appropriately until use in the derivatisation process.

SBD-F 1mg/ml was also prepared but due to a short shelf life and daily runs requiring less than 1 ml per run, the solution contained 1mg of the ammonium salt dissolved in 1 ml of 0.1M potassium tetraborate/2mM disodium EDTA (pH 9.5). The stock standard solutions of cysteine (10mM), cysteinyl-glycine (10mM), glutathione (1mM) and homocysteine (5mM) were prepared in deionised water, along with N-(Mercaptopropionyl)-glycine (10mM) this was treated as the internal standard. These

solutions were aliquoted into cryo-vials and stored at -84ºC until used in the making of the working standards.

Four working standard solutions were prepared by combining the thiol stock standard, along with the internal standard diluted in 0.1M potassium tetraborate/ 2mM disodium EDTA buffer (pH 9.5). The working standards included a blank, low, medium and high concentrations based on previously defined calibration curves. (see table 2-2 for concentrations). The working standard solutions (0 – 3) were stored in 1ml aliquots in the freezer and used within 10 days of preparation.

Table 2-2: Thiol concentrations of the working standard solutions

Working Standard	Cysteine (µmol/L)	Homocysteine (µmol/L)	Cysteinyl-glycine (µmol/L)	Glutathione (µmol/L)
0	0	0	0	0
1	50	5	25	5
2	200	15	100	15
3	400	35	150	20

In order to maintain quality results and minimise variations between runs that could extend over weeks or months, each run contained a duplicated control, known as 'pool plasma'. Pool plasma was collected from healthy volunteers, the separated plasma was pooled into one 50mL falcon tube, and this was vortexed and spun, then aliquoted into (500µl) cryo-vials. The pool plasma control was stored at -80^{o}C until used for each derivatisation. In addition to this use as a quality control, the pool plasma control was used to make a standard curve for each assay run.

2.2.3. Plasma thiol derivatisation

On the day of derivatisation, the first step was to ensure all equipment required was on and ready for use, the water bath was set at 60^{o}C, the HPLC was functioning at 1mL/per min and the peltier was cooled to 8^{o}C. Two identical sets of 1.5ml microcentrifuge tubes were labelled according to standard number, control number, and sample identification. One aliquot of pooled plasma, the four working standards and the study samples were taken out of the freezer (-84°C) to defrost at room temperature for 30 minutes. Once defrosted the pooled plasma aliquot and the study samples were centrifuged at 14,000 rpm for 15 minutes to sediment any fibrin clots.

During this time, 20µl of working standard 0 (blank) was added to the appropriately labelled tubes including the water blank, as well as all the participant samples being analysed. 20µl of working standard 1, 2 and 3 was also added to the appropriate

labelled tubes. Once spun, 60µl of cleared pooled plasma was added to all the standard tubes (60µl of HPLC grade water was added to the blank), and 60µl of the participant samples were added to the corresponding labelled tubes.

Then, 8 µl of the reducing agent TCEP solution was added to all samples before incubating them at room temperature for 30 minutes. 50µl of 0.6M perchloric acid/1mM EDTA solution was then added to the plasma samples, in order to denature the plasma proteins and release the thiols. All tubes were then vigorously vortex mixed and rested at room temperature for 5 minutes. Samples were then centrifuged for 10 minutes at 14,000rpm in order to separate the denatured proteins. 40µl of the clear supernatant was aliquoted off into a new second set of microcentrifuge tubes and 80µl of 0.5M potassium-borate/5mM EDTA solution (pH 10.5) was added. Finally, SBD-F solution was added (40µl) to all tubes and the samples were incubated in a water bath at 60^{o}C for 1 hour.

All samples were cooled to ~5^{o}C, then vortex mixed and centrifuged for 5 minutes. Finally, the samples were transferred into 100µl inserts that were held within brown HPLC autosampler vials, 20µl of each sample was sequentially injected onto the HPLC column by an auto-injector. All plasma samples were derivatised and analysed by HPLC during the same day, with analysis runs lasting up to 16 hours. A typical run consisted of a blank (water), two quality control samples, 4 working standard curve samples and 6 study samples, all samples were derivatised in duplicate and injected on the HPLC column twice. The control samples and the working standards were analysed at the beginning and at the end of each run, in order to control for any drift during the runs. The elution time of each plasma thiol was determined using the pooled plasma samples spiked with known amounts of the pure thiols along with internal standard. See figure 2-1 for typical chromatogram identifying the individual thiols and internal standard.

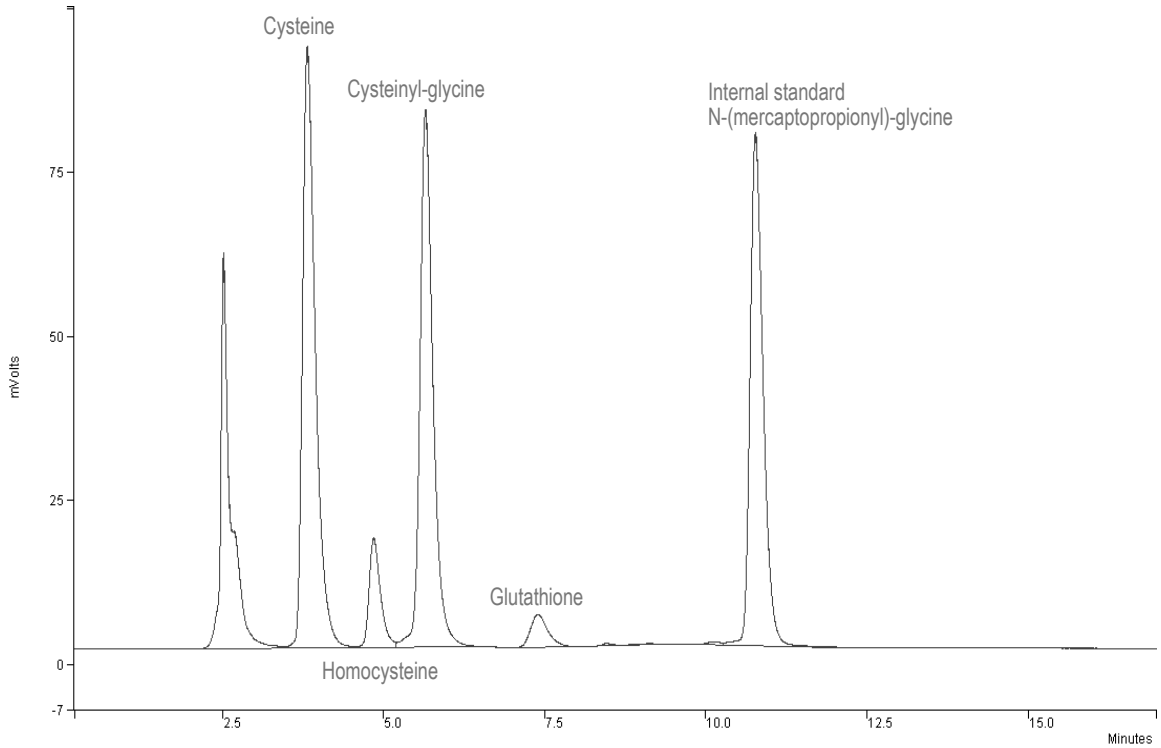

Figure 2-1: Typical chromatogram of the plasma thiols with internal standard

2.2.4. The standard curve and calculation of thiol concentrations

A standard curve was determined for each run by plotting the peak area ratios [peak area of thiol/peak area internal standard] against the known concentration of the thiols in each working standard solution (table 2-2). The standards were derivatised in duplicate and then injected at the start and end of each run and the mean slope values were calculated. The concentration of the thiols in each of the participants' plasma samples was calculated by dividing thiol peak area ratios by the mean standard curve slope of the individual thiols. The final concentrations of the thiols in the participant's plasma samples were derived as the mean value of the sample duplicates. Note; if any results fell outside the standard curve ranges found in table 2-1 then the sample was repeated, or diluted if the duplicate gave the same value (<1% required dilution).

2.2.5. Intra- and inter-assay coefficients of variation for plasma thiols

Intra and inter assay coefficients of variation (CV) for the individual plasma thiols were determined using the quality control plasma samples as shown in tables 2.2.

Table 2-3: Mean thiol value for quality control

		Homocysteine (µmol/L)	Cysteine (µmol/L)	Cysteinyl-glycine (µmol/L)	Glutathione (µmol/L)
Intra	n	21	21	21	21
	Mean (µM)	11.05	288.46	23.21	10.62
	SD	0.17	0.49	0.06	0.16
	CV (%)	1.61	0.17	0.24	1.55
Inter	n	18	18	18	18
	Mean (µM)	11.60	280.75	22.74	10.40
	SD	0.34	6.70	0.63	0.44
	CV (%)	2.93	2.38	2.77	4.23

Note: inter assay variation based on number of runs over 6 months

Based on the above validation it can be determined that this method provides a reliable technique for the analysis of plasma thiols.

2.3. Gene Polymorphism Detection

The studies undertaken in this thesis evaluated eleven key SNPs that are at or close to the nexus of folate and thiol homeostasis;

1. 677C>T MTHFR
2. 1298A>C MTHFR
3. 19bp deletion DHFR
4. 2756A>G MTR
5. 66A>G MTRR
6. 1420C>T SHMT
7. 3'UTR 6bp deletion TS
8. 2R>3R TSER
9. 1947G>A COMT
10. 80G>A RFC
11. 1561C>T GCPII

Polymorphisms were scored using the restriction fragment length polymorphism (RFLP) method, with the exception of 19bp deletion DHFR (nested primer allele-specific strategy) and 2R>3R TSER (tandem repeat polymorphism). The following sections describe RFLP analysis in detail.

2.3.1. Polymerase chain reaction

Polymerase Chain Reaction (PCR) is a technique commonly used to amplify DNA prior to SNP analysis. In this dissertation, gene variants encoding folate related proteins are being examined, although this technique can be applied to a wide range of genetic analyses. Amplification of the target DNA sequence containing the area where the polymorphism occurs was based on the methodology first identified in key research

papers displayed in table 2-4 below. The reagents required for PCR included; Go Taq Green Master mix® (Promega, Annandale, NSW) which is a premixed ready to use solution containing bacterially derived *Taq* DNA polymerase, deoxyribonucleoside triphosphate (dNTPs), $MgCl_2$ and reaction buffers. Invitrogen (Mt Waverly, VIC) supplied custom primers in dehydrated form, later reconstituted and diluted as required. The primer sequences used and the size of the fragments of each gene amplified by PCR are described in table 2-5.

All PCR reactions were performed at a final volume of 30μL in 0.2ml thin wall PCR tubes (Interpath Services, Heidelberg West, VIC). The reaction contained the primers, along with extracted DNA, sterile water and Go Taq Green Master Mix®. For more sensitive reactions, mineral oil was also added. The samples were gently mixed, spun, and placed in a thermal cycler (Bio Rad iCycler™ or MyCycler™ Gladesville, NSW). In general, this combination was heated to between 92-95 °C causing the double-stranded DNA to denature to single strands. A change in temperature to ~56°C then allows the primers to anneal to their complementary strand. The temperature was then raised to 72°C, which is the optimal temperature for Taq polymerase to extend the primers along the annealed strand in the 5'→3' direction using complementary nucleotide bases.

This three-step cycle, which defines PCR, is typically repeated around 35 times, resulting in the newly synthesised DNA strands being separated from the templates and hence doubling the amount of DNA for each PCR cycle performed. This repetition results in an exponential increase in the amount of target sequence producing more than 10^7 copies [928]. The optimal PCR reaction mixture and temperature varied for each polymorphism examined, further details are described in

table 2-6. In an attempt to amplify DNA efficiently and specifically, it was often necessary to optimise the PCR conditions. This was done by either adjusting the primer annealing temperature during thermal cycling or when using a new set of primers, via titration of the magnesium concentration ($MgCl_2$). PCR can often be troublesome, therefore, it is important to ensure an optimal laboratory environment; this includes a high level of cleanliness. The most common problem with PCR is

contamination from other DNA present in the laboratory environment (bacteria, viruses, errant human DNA etc). Therefore, having clean benches, pipettes and equipment is key to avoiding such contamination.

Where possible, DNA sample preparation, reaction mixtures, the PCR process and reaction product analysis was performed in specially designated areas of the laboratory. The reagents for PCR were prepared separately and aliquots were stored away from other DNA samples. A control reaction, omitting template DNA was always performed to confirm the absence of contamination. Fresh gloves were always used for each PCR step, as were displacement pipettes with aerosol filters. All genotyping completed as part of the present thesis was done in the Molecular Nutrition PC2 Laboratory at the University of Newcastle, Ourimbah.

2.3.2. DNA extraction

The first process in gene polymorphism scoring is DNA extraction this was achieved in all studies using the following method. A QIAamp (Qiagen Clifton Hill, VIC) DNA blood mini-kit was used to extract DNA from whole blood using the blood and body fluid spin protocol. Using this protocol, 200µl of whole blood yields approximately 6µl of DNA in 200µl of elution buffer (30ng/µl).

The extraction process begins with 20µl of Qiagen proteinase K (20mg/ml) which was added to 200µl of whole blood, along with 200µl of lysis buffer. The contents of the tube was then mixed by pulse-vortexing for 15 seconds and incubated at 56°C for 10 minutes. After incubation, the samples were centrifuged at 8,000rpm for 30 seconds. Then 200µl of 100% ethanol (Sigma-Aldrich, Castle Hill, NSW) was added to the tubes and again the samples were vortex mixed and spun. The samples were carefully placed into QIAamp spin columns, and centrifuged at 8,000rpm for 1 minute. The collection tubes and filtrate were discarded and replaced with new sterile collection tubes. 500µL of a buffer solution was added to the precipitate, which was then centrifuged at 14,000 rpm for 3 minutes. The QIAamp spin column was transferred into a clean 1.5mL tube and the previous step was repeated to further purify the DNA sample. The QIAamp column was transferred into a clean 1.5mL microcentrifuge tube and 200µL of elution buffer solution was added to the DNA precipitate. The tube was incubated at room temperature for 1-3 minutes and then centrifuged at 8,000rpm for 1 minute. The eluted DNA sample was stored at -20^{o}C until required for the PCR process.

Table 2-4: Key discovery papers

Polymorphism	Key Discovery Papers	Ref
MTHFR 677C>T	Frosst, P., et al., **A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase**. Nat Genet, 1995. 10(1): p. 111-3.	[416]
MTHFR 1298A>C	Weisberg, I., et al., **A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity**. Mol Genet Metab, 1998. 64(3): p. 169-72.	[417]
DHFR 19bp	Johnson, W.G., et al., **New 19 bp deletion polymorphism in intron-1 of dihydrofolate reductase (DHFR): a risk factor for spina bifida acting in mothers during pregnancy?** Am J Med Genet A, 2004. 124A(4): p. 339-45.	[418]
MTR 2756A>G	van der Put, N.M., et al., **Sequence analysis of the coding region of human methionine synthase: relevance to hyperhomocysteinaemia in neural-tube defects and vascular disease**. Qjm, 1997. 90(8): p. 511-7.	[419]
MTRR 66A>G	Wilson, A., et al., **A common variant in methionine synthase reductase combined with low cobalamin (vitamin B12) increases risk for spina bifida.** Mol Genet Metab, 1999. 67(4): p. 317-23.	[420]
RFC 80G>A	Winkelmayer, W.C., et al., **Effects of the glutamate carboxypeptidase II (GCP2 1561C>T) and reduced folate carrier (RFC1 80G>A) allelic variants on folate and total homocysteine levels in kidney transplant patients.** Kidney Int, 2003. 63(6): p. 2280-5.	[421]
GCPII 1561C>T	Devlin, A.M., et al., **Glutamate carboxypeptidase II: a polymorphism associate with lower levels of serum folate and hyperhomocysteinemia.** Hum Mol Genet, 2000. 9(19): p. 2837-44.	[422]
SHMT 1420C>T	Heil, S.G., et al., **Is mutated serine hydroxymethyltransferase (SHMT) involved in the etiology of neural tube defects?** Mol Genet Metab, 2001. 73(2): p. 164-72.	[423]
COMT 1947G>A	Lachman, H.M., et al., **Association of codon 108/158 catechol-O-methyltransferase gene polymorphism with the psychiatric manifestations of velo-cardio-facial syndrome.** Am J Med Genet, 1996. **67**(5): p. 468-72.	[668]
TS 3'UTR 6bp del	Ulrich, C.M., et al., **Searching expressed sequence tag databases: discovery and confirmation of a common polymorphism in the thymidylate synthase gene.** Cancer Epidemiol Biomarkers Prev, 2000. 9(12): p. 1381-5.	[424]
TSER 2R>3R	Horie, N., et al., **Functional analysis and DNA polymorphism of the tandemly repeated sequences in the 5'-terminal regulatory region of the human gene for thymidylate synthase.** Cell Struct Funct, 1995. 20(3): p. 191-7.	[425]

Table 2-5: Primer sequences

Polymorphism	Forward Primer (5'→3')	Reverse Primer (5'→3')	Fragment length (bp)	Ref
MTHFR 677C>T	TGA AGG AGA AGG TGT CTG CGG GA	AGG ACG GTG CGG TGA GAG TG	198	[416]
MTHFR 1298A>C	CTT TGG GGA GCT GAA GGA CTA CTA C	CAC TTT GTG ACC ATT CCG GTT TG	163	[417]
MTR 2756A>G	GGT GTG TTC CCA GCT GTT AGA TG	GAC ACT GAA GAC CTC TGA TTT GAA C	265	[419]
MTRR 66A>G	GCA AAG GCC ATC GCA GAA GAC AT	GTG AAG ATC TGC AGA AAA TCC ATG TA	66	[420]
DHFR 19bp *	1) CCA CGG TCG GGG TAC CTG GG 2) ACG GTC GGG CTG GCC GAC TC	AAA AGG GGA ATC CAG TCG G	113 or 92 or both	[418]
RFC 80G>A	AGT GTC ACC TTC GTC CC	TCC CGC GTG AAG TTC TTG	162	[421]
GCPII 1561C>T	GAG TTG ATT GTA CAC CGC TGA TG	CCA CCT ATG TTT AAC ATA ATA CCT CAA GA	244	[422]
SHMT 1420C>T	AGA GTT CAA GGA GAG ACT GGC AG	GTC AAC AGT TCC CTT TGG AGC	218	[423]
COMT 1947G>A	TAC TGT GGC TAC TCA GCT GTG C	GTG AAC GTG GTG TGA ACA CC	237	[929]
TS 3'UTR 6bp del	CAA ATC TGA GGG AGC TGA GT	CAG ATA AGT GGC AGT ACA GA	152	[607]
TSER 2R>3R *	GTG GCT CCT GCG TTT CCC CC	GGC TCC GAG CCG GCC ACA GGC ATG GCG CGG	243 or 215	[425]

* Do not need to be digested by a restriction endonuclease as allele specific primers are used in this detection method

Table 2-6: Polymerase chain reaction conditions

Polymorphism	Master Mix Go Taq Green ® (ul)	Primer (5pmol)	DNA (ul)	H_20 (ul)	Reaction Volume (ul)	Thermal Cycling Conditions
677C>T MTHFR	15	3	8	1	30	95°C x 2mins; 35 x [95°C x 60secs; 56°C x 60secs; 72°C x 60secs]; 72°C x 7mins
1298A>C MTHFR	15	3	8	1	30	95°C x 5mins,55°C x 2mins, 72°C x 2mins; 35 x [95°C x 75secs; 55°C x 75secs; 72°C x 90secs;]; 72°C x 6mins
19bp del DHFR	15	3	6	N/A	30	94°C x 4mins; 35 x [94°C x 55secs; 62°C x 55secs; 72°C x 55secs]; 72°C x 12mins
2756A>G MTR	15	3	8	1	30	92°C x 2mins; 35 x [92°C x 60secs; 56°C x 60secs; 72°C x 60secs]; 72°C x 7mins
66A>G MTRR	15	3	8	1	30	92°C x 2mins; 35 x [95°C x 60secs; 56°C x 60secs; 72°C x 60secs]; 72°C x 7mins
80G>A RFC	15	3	8	1	30	94°C x 2mins; 35 x [94°C x 30secs; 58°C x 30secs; 72°C x 45secs]; 72°C x 7mins
1561C>T GCPII	15	3	8	1	30	94°C x 3mins; 35 x [94°C x 30secs; 56°C x 60secs; 72°C x 60secs]; 72°C x 7mins
1420C>T SMHT	15	3	8	1	30	95°C x 2mins; 35 x [95°C x 60secs; 56°C x 60secs; 72°C x 60secs]; 72°C x 7mins
1947G>A COMT	15	3	8	1	30	94°C x 3mins; 35 x [94°C x 30secs; 60°C x 30secs; 72°C x 30secs]; 72°C x 7mins
3'UTR6bp del TS	15	3	8	1	30	94°C x 5mins; 30 x [94°C x 30secs; 58°C x 45secs; 72°C x 45secs]; 72°C x 5mins
2R>3R TSER	15	3	6	10% DMSO*	30	94°C x 2mins; 35 x [94°C x 30secs; 63°C x 30secs; 72°C x 30secs]; 72°C x 5mins

* Dimethyl sulfoxide

2.3.3. Restriction enzyme digestion

To enable the detection of folate related polymorphisms, restriction digestion was performed following PCR amplification. Specific restriction endonucleases were employed to produce allele specific fragment sizes. Restriction endonucleases cleave DNA sequences at specific sequences of four to six nucleotides (known as palindromic sequences). Many restriction endonucleases have been isolated in pure form. Varying the amount of restriction endonuclease, the time of incubation, and the amount of amplicon optimises the restriction digestion conditions. Generally, 4 to 8 µl of the amplified PCR product was added to the restriction digestion mixture, which contained the appropriate buffer, bovine serum albumin, the restriction enzyme and water if required. The restriction digestion mixture was incubated for 5 to 7 hours at 37^{o}C depending of the polymorphism.

Nine out the eleven polymorphisms studied required restriction enzyme digestion. DHFR 19bp deletion uses a nested primer allele-specific strategy for genotype scoring and hence there is no need for enzyme digestion. TSER 2R>3R also does not require this process because it is a tandem repeat polymorphism. Details of the restriction digestion and conditions are displayed below in table 2-7.

2.3.4. Electrophoresis, imaging and analysis

Gel electrophoresis is the method by which an amplified DNA sample is mixed in a buffer solution and applied to media; the electric current from one electrode repels the DNA molecules while the other electrode attracts molecules. The gel additionally acts as a “molecular sieve” and separates the DNA fragments depending on size. The gel requires staining, in all case we used ethidium bromide, because it is a DNA intercalator, which fluoresces under UV light. In RFLP determination, gel electrophoresis methodology was used on two occasions.

In the first stage, the PCR amplicon is checked for correct amplification size and to ensure no contamination or non-specific banding occurred during the initial PCR process. This first stage required a 1.5% agarose solution, which was prepared by combining 1.5g of molecular grade low-melting agarose (Bio-Rad, Gladesville, NSW), with 110mls of 1X Tris/Borate/EDTA (TBE) (Sigma-Aldrich, Castle Hill, NSW). This solution was mixed thoroughly and heated in a microwave until the agarose had completely dissolved. Also added to the agarose mixture was 2µL of 10mg/mL ethidium bromide (Sigma-Aldrich, Castle Hill, NSW) this was mixed evenly within the liquid gel,

and cast immediately into a gel tray of an appropriate size with combs for 20 to 40 wells.

Table 2-7: Digestion enzymes and conditions

Polymorphism	Restriction Endonuclease	PCR Product (ul)	Digestion Buffer	Other	Incubation length @ 37°C	Gel Conditions Agarose/ nusieve (%)	Fragments on gel (bp)
677C>T MTHFR	5U *HinfI*	5	10x Buffer B	0.1µg BSA	5 hrs	3	**CC:** 198 **CT:** 198, 175, 23 **TT:** 175, 23
1298A>C MTHFR	2U *MboII*	5	10x Buffer B	0.1µg BSA	7 hrs	4	**AA:** 56, 31, 30, 28,18 **AC:** 84, 56, 31, 30, 28, 18 **CC:** 84, 31, 30, 18
19bp del DHFR	n/a	n/a	n/a	n/a	n/a	n/a	**11:** 113 **12:** 113, 92 **22:** 92
2756A>G MTR	5U *HaeIII*	5	10x Buffer C	0.1µg BSA	5 ½ hrs	2.5	**AA:** 265 **AG:** 265, 180, 85 **GG:** 180, 85
66A>G MTRR	5U *NdeI*	7	10x Buffer D	0.1µg BSA	6 ½ hrs	4	**AA:** 44, 22 **AG:** 66, 44, 22 **GG:** 66
80G>A RFC	5U *CfoI*	5	10x Buffer B	0.1µg BSA	5 hrs	3.5	**GG:** 125, 68, 37 **GA:** 162,125, 68, 37 **AA:** 162, 68
1561C>T GCPII	2U *AccI*	5	10x Buffer G	0.1µg BSA	6 hrs	3	**CC:** 244 **CT:** 244, 141,103 **TT:** 141,103
1420C>T SMHT	3U *EarI*	5	1xTango Buffer	N/A	5 ½ hrs	2.5	**CC:** 131, 86 **CT:** 218, 131, 86 **TT:** 218
1947G>A COMT	6U *NlaIII*	6	1x NEB 4	N/A	3 hrs	4	**GG:** 114, 54, 42 **GA:** 114, 96, 54, 42 **AA:** 96, 54, 42
3'UTR6bp del TS	5U *DraI*	7	10x Buffer B	0.1µg BSA	6 hrs	3	**6/6:** 88, 70 **6/0:** 158,152, 88, 70 **0/0:** 152
2R > 3R TSER	n/a	n/a	n/a	n/a	n/a	n/a	**3R/3R:** 243 **3R/2R:** 243, 215 **2R/2R:** 215

Note: All gels are prepared in 1x TBE stained with ethidium bromide, generally run at 90volts for up to 90minutes. Digestion buffers were supplied with the enzyme.

Once the gel had polymerised, it was placed in an electrophoresis tank (BioRad, Gladesville, NSW) containing 1 x TBE. Each well was loaded with 10µL of the cleaved PCR product. The gel was run on most occasions at 100V for ~ 40 minutes at room temperature, and included 25bp or 100bp DNA ladders (1µg/µl) (Promega, Annandale,

NSW) for fragment size comparisons. Once completed, the gel was immediately analysed under an ultraviolent transilluminator and a digital image of the gel was acquired. This was achieved using the ImageQuant™ 300 Capture Imaging system (GE healthcare, Rydalmere, NSW).

The second stage of gel electrophoresis was used after the restriction enzyme digestion phase; this is the final step in determination of genotype. The same principals apply as before, with the exception of the gel consistency. For most restriction digestion gels we used a combination of molecular grade NuSieve® GTG® (Lonza, Mt Waverly, VIC) and agarose, this was done to achieve best separation between the DNA fragments. See table 2-7 for polymorphism specific conditions, normally the gel concentration ranges from 2.5% to 4%, which at its highest contained 3g of NuSieve® GTG® and 1g of agarose dissolved in 110mls of TBE buffer. The gel was heated, set and stained in the same manner as described above, and there were no changes to the tank buffer conditions. However the current and time applied to the gel were different. Normally to achieve best separation the gel would be left at 90 V for ~ 90 minutes, depending on the polymorphism.

Once again, the gel was immediately analysed under an ultraviolent transilluminator and a digital image of the gel was acquired. The detection of each genotype was determined using the ImageQuant™ TL software (GE healthcare, Rydalmere, NSW). The software has an automated gel analysis function which aids in lane creation, band detection and molecular size quantification. The size of the fragments detectable for wild-types, hetero- and homozygous mutation carriers for each polymorphism studied are also displayed in table 2-7.

2.4. Nutritional Intake Assessment

An important factor in all studies is the determination of the nutrient intake; the Food Frequency Questionnaire (FFQ) is one of the most commonly used tools in epidemiologic studies to assess long-term nutritional exposure. FFQ's are designed to measure a person's usual dietary intake over a defined period of time. Used by researchers in one form or another for nearly 50 years, FFQ's have been considered a useful, if broad, tool. However, FFQ's often come under scrutiny for being inaccurate; FFQ's are prone to measurement error. Cognitively, questions' regarding the usual frequency of food intake are difficult to answer. The number of foods one can ask about is limited and extensive detail about food preparation is not collected. FFQ's generally

query usual portion size, which may not be so problematic for discreet foods like pieces of fruit, but can be quite difficult and highly variable for foods like pasta or vegetables.

2.4.1. Design of the nutritional questionnaire

The FFQ used in this thesis was based on that described by Hoyos [930]. Several questionnaires were used as a guideline for the original design, including; the NSW Health Survey Nutrition Questionnaires [931], the Short Fat Survey [932] and the Commonwealth Scientific and Industrial Research Organisation Diet and Health Booklet [933]. The questionnaire aimed to cover total consumption of all food groups and hence nutrients (a copy of the FFQ used throughout this body of work is found in appendix 1, page 350).

To obtain an estimated daily intake of nutrients, a series of structured questions were asked about all foods groups, this ensured all areas of the diet were examined. The frequency of consumption was categorised to a frequency per time period, i.e. 'so many times' per day, week, month, or year. Similarly, the quantity of food consumed for each food group was requested (often pictures of average serving sizes were given as a guide). To assist in the estimation of food quantity, a number of photographs were used showing specific amounts of common foods eaten, which are often difficult to evaluate/guess in terms of quantity.

At the end of the questionnaire, participants were asked to name any foods that they consume, but which had not been mentioned in the survey. The participants' consumption of vitamin and mineral supplements were also surveyed. The classification of foods into assigned food groups is displayed in table 2-8. The FFQ was administered as an interview to the participants, with each interview taking 30 to 60 minutes. Where possible, carers of individuals with cognitive impairment were interviewed with the participant.

Foodworks™ 3.02 (Xyris Software) was the database package used to assess intakes from the FFQ. This package uses a number of smaller food databases to cover the majority of foods consumed by Australians. These include; AusFoods (brands), Aus Nut (base foods) and the New Zealand – Vitamin and Mineral Supplements 1999 databases. A base template was first designed, which included a lot of common foods for each food group. This saved searching through the food directory for common foods, and allowed easy addition or changing of foods based on each participant's answers on their questionnaire.

During data entry, certain assumptions were made for seasonal foods, due to variations in availability. By way of example, i.e. for stone fruit (i.e. apricot, nectarine, peach, mango, plum) participants were asked how many, what types and how often, seasonal fruits were consumed. The amount was then divided by 3 (average duration of fruits in season i.e. a third of the year) to give an average intake over a year. If it was indicated that the food was consumed in certain seasons, they were recorded for 4 months of the year, unless otherwise stated.

Some people could not recall how often they ate an individual item, this particularly occurred in relation to vegetables. If this occurred participants were asked the types of vegetables they consume (these were ticked), how many cups of cooked vegetables they ate at a time, and how often they ate them. They were also asked if there were any that they ate more often. Takeaway meals such as Chinese/Thai foods were given a list of a number of common foods, unless specific items were suggested by the subject, and were adjusted only by how often they were consumed, this is because some people would have a variety of dishes each time they consumed this type of meal. *Note: restaurant meals such as this were very infrequent in the study populations; level of accuracy should not impact greatly on their average daily consumption/nutrient profiles.*

2.4.2. Determination of dietary folates

Total folate intake (µg per day) was calculated by adding the daily average value from Foodworks™ with any additional amounts from supplemental sources. Synthetic folate in the form of pteroylmonoglutamic acid was estimated by adding any folate containing vitamin supplements with breakfast cereal sources of pteroylmonoglutamic acid and any other known sources of pteroylmonoglutamic acid, such as Sustagen® powder or fortified drinks/juices. Natural folate as 5-methyl-H_4folic acid was estimated by adding together only natural forms of folate from foods such as fruits, vegetables, and grains. *Note: Folate Fortification of bread flours occurred on 19th September 2009 in Australia; all samples were collected prior to this date.*

Table 2-8: 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

2.5. Statistical Analysis of Data

All statistical analysis was conducted using; Microsoft Excel and JMP for Windows (version 4; SAS Institute Inc., Cary, NC, U.S.A). Non-genetic data such as age, gender, BMI and all biochemical indices were examined for descriptive statistics (number, mean, and standard deviation, along with median and interquartile range as appropriate. The prevalence of genotype was calculated as a percentage and allele number ascertained and presented as a count and a frequency. The degree and significance of an allele as risk factor for biochemical/clinical phenotype was calculated using odds ratio with associated 95% confidence intervals the *p* value was obtained using a chi-square test (χ^2) test. Where a frequency was (n) equals less than 5 a fishers-exact test was performed in place of chi-square test.

Significant differences for unpaired data were established using an independent two sample t-test. All B-vitamin related blood metabolite/ thiol concentrations were normalised by $\log_{10}$ transformation prior to analysis of variance (ANOVA), which was used to assess the differences in continuous variables between individual genotypes. Significance (*p* value) was set at <0.05. Since some serum folate, red cell folate, and vitamin B_{12} measurements were surprisingly high, indicative of likely pharmacologic intervention, the decision was made to examine the data in two ways. Firstly, the complete data set was examined unmodified in its native format, and then it was analysed after $\log_{10}$ transformation and removal of all extreme data points (above the upper range of instrumental calibration).

The relationship between a given phenotype and other recorded parameters was analysed by the implementation of an appropriate linear model according to the type of data (i.e. categorical or continuous). Either a standard least squares fit for one or more continuous responses (multiple regression), stepwise linear regression or logistic regression for ordinal response was employed. 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 model 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.

CHAPTER 3

B-VITAMIN NUTRITIONAL GENETICS IN THE ELDERLY

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A DETAILED STUDY OF HYPERTENSIVE AND DEPRESSION PHENOTYPES

3. Overview

Common chronic degenerative diseases are caused by multiple factors related to a person's genetics, lifestyle, and environment. Today, the leading causes of death in Australia are due to a small number of chronic diseases. In fact, 83% of all premature deaths (before age 75) are the result of chronic disease [934]. In 2011, it was reported that there were 1.04 million people aged 65 years and over living in NSW, this represented 14% of the total population [935]. By the year 2031 projections are that this proportion will increase to between 19-21% of the total population [936]. With the majority of people in this age group preferring coastal retirement, the NSW Central Coast is a popular location for this age group to retire too. Currently, 19% of the population of the Central Coast is aged over 65 years [937], which is well above national averages and thus provides an excellent model for how Australia will look demographically in the future. This increase in the ageing population has resulted in the development of 'retirement villages' which are a relatively new phenomenon, not only on the Central Coast, but throughout Australia. There are approximately 1,850 retirement villages in Australia, accommodating around 5% of people over 65, however, growth in the retirement living sector is expected to increase almost threefold over the next four decades [938].

As described in the thesis scope (see page 103), it is appropriate to look at this age group as health costs tend to be primarily spent on this 65+ age range. CVD is the leading cause of death in Australia, and the greatest modifiable risk factor for CVD is high blood pressure [62, 939]. Also, due to significant findings linking increased dietary folate with a decreased risk of hypertension it was appropriate to examine this phenotype for folate nutrigenetic associations within an over 65's population. In addition to this, the prevalence of mental disease is increasing in Australia, with a recent report suggesting anxiety and depression are the second leading cause of disease burden behind coronary heart disease [62, 939]. Another report has suggested that 25% of permanent residential aged care residents have a diagnosed mental disorder which excludes dementia [940]. The reasons for higher levels of mental disorders among this group are not clear, however, there is growing evidence for a role of folate in the ageing brain and its effects on the maintenance and the proper functioning of the central nervous system. With this in mind, the present study aims to explore the relationship between eleven common folate polymorphisms, important indices of folate status, and transsulphuration pathway thiols in both the hypertensive and depression phenotype within an elderly population sample.

3.1. Study Design

3.1.1. Ethics approval

Approval for this study was obtained from the University of Newcastle Human Research Ethics Committee (H-782-0304) and the Northern Sydney Central Coast Health Committee (04/19). Informed written consent was obtained from the participants.

3.1.2. Study recruitment

Recruitment began with selecting suitable retirement villages; in 2004, Alloura Waters' was chosen as the initial collection site. Alloura Waters' is a Living Choice retirement village at Davistown on the N.S.W. Central Coast; it contains approximately 200 units with 295 residents. In 2006, Henry Kendall was selected as the second site for recruitment. Henry Kendall is a lend lease prime-life retirement village situated at Wyoming also on the N.S.W Central Coast, which has approximately 644 residences. Recruitment involved randomly selecting units and proceeding to send an "Invitation to participate" pack. These packs contained information sheets and consent forms which had self-addressed reply-paid envelopes.

After receiving consent to participate in the study, a total of 4 clinics were conducted at the Carer's Unit/offices on the premises of the retirement village or in the participants home if required/agreed too. Total research clinic time for each resident was approximately 5-6 hours, although this was spread out over a several weeks (4-6 weeks). Clinic 1, 2 and 4 involved questionnaires, surveys, anthropometric and blood pressure measurements. Clinic 3 involved the collection of fasting blood samples for pathology. Figure 3-1 shows a detailed overview of what each clinic entailed.

Two hundred and fifty independently living residents volunteered to participate; subjects were excluded if they were less than 65 years old. In additional to this 5 people left during the study and, another 16 either did not give blood or removed themselves from the study. After all exclusions, the total number of retirement village participants was 229 residents. Within this population there were 20 people with saturated serum folate levels, i.e. too high to measure (>45nmol/L) in the specified assay. One person had an extreme level for red cell folate measurement and three people had serum B_{12} values greater than the calibration range indicating a vitamin B_{12} injection was used. Statistical analyses involving these outliers have been performed to both include and exclude where appropriate.

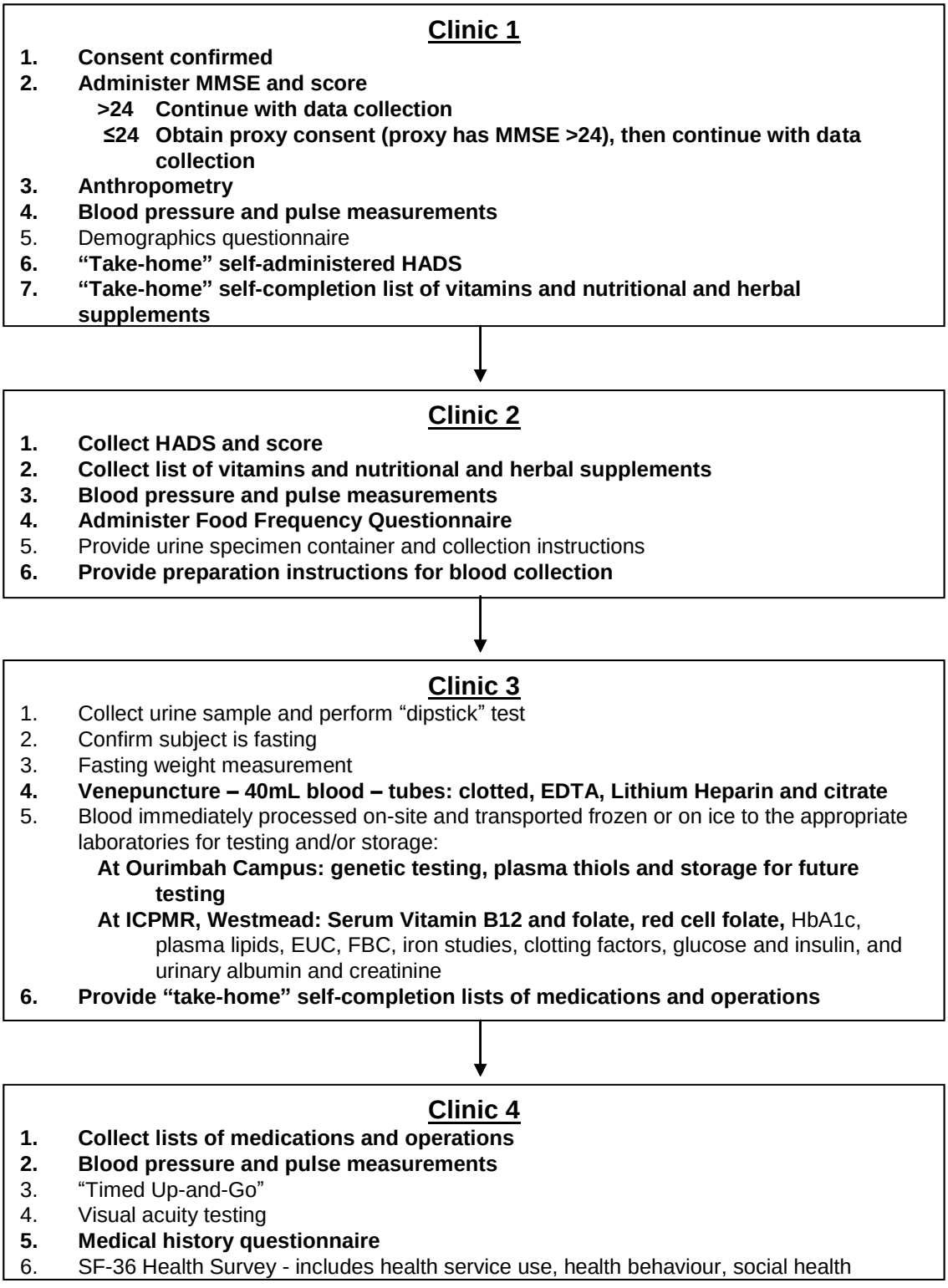

Figure 3-1: Retirement village study clinic protocols and data collection

Note: contains information outside the sphere of this sub project, refer to highlighted text only for relevant information

3.1.3. Hospital anxiety and depression scale; self-administration and scoring

At the end of clinic 1 (figure 3-1), the subjects were provided with a take home hospital anxiety and depression scale (HADS) (see appendix 2 on page 365 for a copy). The HADS is a widely used 14 item self-report scale designed to briefly measure current anxiety and depressive symptomatology. The participants were asked to complete the survey at home, by underlining the answer that best described their feelings and emotions in the past week. The completed HADS questionnaires were collected from participants and checked for completion at the start of study Clinic 2 (figure 3-1).

The scoring of the HADS questionnaire was based on the method developed by the original designers and authors Zigmund and Snaith [941]. Scores on each scale (Anixety (A) or Depression (D)) can be interpreted in ranges; Normal" (0 – 7), "Mild" (8 – 10), "Moderate" (11 – 14) and "Severe" (15 – 21), a score of 8 or more is considered to indicate anxiety or depression [942]. A 2002 review of 747 papers on HADS by Ingvar Bjelland *et al.* [943] determined that the HADS questionnaire performs well in assessing the symptom severity and caseness of anxiety disorders and depression in both somatic, psychiatric, primary care patients and in the general population [943]. Optimal balance between sensitivity and specificity for HADS as a screening instrument was achieved at a cut-off of 8+ for both HADS-A and HADS-D giving sensitivities and specificities for both subscales of approximately 0.80. In addition, a Cronbach's alpha which is commonly used as a measure of the internal consistency or reliability of a psychometric test scores for a sample of examinees was performed on the HADS data (see below for results).

Cronbach's alpha coefficient of internal consistency for HADS index

HADS - Anxiety	HADS - Depression
0.807	0.904

Results displayed above are comparable to Bjelland's review of 747 studies which found Cronbach's alpha values of HADS-A (0.68-0.93) with an overall average of 0.83 and for HADS-D values of (0.67-0.90) with an overall average of 0.82 determining the reliability of scores obtained [943]. In the depression phenotype section of these results, the generally accepted cut-off point of 8 has been used, although a second categorisation using the anxiety subscale to control for co-morbidity has also been examined where appropriate.

3.1.4. Mini-mental state examination; administration and scoring

At study clinic 1 (figure 3-1), informed written consent was confirmed. The participants were screened using the mini-mental state examination (MMSE), administration and scoring of the different sections of MMSE was in accordance with standardised guidelines for MMSE administration [944, 945]. The participants who had a MMSE score greater than 24 continued with the study, the participants who had a MMSE score of 24 or below were identified as having reduced cognitive functioning, and the participant was withdrawn from the study. The data collected on the MMSE scores have not been used in this chapter of analysis, but for the subsequent chapter (chapter 4, page 179) the data has been used as a comparative to people with an established diagnosis of dementia/Alzheimer's disease.

3.1.5. Blood pressure/ pulse rate determination and anthropometrics

Blood pressure measurements were taken on three separate occasions, during clinic 1, 2, and 4, the average of the three was taken as the subject's usual blood pressure. At each of the clinic visits, the subjects' blood pressure was measured while they were in a recumbent position (recumbent blood pressure) after they had been resting for at least 5 minutes, a further blood pressure was taken whilst in the standing position. A standard mercury sphygmomanometer was used and the first systolic blood pressure and fifth diastolic blood pressure Korotkoff sounds were recorded to the nearest 2 mm Hg. Pulse rate per minute in the recumbent position was also recorded during each of the clinic visits; an average of the three recordings determined the final pulse result. Participants were required to undergo some anthropometric measures; these included height (cm), weight (kg), waist (cm), hip (cm) and arm (cm) circumferences. Using the height and weight measurements; BMI was calculated and used in the statistical analysis of the data set.

3.1.6. Food frequency questionnaire

A FFQ was performed during clinic visit 2 (see figure 3-1). Estimated total dietary folic acid intake was determined using Foodworks™ for further details on the questionnaire and dietary estimation see section 2.4 on page 124.

3.1.7. Non-clinical measurements

At the third clinic, each participant donated approximately 30mls of blood, which was collected first thing in the morning by a trained phlebotomist (for specific information

regarding collection see section 2.1.1 on page 107). The blood was separated accordingly and taken to the Molecular Nutrition PC2 Laboratory, University of Newcastle, Ourimbah, NSW and ICPMR at Westmead Hospital Sydney, NSW. With the denoted blood the following methods were undertaken:

- Blood levels of red cell folate, serum folate and vitamin B_{12}, were measured using an automated Access Immunoassay System. For detailed information regarding the automated access immunoassay system, see Chapter 2 section 2.1.2 on page 108.

- Homocysteine, cysteine, cysteinyl-glycine, and glutathione concentrations were determined by HPLC with florescence detection using SBD-F. The full description of the methods employed and the validation process is described in section 2.2 on page 109.

- For gene polymorphism detection, the eleven SNPs were scored using the RFLP method described in full in section 2.3 beginning on page 114.

3.1.8. Statistical analysis

A full description of the statistical methods employed for the entire thesis can be found in section 2.5, page 128. For this chapter, the non-genetic data such as age and gender, and all biochemical indices were examined for general descriptive statistics. In order to assess all blood thiol and B-vitamin concentrations between individual genotypes, data was first normalised by $\log_{10}$ transformation prior to performing an ANOVA, with significance set at $p=<0.05$. As mentioned above, some of the B-vitamin concentrations were exceptionally high, which is indicative of pharmacologic intervention. Due to this, the data was examined in two ways, the complete data set in an unmodified 'native' format, and then analysis was performed after $\log_{10}$ transformation with all extreme data points removed. The results that follow report outcomes according to both approaches.

Also, odds ratio analyses were performed to determine the degree and significance of an allele as a risk factor for each of the phenotypes examined. And finally, the relationships between the two phenotypes and the other recorded parameters was analysed by the implementation of an appropriate linear model according to the type of data i.e. categorical or continuous. This meant the use of stepwise linear regression for the hypertensive phenotype and both stepwise linear and ordinal logistic regression was employed for the depression phenotype.

3.2. Results

3.2.1. Descriptive statistics

The principal reason for studying this cohort was to examine the molecular basis for both hypertensive and depression phenotypes in the elderly. Given this, the first table below outlines simple descriptive data based on age distribution, and includes; number, mean, standard deviation, median, interquartile range and range.

Table 3-1: Descriptive data based on age (years)

	All subjects	Male	Female
n	229	94	139
$\bar{x}$	77	78	77
SD	6	6	7
Median	77	79	77
IQR	73-82	73-82	72-82
Range	65-96	65-92	65-96

n=number, $\bar{x}$ =mean, SD=standard deviation, IQR=interquartile range

The following three tables contain the descriptive statistics for all non-genetic nutritional and blood indices. Table 3-2, table 3-3 and table 3-4 include data for all subjects, males and females respectively. These data sets have a number of extreme values, predominantly within the serum folate, red cell folate and serum vitamin B_{12} values; these are a likely consequence of pharmacological intervention and have not been removed from the following tables. They are however, taken into account in the statistical analysis shown later.

Table 3-2: Data for all subjects; blood metabolites and related indices

Indices	$\bar{x}$ (SD)	Median (IQR)	Range
Serum Vitamin B_{12} (pmol/L)	277.2 (161.9)	240.0 (182.0-317.0)	64.0-1100.0
Serum Folate (nmol/L)	24.55 (11.68)	23.00 (15.00-33.00)	3.00-45.00
Red Cell Folate (nmol/L)	853.5 (359.1)	797.0 (597.8-1063.5)	221.0-2500.0
Homocysteine (µmol/L)	9.42 (2.71)	8.83 (7.63-10.72)	4.57-18.58
Cysteine (µmol/L)	258.3 (32.6)	257.5 (235.7-279.0)	180.9-343.3
Cysteinyl-glycine (µmol/L)	25.93 (4.91)	25.54 (22.90- 28.88)	15.68- 46.75
Glutathione (µmol/L)	10.40 (3.81)	9.97 (7.95- 12.31)	3.17-35.20
Total Dietary Folic Acid (µg/day)	446.8 (196.3)	395.5 (314.2-516.2)	149.6-1197.1
Total Synthetic Folic Acid (µg/day)	120.0 (150.0)	68.6 (14.3- 137.4)	0.0-740.7
Total Natural Folic Acid (µg/day)	326.8 (104.0)	305.6 (259.4-380.8)	114.7-706.6

Table 3-3: Data for male subjects; blood metabolites and related indices

Indices	$\bar{x}$ (SD)	Median (IQR)	Range
Serum Vitamin B_{12} (pmol/L)	259.7 (137.0)	239.5 (174.5-313.8)	64.0- 1018.0
Serum Folate (nmol/L)	23.82 (11.57)	22.00 (14.00-33.00)	7.00-45.00
Red Cell Folate (nmol/L)	857.2 (368.1)	791.5 (604.8-1076.0)	263.0-2500.0
Homocysteine (µmol/L)	10.04 (2.85)	9.46 (7.85-11.55)	4.89-18.58
Cysteine (µmol/L)	256.5 (32.4)	257.6 (230.3-275.5)	186.1-333.8
Cysteinyl-glycine (µmol/L)	26.39 (5.17)	26.30 (23.17 -29.35)	15.68-40.54
Glutathione (µmol/L)	10.08 (3.23)	10.12 (7.94-12.10)	3.50-23.46
Total Dietary Folic Acid (µg/day)	472.6 (217.9)	409.0 (327.3-518.3)	183.8-1197.1
Total Synthetic Folic Acid (µg/day)	132.7 (155.1)	92.8 (45.7-149.4)	0.0-740.7
Total Natural Folic Acid (µg/day)	339.9 (113.5)	306.3 (262.9-407.4)	149.7-706.6

Table 3-4: Data for female subjects; blood metabolites and related indices

Indices	$\bar{x}$ (SD)	Median (IQR)	Range
Serum Vitamin B_{12} (pmol/L)	289.1 (176.2)	244.0 (188.5- 325.0)	78.0-1100.0
Serum Folate (nmol/L)	25.05 (11.76)	24.00 (16.00-34.50)	3.00-45.00
Red Cell Folate (nmol/L)	850.9 (354.2)	797.0 (591.3-1056.3)	221.0-2007.0
Homocysteine (µmol/L)	9.00 (2.54)	8.56 (7.14-10.43)	4.57-17.65
Cysteine (µmol/L)	259.5 (32.8)	257.5 (236.9-280.6)	180.9- 343.3
Cysteinyl-glycine (µmol/L)	25.62 (4.73)	25.06 (22.90- 28.25)	17.07-46.75
Glutathione (µmol/L)	10.61 (4.15)	9.92 (8.05- 12.40)	3.17- 35.20
Total Dietary Folic Acid (µg/day)	429.5 (179.1)	376.9 (305.2-508.3)	149.6- 963.3
Total Synthetic Folic Acid (µg/day)	111.5 (146.4)	57.1 (5.4-121.8)	0.0- 550.0
Total Natural Folic Acid (µg/day)	318.0 (96.5)	305.4 (257.7 373.4)	114.7-600.9

3.2.2. B-vitamin related genetics

The table below outlines the prevalence (n) and percentage for each genotype examined in the population, along with allele number and frequency.

Table 3-5: Complete genetic data; genotype prevalence and allele number

SNP	GENOTYPE Prevalence (%)			ALLELE Number (frequency)	
	Wildtype	Heterozygote	Recessive	Wildtype	Mutant
677C>T MTHFR	107 (47)	96 (42)	26 (11)	310 (0.68)	148 (0.32)
1298A>C MTHFR	106 (46)	105 (46)	18 (8)	317 (0.69)	141 (0.31)
19bp del DHFR	77 (34)	107 (47)	45 (20)	261 (0.57)	197 (0.43)
2756A>G MTR	126 (55)	88 (38)	15 (7)	340 (0.74)	118 (0.26)
66A>G MTRR	39 (17)	116 (51)	74 (32)	194 (0.42)	264 (0.58)
80G>A RFC	69 (30)	104 (45)	56 (24)	242 (0.53)	216 (0.47)
1561C>T GCPII	210 (92)	19 (8)	0 (0)	439 (0.96)	19 (0.04)
1420C>T SHMT	115 (50)	94 (41)	20 (9)	324 (0.71)	134 (0.29)
1947G>A COMT	60 (26)	101 (44)	66 (29)	221 (0.49)	233 (0.51)
3'UTR 6bp del TS	105 (46)	100 (44)	24 (10)	310 (0.68)	148 (0.32)
2R > 3R TSER	60 (26)	126 (55)	43 (19)	242 (0.53)	216 (0.47)

3.2.3. B-vitamin/thiol related nutritional genetics organised by genotype

Complete descriptive data (independent of phenotype) is display in the next four tables for each B-vitamin or thiol related parameter examined - each is categorised by genotype. In order to investigate whether genetic factors can influence variability in blood indices, a one-way ANOVA was performed. All subject data was examined twice, since the data set contains a number of extreme data points (a likely consequence of pharmacologic intervention), and therefore the ANOVA's were performed both with and without these extreme points. The result of ANOVA based on $\log_{10}$ transformed (normalised) data is given in the following four tables, and significance is denoted by either a red asterisk(s) (all $\log_{10}$ transformed values) or a blue asterisk(s) ($\log_{10}$ transformed values with outliers removed).

Note: Statistical results for the untransformed data are displayed as footnotes in each table.

The ANOVA outcomes displayed below show some significance for variability in folate levels with respect to the 677C>T MTHFR SNP and in variability in the thiol-glutathione for 1947G>A COMT and 1561C>T GCPII polymorphisms.

Table 3-6: All data; B-vitamin/thiol related nutritional genetic data by genotype (1 of 4)

Indices		677C>T MTHFR			1298A>C MTHFR			19bp del DHFR		
		Wt (n=107)	Het (n=96)	Rec (n=26)	Wt (n=106)	Het (n=105)	Rec (n=18)	Wt (n=77)	Het (n=107)	Rec (n=45)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**287.5** (163.6)	**261.6** (155.2)	**277.6** (114.2)	**289.9** (164.3)	**267.2** (152.6)	**238.8** (100.8)	**285.9** (182.8)	**272.7** (149.8)	**264.4** (111.9)
	Median (IQR)	259.0 (188.5-336.0)	219.5 (177.5-290.3)	239.0 (198.5-350.5)	243.0 (196.0-100.0)	240.0 (174.0-100.8)	230.0 (178.0-287.3)	244.0 (182.0-318.0)	240.0 (185.0-309.0)	253.0 (180.0-340.0)
	Range	64.0-1100.0	78.0-1100.0	74.0-544.0	74.0-1100.0	81.0-1100.0	64.0-434.0	78.0-1100.0	81.0-1100.0	64.0-620.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**26.87** (12.00)	**22.49** (10.99)	**21.96** (11.16) * *	**23.96** (12.10)	**23.95** (10.97)	**30.56** (11.83)	**24.97** (12.16)	**24.51** (11.09)	**23.53** (12.30)
	Median (IQR)	24.00 (17.00-38.50)	21.00 (13.00-27.25)	21.00 (14.25-25.00)	22.00 (14.00-96.90)	23.00 (15.00-98.50)	26.00 (22.00-44.50)	23.00 (15.00-37.00)	23.00 (16.00-32.50)	23.00 (12.00-33.00)
	Range	3.00-45.00	7.00-45.00	6.00-45.00	6.00-45.00	3.00-45.00	11.00-45.00	6.00-45.00	4.00-45.00	3.00-45.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**865.8** (325.0)	**792.8** (345.2)	**992.7** (471.5) *	**872.8** (422.9)	**809.5** (277.5)	**951.6** (321.2)	**863.7** (307.2)	**861.3** (398.0)	**797.6** (333.3)
	Median (IQR)	825.0 (616.5-1095.3)	730.5 (561.5-1001.5)	923.0 (697.0-1294.8)	792.5 (548.8-102.0)	780.0 (602.0-101.5)	861.0 (750.0-1193.0)	804.0 (648.0-1048.0)	783.5 (548.5-1074.8)	780.0 (536.0-983.0)
	Range	221.0-1726.0	254.0-2500.0	288.0-2007.0	254.0-2500.0	221.0-1534.0	425.0-1550.0	288.0-1650.0	256.0-2500.0	221.0-1534.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**9.21** (2.78)	**9.81** (2.59)	**8.86** (2.75)	**9.16** (2.72)	**9.57** (2.69)	**10.08** (2.72)	**9.24** (2.60)	**9.30** (2.63)	**10.02** (3.03)
	Median (IQR)	8.47 (7.23-10.80)	9.44 (7.86-10.82)	8.27 (7.61-9.68)	8.35 (7.28-82.38)	9.10 (7.87-81.00)	9.31 (8.43-11.43)	8.80 (7.64-10.00)	8.75 (7.62-10.73)	9.21 (7.74-12.17)
	Range	4.76-17.02	5.62-18.58	4.57-16.29	4.57-18.58	4.76-17.65	6.07-15.64	4.57-18.58	4.66-17.65	4.76-17.01
Cysteine (µmol/L)	$\bar{x}$ (SD)	**258.6** (32.6)	**260.4** (32.8)	**249.1** (31.2)	**257.8** (35.6)	**258.9** (30.1)	**257.9** (29.5)	**260.1** (31.3)	**257.6** (35.1)	**256.7** (28.9)
	Median (IQR)	257.3 (237.8-279.3)	260.4 (236.7-279.1)	246.3 (229.9-272.5)	254.6 (230.4-88.8)	258.3 (239.1-88.7)	260.2 (241.4-278.2)	261.2 (235.0-280.6)	257.3 (230.0-278.7)	254.4 (240.6-275.9)
	Range	200.7-333.8	180.9-343.3	186.1-307.9	180.9-343.3	200.7-333.8	205.3-317.8	186.1-326.6	180.9-343.3	204.2-313.0
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**26.25** (5.09)	**25.35** (4.90)	**26.74** (4.09)	**26.15** (5.11)	**25.86** (4.66)	**25.07** (5.30)	**26.08** (5.63)	**25.79** (4.52)	**26.00** (4.59)
	Median (IQR)	25.83 (22.49-29.16)	24.83 (22.91-27.37)	27.56 (23.41-29.57)	25.65 (22.93-76.00)	25.71 (22.90-75.00)	24.00 (21.70-26.62)	24.12 (22.91-27.38)	26.05 (22.90-28.93)	26.17 (22.79-29.05)
	Range	15.68-40.81	15.74-46.75	20.31-34.04	17.04-46.75	15.68-40.81	16.76-35.04	15.74-46.75	15.68-36.08	17.86-36.42
Glutathione (µmol/L)	$\bar{x}$ (SD)	**10.88** (4.66)	**9.98** (2.82)	**9.98** (2.87)	**10.55** (3.49)	**10.22** (4.19)	**10.53** (3.48)	**10.37** (3.79)	**10.46** (4.13)	**10.30** (3.07)
	Median (IQR)	10.05 (8.08-12.60)	9.63 (7.83-11.87)	9.85 (8.05-12.16)	10.31 (8.30-70.00)	9.36 (7.76-70.00)	10.11 (8.69-13.15)	10.27 (8.24-12.04)	9.78 (7.66-12.65)	10.18 (8.54-11.59)
	Range	3.17-35.20	4.09-20.89	5.99-16.81	3.50-27.94	3.17-35.20	4.99-18.30	5.25-35.20	3.17-27.94	4.09-18.30

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 677C>T MTHFR significance is achieved with native (untransformed) data for both serum folate ** ** and red cell folate * **.

Table 3-7: All data; B-vitamin/thiol related nutritional genetic data by genotype (2 of 4)

Indices		2756A>G MTR			66A>G MTRR			1947G>A COMT		
		Wt (n=126)	Het (n=88)	Rec (n=15)	Wt (n=39)	Het (n=116)	Rec (n=74)	Wt (n=60)	Het (n=101)	Rec (n=66)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**257.2** (111.8)	**302.0** (202.7)	**273.3** (131.4)	**262.9** (107.1)	**285.5** (176.0)	**266.5** (141.5)	**263.2** (126.9)	**286.7** (172.1)	**266.0** (152.9)
	Median (IQR)	240.5 (180.0-306.5)	244.5 (197.5-318.5)	221.0 (174.5-350.0)	247.0 (186.5-333.0)	244.0 (182.8-319.3)	233.5 (183.0-293.3)	234.5 (172.0-314.0)	255.0 (198.0-320.0)	223.5 (177.8-291.0)
	Range	64.0-632.0	74.0-1100.0	138.0-557.0	95.0-554.0	64.0-1100.0	81.0-854.0	81.0-702.0	64.0-1100.0	89.0-1100.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**24.36** (11.50)	**24.22** (11.89)	**27.00** (12.17)	**24.77** (11.44)	**25.59** (11.93)	**22.58** (11.27)	**22.92** (11.41)	**25.13** (11.92)	**24.45** (11.39)
	Median (IQR)	23.50 (15.00-32.75)	21.00 (15.00-33.25)	23.00 (16.50-38.50)	23.00 (15.00-33.00)	24.00 (16.00-36.00)	21.50 (14.00-27.00)	20.00 (14.00-26.25)	23.00 (16.00-33.00)	23.00 (15.00-34.75)
	Range	3.00-45.00	6.00-45.00	12.00-45.00	10.00-45.00	4.00-45.00	3.00-45.00	4.00-45.00	3.00-45.00	7.00-45.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**844.8** (343.5)	**826.3** (331.7)	**1025.6** (542.4)	**801.5** (277.7)	**896.9** (389.8)	**800.0** (331.4)	**851.8** (364.6)	**862.0** (367.7)	**827.5** (339.9)
	Median (IQR)	798.0 (591.0-1077.0)	781.5 (611.8-994.8)	973.0 (636.0-1251.5)	780.0 (600.0-995.5)	834.5 (604.3-1104.0)	750.0 (545.0-993.0)	782.0 (601.3-1050.8)	804.0 (602.0-1077.0)	780.0 (550.0-990.0)
	Range	221.0-2007.0	256.0-1996.0	386.0-2500.0	346.0-1424.0	254.0-2500.0	221.0-1726.0	322.0-1996.0	221.0-2500.0	263.0-2007.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**9.34** (2.67)	**9.52** (2.72)	**9.50** (3.16)	**9.47** (2.86)	**9.47** (2.80)	**9.31** (2.51)	**9.29** (2.76)	**9.74** (2.79)	**9.13** (2.54)
	Median (IQR)	8.64 (7.55-10.48)	8.96 (7.65-10.89)	8.75 (8.10-11.29)	8.33 (7.40-11.38)	8.57 (7.64-10.74)	9.29 (7.64-10.48)	8.83 (7.64-9.91)	8.99 (7.80-11.02)	8.76 (7.62-10.69)
	Range	4.66-18.58	4.57-17.65	4.89-15.80	5.56-17.65	4.66-17.02	4.57-18.58	4.57-17.02	4.89-18.58	4.66-17.65
Cysteine (µmol/L)	$\bar{x}$ (SD)	**259.2** (32.6)	**254.6** (32.3)	**271.8** (31.9)	**259.2** (34.2)	**255.5** (32.5)	**262.1** (31.9)	**252.7** (32.8)	**261.9** (31.8)	**258.7** (33.2)
	Median (IQR)	258.6 (238.2-279.2)	253.7 (230.6-278.9)	265.3 (254.6-291.3)	253.7 (236.9-279.3)	254.2 (231.5-278.8)	263.8 (239.4-279.4)	248.0 (232.1-276.7)	261.8 (237.0-279.3)	255.1 (239.5-280.1)
	Range	200.7-343.3	180.9-335.2	229.0-333.8	205.3-325.1	180.9-343.3	204.4-335.2	186.1-332.5	204.4-335.2	180.9-343.3
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**25.45** (4.64)	**26.46** (5.05)	**26.82** (6.15)	**26.90** (6.29)	**25.71** (4.22)	**25.76** (5.12)	**25.66** (5.08)	**26.20** (5.29)	**25.70** (4.23)
	Median (IQR)	25.02 (22.56-27.96)	25.65 (22.93-29.17)	27.68 (24.55-29.88)	25.78 (22.89-29.17)	25.80 (22.98-28.98)	25.00 (22.87-28.38)	25.31 (22.87-29.08)	25.71 (22.79-28.99)	25.53 (23.01-28.19)
	Range	15.74-46.75	18.09-40.81	15.68-37.15	17.04-46.75	15.74-35.04	15.68-39.09	15.74-38.47	15.68-46.75	17.04-36.42
Glutathione (µmol/L)	$\bar{x}$ (SD)	**10.10** (3.15)	**10.77** (4.42)	**10.69** (4.99)	**10.82** (4.81)	**10.44** (3.78)	**10.11** (3.26)	**9.28** (2.67)	**10.89** (4.47)	**10.52** (3.38) *
	Median (IQR)	9.80 (7.96-11.88)	10.29 (8.16-12.60)	9.37 (7.52-13.22)	10.27 (8.02-12.58)	10.11 (7.98-12.26)	9.65 (7.94-12.05)	9.09 (7.35-10.66)	10.38 (8.29-12.70)	10.32 (7.88-12.60)
	Range	4.09-26.40	3.50-35.20	3.17-23.46	4.38-35.20	3.17-27.94	3.50-23.46	3.50-20.89	3.17-35.20	5.36-23.46

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 1947G>A COMT significance is achieved with native (untransformed) data for glutathione*.

Table 3-8: All data; B-vitamin/thiol related nutritional genetic data by genotype (3 of 4)

Indices		3'UTR 6bp del TS			2R > 3R TSER			1420C>T SHMT		
		Wt (n=105)	Het (n=100)	Rec (n=24)	Wt (n=60)	Het (n=126)	Rec (n=43)	Wt (n=115)	Het (n=94)	Rec (n=20)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**275.5** (148.3)	**275.0** (169.6)	**277.7** (123.4)	**260.0** (105.0)	**288.9** (187.1)	**258.0** (98.8)	**286.7** (166.4)	**268.7** (148.0)	**242.7** (115.1)
	Median (IQR)	243.0 (196.0-308.0)	227.0 (180.8-325.3)	261.5 (204.5-365.3)	242.5 (193.5-290.3)	238.5 (182.0-329.8)	247.0 (180.0-305.0)	249.0 (182.5-346.5)	234.5 (196.0-312.5)	212.0 (175.3-290.5)
	Range	89.0-1100.0	64.0-1100.0	74.0-554.0	74.0-688.0	64.0-1100.0	117.0-544.0	64.0-1100.0	81.0-1100.0	102.0-544.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**25.01** (11.82)	**23.81** (11.90)	**24.92** (10.15)	**22.78** (12.25)	**25.78** (11.65)	**23.02** (10.58)	**24.41** (11.77)	**25.11** (12.22)	**21.90** (7.78)
	Median (IQR)	23.00 (15.00-35.00)	21.50 (14.00-33.00)	23.50 (17.00-31.50)	20.50 (12.75-31.25)	24.00 (17.00-35.75)	22.00 (14.00-30.50)	23.00 (14.00-34.00)	23.00 (15.00-35.50)	20.00 (16.75-26.25)
	Range	4.00-45.00	3.00-45.00	8.00-45.00	3.00-45.00	6.00-45.00	4.00-45.00	4.00-45.00	3.00-45.00	12.00-40.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**842.3** (350.8)	**859.3** (382.8)	**840.5** (266.8)	**809.8** (332.3)	**879.3** (380.1)	**818.4** (314.9)	**848.1** (374.6)	**867.4** (351.5)	**774.1** (266.3)
	Median (IQR)	798.5 (581.3-1023.3)	784.5 (597.8-1128.8)	806.5 (606.5-1029.8)	781.5 (591.8-1028.0)	798.0 (605.0-1107.0)	830.0 (601.0-990.5)	790.5 (588.3-1074.8)	798.5 (614.0-1083.5)	771.5 (634.5-838.5)
	Range	254.0-2007.0	221.0-2500.0	471.0-1389.0	221.0-1550.0	256.0-2500.0	254.0-1726.0	254.0-2500.0	221.0-1996.0	421.0-1650.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**9.22** (2.56)	**9.68** (2.75)	**9.21** (3.15)	**9.59** (2.77)	**9.34** (2.66)	**9.40** (2.83)	**9.32** (2.86)	**9.61** (2.53)	**9.13** (2.72)
	Median (IQR)	8.62 (7.63-10.50)	9.34 (7.68-11.36)	8.71 (6.91-9.81)	9.15 (7.63-10.76)	8.65 (7.62-10.55)	8.56 (7.69-10.98)	8.56 (7.49-10.56)	9.05 (7.86-10.83)	8.71 (7.16-10.08)
	Range	4.57-18.58	5.20-17.65	5.56-17.02	4.76-18.58	4.66-17.65	4.57-16.59	4.66-18.58	4.57-17.65	5.68-15.80
Cysteine (µmol/L)	$\bar{x}$ (SD)	**256.1** (33.6)	**259.6** (33.0)	**262.3** (26.7)	**258.9** (35.4)	**259.7** (31.2)	**253.3** (32.7)	**257.8** (34.2)	**259.5** (32.5)	**254.9** (23.1)
	Median (IQR)	258.9 (230.2-274.8)	254.5 (236.7-282.5)	267.6 (239.4-279.1)	258.1 (232.8-279.0)	257.8 (239.3-279.4)	254.2 (229.7-278.8)	254.4 (232.4-278.9)	258.9 (237.4-282.0)	257.2 (237.7-271.1)
	Range	180.9-343.3	186.1-333.8	207.5-322.5	204.2-343.3	180.9-335.2	200.7-317.5	200.7-343.3	180.9-333.8	213.6-297.8
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**25.68** (4.81)	**26.41** (5.10)	**24.98** (4.52)	**25.87** (5.32)	**25.95** (4.53)	**25.95** (5.48)	**25.88** (4.71)	**25.63** (5.07)	**27.62** (5.21)
	Median (IQR)	25.27 (22.99-27.86)	25.98 (22.84-29.70)	24.68 (21.61-26.75)	25.46 (22.14-28.51)	25.74 (23.08-28.96)	25.17 (22.78-28.71)	26.09 (22.90-28.81)	24.74 (22.56-27.88)	27.44 (23.56-31.23)
	Range	15.68-46.75	16.76-40.81	18.58-36.08	16.76-46.75	15.74-39.09	15.68-40.54	15.68-40.54	15.74-46.75	20.31-40.81
Glutathione (µmol/L)	$\bar{x}$ (SD)	**10.66** (3.86)	**10.28** (4.04)	**9.77** (2.31)	**9.90** (2.62)	**10.84** (4.41)	**9.79** (3.15)	**10.52** (3.67)	**10.07** (4.09)	**11.25** (3.18)
	Median (IQR)	10.27 (8.05-12.61)	9.63 (7.94-11.83)	9.50 (7.88-12.00)	9.79 (7.91-12.12)	10.25 (8.28-12.56)	9.47 (7.74-11.97)	10.17 (7.97-12.58)	9.73 (7.60-11.55)	10.15 (8.72-13.51)
	Range	3.17-27.94	3.50-35.20	6.11-14.56	5.25-16.52	3.50-35.20	3.17-20.89	4.09-27.94	3.17-35.20	7.47-18.30

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data log_{10} transformed * Blue asterisk (s) denotes all data log_{10} transformed with outliers removed.

Table 3-9: All data; B-vitamin/thiol related nutritional genetic data by genotype (4 of 4)

Indices		80G>A RFC			1561C>T GCPII		
		Wt (n=69)	Het (n=104)	Rec (n=56)	Wt (n=210)	Het (n=19)	Rec (n=0)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**257.6** (125.9)	**289.4** (178.3)	**271.8** (140.8)	**277.6** (158.7)	**252.6** (107.3)	**0.0** (0.0)
	Median (IQR)	227.0 (197.0-292.0)	246.0 (193.5-323.0)	239.5 (171.5-347.5)	240.5 (185.3-317.0)	246.0 (159.5-315.0)	0.0 (0.0-0.0)
	Range	78.0-854.0	64.0-1100.0	95.0-702.0	64.0-1100.0	106.0-495.0	0.0-0.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**22.28** (11.58)	**25.89** (11.54)	**24.55** (11.78)	**24.16** (11.67)	**28.00** (11.26)	**0.00** (0.00)
	Median (IQR)	19.00 (14.00-30.00)	24.00 (17.00-35.25)	22.50 (14.75-33.50)	22.00 (15.00-33.00)	26.00 (21.00-37.00)	0.00 (0.00-0.00)
	Range	3.00-45.00	4.00-45.00	7.00-45.00	3.00-45.00	7.00-45.00	0.00-0.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**836.7** (358.8)	**862.2** (337.6)	**842.1** (391.4)	**846.2** (353.1)	**886.0** (399.5)	**0.0** (0.0)
	Median (IQR)	750.0 (554.0-1098.0)	812.0 (617.5-1061.0)	789.5 (595.5-1005.0)	789.0 (599.0-1061.0)	844.0 (546.5-1039.5)	0.0 (0.0-0.0)
	Range	221.0-1650.0	288.0-1996.0	254.0-2500.0	221.0-2500.0	378.0-1726.0	0.0-0.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**9.77** (2.73)	**9.21** (2.72)	**9.38** (2.68)	**9.48** (2.74)	**8.76** (2.27)	**0.00** (0.00)
	Median (IQR)	9.37 (7.61-11.68)	8.57 (7.64-10.38)	8.63 (7.63-10.78)	8.87 (7.63-10.74)	8.37 (7.26-10.43)	0.00 (0.00-0.00)
	Range	5.48-17.65	4.57-18.58	4.66-17.01	4.57-18.58	4.89-12.94	0.00-0.00
Cysteine (µmol/L)	$\bar{x}$ (SD)	**262.8** (36.2)	**254.1** (27.4)	**260.6** (36.3)	**257.7** (32.3)	**264.1** (35.9)	**0.0** (0.0)
	Median (IQR)	265.7 (232.0-285.2)	254.3 (236.0-272.1)	256.6 (237.0-282.6)	257.7 (235.8-279.2)	257.5 (236.1-274.6)	0.0 (0.0-0.0)
	Range	186.1-335.2	180.9-326.6	203.0-343.3	180.9-343.3	215.3-333.8	0.0-0.0
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**25.49** (4.42)	**26.35** (5.34)	**25.67** (4.67)	**25.97** (4.80)	**25.51** (6.17)	**0.00** (0.00)
	Median (IQR)	25.17 (22.46-27.97)	25.76 (23.17-29.08)	25.81 (22.87-28.01)	25.70 (22.96-28.68)	24.56 (20.91-29.16)	0.00 (0.00-0.00)
	Range	18.09-40.54	15.68-46.75	15.74-37.15	15.74-46.75	15.68-38.07	0.00-0.00
Glutathione (µmol/L)	$\bar{x}$ (SD)	**9.87** (2.97)	**10.82** (4.51)	**10.28** (3.23)	**10.18** (3.19)	**12.86** (7.67)	**0.00** (0.00) *
	Median (IQR)	9.97 (7.54-11.86)	10.12 (8.14-12.68)	9.85 (8.62-12.04)	10.04 (7.94-12.17)	9.48 (8.21-13.35)	0.00 (0.00-0.00)
	Range	4.09-20.89	3.17-35.20	4.38-23.46	3.17-27.94	6.73-35.20	0.00-0.00

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 1561C>T GCPII an unpaired T-test was performed in place of ANOVA given no recessive genotype was recorded.
For 1561C>T GCPII significance is achieved with native (untransformed) data for glutathione**.

3.2.4. Hypertensive phenotype

In examining the hypertensive phenotype to better understand the molecular basis of this condition, clinical data related to blood pressure has been collected. The most relevant data pertains to recumbent diastolic and systolic blood pressure however, standing diastolic and systolic blood pressure and recumbent and standing pulse rate were also collected. These have all been examined statistically, although they are not all necessarily recorded on every occasion in this thesis. Data has been separated according to hypertension status. This was achieved by organising the results according to the standard index for hypertension; a systolic blood pressure of ≥140 mm Hg or a diastolic blood pressure ≥90 mm Hg.

Displayed below are descriptive statistics for recumbent blood pressure measurement; these include diastolic (mm Hg), systolic (mm Hg) and pulse average (average beats per minute). These measurements are shown for all subjects, males, females and for normotensive and hypertensive groups.

Table 3-10: Hypertensive phenotype; recumbent blood pressure measurements

Measured blood pressure indices		$\bar{x}$ (SD)	Median (IQR)	Range
All (n=229)	Diastolic (mmHg)	70 (8)	69 (63-75)	45-94
	Systolic (mmHg)	131 (12)	132 (124-138)	93-165
	Pulse Average (min)	70 (9)	70 (64-77)	43-92
Males (n=92)	Diastolic (mmHg)	70 (8)	68 (63-76)	48-90
	Systolic (mmHg)	131 (13)	133 (124-139)	102-164
	Pulse Average (min)	69 (9)	68 (63-74)	43-92
Females (n=137)	Diastolic (mmHg)	70 (8)	69 (65-75)	45-94
	Systolic (mmHg)	131 (12)	132 (125-138)	93-165
	Pulse Average (min)	71 (8)	71 (65-78)	51-89
Normotensive (n=178)	Diastolic (mmHg)	68 (7)	67 (63-73)	45-88
	Systolic (mmHg)	127 (10)	128 (122-134)	93-139
	Pulse Average (min)	70 (9)	70 (64-76)	43-92
Hypertensive (n=51)	Diastolic (mmHg)	76 (8)	75 (68-82)	63-94
	Systolic (mmHg)	148 (7)	146 (143-151)	133-165
	Pulse Average (min)	71 (9)	69 (64-78)	56-91

The difference between B-vitamin and related thiols for hypertensive and normotensive phenotypes was examined using an unpaired t-test. Figure 3-2 (below) displays any differences graphically, and shows both the mean and standard deviation (error bars) for each variable. Significance was achieved with serum folate when the data was $\log_{10}$

transformed and extreme values removed. No other significance was detected between phenotypes.

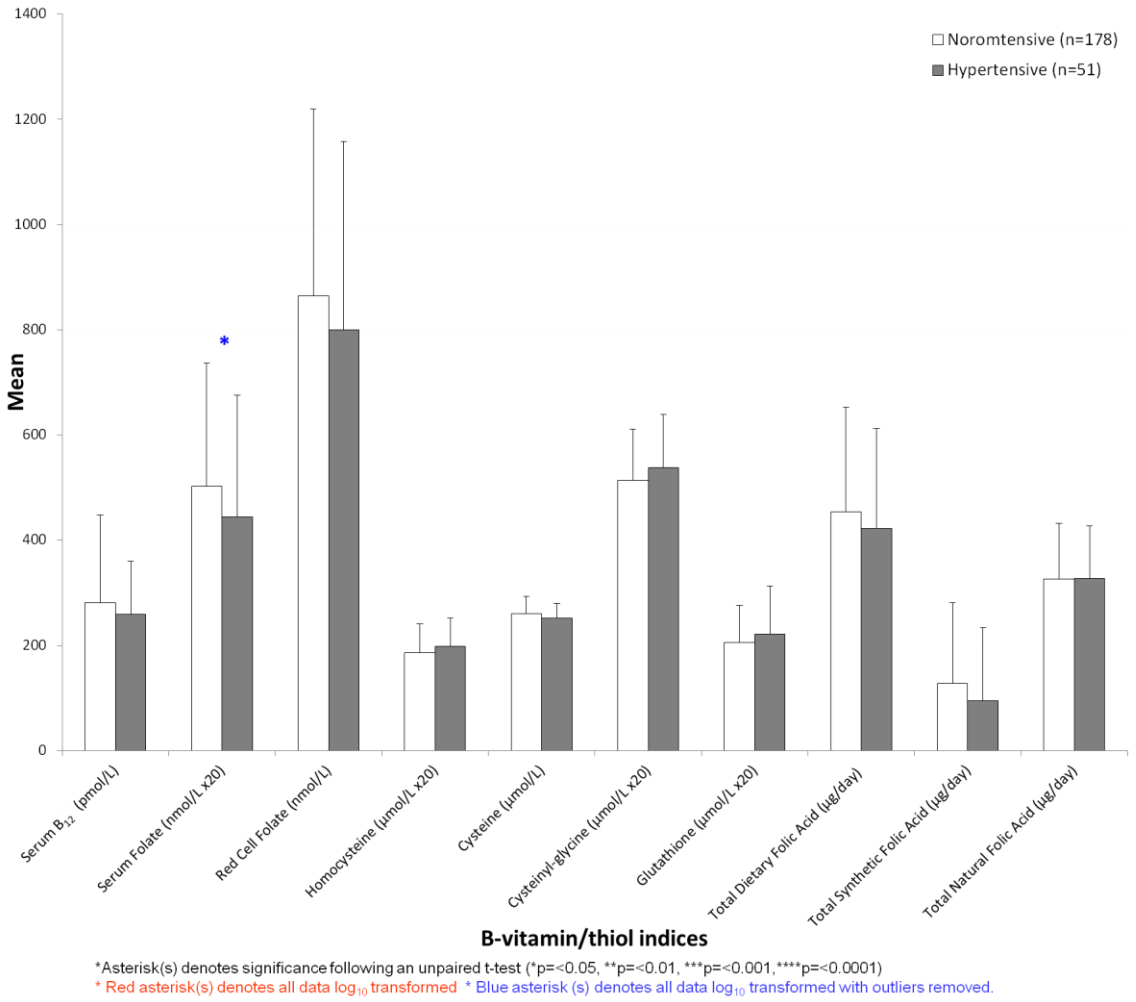

Figure 3-2: Mean and standard deviation values for B-vitamin/thiol measurements comparing hypertensive and normotensive phenotypes.

Means have been compared using an unpaired t-test; asterisk denotes significance level.

On an a priori basis, the hypertensive phenotype is likely to be an important outcome of B-vitamin related genetics. Therefore, each of the eleven genotypes that have been examined, are grouped into normotensive and hypertensive phenotypes. These are shown below where the genotype prevalence (%), allele number (frequency) and carriage of mutant allele are presented.

Table 3-11: Hypertensive phenotype; genotype prevalence and allele number

SNP	PHENOTYPE	GENOTYPE Prevalence (%)			ALLELE Number (frequency)		Carriage of mutant allele (%)
		Wildtype	Heterozygote	Recessive	Wildtype	Mutant	
677C>T MTHFR	Normotensive	85 (48)	78 (44)	15 (8)	248 (0.70)	108 (0.30)	52
	Hypertensive	22 (43)	18 (35)	11 (22)	62 (0.61)	40 (0.39)	57
1298A>C MTHFR	Normotensive	77 (43)	87 (49)	14 (8)	241 (0.68)	115 (0.32)	57
	Hypertensive	29 (57)	18 (35)	4 (8)	76 (0.75)	26 (0.25)	43
19bp del DHFR	Normotensive	61 (34)	79 (44)	38 (21)	201 (0.56)	155 (0.44)	66
	Hypertensive	16 (31)	28 (55)	7 (14)	60 (0.59)	42 (0.41)	69
2756A>G MTR	Normotensive	101 (57)	66 (37)	11 (6)	268 (0.75)	88 (0.25)	43
	Hypertensive	25 (49)	22 (43)	4 (8)	72 (0.71)	30 (0.29)	51
66A>G MTRR	Normotensive	31 (17)	92 (52)	55 (31)	154 (0.43)	202 (0.57)	83
	Hypertensive	19 (37)	24 (47)	8 (16)	40 (0.39)	62 (0.61)	63
80G>A RFC	Normotensive	51 (29)	85 (48)	42 (24)	187 (0.53)	169 (0.47)	71
	Hypertensive	18 (35)	19 (37)	14 (27)	55 (0.54)	47 (0.46)	65
1561C>T GCPII	Normotensive	162 (91)	16 (9)	0 (0)	340 (0.96)	16 (0.04)	9
	Hypertensive	48 (94)	3 (6)	0 (0)	99 (0.97)	3 (0.03)	6
1420C>T SHMT	Normotensive	93 (52)	72 (40)	13 (7)	258 (0.72)	98 (0.28)	48
	Hypertensive	22 (43)	22 (43)	7 (14)	66 (0.65)	36 (0.35)	57
1947G>A COMT	Normotensive	45 (26)	76 (43)	55 (31)	166 (0.47)	186 (0.53)	74
	Hypertensive	15 (29)	25 (49)	11 (22)	55 (0.54)	47 (0.46)	71
3'URT 6bp del TS	Normotensive	86 (48)	73 (41)	19 (11)	245 (0.69)	111 (0.31)	52
	Hypertensive	19 (37)	27 (53)	5 (10)	65 (0.64)	37 (0.36)	63
2R > 3R TSER	Normotensive	47 (26)	96 (54)	35 (20)	190 (0.53)	166 (0.47)	74
	Hypertensive	13 (25)	30 (59)	8 (16)	56 (0.55)	46 (0.45)	75

An odds ratio and the associated 95% confidence interval was calculated in order to assess the degree and significance of risk of individual SNPs for a systolic blood pressure of ≥140 or a diastolic blood pressure ≥90 (i.e. risk of having a hypertensive phenotype). A 2x2 table was then used to carry out a chi-square test and derive a *p* value. This was achieved using the Yates correction, which provides a stricter analysis for small samples. Table 3-12 below, displays the outcome of all these tests for each of the eleven B-vitamin related genetic variants examined.

Clearly, the odds ratio, 95% CI and *p* values show that there is no significant risk of any individual SNP being associated with the hypertensive phenotype as defined in the blood pressure range given in the previous paragraph.

Table 3-12: Hypertensive phenotype; odds ratio and 95% CI along with chi-square test *p* value

SNP	Odds Ratio	95% Confidence Intervals	*p*	x^2
677C>T MTHFR	1.48	0.94 - 2.34	0.1163	2.466
1298A>C MTHFR	0.72	0.44 - 1.18	0.2330	1.422
19bp del DHFR	0.91	0.58 -1.42	0.7554	0.097
2756A>G MTR	1.27	0.78 - 2.07	0.4082	0.684
66A>G MTRR	1.18	0.75 -1.85	0.5386	0.378
80G>A RFC	0.95	0.61 -1.47	0.8903	0.019
1561C>T GCPII	0.64	0.18 - 2.25	0.7778 ^	-
1420C>T SHMT	1.44	0.90 - 2.29	0.1625	1.95
1947G>A COMT	0.76	0.49 - 1.19	0.2753	1.19
3'UTR 6bp del TS	1.26	0.79 - 1.99	0.3954	0.722
2R > 3R TSER	0.94	0.60 - 1.46	0.8719	0.026

^ The *p* value for 1561C>T GCP11 was derived from a fishers-exact test, this was performed in place of chi-square test due to the low frequency of mutant alleles

3.2.5. Hypertensive phenotype; B-vitamin/thiol related nutritional genetic data organised by genotype

All descriptive data is displayed in the next eight tables for each B-vitamin and thiol related parameter examined (*normotensive tables 1 to 4 and hypertensive tables 1 to 4*), each is organised by genotype. In order to investigate whether genetic factors can influence variability in blood indices, a one-way ANOVA was performed. As alluded to earlier, the data set contains a number of extreme data points as a likely consequence of pharmacologic intervention, and therefore the ANOVA's were performed both with and without these extreme values. Similarly, the result of ANOVA based on $\log_{10}$ transformed (normalised) data is given in the following eight tables, and significance is denoted by either a red asterisk(s) (all $\log_{10}$ transformed values) or a blue asterisk(s) ($\log_{10}$ transformed values with outliers removed).

As previously stated, statistical results for untransformed data are displayed as footnotes in each table.

Table 3-13: Normotensive subjects; B-vitamin/thiol related nutritional genetic data by genotype (1 of 4)

Indices		677C>T MTHFR			1298A>C MTHFR			19bp del DHFR		
		Wt (n=85)	Het (n=78)	Rec (n=15)	Wt (n=77)	Het (n=87)	Rec (n=14)	Wt (n=61)	Het (n=79)	Rec (n=38)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**295.1** (175.4)	**271.0** (166.8)	**259.7** (111.2)	**301.7** (178.9)	**268.7** (163.3)	**250.6** (106.2)	**296.3** (197.6)	**280.0** (164.4)	**261.0** (110.2)
	Median (IQR)	259.0 (197.0-349.0)	218.0 (181.3-291.0)	215.0 (197.0-322.5)	246.0 (198.0-334.0)	227.0 (165.5-318.0)	245.0 (188.8-304.5)	245.0 (186.0-318.0)	231.0 (182.0-314.5)	251.0 (198.3-333.5)
	Range	64.0-1100.0	89.0-1100.0	74.0-495.0	74.0-1100.0	81.0-1100.0	64.0-434.0	95.0-1100.0	81.0-1100.0	64.0-620.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**27.42** (12.51)	**23.23** (10.80)	**22.33** (9.12)	**24.92** (11.78)	**24.13** (11.13)	**32.86** (12.44)	**25.41** (11.48)	**26.34** (11.38)	**22.29** (12.41) *
	Median (IQR)	26.00 (17.00-39.00)	22.00 (14.25-28.00)	21.00 (15.50-24.50)	23.00 (15.00-35.00)	23.00 (15.00-33.00)	35.50 (23.00-45.00)	24.00 (17.00-34.00)	24.00 (17.00-36.00)	21.50 (11.25-31.50)
	Range	3.00-45.00	7.00-45.00	10.00-45.00	7.00-45.00	3.00-45.00	11.00-45.00	7.00-45.00	4.00-45.00	3.00-45.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**886.9** (334.5)	**815.3** (357.2)	**988.7** (447.4)	**892.2** (429.2)	**814.1** (275.9)	**1030.4** (314.9)	**888.1** (307.6)	**888.3** (398.3)	**775.4** (332.9)
	Median (IQR)	853.0 (613.8-1120.5)	738.5 (588.0-1012.8)	804.0 (716.0-1227.0)	798.0 (587.0-1145.0)	794.0 (598.5-1003.5)	990.0 (820.0-1205.0)	830.0 (654.0-1062.0)	802.5 (568.3-1096.8)	720.0 (527.3-978.3)
	Range	221.0-1726.0	254.0-2500.0	386.0-2007.0	254.0-2500.0	221.0-1521.0	487.0-1550.0	386.0-1650.0	322.0-2500.0	221.0-1430.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**9.11** (2.77)	**9.59** (2.53)	**8.76** (3.38)	**8.99** (2.76)	**9.49** (2.71)	**9.74** (2.58)	**8.85** (2.49)	**9.29** (2.65)	**10.02** (3.11)
	Median (IQR)	8.53 (7.13-10.36)	9.03 (7.85-10.47)	7.97 (7.09-9.22)	8.13 (7.22-9.88)	8.86 (7.81-10.62)	9.31 (8.17-10.98)	8.45 (7.32-9.70)	8.83 (7.76-10.72)	9.10 (7.66-12.10)
	Range	4.76-17.02	5.62-18.58	4.57-16.29	4.57-18.58	4.76-17.65	6.07-14.76	4.57-18.58	4.66-17.65	4.76-17.01
Cysteine (µmol/L)	$\bar{x}$ (SD)	**260.1** (33.7)	**262.2** (33.5)	**250.4** (34.6)	**259.0** (36.8)	**260.9** (31.5)	**262.3** (30.1)	**258.1** (30.7)	**262.8** (37.1)	**258.2** (31.0)
	Median (IQR)	261.2 (238.2-281.6)	262.5 (239.2-284.4)	254.6 (230.7-273.1)	261.8 (232.0-278.8)	259.7 (239.4-283.6)	264.4 (253.8-280.3)	261.2 (234.2-279.5)	262.4 (237.5-286.7)	257.0 (240.2-279.0)
	Range	200.7-333.8	180.9-343.3	186.1-307.9	180.9-343.3	200.7-333.8	205.3-317.8	186.1-326.6	180.9-343.3	204.2-313.0
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**25.91** (4.83)	**25.34** (5.01)	**26.58** (4.26)	**25.87** (5.06)	**25.65** (4.68)	**25.23** (5.11)	**25.92** (5.49)	**25.52** (4.39)	**25.79** (4.82)
	Median (IQR)	25.82 (22.46-29.04)	24.92 (22.90-27.38)	27.74 (23.31-29.08)	25.20 (22.96-28.88)	25.71 (22.56-28.20)	24.00 (21.75-26.62)	24.12 (22.91-27.38)	26.03 (22.90-28.47)	26.16 (21.84-28.79)
	Range	15.68-40.81	15.74-46.75	20.31-34.04	17.04-46.75	15.68-40.81	16.76-35.04	15.74-46.75	15.68-35.01	17.86-36.42
Glutathione (µmol/L)	$\bar{x}$ (SD)	**10.76** (4.25)	**9.86** (2.76)	**9.51** (2.69)	**10.54** (3.62)	**9.97** (3.48)	**10.52** (3.87)	**9.86** (2.40)	**10.57** (4.46)	**10.26** (2.99)
	Median (IQR)	10.05 (8.12-12.61)	9.71 (7.74-11.79)	8.60 (7.77-12.06)	10.24 (8.34-12.49)	9.42 (7.76-11.62)	10.34 (7.68-13.21)	10.23 (7.76-12.04)	9.78 (7.83-12.63)	10.12 (8.59-11.59)
	Range	3.17-27.94	4.09-20.89	5.99-14.22	3.50-27.94	3.17-26.40	4.99-18.30	5.25-15.10	3.17-27.94	4.09-18.30

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 677C>T MTHFR significance is achieved with native (untransformed) data for serum folate *.
For 1298A>C MTHFR significance is achieved with native (untransformed) data for serum folate *.

Table 3-14: Normotensive subjects; B-vitamin/thiol related nutritional genetic data by genotype (2 of 4)

Indices		2756A>G MTR			66A>G MTRR			1947G>A COMT		
		Wt (n=101)	Het (n=66)	Rec (n=11)	Wt (n=31)	Het (n=92)	Rec (n=55)	Wt (n=45)	Het (n=76)	Rec (n=55)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**259.8** (116.9)	**311.2** (223.9)	**303.7** (138.9)	**262.2** (107.8)	**291.7** (187.5)	**275.6** (158.7)	**270.9** (159.3)	**296.6** (186.4)	**264.4** (142.4)
	Median (IQR)	241.0 (174.0-314.0)	235.5 (199.0-315.5)	243.0 (200.0-393.0)	245.0 (186.5-339.0)	250.0 (189.8-323.5)	229.0 (181.5-293.0)	239.0 (183.5-294.5)	251.0 (199.8-321.3)	220.0 (157.0-318.0)
	Range	64.0-632.0	74.0-1100.0	144.0-557.0	95.0-554.0	64.0-1100.0	81.0-854.0	89.0-1100.0	64.0-1100.0	81.0-702.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**24.65** (11.55)	**25.03** (11.73)	**30.55** (12.27)	**23.55** (10.18)	**26.48** (12.13)	**23.85** (11.63)	**24.56** (11.43)	**26.18** (11.89)	**23.53** (11.48)
	Median (IQR)	24.00 (15.00-33.00)	22.50 (15.00-34.75)	32.00 (22.50-41.00)	23.00 (15.00-32.00)	25.50 (17.00-38.00)	22.00 (14.50-31.00)	23.00 (14.50-35.00)	24.50 (17.00-37.25)	20.00 (14.00-27.00)
	Range	3.00-45.00	8.00-45.00	12.00-45.00	10.00-45.00	4.00-45.00	3.00-45.00	7.00-45.00	3.00-45.00	4.00-45.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**850.3** (340.1)	**842.6** (318.7)	**1117.5** (596.7)	**788.5** (267.7)	**903.8** (388.6)	**839.6** (340.1)	**830.8** (348.1)	**895.7** (376.4)	**849.9** (342.0)
	Median (IQR)	803.5 (595.8-1076.5)	787.0 (613.3-1014.3)	1015.0 (737.5-1341.0)	780.0 (600.0-923.5)	841.5 (604.3-1104.0)	793.0 (564.8-1057.0)	781.5 (553.5-997.5)	850.5 (611.0-1099.3)	784.0 (602.0-1048.0)
	Range	221.0-2007.0	396.0-1726.0	386.0-2500.0	418.0-1424.0	254.0-2500.0	221.0-1726.0	263.0-2007.0	221.0-2500.0	322.0-1726.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**9.35** (2.78)	**9.36** (2.70)	**8.40** (2.35)	**9.44** (2.93)	**9.37** (2.79)	**9.09** (2.52)	**8.88** (2.29)	**9.71** (2.90)	**9.20** (2.88)
	Median (IQR)	8.67 (7.57-10.36)	8.63 (7.64-10.68)	8.37 (7.31-8.99)	8.33 (7.66-11.18)	8.55 (7.63-10.40)	8.86 (7.51-10.36)	8.58 (7.63-10.11)	8.79 (7.77-11.06)	8.62 (7.49-9.88)
	Range	4.66-18.58	4.57-17.65	4.89-12.46	5.56-17.65	4.66-17.02	4.57-18.58	4.66-17.65	4.89-18.58	4.57-17.02
Cysteine (µmol/L)	$\bar{x}$ (SD)	**260.1** (33.1)	**258.0** (34.5)	**274.5** (32.9)	**258.7** (33.7)	**258.1** (33.9)	**264.6** (33.4)	**261.9** (34.6)	**263.6** (32.1)	**253.7** (34.6)
	Median (IQR)	261.6 (238.4-280.6)	260.0 (232.5-282.0)	267.7 (256.5-291.3)	255.0 (236.5-279.3)	259.5 (235.3-281.5)	264.4 (242.7-283.2)	258.9 (242.6-283.6)	264.9 (238.8-286.1)	257.3 (230.3-278.1)
	Range	200.7-343.3	180.9-335.2	229.0-333.8	205.3-325.1	180.9-343.3	204.4-335.2	180.9-343.3	204.4-335.2	186.1-332.5
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**25.37** (4.60)	**26.19** (5.02)	**26.03** (6.28)	**26.37** (5.86)	**25.60** (4.21)	**25.54** (5.29)	**25.80** (4.46)	**25.71** (5.12)	**25.53** (5.05)
	Median (IQR)	25.06 (22.87-27.86)	25.51 (22.90-28.86)	27.68 (22.84-29.55)	25.78 (22.89-28.47)	25.62 (22.98-28.30)	24.60 (22.76-28.26)	25.70 (22.98-28.13)	25.12 (22.70-27.85)	24.78 (22.94-29.05)
	Range	15.74-46.75	18.09-40.81	15.68-34.04	17.04-46.75	15.74-35.04	15.68-39.09	17.04-36.42	15.68-46.75	15.74-38.47
Glutathione (µmol/L)	$\bar{x}$ (SD)	**9.98** (3.13)	**10.67** (3.80)	**10.43** (5.55)	**9.83** (2.44)	**10.59** (3.98)	**9.96** (3.34)	**10.44** (3.37)	**10.57** (3.92)	**9.31** (2.92)
	Median (IQR)	9.67 (7.82-11.49)	10.41 (8.22-12.69)	8.60 (7.47-12.92)	10.05 (8.02-11.62)	10.25 (8.21-12.33)	9.67 (7.56-12.21)	10.35 (8.00-12.55)	10.24 (8.21-12.43)	9.33 (7.32-10.70)
	Range	4.09-26.40	3.50-27.94	3.17-23.46	4.38-15.37	3.17-27.94	3.50-23.46	5.36-23.46	3.17-27.94	3.50-20.89

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data log_{10} transformed * Blue asterisk (s) denotes all data log_{10} transformed with outliers removed.

Note: For 2756A>G MTR significance is achieved with native (untransformed) data for red cell folate*.

Table 3-15: Normotensive subjects; B-vitamin/thiol related nutritional genetic data by genotype (3 of 4)

Indices		3'UTR 6bp del TS			2R > 3R TSER			1420C>T SHMT		
		Wt (n=86)	Het (n=73)	Rec (n=19)	Wt (n=47)	Het (n=96)	Rec (n=35)	Wt (n=93)	Het (n=72)	Rec (n=13)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**284.3** (159.6)	**283.2** (185.0)	**262.7** (128.0)	**248.3** (106.0)	**307.6** (204.5)	**254.8** (94.0)	**293.4** (178.3)	**277.8** (160.3)	**217.8** (98.4)
	Median (IQR)	245.0 (196.3-311.0)	227.0 (182.0-344.0)	245.0 (174.0-326.5)	240.0 (184.0-280.5)	240.0 (197.8-365.3)	247.0 (180.5-303.0)	255.0 (183.0-349.0)	241.0 (196.8-314.8)	200.0 (177.0-227.0)
	Range	89.0-1100.0	64.0-1100.0	74.0-554.0	74.0-688.0	64.0-1100.0	117.0-495.0	64.0-1100.0	81.0-1100.0	102.0-495.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**25.17** (11.78)	**25.25** (12.19)	**24.74** (9.55)	**24.49** (12.29)	**26.43** (11.62)	**22.57** (10.78)	**24.66** (11.44)	**26.33** (12.40)	**22.23** (8.82)
	Median (IQR)	23.00 (15.25-35.00)	23.00 (14.00-37.00)	24.00 (17.00-32.00)	22.00 (15.00-33.50)	24.00 (17.00-38.00)	21.00 (14.00-30.50)	23.00 (15.00-35.00)	24.00 (16.75-39.75)	21.00 (15.00-26.00)
	Range	4.00-45.00	3.00-45.00	9.00-40.00	3.00-45.00	8.00-45.00	4.00-45.00	4.00-45.00	3.00-45.00	12.00-40.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**857.6** (341.6)	**888.8** (398.0)	**797.1** (240.1)	**802.2** (323.7)	**906.3** (377.3)	**832.3** (333.7)	**850.6** (389.9)	**888.0** (320.8)	**825.5** (310.8)
	Median (IQR)	803.0 (614.0-1048.0)	815.0 (598.0-1145.0)	783.0 (597.0-985.0)	783.0 (591.5-1005.0)	812.0 (613.5-1132.5)	830.0 (601.0-1003.0)	782.5 (555.5-1075.5)	803.5 (628.5-1091.0)	820.0 (683.0-864.0)
	Range	254.0-2007.0	221.0-2500.0	471.0-1266.0	221.0-1550.0	346.0-2500.0	254.0-1726.0	254.0-2500.0	221.0-1588.0	421.0-1650.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**9.09** (2.61)	**9.45** (2.69)	**9.63** (3.38)	**9.67** (3.00)	**9.11** (2.56)	**9.29** (2.79)	**9.21** (2.92)	**9.52** (2.62)	**8.64** (1.60)
	Median (IQR)	8.54 (7.61-10.32)	8.67 (7.64-10.88)	8.86 (7.48-10.07)	9.10 (7.63-10.91)	8.55 (7.60-10.05)	8.56 (7.74-10.65)	8.50 (7.41-10.43)	8.87 (7.79-10.55)	8.96 (7.24-9.88)
	Range	4.57-18.58	5.20-17.65	5.56-17.02	4.76-18.58	4.66-17.65	4.57-16.59	4.66-18.58	4.57-17.65	6.12-10.93
Cysteine (µmol/L)	$\bar{x}$ (SD)	**258.3** (34.0)	**262.2** (35.5)	**261.2** (24.4)	**261.9** (36.2)	**261.7** (33.6)	**253.8** (30.2)	**258.5** (33.8)	**262.8** (35.1)	**258.6** (23.8)
	Median (IQR)	261.7 (235.3-275.3)	259.7 (235.7-287.0)	264.7 (245.9-280.1)	261.8 (237.3-280.7)	262.5 (239.6-285.3)	258.4 (232.0-277.3)	257.5 (233.7-279.0)	263.1 (238.5-286.5)	262.6 (244.1-273.4)
	Range	180.9-343.3	186.1-333.8	207.5-294.1	204.2-343.3	180.9-335.2	200.7-309.2	200.7-343.3	180.9-333.8	220.8-297.8
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**25.57** (4.78)	**26.13** (5.17)	**24.74** (3.88)	**25.62** (5.52)	**25.84** (4.58)	**25.51** (4.77)	**25.49** (4.45)	**25.75** (5.23)	**27.14** (5.60)
	Median (IQR)	25.37 (23.01-27.91)	25.82 (22.79-29.05)	24.56 (22.41-26.39)	24.56 (22.44-27.44)	25.78 (23.04-28.91)	25.17 (22.78-28.15)	26.03 (22.87-28.43)	24.71 (22.89-28.19)	27.38 (23.38-27.86)
	Range	15.68-46.75	16.76-40.81	18.58-31.80	16.76-46.75	15.74-39.09	15.68-38.47	15.68-38.47	15.74-46.75	20.31-40.81
Glutathione (µmol/L)	$\bar{x}$ (SD)	**10.54** (3.99)	**10.05** (3.29)	**9.80** (2.34)	**9.86** (2.51)	**10.70** (4.00)	**9.59** (3.41)	**10.45** (3.82)	**10.05** (3.35)	**10.08** (2.85)
	Median (IQR)	10.25 (7.80-12.47)	9.62 (8.12-11.81)	10.04 (8.12-11.40)	9.94 (8.10-11.87)	10.25 (8.32-12.42)	8.92 (7.45-12.16)	10.04 (7.90-12.70)	10.12 (7.74-11.97)	9.19 (8.29-10.49)
	Range	3.17-27.94	3.50-23.46	6.11-14.56	5.25-15.37	3.50-27.94	3.17-20.89	4.09-27.94	3.17-23.46	7.47-18.30

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Table 3-16: Normotensive subjects; B-vitamin/thiol related nutritional genetic data by genotype (4 of 4)

Indices		80G>A RFC			1561C>T GCPII		
		Wt (n=51)	Het (n=85)	Rec (n=42)	Wt (n=162)	Het (n=16)	Rec (n=0)
Serum Vitamin B_{12} (pmol/L)	$\bar{X}$ (SD)	**259.7** (135.7)	**297.5** (193.7)	**275.8** (140.7)	**285.4** (171.1)	**242.9** (111.9)	**0.0** (0.0)
	Median (IQR)	215.0 (192.5-290.5)	245.0 (192.0-349.0)	254.0 (168.3-354.5)	241.0 (189.0-330.0)	226.5 (151.8-295.8)	0.0 (0.0-0.0)
	Range	108.0-854.0	64.0-1100.0	95.0-702.0	64.0-1100.0	106.0-495.0	0.0-0.0
Serum Folate (nmol/L)	$\bar{X}$ (SD)	**22.98** (11.79)	**26.58** (11.13)	**24.93** (12.47)	**24.79** (11.73)	**28.88** (10.78)	**0.00** (0.00)
	Median (IQR)	19.00 (14.00-32.50)	25.00 (19.00-35.00)	21.50 (14.25-36.00)	23.00 (15.00-33.75)	26.50 (21.50-37.50)	0.00 (0.00-0.00)
	Range	3.00-45.00	4.00-45.00	7.00-45.00	3.00-45.00	12.00-45.00	0.00-0.00
Red Cell Folate (nmol/L)	$\bar{X}$ (SD)	**846.7** (364.4)	**877.2** (314.9)	**858.5** (426.9)	**855.0** (352.4)	**954.8** (396.5)	**0.0** (0.0)
	Median (IQR)	802.0 (598.5-1086.5)	820.5 (646.5-1072.8)	789.5 (588.0-1013.0)	799.0 (599.0-1068.0)	955.5 (662.8-1068.3)	0.0 (0.0-0.0)
	Range	221.0-1650.0	322.0-1726.0	254.0-2500.0	221.0-2500.0	386.0-1726.0	0.0-0.0
Homocysteine (µmol/L)	$\bar{X}$ (SD)	**9.57** (2.76)	**9.10** (2.80)	**9.36** (2.54)	**9.38** (2.75)	**8.44** (2.33)	**0.00** (0.00)
	Median (IQR)	8.86 (7.49-11.13)	8.47 (7.55-9.98)	8.79 (7.86-10.34)	8.78 (7.63-10.46)	8.05 (6.86-9.24)	0.00 (0.00-0.00)
	Range	6.07-17.65	4.57-18.58	4.66-17.01	4.57-18.58	4.89-12.94	0.00-0.00
Cysteine (µmol/L)	$\bar{X}$ (SD)	**262.9** (39.1)	**256.9** (28.1)	**263.6** (36.9)	**259.8** (33.2)	**264.4** (39.1)	**0.0** (0.0)
	Median (IQR)	266.7 (228.4-291.4)	257.5 (239.1-275.9)	263.6 (239.8-286.8)	261.7 (237.2-281.8)	257.4 (233.1-281.0)	0.0 (0.0-0.0)
	Range	186.1-335.2	180.9-326.6	204.2-343.3	180.9-343.3	215.3-333.8	0.0-0.0
Cysteinyl-glycine (µmol/L)	$\bar{X}$ (SD)	**25.05** (3.76)	**26.27** (5.52)	**25.39** (4.56)	**25.81** (4.77)	**24.78** (5.73)	**0.00** (0.00)
	Median (IQR)	25.17 (22.72-27.44)	25.70 (23.07-29.05)	25.38 (22.42-27.76)	25.70 (22.93-28.01)	24.32 (20.26-29.16)	0.00 (0.00-0.00)
	Range	18.09-34.64	15.68-46.75	15.74-36.42	15.74-46.75	15.68-34.04	0.00-0.00
Glutathione (µmol/L)	$\bar{X}$ (SD)	**10.01** (2.98)	**10.42** (3.99)	**10.25** (3.35)	**10.14** (3.28)	**11.48** (5.71)	**0.00** (0.00)
	Median (IQR)	10.27 (7.76-11.90)	9.67 (7.99-12.65)	9.63 (8.33-12.21)	10.05 (7.96-12.08)	9.28 (8.13-12.62)	0.00 (0.00-0.00)
	Range	4.09-20.89	3.17-27.94	4.38-23.46	3.17-27.94	6.73-26.40	0.00-0.00

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 1561C>T GCPII an unpaired T-test was performed in place of ANOVA given no recessive genotype was recorded.

Table 3-17: Hypertensive subjects; B-vitamin/thiol related nutritional genetic data by genotype (1 of 4)

Indices		677C>T MTHFR			1298A>C MTHFR			19bp del DHFR		
		Wt (n=22)	Het (n=18)	Rec (n=11)	Wt (n=29)	Het (n=18)	Rec (n=4)	Wt (n=16)	Het (n=28)	Rec (n=7)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**258.1** (104.5)	**220.7** (80.3)	**302.0** (119.0)	**258.6** (114.2)	**260.2** (87.3)	**197.5** (75.7)	**246.1** (105.1)	**251.9** (97.2)	**283.0** (128.2)
	Median (IQR)	271.0 (181.3-313.3)	225.0 (172.5-270.0)	249.0 (231.0-382.5)	240.0 (194.0-317.0)	267.0 (188.3-299.5)	192.0 (160.0-229.5)	241.0 (173.0-303.5)	240.0 (195.5-294.5)	291.0 (178.0-340.0)
	Range	102.0-508.0	78.0-368.0	138.0-544.0	78.0-544.0	146.0-508.0	112.0-294.0	78.0-484.0	89.0-544.0	146.0-508.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**24.73** (9.73)	**19.28** (11.56)	**21.45** (13.94) *	**21.41** (12.78)	**23.11** (10.46)	**22.50** (3.42)	**23.31** (14.79)	**19.36** (8.45)	**30.29** (9.89)
	Median (IQR)	22.50 (18.00-27.00)	15.00 (10.25-25.50)	21.00 (8.50-28.50)	20.00 (10.00-26.00)	18.50 (16.25-29.25)	23.00 (21.00-24.50)	18.50 (11.25-38.50)	18.00 (13.75-24.25)	27.00 (24.00-37.00)
	Range	12.00-45.00	8.00-45.00	6.00-45.00	6.00-45.00	9.00-45.00	18.00-26.00	6.00-45.00	7.00-45.00	18.00-45.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**785.2** (278.3)	**695.3** (275.1)	**998.2** (524.8)	**821.3** (408.3)	**787.3** (292.7)	**695.5** (198.4)	**770.6** (296.9)	**786.1** (394.2)	**918.6** (333.2)
	Median (IQR)	703.0 (634.0-887.3)	654.0 (531.5-786.8)	983.0 (629.0-1253.5)	753.0 (530.0-1077.0)	698.5 (644.5-836.5)	728.5 (636.5-787.5)	722.5 (640.0-990.5)	699.0 (517.0-950.3)	849.0 (729.0-1026.5)
	Range	425.0-1534.0	256.0-1389.0	288.0-1996.0	256.0-1996.0	433.0-1534.0	425.0-900.0	288.0-1336.0	256.0-1996.0	536.0-1534.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**9.59** (2.85)	**10.74** (2.73)	**8.99** (1.70)	**9.61** (2.60)	**9.97** (2.64)	**11.26** (3.29)	**10.75** (2.54)	**9.33** (2.62)	**9.99** (2.81)
	Median (IQR)	8.45 (7.67-11.65)	10.64 (9.15-12.24)	8.99 (7.97-10.09)	9.42 (7.56-11.45)	9.77 (8.01-11.65)	10.48 (8.89-12.85)	10.17 (9.03-12.00)	8.55 (7.36-10.90)	10.50 (7.81-12.18)
	Range	5.48-15.64	6.83-15.80	5.68-11.83	5.68-15.12	5.48-15.80	8.44-15.64	6.83-15.80	5.48-15.64	6.00-13.46
Cysteine (µmol/L)	$\bar{x}$ (SD)	**252.7** (28.1)	**252.7** (29.5)	**247.2** (27.3)	**254.3** (32.5)	**249.0** (20.4)	**242.3** (24.0)	**267.7** (33.2)	**242.9** (24.0)	**248.9** (10.0) **
	Median (IQR)	248.2 (238.2-268.3)	249.3 (233.8-265.5)	232.9 (230.0-264.2)	249.9 (230.1-267.0)	247.4 (239.5-263.6)	238.7 (231.9-249.1)	261.3 (238.6-291.4)	239.1 (226.2-259.9)	244.5 (241.0-254.2)
	Range	211.0-322.5	203.0-317.5	206.6-292.9	206.6-322.5	203.0-279.3	217.1-274.8	227.7-322.5	203.0-292.9	240.5-267.0
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**27.56** (5.94)	**25.41** (4.51)	**26.96** (4.03)	**26.87** (5.27)	**26.83** (4.60)	**24.50** (6.75)	**26.70** (6.31)	**26.53** (4.87)	**27.19** (3.07)
	Median (IQR)	27.40 (23.04-31.67)	24.74 (23.03-26.26)	27.38 (24.16-29.94)	26.05 (22.53-29.71)	25.64 (24.23-28.98)	23.77 (21.49-26.77)	24.98 (22.81-26.84)	26.19 (23.35-29.82)	28.70 (25.25-29.39)
	Range	17.07-38.07	19.79-40.54	20.63-33.27	18.61-40.54	21.13-38.07	17.07-33.40	20.63-40.54	17.07-36.08	22.06-30.29
Glutathione (µmol/L)	$\bar{x}$ (SD)	**11.34** (6.11)	**10.46** (3.09)	**10.62** (3.12)	**10.58** (3.16)	**11.42** (6.65)	**10.57** (1.91)	**12.29** (6.68)	**10.17** (3.06)	**10.48** (3.73)
	Median (IQR)	10.12 (7.93-12.58)	9.19 (8.15-11.75)	10.45 (8.57-12.36)	10.56 (8.21-12.19)	9.35 (7.61-13.50)	10.10 (9.64-11.03)	10.97 (8.99-12.06)	9.78 (7.48-12.71)	10.28 (8.18-11.83)
	Range	4.87-35.20	6.91-17.21	6.01-16.81	4.87-17.21	5.86-35.20	8.81-13.28	6.01-35.20	4.87-16.52	5.86-17.21

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data log_{10} transformed * Blue asterisk (s) denotes all data log_{10} transformed with outliers removed.

Note: For 19bp del DHFR significance is achieved with native (untransformed) data for both cysteine ** and serum folate *.

Table 3-18: Hypertensive subjects; B-vitamin/thiol related nutritional genetic data by genotype (2 of 4)

Indices		2756A>G MTR			66A>G MTRR			1947G>A COMT		
		Wt (n=25)	Het (n=22)	Rec (n=4)	Wt (n=8)	Het (n=24)	Rec (n=19)	Wt (n=15)	Het (n=25)	Rec (n=11)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**246.9** (89.9)	**274.7** (118.3)	**189.5** (59.9)	**265.8** (111.7)	**262.0** (122.9)	**240.0** (68.7)	**259.7** (64.2)	**256.7** (116.3)	**241.9** (118.8)
	Median (IQR)	240.0 (198.0-292.0)	262.0 (194.5-321.5)	172.0 (162.0-199.5)	270.5 (200.8-289.3)	240.0 (177.5-318.5)	238.0 (195.0-291.0)	240.0 (220.5-303.5)	267.0 (194.0-320.0)	185.0 (173.0-265.5)
	Range	78.0-484.0	89.0-544.0	138.0-276.0	129.0-484.0	78.0-544.0	102.0-368.0	174.0-417.0	78.0-508.0	146.0-544.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**23.16** (11.44)	**21.77** (12.32)	**17.25** (3.95)	**29.50** (15.27)	**22.17** (10.68)	**18.89** (9.46)	**21.07** (11.40)	**21.92** (11.64)	**23.91** (11.73)
	Median (IQR)	23.00 (15.00-27.00)	18.00 (13.50-30.00)	16.50 (14.00-19.75)	30.00 (17.75-45.00)	18.50 (15.00-27.00)	18.00 (11.00-24.00)	18.00 (13.50-25.50)	20.00 (14.00-27.00)	22.00 (15.50-29.50)
	Range	7.00-45.00	6.00-45.00	14.00-22.00	10.00-45.00	7.00-45.00	6.00-43.00	7.00-45.00	6.00-45.00	9.00-45.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**823.1** (363.0)	**777.4** (371.7)	**772.8** (264.9)	**851.8** (328.5)	**870.5** (401.7)	**687.6** (284.2)	**857.4** (438.7)	**759.5** (325.7)	**811.2** (311.2)
	Median (IQR)	749.0 (530.0-1077.0)	713.5 (570.3-905.5)	645.0 (638.5-779.3)	904.0 (614.8-1111.8)	713.5 (617.3-1052.3)	699.0 (484.5-797.5)	750.0 (588.5-1021.0)	699.0 (521.0-1013.0)	695.0 (592.5-891.0)
	Range	346.0-1534.0	256.0-1996.0	631.0-1170.0	346.0-1236.0	425.0-1996.0	256.0-1389.0	378.0-1996.0	256.0-1389.0	505.0-1534.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**9.32** (2.18)	**10.01** (2.80)	**12.51** (3.41)	**9.60** (2.77)	**9.87** (2.88)	**9.96** (2.42)	**9.55** (2.43)	**9.83** (2.48)	**10.38** (3.39)
	Median (IQR)	8.47 (7.43-11.45)	9.77 (7.86-11.66)	12.82 (10.20-15.14)	9.66 (7.02-11.76)	8.81 (7.83-11.95)	9.76 (8.73-10.76)	9.42 (7.97-10.80)	9.76 (7.87-10.90)	11.45 (7.45-12.49)
	Range	6.00-13.88	5.48-15.64	8.63-15.80	6.36-13.46	5.48-15.80	6.00-15.12	6.00-14.92	6.83-15.64	5.48-15.80
Cysteine (µmol/L)	$\bar{x}$ (SD)	**255.8** (31.1)	**244.3** (22.3)	**264.4** (32.2)	**261.2** (38.4)	**245.6** (24.8)	**254.9** (26.6)	**249.5** (27.8)	**256.6** (30.9)	**242.8** (19.5)
	Median (IQR)	254.4 (237.0-271.1)	239.8 (230.3-253.1)	260.0 (248.5-275.8)	245.4 (237.3-277.8)	243.0 (230.0-255.3)	256.0 (234.8-272.5)	240.8 (232.8-262.8)	254.6 (230.7-273.4)	240.6 (234.6-258.4)
	Range	203.0-322.5	206.6-292.9	230.2-307.4	219.9-322.5	203.0-314.2	206.6-307.4	213.6-307.4	206.6-322.5	203.0-267.0
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**25.80** (4.88)	**27.24** (5.19)	**28.99** (6.06)	**28.93** (7.85)	**26.14** (4.31)	**26.39** (4.64)	**26.04** (5.32)	**27.69** (5.62)	**25.21** (2.90)
	Median (IQR)	24.80 (22.35-28.87)	26.21 (23.73-30.26)	27.79 (25.21-31.57)	26.89 (24.00-34.81)	26.39 (23.01-29.72)	25.27 (23.77-27.40)	25.93 (22.33-29.52)	26.05 (24.68-30.17)	24.56 (23.42-27.58)
	Range	17.07-40.54	18.61-38.07	23.23-37.15	18.61-40.54	17.07-33.40	20.79-37.15	17.07-37.15	19.79-40.54	21.13-29.76
Glutathione (µmol/L)	$\bar{x}$ (SD)	**10.62** (3.25)	**11.07** (6.00)	**11.38** (3.52)	**14.67** (8.89)	**9.86** (2.86)	**10.56** (3.06)	**9.19** (1.82)	**11.88** (5.81)	**10.91** (3.58)
	Median (IQR)	10.28 (8.21-12.57)	9.50 (8.07-12.44)	11.35 (9.89-12.84)	12.58 (10.33-15.07)	9.78 (7.23-11.97)	9.56 (8.94-11.56)	8.92 (7.88-9.94)	11.24 (8.96-13.28)	10.28 (8.16-13.20)
	Range	4.87-17.21	5.86-35.20	7.13-15.69	7.28-35.20	5.86-15.69	4.87-17.21	6.93-13.27	4.87-35.20	6.44-17.21

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data log_{10} transformed * Blue asterisk (s) denotes all data log_{10} transformed with outliers removed.

Note: For 66A>G MTRR significance is achieved with native (untransformed) data for glutathione*.

Table 3-19: Hypertensive subjects; B-vitamin/thiol related nutritional genetic data by genotype (3 of 4)

Indices		3'UTR 6bp del TS			2R > 3R TSER			1420C>T SHMT		
		Wt (n=19)	Het (n=27)	Rec (n=5)	Wt (n=13)	Het (n=30)	Rec (n=8)	Wt (n=22)	Het (n=22)	Rec (n=7)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**235.6** (69.5)	**252.7** (118.5)	**334.6** (93.6)	**302.1** (93.2)	**229.0** (95.1)	**271.9** (124.4) ‡	**258.7** (100.5)	**239.1** (94.5)	**288.9** (137.1)
	Median (IQR)	238.0 (189.0-293.0)	231.0 (177.0-321.5)	288.0 (275.0-368.0)	267.0 (231.0-368.0)	228.5 (157.3-291.8)	241.0 (179.0-305.8)	246.5 (183.5-293.3)	227.5 (187.8-295.5)	292.0 (218.5-318.5)
	Range	112.0-357.0	78.0-544.0	258.0-484.0	194.0-484.0	78.0-508.0	174.0-544.0	78.0-484.0	89.0-508.0	112.0-544.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**24.26** (12.32)	**19.93** (10.31)	**25.60** (13.46)	**16.62** (10.32)	**23.70** (11.71)	**25.00** (10.10) *	**23.36** (13.33)	**21.09** (10.90)	**21.29** (5.94)
	Median (IQR)	24.00 (15.00-27.00)	18.00 (12.50-24.00)	23.00 (22.00-30.00)	14.00 (10.00-20.00)	22.50 (15.25-29.25)	24.00 (17.50-28.25)	22.50 (12.50-27.00)	18.00 (13.25-25.75)	19.00 (17.50-23.50)
	Range	6.00-45.00	7.00-45.00	8.00-45.00	7.00-45.00	6.00-45.00	14.00-45.00	6.00-45.00	7.00-45.00	16.00-32.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**773.7** (392.0)	**779.4** (332.3)	**1005.4** (327.0)	**837.5** (374.1)	**794.1** (382.7)	**757.6** (220.9)	**837.4** (310.1)	**800.0** (439.6)	**678.6** (122.5)
	Median (IQR)	707.0 (528.5-941.5)	698.0 (580.5-897.0)	1059.0 (699.0-1236.0)	749.0 (641.0-1171.0)	698.5 (531.5-1005.5)	789.5 (609.8-886.5)	797.5 (647.8-1072.5)	680.5 (481.0-962.3)	698.0 (597.5-728.0)
	Range	288.0-1996.0	256.0-1534.0	644.0-1389.0	355.0-1509.0	256.0-1996.0	425.0-1098.0	288.0-1534.0	256.0-1996.0	521.0-880.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**9.84** (2.31)	**10.30** (2.88)	**7.61** (1.38)	**9.30** (1.75)	**10.10** (2.87)	**9.90** (3.13)	**9.81** (2.59)	**9.87** (2.27)	**10.03** (4.11)
	Median (IQR)	9.42 (8.14-11.20)	10.47 (7.98-12.16)	6.92 (6.83-8.19)	9.47 (8.08-10.47)	9.91 (7.86-11.88)	10.06 (7.52-11.84)	9.61 (7.61-11.62)	9.75 (8.17-11.65)	8.47 (7.28-12.85)
	Range	7.06-15.64	5.48-15.80	6.36-9.76	6.83-12.31	5.48-15.80	5.68-14.92	6.00-14.92	5.48-15.12	5.68-15.80
Cysteine (µmol/L)	$\bar{x}$ (SD)	**246.1** (30.6)	**252.6** (23.9)	**266.5** (37.3)	**248.3** (31.2)	**253.1** (21.4)	**251.1** (44.4)	**255.3** (36.5)	**249.0** (19.2)	**247.9** (21.7)
	Median (IQR)	240.5 (224.9-270.9)	250.3 (238.1-260.9)	271.6 (237.4-273.4)	237.0 (230.2-257.2)	253.2 (239.5-265.5)	235.3 (216.2-286.3)	242.6 (231.2-276.9)	249.3 (234.8-262.7)	256.0 (233.6-261.8)
	Range	203.0-317.5	206.6-314.2	227.7-322.5	206.6-322.5	211.0-314.2	203.0-317.5	203.0-322.5	217.1-286.1	213.6-274.8
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**26.16** (5.04)	**27.18** (4.92)	**25.88** (6.97)	**26.77** (4.63)	**26.31** (4.43)	**27.88** (8.04)	**27.50** (5.53)	**25.25** (4.57)	**28.53** (4.66)
	Median (IQR)	25.27 (23.77-26.92)	26.05 (23.42-29.97)	24.80 (20.79-29.11)	26.33 (21.69-30.17)	25.27 (23.44-28.98)	25.51 (23.09-33.39)	26.27 (23.24-29.60)	24.75 (21.55-27.12)	28.87 (24.19-32.77)
	Range	17.07-40.54	19.79-38.07	18.61-36.08	20.63-33.72	18.61-38.07	17.07-40.54	18.61-40.54	17.07-38.07	23.23-33.72
Glutathione (µmol/L)	$\bar{x}$ (SD)	**11.19** (3.26)	**10.88** (5.64)	**9.65** (2.45)	**10.05** (3.11)	**11.30** (5.59)	**10.64** (1.43)	**10.81** (3.01)	**10.13** (6.02)	**13.42** (2.68) ‡
	Median (IQR)	10.28 (9.14-13.54)	9.65 (7.62-11.57)	8.96 (7.54-11.89)	9.36 (7.83-12.52)	10.06 (7.86-13.11)	10.12 (9.85-10.94)	10.94 (9.04-12.56)	8.87 (7.28-10.49)	14.18 (11.62-15.19)
	Range	4.87-17.21	5.86-35.20	7.28-12.57	6.01-16.52	4.87-35.20	9.35-13.81	4.87-17.21	5.86-35.20	9.65-16.52

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.
‡ denotes approaching significance

Note: For 2R > 3R TSER significance is achieved with native (untransformed) data for serum folate*.

Table 3-20: Hypertensive subjects; B-vitamin/thiol related nutritional genetic data by genotype (4 of 4)

Indices		80G>A RFC			1561C>T GCPII		
		Wt (n=18)	Het (n=19)	Rec (n=14)	Wt (n=48)	Het (n=3)	Rec (n=0)
Serum Vitamin B_{12} (pmol/L)	$\bar{X}$ (SD)	**251.5** (96.0)	**253.3** (71.6)	**259.6** (145.8)	**251.3** (104.0)	**304.3** (70.7)	**0.0** (0.0)
	Median (IQR)	249.0 (203.3-315.8)	249.0 (205.5-302.5)	197.0 (175.5-293.3)	240.0 (180.0-296.0)	332.0 (278.0-344.5)	0.0 (0.0-0.0)
	Range	78.0-417.0	129.0-409.0	102.0-544.0	78.0-544.0	224.0-357.0	0.0-0.0
Serum Folate (nmol/L)	$\bar{X}$ (SD)	**20.28** (11.02)	**22.84** (13.12)	**23.43** (9.77)	**22.02** (11.33)	**23.33** (15.18)	**0.00** (0.00)
	Median (IQR)	18.00 (12.75-24.75)	19.00 (13.50-32.00)	23.50 (18.50-27.00)	19.50 (14.00-27.00)	26.00 (16.50-31.50)	0.00 (0.00-0.00)
	Range	7.00-45.00	6.00-45.00	8.00-45.00	6.00-45.00	7.00-37.00	0.00-0.00
Red Cell Folate (nmol/L)	$\bar{X}$ (SD)	**808.3** (351.1)	**795.9** (427.7)	**792.7** (264.5)	**817.0** (357.7)	**519.0** (133.3)	**0.0** (0.0)
	Median (IQR)	680.5 (531.5-1076.8)	698.0 (533.5-948.5)	774.5 (696.0-881.5)	734.5 (609.8-1024.5)	536.0 (457.0-589.5)	0.0 (0.0-0.0)
	Range	433.0-1509.0	288.0-1996.0	256.0-1236.0	256.0-1996.0	378.0-643.0	0.0-0.0
Homocysteine (µmol/L)	$\bar{X}$ (SD)	**10.32** (2.63)	**9.75** (2.32)	**9.43** (3.16)	**9.83** (2.72)	**10.46** (0.71)	**0.00** (0.00)
	Median (IQR)	10.54 (8.56-11.97)	9.72 (7.80-10.66)	8.31 (7.21-11.59)	9.44 (7.73-11.82)	10.50 (10.11-10.82)	0.00 (0.00-0.00)
	Range	5.48-15.64	6.36-15.80	5.68-15.12	5.48-15.80	9.72-11.14	0.00-0.00
Cysteine (µmol/L)	$\bar{X}$ (SD)	**262.5** (27.1)	**241.1** (19.9)	**251.5** (34.2)	**250.8** (28.6)	**262.8** (11.0)	**0.0** (0.0)
	Median (IQR)	260.9 (243.2-274.5)	240.5 (230.0-253.1)	250.1 (231.9-262.9)	245.3 (230.6-265.4)	267.0 (258.6-269.1)	0.0 (0.0-0.0)
	Range	223.3-317.5	206.6-286.1	203.0-322.5	203.0-322.5	250.3-271.1	0.0-0.0
Cysteinyl-glycine (µmol/L)	$\bar{X}$ (SD)	**26.75** (5.87)	**26.71** (4.56)	**26.51** (5.08)	**26.50** (4.91)	**29.43** (8.31)	**0.00** (0.00)
	Median (IQR)	25.04 (21.86-29.98)	25.86 (24.62-29.32)	25.99 (23.73-29.08)	25.64 (23.16-29.26)	28.70 (25.10-33.39)	0.00 (0.00-0.00)
	Range	19.79-40.54	18.61-38.07	17.07-37.15	17.07-40.54	21.50-38.07	0.00-0.00
Glutathione (µmol/L)	$\bar{X}$ (SD)	**9.48** (2.99)	**12.58** (6.20)	**10.37** (2.96)	**10.29** (2.90)	**20.21** (13.74)	**0.00** (0.00) *
	Median (IQR)	8.81 (7.28-11.29)	11.08 (9.14-14.38)	10.24 (9.16-11.73)	9.94 (7.91-12.27)	17.21 (12.71-26.21)	0.00 (0.00-0.00)
	Range	6.01-17.21	7.13-35.20	4.87-16.52	4.87-16.81	8.21-35.20	0.00-0.00

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 1561C>T GCPII an unpaired T-test was performed in place of ANOVA given no recessive genotype was recorded.
For 1561C>T GCPII significance is achieved with native (untransformed) data for glutathione**.

The significant outcomes from one-way ANOVA's are overlayed on the tables above, as indicated by red boxes and asterisks(s) for levels of significance. People with healthy blood pressure levels (normotensive) exhibited some variability in serum folate levels according to the 19bp DHFR deletion genotype. Serum folate levels were also influenced by 677C>T MTHFR and 2R>3R TSER genes, although this significance was only found in the hypertensive group. In addition to these findings for the hypertensive group; variability existed in cysteine levels according to the 19bp DHFR deletion genotype and in glutathione according to 1561C>T GCPII genotype. Note: there were nil recessive genotypes recorded for 1561C>T GCPII, therefore a t-test was performed in place of a one-way ANOVA.

3.2.6. Analysis of combined nutritional biochemistry and genetic data sets to establish any relationship to hypertension

The relationship between hypertensive phenotype and other recorded parameters was analysed by implementation of an appropriate linear model according to the type of data (i.e. categorical or continuous). In the context of continuous blood pressure measurements, this meant that either a standard least squares fit for one or more continuous responses (multiple regression), or stepwise linear regression was employed. 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 model 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. r^2 is reported and is the proportion of the variation in the response that can be attributed to terms in the model as opposed to random error.

The parameter estimate is given, and lists the parameter estimates of the linear model. The prediction formula is the linear combination of these estimates with the values of their corresponding variables. The T value refers to the test statistics for the hypothesis that each parameter is zero. It is the ratio of the parameter estimate to its standard error. If the hypothesis is true, then this statistic has a student's t-distribution. Looking for a t-ratio greater than 2 in absolute value is a common rule of thumb for judging significance because it approximates to the 0.05 significance level.

Several linear models have been examined using stepwise regression based on an *a priori* understanding of possible biochemical, genetic and physiologic relationships. The outcomes from applying these linear models are defined in the following tables. Stepwise regression analysis was performed taking account of the following factors:

age, BMI, gender, dietary folic acid intake, serum B_{12}, serum folate, red cell folate, cysteine, homocysteine, cysteinyl-glycine, glutathione, and eleven B-vitamin related genetic mutations. Native data contains all data points (black text); log_{10} transformed data has had extreme data points removed (blue text). The results of this stepwise regression analysis are presented in the table below.

Table 3-21: Stepwise regression; model for all genetic, metabolic, and physiologic variables

			Native data				Transformed data			
Position	**Measurement**	**Variable**	**R^2**	**Estimate**	**T value**	***p* value**	**R^2**	**Estimate**	**T value**	***p* value**
Recumbent	Diastolic (mm Hg)	Age (yrs)	0.2868	-0.195	-2.24	0.0259*	0.2894	-0.212	-2.33	0.0267*
		BMI (weight)/height^2)		0.384	2.96	0.0034**		0.162	3.23	0.0014**
		Serum Vitamin B_{12} (pmol/L)		-0.006	-2.07	0.0396*				-
		Red Cell Folate (nmol/L)				-		-0.050	-2.72	0.0071**
		Cysteine (µmol/L)		-0.050	-3.01	0.0029**		-0.202	-3.28	0.0012**
		Cysteinyl-glycine (µmol/L)		0.257	2.51	0.0130*		0.096	2.46	0.0147*
		Total Dietary Folic Acid (µg/day)		0.005	1.90	0.0587 ‡		0.041	2.18	0.0306*
		677C>T MTHFR		-1.797	-2.27	0.0242*		-0.011	-2.48	0.0139*
		1298A>C MTHFR		-2.250	-2.37	0.0185*		-0.015	-2.59	0.0102*
		1420C>T SHMT		1.888	2.22	0.0273*		0.012	2.33	0.0207*
	Systolic (mm Hg)	Serum Vitamin B_{12} (pmol/L)	0.1024	-0.012	-2.32	0.0210*	0.0877	-0.028	-2.03	0.0438*
		1561C>T GCPII		-5.687	-1.95	0.0530 ‡				-
		19bp del DHFR		2.094	2.05	0.0416*		0.007	2.17	0.0311*
	Pulse rate (min)	Gender	0.0643	1.321	2.22	0.0274*	0.0774	0.008	2.39	0.0179*
		Cysteine (µmol/L)		-0.035	-1.94	0.0534 ‡				-
		1420C>T SMHT				-		0.007	2.00	0.0470*
Standing	Diastolic (mm Hg)	Age (yrs)	0.2383	-0.348	-3.75	0.0002***	0.2446	-0.389	-3.79	0.0002***
		BMI (weight)/height2)		0.429	3.06	0.0025**		0.170	3.11	0.0021**
		Cysteine (µmol/L)		-0.053	-2.98	0.0032**		-0.196	-2.96	0.0034**
	Systolic (mm Hg)	BMI (weight)/height2)	0.1434	0.510	2.37	0.0185*	0.1316	0.125	2.76	0.0063**
		Cysteine (µmol/L)		-0.061	-2.32	0.0212*		-0.120	-2.24	0.0262*
		19bp del DHFR		2.599	2.42	0.0161*		0.008	2.38	0.0182*
	Pulse rate (min)	Gender	0.0839	1.654	2.63	0.0091**	0.1011	0.009	2.50	0.0132*
		Red Cell Folate (nmol/L)				-		-0.057	-2.00	0.0473*
		3'URT 6bp del TS		-2.030	-2.00	0.0466*				-

* Asterisk(s) denotes significance (* p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡ denotes approaching significance
Black = Native data including all data points. Blue = log_{10} transformed data with extreme data points removed.

Data in table 3-21 indicates several parameters predict blood pressure; the most notable of these are as follows. Perhaps unsurprisingly, both age and BMI were found to be important determinants of both recumbent and standing diastolic blood pressure. Other notable associations were also uncovered, and include a clear effect of both folate (red cell folate, 677C>T & 1298A>C MTHFR and 1420C>T SHMT genotypes), and thiol (cysteine, cysteinyl-glycine) metabolism on recumbent diastolic blood pressure. Lesser effects were apparent for recumbent systolic blood pressure and pulse rate. Overall, the greatest effect of this area of metabolism on recumbent blood pressure would seem to be on diastolic indices. This extends even too total dietary folic acid intake influencing recumbent diastolic blood pressure. Cysteine stands out as being a particularly significant correlate of both recumbent and standing diastolic and systolic blood pressures, which is interesting and was unexpected.

Given that folate and thiol metabolism, including B-vitamin related SNPs can predict blood pressure as shown in table 3-21, there is clear merit to examining a linear model comprising only genetic data (B-vitamin related SNPs) as putative predictors of blood pressure. Table 3-22 provides this information for eleven gene variants using stepwise regression analysis.

Table 3-22: Stepwise regression; model for genetic data only (eleven variants)

			Native data				Transformed data			
Position	**Measurement**	**Variable**	**R^2**	**Estimate**	**T value**	***p* value**	**R^2**	**Estimate**	**T value**	***p* value**
Recumbent	Diastolic (mm Hg)	677C>T MTHFR	0.0655	-1.955	-2.30	0.0224 *	0.0650	-0.011	-2.07	0.0397*
		1298A>C MTHFR		-1.990	-1.94	0.0541 ‡		-0.012	-1.97	0.0504 ‡
	Systolic (mm Hg)	19bp del DHFR	0.0643	1.926	1.86	0.0644 ‡				-
		1561C>T GCPII		-5.608	-1.89	0.0595 ‡				-
	Pulse rate (min)	-				-				-
Standing	Diastolic (mm Hg)	-				-				-
	Systolic (mm Hg)	19bp del DHFR	0.0791	2.509	2.30	0.0222 *	0.0868	0.009	2.45	0.0152*
		1561C>T GCPII		-6.759	-2.17	0.0310 *		-0.021	-2.07	0.0397*
		677C>T MTHFR		-2.545	-1.89	0.0601 ‡		-0.009	-2.02	0.0443*
	Pulse rate (min)	3'URT 6bp del TS	0.0369	-1.916	-1.87	0.0628 ‡				-

* Asterisk(s) denotes significance (* p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡ denotes approaching significance
Black = Native data including all data points. Blue = $\log_{10}$ transformed data with extreme data points removed.

Data analysis clearly shows that only the 677C>T MTHFR variant contributes to recumbent diastolic blood pressure. However, three variant genes contribute to standing systolic blood pressure – 19bp DHFR deletion, 1561C>T GCPII and 677C>T MTHFR. A tukey's HSD post hoc test was performed to determine the statistical difference between genotypes. For 677C>T MTHFR the difference lies between CC and TT genotypes for both recumbent diastolic and standing systolic blood pressures. For the 19bp-DHFR mutation, a statistically significant difference exists between the 22 (del/del) recessive genotype and both the 12 (heterozygote (ins/del)) and 11 (wild-type (ins/ins)) genotypes.

The next linear model derived from a stepwise regression analysis was based on non-B vitamin related 'population' indices – age, BMI, and gender. As with previous models, data is either native, or contains $\log_{10}$ transformed data with extreme data points removed (see table 3-23 below).

Table 3-23: Stepwise regression; model for basic population information – age, body mass index and gender

			Native data				Transformed data			
Position	**Measurement**	**Variable**	**R^2**	**Estimate**	**T value**	***p* value**	**R^2**	**Estimate**	**T value**	***p* value**
Recumbent	Diastolic (mm Hg)	Age (yrs)	0.1191	-0.352	-4.30	<0.0001****	0.1208	-0.389	-4.30	<0.0001****
		BMI (weight)/height^2)		0.312	2.40	0.0174*		0.126	2.51	0.0126*
	Systolic (mm Hg)	-				-				-
	Pulse rate (min)	Gender	0.0164	1.164	1.94	0.0532 ‡	0.0189	0.007	2.09	0.0377*
Standing	Diastolic (mm Hg)	Age (yrs)	0.1599	-0.453	-5.28	<0.0001****	0.1602	-0.491	-5.19	<0.0001****
		BMI (weight)/height^2)		0.364	2.64	0.0087*		0.151	2.85	0.0048**
	Systolic (mm Hg)	BMI (weight)/height^2)	0.0172	0.434	2.00	0.0471*	0.0189	0.095	2.09	0.0374*
	Pulse rate (min)	Gender	0.0323	1.724	2.76	0.0063**	0.0353	0.010	2.88	0.0043**

* Asterisk(s) denotes significance (* p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡ denotes approaching significance
Black = Native data including all data points. Blue = $\log_{10}$ transformed data with extreme data points removed.

When three basic population descriptors - age, BMI and gender are examined using stepwise regression, unsurprisingly, age is a highly significant factor in both recumbent and standing diastolic blood pressure. BMI is also a fairly significant predictor of blood pressure, and gender appears to be a factor in both recumbent and standing pulse rate.

While gene-nutrient interactions are extremely important in the biochemical and clinical phenotype associated with high blood pressure, it is important to examine the data independent of genetic variants. Table 3-24 provides the significant output following stepwise regression analysis of all B-vitamin related blood metabolites and thiols combined – i.e. no genetic/dietary data. The following parameters were examined; serum vitamin B_{12}, serum folate, red cell folate, cysteine, homocysteine, cysteinyl-glycine and glutathione.

Table 3-24: Stepwise regression; model for all B-vitamin related blood metabolites and thiols combined

			Native data				Transformed data			
Position	**Measurement**	**Variable**	**R^2**	**Estimate**	**T value**	***p* value**	**R^2**	**Estimate**	**T value**	***p* value**
Recumbent	Diastolic (mm Hg)	Cysteine (µmol/L)	0.1264	-0.064	-4.04	<0.0001****	0.1285	-0.228	-3.83	0.0002***
		Cysteinyl-glycine (µmol/L)		0.314	2.98	0.0032**		0.124	3.07	0.0024**
	Systolic (mm Hg)	Serum Vitamin B_{12} (pmol/L)	0.0412	-0.012	-2.31	0.0216*	0.0362	-0.030	-2.10	0.0368*
	Pulse rate (min)	Red Cell Folate (nmol/L)				-	0.0491	-0.048	-2.21	0.0279*
Standing	Diastolic (mm Hg)	Cysteine (µmol/L)	0.1084	-0.071	-4.16	<0.0001 ****	0.1291	-0.269	-3.83	0.0002***
		Cysteinyl-glycine (µmol/L)		0.318	2.79	0.0057**		0.126	2.81	0.0054**
	Systolic (mm Hg)	Cysteine (µmol/L)	0.0344	-0.050	-1.90	0.0586 ‡	0.0260			-
		Serum Vitamin B_{12} (pmol/L)		-0.010	-1.93	0.0547 ‡		-0.028	-1.83	0.0690 ‡
	Pulse rate (min)	-				-				-

* Asterisk(s) denotes significance (* p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡ denotes approaching significance
Black = Native data including all data points. Blue = $\log_{10}$ transformed data with extreme data points removed.

Table 3-24 provides a very interesting outcome indicating that transsulphuration metabolites – cysteine and cysteinyl-glycine predict recumbent and standing diastolic blood pressure, and in a highly statistically relevant fashion. To a lesser extent, vitamin B_{12} and folate also seem relevant factors in blood pressure and pulse rate.

Given the above findings, all transsulphuration thiol metabolites were examined collectively by stepwise regression. This included cysteine, homocysteine, cysteinyl-glycine, and glutathione. Again, as with previous models data is either native, or contains $\log_{10}$ transformed data with extreme data points removed (see table 3-25).

Table 3-25: Stepwise regression; model for thiol transsulphuration pathway metabolites

			Native data				Transformed data			
Position	**Measurement**	**Variable**	**R^2**	**Estimate**	**T value**	***p* value**	**R^2**	**Estimate**	**T value**	***p* value**
Recumbent	Diastolic (mm Hg)	Cysteine (µmol/L)	0.1145	-0.066	-4.22	<0.0001 ****	0.1246	-0.232	-3.95	0.0001****
		Cysteinyl-glycine (µmol/L)		0.304	2.87	0.0044**		0.141	3.39	0.0008***
	Systolic (mm Hg)	Cysteine (µmol/L)	0.0243	-0.061	-2.31	0.0217 *	0.0142	-0.091	-1.81	0.0720 ‡
	Pulse rate (min)					-				-
Standing	Diastolic (mm Hg)	Cysteine (µmol/L)	0.1084	-0.071	-4.16	<0.0001 ****	0.1161	-0.240	-3.79	0.0002***
		Cysteinyl-glycine (µmol/L)		0.318	2.79	0.0057**		0.150	3.34	0.0010***
	Systolic (mm Hg)	Cysteine (µmol/L)	0.0185	-0.055	-2.07	0.0396*	0.0137	-0.096	-1.78	0.0770 ‡
	Pulse rate (min)	-				-				-

* Asterisk(s) denotes significance (* p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡ denotes approaching significance
Black = Native data including all data points. Blue = $\log_{10}$ transformed data with extreme data points removed.

As was established in table 3-24, the model output presented in table 3-25 similarly shows that the two transsulphuration metabolites – cysteine and cysteinyl-glycine are statistical significant in predicting diastolic blood pressure in both the recumbent and standing positions. The effect shown is in fact highly significant and merits detailed discussion, see page 253.

For completion, the B-vitamin related blood metabolites were also examined as a discrete entity. The stepwise regression model was derived from data that was either native, or contained $\log_{10}$ transformed data, but with any extreme data points removed in line with the former tables. Stepwise regression was performed on serum vitamin B_{12}, serum folate, and red cell folate.

Table 3-26: Stepwise regression; model for B-vitamin related blood metabolites

			Native data				Transformed data			
Position	**Measurement**	**Variable**	**R^2**	**Estimate**	**T value**	***p* value**	**R^2**	**Estimate**	**T value**	***p* value**
Recumbent	Diastolic (mm Hg)	Red Cell Folate (nmol/L)				-	0.0202	-0.040	-2.15	0.0326*
	Systolic (mm Hg)	Serum Vitamin B_{12} (pmol/L)	0.0266	-0.013	-2.49	0.0136*	0.0166	-0.028	-1.95	0.0529 ‡
	Pulse rate (min)	Red Cell Folate (nmol/L)				-	0.0240	-0.047	-2.19	0.0293*
Standing	Diastolic (mm Hg)	-				-				-
	Systolic (mm Hg)	Serum Vitamin B_{12} (pmol/L)	0.0190	-0.011	-2.10	0.0369*	0.0141	-0.027	-1.79	0.0741 ‡
	Pulse rate (min)	-				-				-

* Asterisk(s) denotes significance (* p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡ denotes approaching significance
Black = Native data including all data points. Blue = $\log_{10}$ transformed data with extreme data points removed.

Previously (table 3-24), vitamin B_{12} and red cell folate were examined as part of a model containing thiol transsulphuration metabolites as potential predictors of recumbent blood pressure. However, in the present analysis (table 3-26), which omits

the transsulphuration metabolites, red cell folate was the only parameter seen as impacting on recumbent diastolic blood pressure and pulse.

In the stepwise regression analysis shown in table 3-21, total dietary folic acid was seen as influencing diastolic blood pressure. Given this finding, stepwise regression analysis has also been used to explore any potential relationship between the three indices of dietary folic acid – 'total intake', 'synthetic folic acid in the form of pteroylmonoglutamic acid' and 'natural folic acid as 5-methyl-H_4folic acid' and blood pressure. The outcome is presented in table 3-27 below.

Table 3-27: Stepwise regression; model for dietary folic acid

			Native data				Transformed data			
Position	**Measurement**	**Variable**	**R^2**	**Estimate**	**T value**	***p* value**	**R^2**	**Estimate**	**T value**	***p* value**
Recumbent	Diastolic (mm Hg)	Total Natural Folic Acid (µg/day)	0.0167	0.010	1.96	0.0513 ‡	0.0171	0.049	1.98	0.0485*
	Systolic (mm Hg)	-				-				-
	Pulse rate (min)	-				-				-
Standing	Diastolic (mm Hg)	Total Natural Folic Acid (µg/day)	0.0132	0.009	1.75	0.0823 ‡				-
	Systolic (mm Hg)	Total Natural Folic Acid (µg/day)	0.0142	0.015	1.81	0.0711 ‡	0.0189	0.045	2.10	0.0373*
	Pulse rate (min)	-				-				-

* Asterisk(s) denotes significance (* p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡ denotes approaching significance
Black = Native data including all data points. Blue = $\log_{10}$ transformed data with extreme data points removed.

Although total dietary folic acid was previously shown to influence diastolic blood pressure (table 3-21), when examined without transsulphuration metabolites, without B-vitamin related blood metabolites and without gene variants; total natural folic acid (i.e. 5-methyl-H_4folic acid) was seen as predicting recumbent diastolic and standing systolic blood pressures. Synthetic folic acid (supplemental pteroylmonoglutamic acid) intakes did not indicate any relationship to blood pressure.

The salient findings relating B-vitamins/thiols to the hypertensive phenotype are presented in the summary of results at the end of this chapter. The discussion of these results is found in chapter 6, page 253. The next examination of the data focuses on depression in this same elderly cohort.

3.2.7. Depression phenotype

The HADS index is a widely used 14 item self-report scale designed to briefly measure current anxiety and depressive symptomatology. As described in the clinical section (see page 134) the Cronbach's alpha outcome (A-0.80, D-0.90) provides evidence of the high reliability of the data scores obtained, and is comparable with the outcomes described by Bjelland's review of 747 studies reporting average Cronbach's alpha of A-0.83 and D-0.82 [943]. The scoring of the HADS questionnaire was based on the

method developed by the original authors Zigmund and Snaith [941], where a score of 8 or more was considered to indicate anxiety or depression. [943].

The data presented in this section will focus on the depression scores obtained, although some analysis has been performed to include anxiety by way of controlling for co-morbidity. The depression phenotype is defined as a HADS-D score of 8 or more, while control subjects were defined as having a HADS-D of less than 8. Where appropriate, the data was repeated in order to control for co-morbidity. This defined depression as a HADS-D score of 8 or more restricted to a HADS-A score less than 8, with the controls containing any subject which did not meet the depressed categorisation. Given the low numbers achieved in both categories, the data is generally presented based on the original author's recommendations of either above or below a score of 8. Displayed below are the descriptive statistics for HADS. Data is presented for all subjects, males, females, and for those not depressed (control) and those found to be depressed according to the above criteria.

Table 3-28: Depression phenotype; HADS scores

Measured Scores		$\bar{x}$ (SD)	Median (IQR)	Range
All (n=229)	HADS - Anxiety	5 (3)	5 (2-7)	0-16
	HADS - Depression	3 (3)	3 (1-4)	0-14
	HADS - Total	8 (5)	7 (4-11)	0-28
Males (n=92)	HADS - Anxiety	4 (3)	4 (2-5)	0-12
	HADS - Depression	4 (3)	3 (1-5)	0-13
	HADS - Total	8 (5)	6 (4-11)	0-22
Females (n=137)	HADS - Anxiety	6 (4)	5 (3-8)	0-16
	HADS - Depression	3 (3)	2 (1-4)	0-14
	HADS - Total	9 (6)	7 (5-12)	1-28
Control (n=212)	HADS - Anxiety	5 (3)	4 (2-6)	0-14
	HADS - Depression	3 (2)	2 (1-4)	0-7
	HADS - Total	7 (4)	7 (4-10)	0-20
Depression (n=17)	HADS - Anxiety	9 (4)	8 (6-12)	1-16
	HADS - Depression	10 (2)	10 (8-11)	8-14
	HADS - Total	19 (5)	19 (16-22)	9-28

The difference in the mean value between B-vitamins and related thiols for depression and control phenotypes was examined using an unpaired t-test. Figure 3-3 below displays any differences graphically, and shows both the mean and standard deviation (error bars) for each variable examined. Significance was achieved for red cell folate and cysteinyl-glycine when the data was $\log_{10}$ transformed to normalise the distribution, and outliers at or beyond the calibration range were removed. No other points of significance were detected between phenotypes.

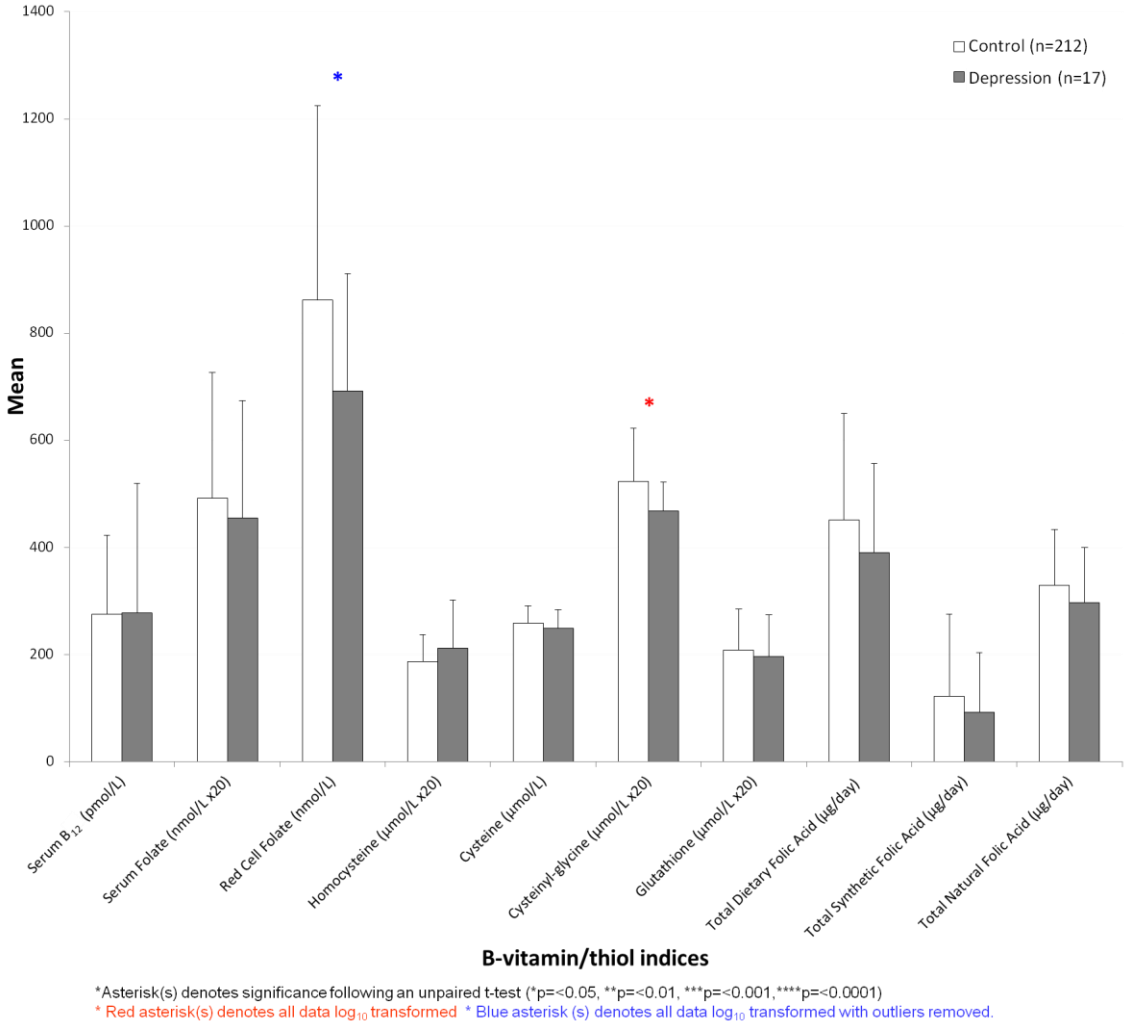

Figure 3-3: Mean and standard deviation values for B-vitamin/thiol measurements comparing the depression phenotype with controls.

Means have been compared using an unpaired t-test; asterisk denotes significance level.

The depression phenotype may be modified by B-vitamin related genetics, therefore the impact of each of the eleven genotypes that have been examined are displayed in the table below. The table describes the genotype prevalence (%), allele number (frequency) and carriage of mutant allele for each phenotype. The phenotypes are defined as; control (HADS-D score less than 8) and depression (HADS-D score of 8 or more).

Table 3-29: Depression phenotype; genotype prevalence, allele number and carriage of mutant allele

SNP	PHENOTYPE	GENOTYPE Prevalence (%)			ALLELE Number (frequency)		Carriage of mutant allele (%)
		Wildtype	Heterozygote	Recessive	Wildtype	Mutant	
677C>T MTHFR	Control	101 (48)	89 (42)	22 (10)	291 (0.69)	133 (0.31)	52
	Depression	6 (35)	7 (41)	4 (24)	19 (0.56)	15 (0.44)	65
1298A>C MTHFR	Control	99 (47)	97 (46)	16 (8)	295 (0.70)	129 (0.30)	53
	Depression	7 (41)	8 (47)	2 (12)	22 (0.65)	12 (0.35)	59
19bp del DHFR	Control	69 (33)	101 (48)	42 (20)	239 (0.56)	185 (0.44)	67
	Depression	8 (47)	6 (35)	3 (18)	22 (0.65)	12 (0.35)	53
2756A>G MTR	Control	120 (57)	79 (37)	13 (6)	319 (0.75)	105 (0.25)	43
	Depression	6 (35)	9 (53)	2 (12)	21 (0.62)	13 (0.38)	65
66A>G MTRR	Control	37 (17)	105 (50)	70 (33)	179 (0.42)	245 (0.58)	83
	Depression	2 (12)	11 (65)	4 (24)	15 (0.44)	19 (0.56)	88
80G>A RFC	Control	64 (30)	94 (44)	54 (25)	222 (0.53)	202 (0.47)	70
	Depression	5 (29)	10 (59)	2 (12)	20 (0.59)	14 (0.41)	71
1561C>T GCPII	Control	193 (91)	19 (9)	0 (0)	405 (0.96)	19 (0.04)	9
	Depression	17 (100)	0 (0)	0 (0)	34 (1.00)	0 (0.00)	0
1420C>T SHMT	Control	107 (50)	88 (42)	17 (8)	302 (0.71)	122 (0.29)	50
	Depression	8 (47)	6 (35)	3 (18)	22 (0.65)	12 (0.35)	53
1947G>A COMT	Control	57 (27)	93 (44)	60 (29)	207 (0.49)	213 (0.51)	73
	Depression	3 (18)	8 (47)	6 (35)	14 (0.41)	20 (0.59)	82
3'URT 6bp del TS	Control	96 (45)	94 (44)	22 (10)	286 (0.67)	138 (0.33)	55
	Depression	9 (53)	6 (35)	2 (12)	24 (0.70)	10 (0.30)	47
2R > 3R TSER	Control	57 (27)	118 (56)	37 (17)	232 (0.55)	192 (0.45)	73
	Depression	3 (18)	8 (41)	6 (35)	14 (0.41)	20 (0.59)	82

Using the data displayed above, an odds ratio and 95% confidence interval was calculated in order to assess the degree and significance of risk of an individual SNP for depression.

A 2x2 table was then used to carry out a chi-square test and derive a *p* value. This was achieved using the Yates correction, which provides a stricter analysis for small samples. However, when an allele frequency was less than 5 a fisher-exact test was performed in place of a chi-square test. The table below displays the outcomes of all tests for each of the eleven B-vitamin related genetic variants examined. No association between depression and any individual SNP was detected. The outcomes described in the table 3-30 below were also repeated for the co-morbid categorisation; the results indicated no new points of significance.

Table 3-30: Depression phenotype; odds ratio and 95% CI along with chi-square test *p* value

SNP	Odds Ratio	95% Confidence Intervals	*p*	χ^2
677C>T MTHFR	1.73	0.85 - 3.50	0.1806	1.793
1298A>C MTHFR	1.25	0.60 - 2.60	0.6901	0.159
19bp del DHFR	0.70	0.34 - 1.46	0.4444	0.585
2756A>G MTR	1.88	0.91 - 3.89	0.1274	2.324
66A>G MTRR	0.93	0.46 - 1.87	0.9717	0.001
80G>A RFC	0.77	0.38 - 1.56	0.5836	0.300
1561C>T GCPII	0.30	0.01 - 5.09	0.3837 ^	-
1420C>T SHMT	1.35	0.65 - 2.81	0.5430	0.370
1947G>A COMT	1.39	0.68 - 2.82	0.4644	0.535
3'UTR 6bp del TS	0.86	0.40 - 1.86	0.8528	0.034
2R > 3R TSER	1.73	0.85 - 3.51	0.1787	1.809

^ The *p* value for 1561C>T GCP11 was derived from a fishers-exact test, this was performed in place of chi-square test due to the low frequency of mutant alleles

3.2.8. Depression phenotype; B-vitamin/thiol related nutritional genetic data organised by genotype

All descriptive data is displayed in the next eight tables for each B-vitamin and thiol related parameter examined (*control phenotype tables 1 to 4 and depression phenotype tables 1 to 4*). In all cases each phenotype is organised by genotype. In order to investigate whether genetic factors can influence variability in blood indices, a one-way ANOVA was performed. As alluded to previously, the data set contains a number of extreme data points, and therefore the ANOVA's were performed both with and without these extreme values. In addition to this, the data was treated both as 'native' and '$\log_{10}$ transformed'. Significance is denoted by either a red asterisk(s) (all $\log_{10}$ transformed values) or a blue asterisk(s) ($\log_{10}$ transformed values with outliers removed). *Note: results of significant ANOVA's on native data are displayed as footnotes in each table.*

Table 3-31: Control; B-vitamin/thiol related nutritional genetic data by genotype (1 of 4)

Indices		677C>T MTHFR			1298A>C MTHFR			19bp del DHFR		
		Wt (n=101)	Het (n=89)	Rec (n=22)	Wt (n=99)	Het (n=97)	Rec (n=16)	Wt (n=69)	Het (n=101)	Rec (n=42)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**283.1** (144.6)	**268.5** (158.4)	**267.1** (108.0)	**290.8** (166.8)	**264.5** (130.9)	**244.9** (95.0)	**283.3** (162.2)	**273.5** (150.2)	**266.6** (110.7)
	Median (IQR)	259.0 (197.0-332.0)	226.0 (182.0-291.0)	231.0 (196.5-345.3)	241.0 (196.0-336.5)	244.0 (182.0-318.0)	230.0 (180.0-273.8)	245.0 (196.0-332.0)	240.0 (188.0-307.0)	251.0 (184.5-335.8)
	Range	81.0-1100.0	78.0-1100.0	74.0-495.0	74.0-1100.0	81.0-1100.0	106.0-434.0	78.0-1100.0	81.0-1100.0	74.0-620.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**27.04** (11.99)	**22.48** (11.12)	**22.09** (11.21) * *	**24.28** (12.23)	**23.76** (10.87)	**31.81** (11.97) *	**25.39** (12.38)	**24.41** (10.99)	**23.83** (12.56)
	Median (IQR)	24.00 (17.00-39.00)	21.00 (13.00-27.00)	21.00 (14.25-24.75)	22.00 (14.00-34.50)	22.00 (15.00-33.00)	29.00 (23.50-45.00)	24.00 (14.00-37.00)	22.00 (16.00-33.00)	23.00 (12.50-33.00)
	Range	3.00-45.00	7.00-45.00	7.00-45.00	7.00-45.00	3.00-45.00	11.00-45.00	7.00-45.00	7.00-45.00	3.00-45.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**875.3** (327.7)	**802.4** (354.1)	**1045.6** (481.4) * *	**886.3** (430.0)	**817.6** (281.4)	**992.7** (314.7)	**884.6** (311.5)	**869.1** (403.9)	**809.5** (338.9)
	Median (IQR)	834.5 (621.5-1107.0)	732.0 (587.0-1013.0)	957.5 (761.8-1388.5)	796.0 (557.5-1145.0)	780.0 (602.0-1006.0)	900.0 (785.0-1199.0)	821.0 (652.0-1073.0)	776.5 (549.5-1090.3)	788.0 (572.3-996.5)
	Range	221.0-1726.0	254.0-2500.0	355.0-2007.0	254.0-2500.0	221.0-1534.0	425.0-1550.0	378.0-1650.0	256.0-2500.0	221.0-1534.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**9.11** (2.64)	**9.64** (2.25)	**9.06** (2.83)	**9.15** (2.60)	**9.47** (2.46)	**9.52** (2.32)	**9.03** (2.21)	**9.21** (2.47)	**10.10** (2.92)
	Median (IQR)	8.44 (7.32-10.36)	9.47 (7.86-10.75)	8.50 (7.77-9.68)	8.45 (7.34-10.49)	9.19 (7.87-10.75)	9.12 (8.34-10.57)	8.80 (7.64-9.88)	8.67 (7.61-10.72)	9.39 (7.78-12.11)
	Range	4.89-17.02	5.62-15.45	4.57-16.29	4.57-16.29	4.89-17.02	6.07-15.64	4.57-15.45	4.66-17.02	6.00-17.01
Cysteine (µmol/L)	$\bar{x}$ (SD)	**259.4** (32.5)	**260.2** (32.9)	**252.5** (29.9)	**258.7** (34.9)	**259.1** (30.6)	**260.7** (28.1)	**260.3** (30.7)	**258.1** (35.5)	**259.3** (27.5)
	Median (IQR)	257.3 (238.2-279.3)	259.3 (236.1-279.0)	254.6 (230.6-272.5)	254.6 (232.4-278.8)	258.3 (239.1-279.5)	260.2 (243.3-279.6)	259.7 (235.0-280.6)	257.3 (229.9-278.8)	254.8 (241.4-278.1)
	Range	202.0-333.8	180.9-343.3	206.6-307.9	180.9-343.3	202.0-333.8	207.1-317.8	208.3-326.6	180.9-343.3	204.2-313.0
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**26.38** (5.15)	**25.60** (4.97)	**27.14** (4.30)	**26.36** (5.18)	**26.04** (4.73)	**25.23** (5.62)	**26.40** (5.83)	**25.84** (4.61)	**26.40** (4.46)
	Median (IQR)	25.93 (22.53-29.23)	25.06 (22.94-27.38)	28.50 (23.68-30.05)	25.93 (22.94-29.16)	25.86 (22.94-28.50)	24.27 (21.25-27.82)	24.56 (22.91-28.44)	26.28 (22.87-28.99)	26.25 (23.13-29.59)
	Range	15.68-40.81	15.74-46.75	20.31-34.04	17.04-46.75	15.68-40.81	16.76-35.04	15.74-46.75	15.68-36.08	17.86-36.42
Glutathione (µmol/L)	$\bar{x}$ (SD)	**10.96** (4.69)	**10.09** (2.74)	**9.50** (2.64)	**10.58** (3.46)	**10.27** (4.22)	**10.63** (3.39)	**10.19** (3.80)	**10.55** (4.15)	**10.59** (2.91)
	Median (IQR)	10.05 (8.12-12.58)	10.17 (7.94-11.89)	8.76 (7.66-12.12)	10.35 (8.32-12.51)	9.42 (7.76-11.65)	10.11 (8.77-12.90)	10.23 (8.21-11.81)	9.92 (7.77-12.77)	10.25 (8.78-12.34)
	Range	3.50-35.20	5.36-20.89	5.99-14.22	3.50-27.94	4.38-35.20	4.99-18.30	5.25-35.20	3.50-27.94	5.86-18.30

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 677C>T MTHFR significance is achieved with native (untransformed) data for serum folate * ** and red cell folate * **.
For 1298A>C MTHFR significance is achieved with native (untransformed) data for serum folate *.

Table 3-32: Control; B-vitamin/thiol related nutritional genetic data by genotype (2 of 4)

Indices		2756A>G MTR			66A>G MTRR			1947G>A COMT		
		Wt (n=120)	Het (n=79)	Rec (n=13)	Wt (n=37)	Het (n=105)	Rec (n=70)	Wt (n=57)	Het (n=93)	Rec (n=60)
Serum Vitamin B_{12} (pmol/L)	$\bar{X}$ (SD)	**260.3** (111.8)	**295.9** (189.0)	**288.8** (134.9)	**264.6** (107.0)	**282.9** (160.9)	**269.5** (144.3)	**267.6** (152.8)	**282.5** (155.1)	**267.7** (128.5)
	Median (IQR)	241.0 (181.8-309.5)	244.0 (197.0-315.0)	243.0 (180.0-382.0)	247.0 (188.0-332.0)	255.0 (192.0-318.0)	233.5 (188.0-294.8)	232.5 (180.8-291.0)	255.0 (198.0-320.0)	240.0 (180.0-317.0)
	Range	78.0-632.0	74.0-1100.0	138.0-557.0	95.0-554.0	74.0-1100.0	81.0-854.0	89.0-1100.0	74.0-1100.0	81.0-702.0
Serum Folate (nmol/L)	$\bar{X}$ (SD)	**24.72** (11.54)	**24.16** (12.09)	**26.38** (12.07)	**24.81** (11.63)	**25.58** (12.05)	**23.06** (11.30)	**24.15** (11.64)	**25.34** (11.96)	**23.40** (11.39)
	Median (IQR)	24.00 (15.00-33.00)	21.00 (14.50-34.50)	23.00 (14.00-38.00)	23.00 (14.00-33.00)	24.00 (16.00-36.00)	22.00 (14.00-27.75)	22.50 (13.75-35.00)	23.00 (16.00-35.00)	20.00 (14.00-27.00)
	Range	3.00-45.00	7.00-45.00	12.00-45.00	10.00-45.00	7.00-45.00	3.00-45.00	7.00-45.00	3.00-45.00	7.00-45.00
Red Cell Folate (nmol/L)	$\bar{X}$ (SD)	**856.7** (344.6)	**838.1** (341.2)	**1060.7** (574.1)	**805.3** (278.1)	**914.3** (401.7)	**813.7** (331.6)	**835.6** (352.9)	**878.2** (372.7)	**863.7** (366.2)
	Median (IQR)	802.0 (597.5-1092.5)	755.0 (610.5-1018.5)	1015.0 (631.0-1333.0)	780.0 (602.0-1000.0)	847.0 (602.0-1140.0)	753.0 (597.0-1006.0)	769.0 (549.0-1034.0)	821.0 (624.0-1098.0)	784.0 (602.0-1059.0)
	Range	221.0-2007.0	256.0-1996.0	386.0-2500.0	346.0-1424.0	254.0-2500.0	221.0-1726.0	263.0-2007.0	221.0-2500.0	346.0-1996.0
Homocysteine (µmol/L)	$\bar{X}$ (SD)	**9.24** (2.45)	**9.50** (2.57)	**9.07** (2.85)	**9.29** (2.59)	**9.42** (2.63)	**9.21** (2.30)	**9.03** (2.13)	**9.61** (2.63)	**9.25** (2.67)
	Median (IQR)	8.64 (7.57-10.41)	9.19 (7.67-10.90)	8.63 (7.97-10.72)	8.33 (7.22-11.33)	8.56 (7.66-10.57)	9.29 (7.64-10.47)	8.79 (7.63-10.57)	8.99 (7.80-10.95)	8.86 (7.64-9.88)
	Range	4.66-17.02	4.57-16.59	4.89-14.92	5.56-16.29	4.66-17.02	4.57-15.12	4.66-13.46	4.89-16.59	4.57-17.02
Cysteine (µmol/L)	$\bar{X}$ (SD)	**260.1** (31.8)	**255.0** (32.6)	**273.2** (34.3)	**258.2** (34.8)	**257.0** (32.4)	**262.6** (31.1)	**258.9** (34.5)	**262.5** (31.3)	**254.5** (31.7)
	Median (IQR)	259.1 (239.4-279.3)	253.3 (231.1-278.9)	267.7 (254.6-307.2)	253.6 (236.8-273.4)	254.2 (232.9-279.0)	263.8 (240.7-279.4)	256.5 (238.9-280.9)	261.8 (237.9-279.3)	248.7 (232.9-278.1)
	Range	203.0-343.3	180.9-335.2	229.0-333.8	205.3-325.1	180.9-343.3	206.6-335.2	180.9-343.3	206.6-335.2	202.0-332.5
Cysteinyl-glycine (µmol/L)	$\bar{X}$ (SD)	**25.60** (4.63)	**26.79** (5.21)	**27.09** (6.56)	**27.04** (6.42)	**25.93** (4.30)	**25.95** (5.14)	**26.02** (4.28)	**26.46** (5.37)	**25.65** (5.19)
	Median (IQR)	25.17 (22.81-27.98)	26.16 (22.93-30.13)	28.88 (25.86-30.05)	25.83 (22.86-29.17)	26.31 (22.99-29.01)	25.04 (22.91-28.65)	25.97 (23.05-28.50)	25.82 (22.86-29.11)	25.42 (22.65-29.15)
	Range	15.74-46.75	18.09-40.81	15.68-37.15	17.04-46.75	15.74-35.04	15.68-39.09	17.04-36.42	15.68-46.75	15.74-38.47
Glutathione (µmol/L)	$\bar{X}$ (SD)	**10.21** (3.12)	**10.72** (4.56)	**10.88** (4.71)	**10.90** (4.89)	**10.47** (3.80)	**10.17** (3.14)	**10.61** (3.42)	**11.03** (4.42)	**9.16** (2.66) **
	Median (IQR)	9.95 (8.10-11.90)	10.24 (7.97-12.62)	9.37 (7.90-11.89)	10.27 (8.26-12.58)	10.18 (7.95-12.22)	9.73 (8.07-12.05)	10.44 (7.75-12.62)	10.38 (8.72-12.70)	9.04 (7.32-10.49)
	Range	4.38-26.40	3.50-35.20	6.09-23.46	4.38-35.20	4.99-27.94	3.50-23.46	5.36-23.46	4.87-35.20	3.50-20.89

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 1947G>A COMT significance is achieved with native (untransformed) data for glutathione*.

Table 3-33: Control; B-vitamin/thiol related nutritional genetic data by genotype (3 of 4)

Indices		3'UTR 6bp del TS			2R > 3R TSER			1420C>T SHMT		
		Wt (n=96)	Het (n=94)	Rec (n=22)	Wt (n=57)	Het (n=118)	Rec (n=37)	Wt (n=107)	Het (n=88)	Rec (n=17)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**278.5** (153.0)	**268.9** (147.0)	**288.6** (122.0)	**261.1** (106.0)	**288.6** (175.0)	**254.8** (90.6)	**282.9** (150.6)	**273.4** (151.0)	**237.5** (92.0)
	Median (IQR)	242.0 (197.8-307.3)	229.0 (182.3-322.3)	266.0 (222.8-365.8)	244.0 (194.0-290.0)	239.5 (188.5-338.0)	247.0 (181.0-302.0)	249.0 (184.0-350.5)	239.5 (196.8-313.3)	215.0 (182.0-290.0)
	Range	89.0-1100.0	78.0-1100.0	74.0-554.0	74.0-688.0	78.0-1100.0	117.0-495.0	74.0-1100.0	81.0-1100.0	112.0-495.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**25.48** (11.72)	**23.88** (12.17)	**23.95** (9.96)	**22.63** (12.51)	**26.09** (11.72)	**22.95** (10.03) * *	**24.84** (11.78)	**25.15** (12.37)	**20.41** (6.49)
	Median (IQR)	24.00 (15.75-35.25)	21.50 (14.00-33.00)	23.00 (17.00-29.50)	20.00 (12.00-32.00)	24.00 (17.00-36.75)	22.00 (14.00-27.00)	23.00 (14.50-35.00)	23.00 (15.00-36.25)	20.00 (16.00-25.00)
	Range	7.00-45.00	3.00-45.00	8.00-45.00	3.00-45.00	8.00-45.00	7.00-45.00	7.00-45.00	3.00-45.00	12.00-36.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**856.9** (355.0)	**872.1** (389.6)	**843.5** (278.9)	**814.0** (338.9)	**896.8** (383.8)	**827.4** (323.8)	**866.4** (376.4)	**874.8** (359.8)	**771.9** (287.0)
	Median (IQR)	798.0 (613.5-1060.5)	790.5 (598.3-1143.8)	812.0 (603.5-1049.3)	780.0 (592.0-1061.0)	802.0 (613.0-1125.0)	789.0 (615.0-1015.0)	797.5 (599.8-1098.0)	800.5 (612.5-1112.3)	749.0 (591.0-830.0)
	Range	254.0-2007.0	221.0-2500.0	471.0-1389.0	221.0-1550.0	256.0-2500.0	254.0-1726.0	254.0-2500.0	221.0-1996.0	421.0-1650.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**9.09** (2.29)	**9.58** (2.53)	**9.28** (3.29)	**9.53** (2.49)	**9.18** (2.45)	**9.49** (2.77)	**9.21** (2.66)	**9.53** (2.37)	**9.01** (2.28)
	Median (IQR)	8.59 (7.63-10.48)	9.34 (7.78-11.18)	8.71 (6.90-9.92)	9.19 (7.64-10.72)	8.62 (7.62-10.46)	8.80 (7.74-11.02)	8.56 (7.55-10.47)	9.15 (7.87-10.82)	8.96 (7.24-10.06)
	Range	4.57-15.64	5.20-16.59	5.56-17.02	6.07-17.01	4.66-17.02	4.57-16.59	4.66-17.01	4.57-17.02	6.12-15.64
Cysteine (µmol/L)	$\bar{x}$ (SD)	**256.5** (33.5)	**260.7** (32.3)	**263.1** (27.8)	**258.1** (35.4)	**260.5** (30.9)	**255.6** (32.7)	**258.8** (33.6)	**260.0** (32.5)	**255.7** (24.2)
	Median (IQR)	258.7 (230.2-276.0)	254.5 (239.1-283.9)	271.0 (238.7-281.1)	258.9 (232.7-278.8)	257.8 (239.7-279.4)	254.2 (230.2-279.3)	254.6 (234.0-278.9)	258.6 (236.8-282.7)	258.3 (237.9-273.4)
	Range	180.9-343.3	206.6-333.8	207.5-322.5	204.2-343.3	180.9-335.2	203.0-317.5	202.0-343.3	180.9-333.8	213.6-297.8
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**25.86** (4.88)	**26.63** (5.19)	**25.19** (4.66)	**26.16** (5.30)	**26.11** (4.63)	**26.14** (5.75)	**26.10** (4.72)	**25.73** (5.21)	**28.37** (5.32)
	Median (IQR)	25.66 (23.06-28.01)	26.16 (22.80-29.75)	25.15 (21.33-27.37)	25.82 (22.86-28.74)	25.88 (22.99-29.03)	25.06 (22.46-28.98)	26.20 (22.96-28.93)	24.79 (22.36-28.99)	27.49 (24.23-32.13)
	Range	15.68-46.75	16.76-40.81	18.58-36.08	16.76-46.75	15.74-39.09	15.68-40.54	15.68-40.54	15.74-46.75	20.31-40.81
Glutathione (µmol/L)	$\bar{x}$ (SD)	**10.74** (3.80)	**10.27** (4.08)	**9.87** (2.39)	**10.00** (2.64)	**10.80** (4.45)	**9.98** (3.00)	**10.57** (3.63)	**10.14** (4.13)	**11.24** (3.18)
	Median (IQR)	10.28 (8.21-12.59)	9.52 (7.99-11.63)	10.10 (7.67-12.22)	10.17 (7.94-12.22)	10.21 (8.28-12.47)	9.47 (7.76-11.86)	10.17 (8.05-12.58)	9.85 (7.67-11.51)	10.35 (8.78-13.28)
	Range	4.87-27.94	3.50-35.20	6.11-14.56	5.25-16.52	3.50-35.20	5.99-20.89	4.38-27.94	3.50-35.20	7.47-18.30

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data log_{10} transformed * Blue asterisk (s) denotes all data log_{10} transformed with outliers removed.

Table 3-34: Control; B-vitamin/thiol related nutritional genetic data by genotype (4 of 4)

Indices		80G>A RFC			1561C>T GCPII			
		Wt (n=64)	Het (n=94)	Rec (n=54)	Wt (n=193)	Het (n=19)	Rec (n=0)	
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**257.6** (129.1)	**290.5** (163.1)	**269.9** (136.5)	**277.5** (150.3)	**252.6** (107.3)	**0.0** (0.0)	
	Median (IQR)	223.5 (194.8-290.5)	260.5 (198.8-329.0)	239.5 (175.5-337.5)	241.0 (194.0-317.0)	246.0 (159.5-315.0)	0.0 (0.0-0.0)	
	Range	78.0-854.0	74.0-1100.0	95.0-702.0	74.0-1100.0	106.0-495.0	0.0-0.0	
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**22.56** (11.82)	**26.29** (11.53)	**24.13** (11.76)	**24.28** (11.75)	**28.00** (11.26)	**0.00** (0.00)	
	Median (IQR)	19.00 (13.75-30.50)	24.00 (17.00-36.75)	22.00 (14.25-33.00)	22.00 (15.00-33.00)	26.00 (21.00-37.00)	0.00 (0.00-0.00)	
	Range	3.00-45.00	7.00-45.00	7.00-45.00	3.00-45.00	7.00-45.00	0.00-0.00	
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**848.3** (366.4)	**883.9** (340.2)	**841.6** (398.7)	**860.0** (359.8)	**886.0** (399.5)	**0.0** (0.0)	
	Median (IQR)	752.5 (587.0-1100.8)	820.0 (641.0-1087.0)	782.5 (592.5-1013.0)	791.5 (602.0-1098.0)	844.0 (546.5-1039.5)	0.0 (0.0-0.0)	
	Range	221.0-1650.0	346.0-1996.0	254.0-2500.0	221.0-2500.0	378.0-1726.0	0.0-0.0	
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**9.68** (2.52)	**9.00** (2.39)	**9.47** (2.67)	**9.38** (2.53)	**8.76** (2.27)	**0.00** (0.00)	
	Median (IQR)	9.37 (7.79-11.51)	8.50 (7.63-10.04)	8.65 (7.69-10.84)	8.91 (7.64-10.70)	8.37 (7.26-10.43)	0.00 (0.00-0.00)	
	Range	5.48-16.59	4.57-17.02	4.66-17.01	4.57-17.02	4.89-12.94	0.00-0.00	
Cysteine (µmol/L)	$\bar{x}$ (SD)	**264.3** (35.4)	**254.2** (26.7)	**261.2** (36.7)	**258.5** (32.1)	**264.1** (35.9)	**0.0** (0.0)	
	Median (IQR)	265.9 (232.6-289.9)	254.3 (236.3-272.1)	259.8 (237.4-284.8)	258.0 (236.8-279.3)	257.5 (236.1-274.6)	0.0 (0.0-0.0)	
	Range	202.0-335.2	180.9-326.6	203.0-343.3	180.9-343.3	215.3-333.8	0.0-0.0	
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**25.70** (4.49)	**26.64** (5.45)	**25.75** (4.74)	**26.19** (4.88)	**25.51** (6.17)	**0.00** (0.00)	
	Median (IQR)	25.60 (22.43-28.12)	26.03 (23.24-29.21)	25.88 (22.64-28.17)	25.83 (22.97-28.98)	24.56 (20.91-29.16)	0.00 (0.00-0.00)	
	Range	18.09-40.54	15.68-46.75	15.74-37.15	15.74-46.75	15.68-38.07	0.00-0.00	
Glutathione (µmol/L)	$\bar{x}$ (SD)	**9.88** (2.91)	**10.90** (4.54)	**10.32** (3.28)	**10.21** (3.13)	**12.86** (7.67)	**0.00** (0.00)	*
	Median (IQR)	9.76 (7.70-11.58)	10.21 (8.16-12.67)	9.98 (8.66-12.34)	10.05 (7.95-12.09)	9.48 (8.21-13.35)	0.00 (0.00-0.00)	
	Range	5.25-20.89	3.50-35.20	4.38-23.46	3.50-27.94	6.73-35.20	0.00-0.00	

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data log_{10} transformed * Blue asterisk (s) denotes all data log_{10} transformed with outliers removed.

Note: For 1561C>T GCPII an unpaired T-test was performed in place of ANOVA given no recessive genotype was recorded.
For 1561C>T GCPII significance is achieved with native (untransformed) data** for glutathione.

Table 3-35: Depression; B-vitamin/thiol related nutritional genetic data by genotype (1 of 4)

Indices		677C>T MTHFR			1298A>C MTHFR			19bp del DHFR		
		Wt (n=6)	Het (n=7)	Rec (n=4)	Wt (n=7)	Het (n=8)	Rec (n=2)	Wt (n=8)	Het (n=6)	Rec (n=3)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**361.5** (376.8)	**173.4** (62.1)	**335.5** (147.7)	**277.7** (134.7)	**300.0** (331.7)	**190.5** (178.9)	**308.0** (326.8)	**259.5** (156.4)	**234.7** (150.6)
	Median (IQR)	246.0 (166.8-341.0)	157.0 (141.0-191.0)	291.5 (240.5-386.5)	249.0 (186.0-312.5)	172.5 (155.0-246.3)	190.5 (127.3-253.8)	192.5 (156.3-266.0)	193.5 (166.8-303.5)	291.0 (177.5-320.0)
	Range	64.0-1100.0	102.0-291.0	215.0-544.0	154.0-544.0	102.0-1100.0	64.0-317.0	102.0-1100.0	128.0-544.0	64.0-349.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**24.00** (12.88)	**22.57** (9.88)	**21.25** (12.53)	**19.43** (9.80)	**26.25** (12.76)	**20.50** (2.12)	**21.38** (10.00)	**26.33** (13.71)	**19.33** (8.33)
	Median (IQR)	24.00 (19.75-29.00)	20.00 (18.00-26.00)	23.50 (13.50-31.25)	20.00 (13.00-26.00)	28.00 (18.50-33.25)	20.50 (19.75-21.25)	19.50 (18.25-23.25)	31.00 (20.50-31.75)	22.00 (16.00-24.00)
	Range	4.00-43.00	10.00-40.00	6.00-32.00	6.00-32.00	4.00-43.00	19.00-22.00	6.00-40.00	4.00-43.00	10.00-26.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**707.3** (245.8)	**671.7** (170.0)	**701.8** (310.7)	**682.4** (254.0)	**711.3** (215.6)	**643.0** (220.6)	**683.5** (201.4)	**732.0** (273.5)	**631.0** (212.5)
	Median (IQR)	807.0 (565.0-860.0)	649.0 (531.5-806.5)	764.0 (558.0-907.8)	648.0 (531.5-893.5)	799.0 (604.3-841.3)	643.0 (565.0-721.0)	724.0 (619.0-818.8)	831.5 (548.3-929.5)	531.0 (509.0-703.0)
	Range	322.0-946.0	470.0-907.0	288.0-991.0	288.0-991.0	322.0-946.0	487.0-799.0	288.0-907.0	322.0-991.0	487.0-875.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**10.85** (4.59)	**12.00** (5.09)	**7.73** (2.18)	**9.24** (4.40)	**10.78** (4.79)	**14.57** (0.26)	**11.11** (4.65)	**10.76** (4.68)	**8.85** (4.97)
	Median (IQR)	11.57 (7.43-14.67)	8.86 (7.84-16.72)	7.32 (6.28-8.78)	7.73 (6.94-9.39)	8.81 (7.71-15.53)	14.57 (14.48-14.67)	9.28 (7.55-15.02)	8.81 (8.32-13.80)	7.41 (6.08-10.90)
	Range	4.76-15.45	7.41-18.58	5.68-10.61	5.68-18.58	4.76-17.65	14.39-14.76	6.47-18.58	5.68-17.65	4.76-14.39
Cysteine (µmol/L)	$\bar{x}$ (SD)	**244.1** (33.4)	**263.5** (34.4)	**230.0** (35.5)	**243.9** (44.7)	**256.4** (26.0)	**235.5** (42.6)	**258.4** (38.2)	**250.3** (30.7)	**220.2** (26.5)
	Median (IQR)	255.7 (216.7-264.4)	264.8 (253.6-275.2)	230.4 (219.1-241.3)	230.7 (217.3-269.0)	257.9 (251.8-269.4)	235.5 (220.4-250.6)	265.1 (249.1-269.6)	256.4 (235.6-270.0)	205.3 (204.9-228.1)
	Range	200.7-281.6	204.4-317.7	186.1-273.2	186.1-317.7	200.7-285.2	205.3-265.7	186.1-317.7	200.7-285.2	204.4-250.8
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**24.04** (3.51)	**22.20** (2.26)	**24.54** (1.32)	**23.12** (2.83)	**23.56** (3.07)	**23.74** (0.42)	**23.30** (2.00)	**24.97** (2.62)	**20.51** (2.56) *
	Median (IQR)	23.74 (21.98-26.20)	23.23 (20.80-23.30)	24.39 (23.53-25.40)	23.62 (21.50-25.18)	23.23 (21.79-24.26)	23.74 (23.60-23.89)	23.32 (22.80-24.33)	24.39 (23.33-26.48)	19.34 (19.05-21.39)
	Range	19.34-28.99	18.76-25.20	23.27-26.09	18.76-26.09	19.34-28.99	23.45-24.04	19.72-26.09	21.89-28.99	18.76-23.45
Glutathione (µmol/L)	$\bar{x}$ (SD)	**9.46** (4.34)	**8.54** (3.61)	**12.64** (3.00)	**10.09** (4.12)	**9.62** (3.96)	**9.75** (5.59)	**11.88** (3.54)	**8.94** (3.65)	**6.15** (2.24) ‡
	Median (IQR)	10.43 (6.49-13.00)	8.54 (6.69-9.03)	12.06 (11.44-13.26)	9.65 (7.97-12.06)	8.64 (8.10-12.54)	9.75 (7.78-11.73)	12.18 (9.13-14.20)	9.19 (7.26-11.47)	5.80 (4.95-7.17)
	Range	3.17-13.71	4.09-15.69	9.65-16.81	4.09-16.81	3.17-15.69	5.80-13.71	6.61-16.81	3.17-13.22	4.09-8.54

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data log_{10} transformed * Blue asterisk (s) denotes all data log_{10} transformed with outliers removed.
‡ denotes approaching significance

Note: For 19bp del DHFR significance is achieved with native (untransformed) data for cysteinyl-glycine *.

Table 3-36: Depression; B-vitamin/thiol related nutritional genetic data by genotype (2 of 4)

Indices		2756A>G MTR			66A>G MTRR			1947G>A COMT		
		Wt (n=6)	Het (n=9)	Rec (n=2)	Wt (n=2)	Het (n=11)	Rec (n=4)	Wt (n=3)	Het (n=8)	Rec (n=6)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ *(SD)*	**196.5** (104.1)	**355.7** (308.1)	**172.5** (3.5)	**231.0** (145.7)	**310.2** (293.6)	**212.8** (68.3)	**178.7** (31.7)	**335.5** (322.2)	**250.8** (167.8)
	Median (IQR)	160.5 (154.8-259.3)	249.0 (212.0-334.0)	172.5 (171.3-173.8)	231.0 (179.5-282.5)	212.0 (167.0-333.0)	203.0 (156.3-259.5)	164.0 (160.5-189.5)	270.0 (169.8-321.3)	191.0 (138.5-314.8)
	Range	64.0-349.0	102.0-1100.0	170.0-175.0	128.0-334.0	64.0-1100.0	154.0-291.0	157.0-215.0	64.0-1100.0	102.0-544.0
Serum Folate (nmol/L)	$\bar{x}$ *(SD)*	**17.17** (8.35)	**24.67** (10.68)	**31.00** (16.97)	**24.00** (9.90)	**25.64** (11.22)	**14.25** (7.41)	**13.67** (8.74)	**22.63** (11.93)	**27.50** (8.64)
	Median (IQR)	20.50 (12.50-21.75)	30.00 (17.00-31.00)	31.00 (25.00-37.00)	24.00 (20.50-27.50)	26.00 (19.00-31.50)	15.00 (9.00-20.25)	16.00 (10.00-18.50)	21.00 (16.75-30.25)	28.50 (20.75-31.75)
	Range	4.00-26.00	6.00-40.00	19.00-43.00	17.00-31.00	4.00-43.00	6.00-21.00	4.00-21.00	6.00-43.00	17.00-40.00
Red Cell Folate (nmol/L)	$\bar{x}$ *(SD)*	**609.0** (231.9)	**722.7** (219.3)	**797.5** (210.0)	**730.5** (368.4)	**730.4** (188.1)	**564.5** (255.6)	**625.7** (293.1)	**673.6** (249.2)	**747.8** (160.4)
	Median (IQR)	531.5 (498.0-789.3)	799.0 (648.0-830.0)	797.5 (723.3-871.8)	730.5 (600.3-860.8)	799.0 (648.5-852.5)	531.5 (470.3-625.8)	648.0 (485.0-777.5)	665.5 (520.0-847.8)	806.5 (682.5-863.8)
	Range	322.0-907.0	288.0-991.0	649.0-946.0	470.0-991.0	322.0-946.0	288.0-907.0	322.0-907.0	288.0-991.0	470.0-880.0
Homocysteine (µmol/L)	$\bar{x}$ *(SD)*	**11.39** (5.48)	**9.68** (4.03)	**12.27** (4.98)	**12.91** (6.71)	**9.99** (4.24)	**11.08** (5.20)	**9.88** (4.86)	**11.21** (4.22)	**10.12** (5.36)
	Median (IQR)	11.06 (7.49-15.18)	8.17 (6.99-10.61)	12.27 (10.51-14.03)	12.91 (10.54-15.28)	8.75 (6.73-14.57)	9.17 (7.65-12.60)	7.73 (7.10-11.59)	9.68 (7.98-14.48)	8.41 (6.25-14.06)
	Range	4.76-18.58	5.68-17.65	8.75-15.80	8.17-17.65	4.76-15.80	7.41-18.58	6.47-15.45	6.99-18.58	4.76-17.65
Cysteine (µmol/L)	$\bar{x}$ *(SD)*	**240.6** (46.4)	**251.1** (31.4)	**263.0** (3.3)	**279.2** (8.5)	**241.2** (31.1)	**254.4** (48.9)	**217.2** (41.9)	**254.9** (39.1)	**256.5** (18.2)
	Median (IQR)	228.1 (204.7-261.3)	255.2 (230.7-273.2)	263.0 (261.8-264.2)	279.2 (276.2-282.2)	252.1 (217.7-263.0)	247.7 (224.1-278.0)	200.7 (193.4-232.7)	263.2 (224.4-275.3)	253.6 (251.1-262.8)
	Range	200.7-317.7	186.1-285.2	260.7-265.3	273.2-285.2	186.1-281.6	204.4-317.7	186.1-264.8	204.4-317.7	230.1-285.2
Cysteinyl-glycine (µmol/L)	$\bar{x}$ *(SD)*	**22.58** (4.05)	**23.57** (1.44)	**25.07** (2.61)	**24.20** (1.37)	**23.60** (2.57)	**22.44** (3.73)	**25.82** (2.91)	**23.20** (2.96)	**22.45** (1.64)
	Median (IQR)	21.59 (19.44-24.76)	23.38 (23.23-24.04)	25.07 (24.15-26.00)	24.20 (23.71-24.68)	23.38 (22.56-23.83)	22.46 (19.48-25.42)	25.20 (24.23-27.09)	23.74 (21.05-25.40)	23.23 (22.22-23.34)
	Range	18.76-28.99	21.49-26.09	23.23-26.92	23.23-25.17	19.34-28.99	18.76-26.09	23.27-28.99	18.76-26.92	19.34-23.62
Glutathione (µmol/L)	$\bar{x}$ *(SD)*	**7.93** (3.20)	**11.18** (3.08)	**9.43** (8.85)	**9.42** (3.75)	**10.13** (3.69)	**9.21** (5.50)	**11.53** (2.00)	**9.32** (5.02)	**9.65** (3.10)
	Median (IQR)	7.58 (6.00-9.13)	12.04 (8.73-12.31)	9.43 (6.30-12.56)	9.42 (8.10-10.75)	9.65 (8.54-12.77)	7.97 (5.98-11.20)	12.04 (10.68-12.63)	9.34 (5.38-12.66)	8.64 (8.54-9.42)
	Range	4.09-13.22	6.77-16.81	3.17-15.69	6.77-12.07	3.17-15.69	4.09-16.81	9.33-13.22	3.17-16.81	6.77-15.69

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Table 3-37: Depression; B-vitamin/thiol related nutritional genetic data by genotype (3 of 4)

Indices		3'UTR 6bp del TS			2R > 3R TSER			1420C>T SHMT		
		Wt (n=9)	Het (n=6)	Rec (n=2)	Wt (n=3)	Het (n=8)	Rec (n=6)	Wt (n=8)	Het (n=6)	Rec (n=3)
Serum Vitamin B_{12} (pmol/L)	$\bar{X}$ (SD)	**243.3** (81.8)	**370.2** (394.8)	**157.0** (77.8)	**238.3** (100.1)	**293.1** (336.2)	**277.5** (149.9)	**338.1** (323.0)	**200.7** (65.9)	**272.0** (238.0)
	Median (IQR)	249.0 (164.0-317.0)	192.5 (138.5-461.8)	157.0 (129.5-184.5)	212.0 (183.0-280.5)	192.5 (121.5-266.0)	233.0 (166.8-323.3)	270.0 (161.5-337.8)	193.5 (161.5-214.3)	170.0 (136.0-357.0)
	Range	154.0-349.0	64.0-1100.0	102.0-212.0	154.0-349.0	64.0-1100.0	157.0-544.0	64.0-1100.0	128.0-317.0	102.0-544.0
Serum Folate (nmol/L)	$\bar{X}$ (SD)	**20.00** (12.45)	**22.67** (6.80)	**35.50** (6.36)	**25.67** (5.51)	**21.13** (10.12)	**23.50** (14.68)	**18.63** (10.68)	**24.50** (10.54)	**30.33** (10.60)
	Median (IQR)	20.00 (10.00-26.00)	20.50 (17.50-28.00)	35.50 (33.25-37.75)	26.00 (23.00-28.50)	19.00 (16.75-24.00)	26.00 (12.75-31.75)	21.00 (9.00-27.00)	20.00 (17.50-28.50)	32.00 (25.50-36.00)
	Range	4.00-43.00	16.00-32.00	31.00-40.00	20.00-31.00	6.00-40.00	4.00-43.00	4.00-31.00	16.00-43.00	19.00-40.00
Red Cell Folate (nmol/L)	$\bar{X}$ (SD)	**687.9** (273.1)	**658.2** (166.6)	**806.5** (33.2)	**730.0** (177.5)	**623.3** (195.0)	**762.8** (271.4)	**605.1** (259.5)	**758.8** (176.0)	**786.3** (121.5)
	Median (IQR)	799.0 (531.0-907.0)	648.5 (527.3-773.5)	806.5 (794.8-818.3)	783.0 (657.5-829.0)	648.5 (482.8-803.0)	893.5 (618.3-936.3)	531.5 (445.8-830.0)	791.0 (681.8-880.0)	830.0 (739.5-855.0)
	Range	288.0-991.0	470.0-880.0	783.0-830.0	532.0-875.0	288.0-830.0	322.0-991.0	288.0-991.0	470.0-946.0	649.0-880.0
Homocysteine (µmol/L)	$\bar{X}$ (SD)	**10.69** (4.56)	**11.16** (5.36)	**8.41** (0.65)	**10.74** (7.10)	**11.83** (4.37)	**8.87** (3.39)	**10.79** (4.85)	**10.71** (4.44)	**9.81** (5.31)
	Median (IQR)	8.75 (7.73-14.76)	10.69 (6.60-15.44)	8.41 (8.18-8.63)	8.86 (6.81-13.72)	12.50 (7.71-15.02)	7.95 (7.49-8.61)	9.39 (7.30-14.65)	8.81 (7.99-13.28)	7.95 (6.81-11.87)
	Range	4.76-18.58	5.68-17.65	7.95-8.86	4.76-18.58	6.47-17.65	5.68-15.45	4.76-18.58	6.47-17.65	5.68-15.80
Cysteine (µmol/L)	$\bar{X}$ (SD)	**252.1** (36.3)	**242.3** (41.4)	**253.6** (2.2)	**273.5** (38.2)	**246.9** (36.1)	**239.0** (31.8)	**245.5** (42.8)	**252.4** (34.3)	**250.2** (18.1)
	Median (IQR)	260.7 (230.7-265.7)	247.7 (211.5-277.5)	253.6 (252.9-254.4)	252.1 (251.5-284.9)	260.3 (224.4-269.6)	245.4 (210.9-263.8)	240.8 (205.1-275.3)	262.7 (254.3-265.4)	255.2 (242.7-260.3)
	Range	200.7-317.7	186.1-285.2	252.1-255.2	250.8-317.7	186.1-285.2	200.7-273.2	200.7-317.7	186.1-285.2	230.1-265.3
Cysteinyl-glycine (µmol/L)	$\bar{X}$ (SD)	**23.80** (3.67)	**23.05** (0.78)	**22.63** (1.05)	**20.32** (1.37)	**23.52** (1.27)	**24.78** (3.47) *	**22.88** (3.68)	**24.09** (1.76)	**23.41** (0.20)
	Median (IQR)	25.17 (19.72-26.09)	23.25 (23.23-23.40)	22.63 (22.26-23.00)	19.72 (19.53-20.80)	23.32 (23.23-23.60)	25.18 (24.01-26.49)	22.47 (19.63-25.40)	23.65 (23.24-24.91)	23.38 (23.30-23.50)
	Range	18.76-28.99	21.49-23.62	21.89-23.38	19.34-21.89	21.49-26.09	18.76-28.99	18.76-28.99	21.89-26.92	23.23-23.62
Glutathione (µmol/L)	$\bar{X}$ (SD)	**9.73** (4.61)	**10.38** (3.72)	**8.63** (0.14)	**7.96** (1.17)	**11.46** (4.06)	**8.59** (4.12)	**9.93** (4.35)	**8.96** (3.75)	**11.29** (3.85)
	Median (IQR)	9.33 (6.61-13.22)	10.84 (7.49-12.24)	8.63 (8.59-8.68)	8.54 (7.58-8.64)	12.18 (8.10-14.20)	9.49 (5.40-11.47)	10.31 (6.41-12.54)	9.03 (7.26-11.36)	9.65 (9.09-12.67)
	Range	3.17-16.81	5.80-15.69	8.54-8.73	6.61-8.73	5.80-16.81	3.17-13.22	4.09-16.81	3.17-13.71	8.54-15.69

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 2R > 3R TSER significance is achieved with native (untransformed) data for cysteinyl-glycine*.

Table 3-38: Depression; B-vitamin/thiol related nutritional genetic data by genotype (4 of 4)

Indices		80G>A RFC			1561C>T GCPII		
		Wt (n=5)	Het (n=10)	Rec (n=2)	Wt (n=17)	Het (n=0)	Rec (n=0)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**257.0** (85.3)	**279.4** (297.6)	**323.0** (312.5)	**277.9** (241.1)	**0.0** (0.0)	**0.0** (0.0)
	Median (IQR)	291.0 (215.0-317.0)	172.5 (158.8-239.8)	323.0 (212.5-433.5)	212.0 (157.0-317.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
	Range	128.0-334.0	64.0-1100.0	102.0-544.0	64.0-1100.0	0.0-0.0	0.0-0.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**18.60** (7.70)	**22.20** (11.53)	**36.00** (5.66)	**22.76** (10.91)	**0.00** (0.00)	**0.00** (0.00)
	Median (IQR)	17.00 (16.00-19.00)	21.50 (19.25-29.00)	36.00 (34.00-38.00)	21.00 (17.00-31.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)
	Range	10.00-31.00	4.00-43.00	32.00-40.00	4.00-43.00	0.00-0.00	0.00-0.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**687.8** (210.9)	**660.4** (242.0)	**855.0** (35.4)	**691.4** (219.3)	**0.0** (0.0)	**0.0** (0.0)
	Median (IQR)	648.0 (531.0-799.0)	716.0 (498.3-860.0)	855.0 (842.5-867.5)	783.0 (531.0-875.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
	Range	470.0-991.0	288.0-946.0	830.0-880.0	288.0-991.0	0.0-0.0	0.0-0.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**10.89** (4.99)	**11.19** (4.56)	**6.81** (1.60)	**10.59** (4.48)	**0.00** (0.00)	**0.00** (0.00)
	Median (IQR)	8.17 (7.41-14.76)	9.74 (7.99-15.18)	6.81 (6.25-7.38)	8.75 (7.41-14.76)	0.00 (0.00-0.00)	0.00 (0.00-0.00)
	Range	6.47-17.65	4.76-18.58	5.68-7.95	4.76-18.58	0.00-0.00	0.00-0.00
Cysteine (µmol/L)	$\bar{x}$ (SD)	**242.9** (44.5)	**253.0** (34.7)	**242.7** (17.7)	**248.8** (34.9)	**0.0** (0.0)	**0.0** (0.0)
	Median (IQR)	265.7 (204.4-273.2)	256.4 (235.7-265.2)	242.7 (236.4-248.9)	255.2 (230.1-265.7)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
	Range	186.1-285.2	200.7-317.7	230.1-255.2	186.1-317.7	0.0-0.0	0.0-0.0
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**22.89** (2.44)	**23.63** (3.15)	**23.50** (0.17)	**23.40** (2.68)	**0.00** (0.00)	**0.00** (0.00)
	Median (IQR)	23.27 (23.23-24.04)	23.34 (21.59-25.86)	23.50 (23.44-23.56)	23.38 (21.89-25.17)	0.00 (0.00-0.00)	0.00 (0.00-0.00)
	Range	18.76-25.17	19.34-28.99	23.38-23.62	18.76-28.99	0.00-0.00	0.00-0.00
Glutathione (µmol/L)	$\bar{x}$ (SD)	**9.74** (4.10)	**10.02** (4.40)	**9.09** (0.79)	**9.83** (3.90)	**0.00** (0.00)	**0.00** (0.00)
	Median (IQR)	12.04 (6.77-12.07)	9.03 (7.09-13.00)	9.09 (8.81-9.37)	9.33 (6.77-12.31)	0.00 (0.00-0.00)	0.00 (0.00-0.00)
	Range	4.09-13.71	3.17-16.81	8.54-9.65	3.17-16.81	0.00-0.00	0.00-0.00

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 1561C>T GCPII no unpaired T-test was performed given no heterozygote and recessive genotypes were recorded.

As was the case with the hypertensive phenotype, significant outcomes from one way-ANOVA's are overlayed on the tables above, as indicated by red boxes and asterisks(s) for levels of significance. Participants with no depression showed variability among serum folate levels for 677C>T & 1298A>C MTHFR and 2R>3R TSER genotypes. Blood levels of glutathione also varied significantly among the 1947G>A COMT and 1561C>T GCPII genotypes for this same healthy population. For the depression phenotype however, cysteinyl-glycine demonstrated variability for the 19bp deletion DHFR and the 2R>3R TSER polymorphisms. The small sample size of this cohort warrants confirmation in larger populations.

3.2.9. Analysis of combined nutritional biochemistry and genetic data to establish any relationship to depression

The nature of the HADS questionnaire data collected provides the ability to examine the data in two ways; this is because the data could be considered either numeric continuous data or as categorical data. Current research treats the data both ways and there does not appear to be a consistent approach in the literature, therefore both methods have been used in this section in order to analyse the data present. Numeric continuous data was explored using stepwise regression analysis. Categorical data was analysed using ordinal logistic regression with the Wald χ^2 one-step linear approximation to the likelihood ratio test used to determine *p* value. The categorical method of analysis employed the cut off points of above/below 8 as described earlier.

As previously performed when analysing the hypertensive phenotype, several linear models have been examined using stepwise regression based on an *a priori* understanding of possible biochemical, genetic and physiologic relationships. Stepwise regression analysis was performed taking account of the following factors: age, BMI, gender, total dietary folic acid intake, serum B_{12}, serum folate, red cell folate, cysteine, homocysteine, cysteinyl-glycine, glutathione, and eleven B-vitamin related genotypes. Data described below are treated as numeric continuous variables for the HADS-D score; native data contains all data points (black text); $\log_{10}$ transformed data has had extreme data points removed (blue text). The first stepwise regression analysis performed included all variables and is presented in the table below.

Table 3-39: Stepwise regression; model for all genetic, metabolic and physiologic variables

	Native data				Transformed data			
Variable	**R^2**	**Estimate**	**T value**	***p* value**	**R^2**	**Estimate**	**T value**	***p* value**
Cysteinyl-glycine (µmol/L)	0.0851	-0.096	-2.69	0.0077**	0.0666	-5.416	-2.55	0.0115*

* Asterisk(s) denotes significance (* p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡ denotes approaching significance
Black = Native data including all data points. Blue = log_{10} transformed data with extreme data points removed.

Data in table 3-39 indicates that out of all variables examined; only cysteinyl-glycine predicts depression. Stepwise regression was repeated to include only the eleven genetic variants; however, no SNPs exhibited any relationship to depression.

The next stepwise regression model examined non-B vitamin related 'population' indices – age, BMI, and gender. As with previous models, data is either native, or contains log_{10} transformed data with any extreme data points removed.

Table 3-40: Stepwise regression; model for basic population information – age, body mass index, and gender

	Native data				Transformed data			
Variable	**R^2**	**Estimate**	**T value**	***p* value**	**R^2**	**Estimate**	**T value**	***p* value**
Age (yrs)	0.0164	0.052	1.95	0.0524 ‡	0.0163	9.311	1.94	0.0537 ‡

* Asterisk(s) denotes significance (* p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡ denotes approaching significance
Black = Native data including all data points. Blue = log_{10} transformed data with extreme data points removed.

When three basic population descriptors - age, BMI and gender are examined using stepwise regression, age approaches being a significant factor in depression. Although the outcomes of this stepwise model are not statistically significant, the outcome is worthy of note given the well-recognised association of age and depression [946-948].

While there was no relationship with depression and any of the genetic variants examined, it is important to further examine the relationship between B-vitamins and thiols in relation to depression. Table 3-41 below, provides a significant finding following stepwise regression analysis of all B-vitamin related blood metabolites and thiols combined (i.e. no genetic/dietary data).

Table 3-41: Stepwise regression; model for all B-vitamin related blood metabolites and thiols combined

	Native data				Transformed data			
Variable	**R^2**	**Estimate**	**T value**	***p* value**	**R^2**	**Estimate**	**T value**	***p* value**
Cysteinyl-glycine (µmol/L)	0.0391	-0.098	-2.79	0.0057**	0.0413	-5.417	-2.54	0.0118*

* Asterisk(s) denotes significance (* p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡ denotes approaching significance
Black = Native data including all data points. Blue = log_{10} transformed data with extreme data points removed.

Although the following parameters were examined; serum vitamin B_{12}, serum folate, red cell folate, cysteine, homocysteine, cysteinyl-glycine and glutathione, only the transsulphuration metabolite – cysteinyl-glycine predicts depression but in a highly statistically significant fashion. Given this finding (table 3-41), all transsulphuration thiol metabolites were examined collectively by stepwise regression. This included cysteine, homocysteine, cysteinyl-glycine, and glutathione. Again as with previous models, data is either native, or contains $\log_{10}$ transformed data with extreme data points removed.

Table 3-42: Stepwise regression; model for thiol transsulphuration pathway metabolites

	Native data				Transformed data			
Variable	**R^2**	**Estimate**	**T value**	***p* value**	**R^2**	**Estimate**	**T value**	***p* value**
Cysteinyl-glycine (µmol/L)	0.0391	-0.098	-2.79	0.0057**	0.0348	-6.127	-2.86	0.0046**

* Asterisk(s) denotes significance (* p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡ denotes approaching significance
Black = Native data including all data points. Blue = $\log_{10}$ transformed data with extreme data points removed.

Similar to table 3-39 and table 3-41 the model output presented in table 3-42 above supports the view that the transsulphuration metabolite – cysteinyl-glycine is statistical significant in predicting depression. The effect shown remains highly significant, and merits detailed discussion (see page 261 to 267).

For completion, the B-vitamin related blood metabolites were also examined as a discrete entity. Stepwise regression was performed on serum vitamin B_{12}, serum folate, and red cell folate and no relationship was seen with the depression phenotype. This is despite the unpaired t-test performed previously (figure 3-3) showing a difference for red cell folate between controls and depressed cases. Finally, stepwise regression for total dietary folic acid (5-methyl-H_4folic acid plus pteroylmonoglutamic acid), total natural folic acid (5-methyl-H_4folic acid), and synthetic folic acid (supplemental pteroylmonoglutamic acid) intakes did not indicate any relationship to depression.

Following analysis of the depression phenotype data as a continuous numeric variable, it was subsequently examined as a categorical variable. Its relationship to B-vitamin nutritional biochemistry and genetic data has involved treatment of the HADS scores as categorical data. The defined cut-off for the depression phenotype is above 8 (depression) and below 8 (controls). Ordinal logistic regression was performed and included the following factors, age, BMI, gender, dietary folic acid intake, serum B_{12}, serum folate, red cell folate, cysteine, homocysteine, cysteinyl-glycine, glutathione and eleven B-vitamin related genotypes. As previously, data presented is native and contains all data points (black text); $\log_{10}$ transformed data has had extreme data

points removed (blue text). The first ordinal logistic regression analysis performed included all variables and is presented in the table below.

Table 3-43: Ordinal logistic regression; model for all genetic, metabolic and physiologic variables

	Native data			Transformed data		
Variable	**R^2**	**Chi-squared**	***p* value**	**R^2**	**Chi-squared**	***p* value**
Red Cell Folate (nmol/L)	0.4620	4.66	0.0309*	0.4202		-
Cysteine (µmol/L)		3.94	0.0470*			-
Cysteinyl-glycine (µmol/L)		3.91	0.0480*		3.87	0.0493*
677C>T MTHFR		6.13	0.0465*			-

* Asterisk(s) denotes significance (* p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡ denotes approaching significance
Black = Native data including all data points. Blue = $\log_{10}$ transformed data with extreme data points removed.

As can be seen in table 3-43 the results repeat the previous outcomes showing cysteinyl-glycine as having a significant relationship with depression. Ordinal logistic regression was repeated to include only the eleven genetic variants. As previously, no SNP exhibited a relationship with depression, this is also consistent with the outcomes of odds ratio and chi-square tests displayed in table 3-30 (page 164).

The next ordinal logistic regression model was based on non-B vitamin related 'population' indices – age, BMI, and gender. No significance was detected, and is noticeably different to the numeric data outcome, which showed age as being almost significant when examined as a predictor of depression.

Given the cysteinyl-glycine relationship identified in the previous model, examination of B-vitamins and thiols without gene variants or population indices was performed next. The table below describes the outcomes of ordinal logistic regression analysis on the following parameters: serum vitamin B_{12}, serum folate, red cell folate, cysteine, homocysteine, cysteinyl-glycine and glutathione.

Table 3-44: Ordinal logistic regression; model for all B-vitamin related blood metabolites and thiols combined

	Native data			Transformed data		
Variable	**R^2**	**Chi-squared**	***p* value**	**R^2**	**Chi-squared**	***p* value**
Serum Vitamin B_{12} (pmol/L)	0.1647	4.31	0.0378*	0.1268		-
Cysteine (µmol/L)		4.82	0.0282*		4.29	0.0383*
Homocysteine (µmol/L)		5.33	0.0209*			-
Cysteinyl-glycine (µmol/L)		5.71	0.0169*		3.94	0.0471*

* Asterisk(s) denotes significance (* p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡ denotes approaching significance
Black = Native data including all data points. Blue = $\log_{10}$ transformed data with extreme data points removed.

When the parameters are examined independently of genes and population indices, both cysteine and cysteinyl-glycine show a clear relationship to depression. To further explore this thiol relationship, the next ordinal logistic regression performed included only the transsulphuration metabolites, outcomes are displayed in table 3-45 below.

Table 3-45: Ordinal logistic regression; model for thiol transsulphuration pathway metabolites

	Native data			Transformed data		
Variable	**R^2**	**Chi-squared**	***p* value**	**R^2**	**Chi-squared**	***p* value**
Cysteine (µmol/L)	0.1120	4.68	0.0305*	0.0862	4.11	0.0427*
Homocysteine (µmol/L)		5.50	0.0190*			-
Cysteinyl-glycine (µmol/L)		5.51	0.0189*		4.87	0.0273*

* Asterisk(s) denotes significance (* p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡ denotes approaching significance
Black = Native data including all data points. Blue = $\log_{10}$ transformed data with extreme data points removed.

Table 3-45 shows that with the removal of the B-vitamin metabolites, the relationship between depression and cysteine/cysteinyl-glycine remains. To ensure all areas are covered the next table below describes the B-vitamin related blood metabolites only. The following parameters, serum vitamin B_{12}, serum folate, and red cell folate were used to perform ordinal logistic regression analysis.

Table 3-46: Ordinal logistic regression; model for B-vitamin related blood metabolites

	Native data			Transformed data		
Variable	**R^2**	**Chi-squared**	***p* value**	**R^2**	**Chi-squared**	***p* value**
Red Cell Folate (nmol/L)	0.0495	4.59	0.0321*			-

* Asterisk(s) denotes significance (* p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡ denotes approaching significance
Black = Native data including all data points. Blue = $\log_{10}$ transformed data with extreme data points removed.

Red cell folate appears to have a significant relationship to depression although this relationship is not sustained when the data is transformed and the individuals with pharmacological intervention are removed (outliers). For completion, ordinal logistic regression was performed for total dietary folic acid (5-methyl-H_4folic acid plus pteroylmonoglutamic acid), total natural folic acid (5-methyl-H_4folic acid) and synthetic folic acid (supplemental pteroylmonoglutamic acid). In all cases dietary folate intake did not exhibit any relationship with depression. The overall findings relating B-vitamins/thiols to the depression phenotype are summarized below.

Summary of results for B-vitamin nutritional genetics in the elderly; a detailed study of hypertensive and depression phenotypes

Data indicates several parameters predict blood pressure; the most notable of these are the non-B vitamin related 'population' indices – naturally, age is a highly significant factor in both recumbent and standing diastolic blood pressure. BMI and gender are also fairly significant predictors of blood pressure and pulse rate.

Data analysis clearly shows that the 677C>T MTHFR gene variant contributes to recumbent diastolic blood pressure, as does, total dietary folic acid. Other notable associations also uncovered were the transsulphuration metabolites – cysteine and cysteinyl-glycine; these both stand out as being particularly significant correlates of both recumbent and standing diastolic and systolic blood pressures.

In examining the depression phenotype, there is a clear relationship with cysteinyl-glycine, this was evident when data was treated as being either numeric or categoric. Additionally, the closely related metabolite cysteine presented as having an association with depression when the categorical definitions of above and below a score of 8 for HADS-D were used.

CHAPTER 4

B-VITAMIN NUTRITIONAL GENETICS IN THE ELDERLY

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RISK FOR ALZHEIMER'S DISEASE

4. Overview

The term dementia describes a syndrome associated with a range of diseases which are characterised by the impairment of brain function; this may include impairment of memory, language, perception and cognitive skills [949]. AD is the most common form of dementia occurring for more than 75% of all dementia cases [127]. It is specifically recognised by short-term memory loss, apathy, and depression in the early stages. Onset can be gradual and the decline is progressive [127]. The AD form of dementia is a major health problem among older people, particularly women, and it is often considered not a natural part of ageing, although there is some evidence to support that the opposite may be true [950]. As outlined in chapter 1, AD is the third leading cause of death in women and the sixth leading cause of death men in Australia (table 1-2, page 20) [62, 114]. With Australia's changing demographic profile the future burden of AD will be significantly greater, especially when compared to CVD, as AD requires more in terms of direct healthcare costs and in resources for effective and appropriate care for existing suffers [144]. The number of people with dementia of all types is projected to triple in Australia to around 900,000 by the year 2050 [143].

With prevention in mind, oxidative stress is known to be involved in the pathogenesis of AD. Mild cognitive impairment concurrent with oxidative damage and is recognised as one of the earliest changes in AD development [184]. Oxidative stress can be induced by abnormal transsulphuration pathway function, where the balance between the production of glutathione from homocysteine is disrupted [361]. Elevated homocysteine has a direct excitotoxic effect on the brain, and high concentrations have been significantly associated with AD diagnosis [133]. In addition to this, studies have also linked low folate and vitamin B_{12} status to a higher incidence of AD [136, 138, 139, 184, 951, 952]. With this knowledge, the present study aims to explore the relationship between transsulphuration pathway thiols, important indices of folate status, eleven folate-related common polymorphisms, and the AD phenotype. A sample elderly population has been recruited via a local neurology clinic, and the subjects from the elderly cohort recruited in chapter 3 have been used as a control comparator. The selection of control subjects was of those whose MMSE score reflected the specified threshold for cognitive function.

4.1. Study Design

4.1.1. Ethics approval

Approval for this study was obtained from the University of Newcastle Human Research Ethics Committee (H-2008-0418) and the Northern Sydney Central Coast Health Committee (06/224). Informed written consent was obtained from the participants.

4.1.2. Study recruitment

Patients with AD were recruited from the clinical practices of neurologists associated with the study, principally Dr Jonathan Sturm and Dr Scott Whyte who largely provide clinical services for the entire Central Coast region, NSW. Due to the absence of a specialised hospital-based dementia clinical service, these two neurologists review large numbers of community-based patients with cognitive impairment and thus enabled ease of recruitment. Study information sheets and consent forms were sent out to eligible participants inviting them to take part in the study.

After receiving initial consent forms, the recruited participants attended a one hour clinical assessment conducted by the neurologist; during the interview consent was confirmed. Where a subject was unable to provide informed consent due to severe cognitive impairment, consent was obtained from the patient's career or a person responsible for the participant. A protocol approved by the Human Research Ethics Committees of the University of Newcastle and Central Coast Health used previously for the Retirement Village project was adhered too.

In total, ninety three participants were recruited into the study. These dementia cases were compared to the cohort of healthy retirement village residents recruited in the previous chapter. These control participants were considered to be free of any cognitive impairment after undergoing the MMSE, which eliminated participants from that study who had reduced cognitive functioning (see chapter 3, section 3.1.4 page 134 for more information).

4.1.3. Clinical assessment and neuropsychological testing

The standard clinical criteria established by the National Institute of Neurological and Communicative Disorders and Stroke - Alzheimer 's Disease and Related Disorders Association (NINCDS-ADRDA) was used in this study. This criterion classifies AD into 'definite', 'probable', and 'possible' levels of diagnostic certainty. Probable AD is the

maximum level of certainty possible without pathological confirmation. This category requires the insidious onset and progressive decline in memory with involvement of at least one other cognitive domain that is established by clinical examination and confirmed by neuropsychological tests [953]. It should be noted that a definitive diagnosis of AD can only be made through a biopsy or autopsy [953]. The NINCDS-ADRDA criterion has been shown to have a predictive accuracy of up to 90% [954].

During the clinical assessment, eligible participants were defined by NINCDS-ADRDA criteria for probable AD. In addition to this, both the Diagnostic and Statistical Manual of Mental Disorders fourth edition (DSM-IV) and the Hachinski Ischaemic scale were also undertaken for eligible participants for confirmation of AD diagnosis. The full clinical assessment also required participants to undergo the MMSE, HADS (see page 133 and 134 for more information) and an Alzheimer's Disease Assessment Scale Cognitive Subsection (ADAS-Cog) which evaluates cognitive impairment. The results of all these tests, along with date of birth, gender, history of disease/symptom onset date and brief medical history were all recorded into a confidential study database.

4.1.4. Food frequency questionnaire

A FFQ was also performed during the clinical assessment, if a subject was unable to provide adequate responses the patient's career was able to assist with knowledge of food intake. Estimated total dietary folic acid intake was determined using Foodworks™ for further details on the questionnaire and dietary estimation see section 2.4 on page 124.

4.1.5. Non-clinical measurements

During the clinical assessment approximately 20mls of fasting blood was collected from each participant (for further information see 2.1.1 on page 107). The blood was separated accordingly and distributed to the Molecular Nutrition PC2 Laboratory, University of Newcastle, Ourimbah and ICPMR at Westmead Hospital Sydney. With the appropriately prepared blood, the following analyses were undertaken:

- Blood levels of red cell folate, serum folate and vitamin B_{12}, were measured using an automated Access Immunoassay System. For detailed information regarding the automated access immunoassay system, see chapter 2 section 2.1.2 on page 108. As with the previous study above, results from these tests included outliers. Statistical analysis has therefore been performed to both include and exclude these as appropriate.

- Homocysteine, cysteine, cysteinyl-glycine, and glutathione concentrations were determined by HPLC with florescence detection using SBD-F. The full description of the methods employed and the validation process is described in section 2.2 on page 109.

- For gene polymorphism detection, the eleven SNPs were scored using the RFLP methods described in full in section 2.3 beginning on page 114.

4.1.6. Statistical analysis

General descriptive statistics for the non-genetic data such as age and gender, and all biochemical indices were examined for AD cases. B-vitamin/thiol measurement means have been compared using an unpaired t-test. The assessment of variation in all blood thiol and B-vitamin concentrations between individual genotypes for both the AD phenotype and controls combined, as well as the AD cohort alone was done by performing a one-way ANOVA. Data was first normalised by $\log_{10}$ transformation prior to analysis and significance set at $p=<0.05$. Also odds ratio analyses were performed to determine the degree and significance of an allele as a risk factor for AD. And finally, the relationships between the AD cohort and controls and the other recorded parameters was examined by the implementation of ordinal logistic regression analysis with the Wald χ^2 one-step linear approximation to the likelihood ratio test used to determine a significant *p* value. These models will be performed based on an *a priori* understanding of biochemical, genetic and physiologic relationships for this dementia phenotype.

As mentioned above, some of the B-vitamin concentrations were exceptionally high which is indicative of pharmacologic intervention. Due to this, the data was examined in two ways, the complete data set in an unmodified 'native' format, and then analysis was performed after $\log_{10}$ transformation with all extreme data points removed. The results displayed below report outcomes according to both approaches. A full description of all statistical methods employed for the entire thesis can be found in section 2.5, page 128.

4.2. Results

As previously mentioned in the study design section above, the data presented in this chapter combines a small AD patient cohort with the healthy retirement village cohort (see chapter 3). A comparison of the folate related parameters and genetics in relation to risk for AD is present below.

4.2.1. Descriptive statistics

The first table below outlines simple descriptive data based on age distribution, and includes number, mean, standard deviation, median, interquartile range and range. This data is limited to the Alzheimer's population alone, and is comparable to table 3-1 in chapter 3 where the healthy retirement village cohort data is presented. This healthy retirement village population represents the control data and is used as a comparator for the AD data presented in this section.

Table 4-1: Descriptive data based on age (years)

	All subjects	Male	Female
n	93	40	53
$\bar{x}$	79	78	79
SD	6	6	6
Median	80	79	81
IQR	75-82	75-82	76-83
Range	65-90	65-90	65-90

n=number, $\bar{x}$=mean, SD=standard deviation, IQR=interquartile range

4.2.2. B-vitamin metabolites and related indices

The following three tables contain the descriptive statistics for all non-genetic nutritional and blood indices. Table 4-2, table 4-3 and table 4-4 contain data for all AD subjects, males and females respectively. Unfortunately, there are some missing values for the dietary intake. It is particularly difficult to ascertain estimate intake in an individual with cognitive impairment, even with a carer present. From the ninety three people present in this cohort, eleven participants failed to perform the FFQ because the degree of their dementia prevented meaningful interaction with the interviewer. In addition to this, there were also a number of extreme values, predominantly within the serum folate, red cell folate and serum vitamin B_{12} indices. It is increasingly likely that dementia patients such as these maybe receiving clinically prescribed B-vitamin supplements. As previously mentioned, these values are therefore likely due to pharmacological intervention.

Table 4-2: Data for all Alzheimer's disease cases; blood metabolites and related indices

Indices	$\bar{x}$ (SD)	Median (IQR)	Range
Serum Vitamin B_{12} (pmol/L)	271.5 (163.2)	224.0 (175.0-292.0)	100.0-1100.0
Serum Folate (nmol/L)	21.91 (12.06)	19.00 (13.00-30.00)	4.00-45.00
Red Cell Folate (nmol/L)	874.7 (475.1)	780.5 (595.5-1034.8)	148.0-2500.0
Homocysteine (µmol/L)	11.65 (4.51)	10.79 (8.29-13.48)	5.91-34.13
Cysteine (µmol/L)	269.0 (43.4)	259.8 (245.1-288.7)	185.8-436.4
Cysteinyl-glycine (µmol/L)	22.85 (4.43)	21.95 (19.77-25.08)	16.03-35.47
Glutathione (µmol/L)	11.44 (4.80)	10.79 (8.28-13.41)	5.10-38.14
Total Dietary Folic Acid (µg/day) ^	380.1 (146.0)	330.3 (289.2-439.2)	162.4-976.6
Total Synthetic Folic Acid (µg/day) ^	103.6 (104.1)	97.0 (52.4-99.9)	0.0-557.1
Total Natural Folic Acid (µg/day) ^	276.5 (91.0)	252.6 (206.3-338.3)	72.7-485.5

^ Note: 11 participants failed to perform the FFQ satisfactorily, descriptive statistics for dietary folates is based on n = 82

Table 4-3: Data for male Alzheimer's disease cases; blood metabolites and related indices

Indices	$\bar{x}$ (SD)	Median (IQR)	Range
Serum Vitamin B_{12} (pmol/L)	277.6 (203.5)	213.0 (164.8-286.3)	100.0-1100.0
Serum Folate (nmol/L)	18.90 (8.75)	17.00 (12.75-26.25)	5.00-37.00
Red Cell Folate (nmol/L)	884.9 (435.9)	845.0 (632.0-1035.5)	148.0-2500.0
Homocysteine (µmol/L)	11.90 (3.59)	11.73 (8.93-14.32)	6.58-18.46
Cysteine (µmol/L)	267.9 (38.1)	259.4 (243.9-296.6)	191.6-365.8
Cysteinyl-glycine (µmol/L)	23.42 (4.76)	22.74 (19.67-25.69)	16.07-35.21
Glutathione (µmol/L)	12.05 (6.11)	10.79 (8.28-14.42)	5.10-38.14
Total Dietary Folic Acid (µg/day) ^	442.4 (169.9)	413.3 (323.3-518.7)	209.6-976.6
Total Synthetic Folic Acid (µg/day) ^	134.7 (134.9)	99.9 (69.0-166.8)	0.0-557.1
Total Natural Folic Acid (µg/day) ^	307.7 (90.4)	323.3 (229.7-378.9)	145.4-482.6

^ Note: 3 male participants failed to perform the FFQ satisfactorily, descriptive statistics for dietary folates is based on n = 37

Table 4-4: Data for female Alzheimer's disease cases; blood metabolites and related indices

Indices	$\bar{x}$ (SD)	Median (IQR)	Range
Serum Vitamin B_{12} (pmol/L)	266.8 (126.6)	224.0 (182.0- 308.0)	108.0-615.0
Serum Folate (nmol/L)	24.19 (13.69)	20.00 (14.00-33.00)	4.00- 45.00
Red Cell Folate (nmol/L)	867.3 (505.9)	771.0 (522.0-1019.0)	245.0-2500.0
Homocysteine (µmol/L)	11.45 (5.12)	10.27 (7.94-12.86)	5.91- 34.13
Cysteine (µmol/L)	269.8 (47.3)	259.8 (246.4- 286.2)	185.8-436.4
Cysteinyl-glycine (µmol/L)	22.42 (4.15)	21.84 (19.77- 24.73)	16.03-35.47
Glutathione (µmol/L)	10.98 (3.49)	10.56 (8.12-12.74)	5.19- 21.49
Total Dietary Folic Acid (µg/day) ^	328.9 (98.5)	304.6 (275.5- 370.2)	162.4- 560.3
Total Synthetic Folic Acid (µg/day) ^	78.0 (59.7)	77.5 (39.5- 99.9)	0.0-302.1
Total Natural Folic Acid (µg/day) ^	250.9 (84.1)	233.7 (196.4-294.6)	72.7-485.5

^ Note: 8 female participants failed to perform the FFQ satisfactorily, descriptive statistics for dietary folates is based on n = 45

The difference in the mean value between B-vitamin and related thiols for control and AD phenotypes was examined using an unpaired t-test. Figure 4-1 below displays any differences graphically, and shows both the mean and standard deviation (error bars) for each variable examined; significance was achieved for serum folate, homocysteine, cysteine, cysteinyl-glycine, glutathione, total dietary folic acid and total natural folic acid intakes when the data was $\log_{10}$ transformed and outliers removed. In the majority of indices, the AD cohort reflects a change in the level that moves towards a sub-optimal status in the measured metabolites.

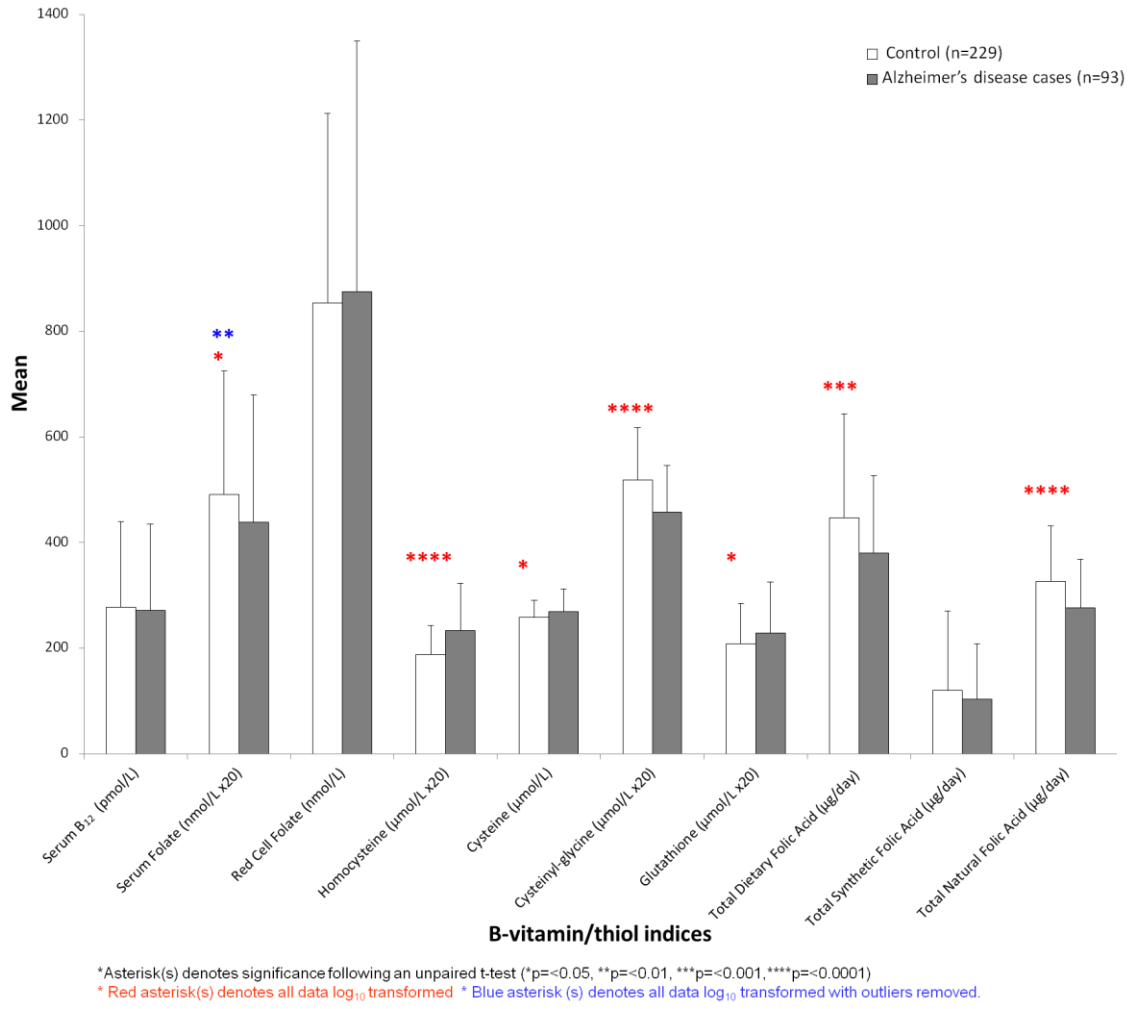


Figure 4-1: Mean and standard deviation values for B-vitamin/thiol measurements comparing Alzheimer's disease cases and controls.

Means have been compared using an unpaired t-test; asterisk denotes significance level.

4.2.3. B-vitamin related genetics (prevalence)

The table below outlines the prevalence (n) and the percentage for each genotype examined in the AD population, along with allele number and frequency.

Table 4-5: Complete genetic data for the Alzheimer's disease cohort; genotype prevalence and allele number

SNP	GENOTYPE Prevalence (%)			ALLELE Number (frequency)	
	Wildtype	Heterozygote	Recessive	Wildtype	Mutant
677C>T MTHFR	38 (41)	48 (51)	7 (8)	124 (0.67)	62 (0.33)
1298A>C MTHFR	40 (43)	42 (45)	11 (12)	122 (0.66)	64 (0.34)
19bp del DHFR	33 (35)	46 (50)	14 (15)	112 (0.60)	74 (0.40)
2756A>G MTR	64 (69)	26 (28)	3 (3)	154 (0.83)	32 (0.17)
66A>G MTRR	13 (14)	48 (52)	32 (34)	74 (0.40)	112 (0.60)
80G>A RFC	31 (33)	43 (46)	19 (21)	105 (0.56)	81 (0.44)
1561C>T GCPII	81 (87)	12 (13)	0 (0)	174 (0.94)	12 (0.06)
1420C>T SHMT	51 (55)	39 (42)	3 (3)	141 (0.76)	45 (0.24)
1947G>A COMT	28 (30)	40 (43)	25 (27)	96 (0.52)	90 (0.48)
3'UTR 6bp del TS	47 (50)	36 (39)	10 (11)	130 (0.70)	56 (0.30)
2R > 3R TSER	27 (29)	48 (52)	18 (19)	102 (0.55)	84 (0.45)

4.2.4. B-vitamin/thiol related nutritional genetics organised by genotype

Complete descriptive data (independent of phenotype, i.e. control population from chapter 3 combined with AD cases) is display in the next four tables for each B-vitamin or thiol related parameter examined - each is categorised by genotype. In order to investigate whether genetic factors can influence variability in blood indices, a one-way ANOVA was performed. All subject data was examined twice, since the data set contains a number of extreme data points (a likely consequence of pharmacologic intervention), and therefore the ANOVA's were performed both with and without these extreme points. The result of ANOVA based on $\log_{10}$ transformed (normalised) data is given in the following four tables, and significance is denoted by either a red asterisk(s) (all $\log_{10}$ transformed values) or a blue asterisk(s) ($\log_{10}$ transformed values with outliers removed). *Note: Statistical results for the untransformed data are displayed as footnotes in each table*.

Table 4-6: All data; B-vitamin/thiol related nutritional genetic data by genotype (1 of 4)

Indices		677C>T MTHFR			1298A>C MTHFR			19bp del DHFR		
		Wt (n=145)	Het (n=144)	Rec (n=33)	Wt (n=146)	Het (n=147)	Rec (n=29)	Wt (n=110)	Het (n=153)	Rec (n=59)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**271.8** (151.2)	**267.6** (156.1)	**314.9** (185.1)	**293.7** (168.5)	**266.7** (153.7)	**215.8** (86.8) * *	**289.3** (174.6)	**262.3** (140.7)	**277.7** (163.9)
	Median (IQR)	239.0 (180.0-318.0)	224.0 (174.0-291.0)	261.0 (200.0-358.0)	247.5 (196.5-350.3)	227.0 (170.5-314.0)	207.0 (176.0-259.0)	245.0 (182.0-344.8)	224.0 (180.0-292.0)	249.0 (187.5-325.0)
	Range	64.0-1100.0	78.0-1100.0	74.0-1100.0	74.0-1100.0	81.0-1100.0	64.0-434.0	78.0-1100.0	81.0-1100.0	64.0-1100.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**25.50** (12.09)	**22.35** (11.48)	**22.03** (11.30)	**24.18** (12.28)	**23.01** (11.31)	**25.17** (12.12)	**24.89** (12.31)	**23.24** (11.25)	**22.86** (12.33)
	Median (IQR)	23.00 (15.00-37.00)	20.00 (13.00-29.25)	21.00 (14.00-25.00)	21.00 (14.25-33.75)	21.00 (13.00-31.00)	22.00 (17.00-32.00)	22.50 (14.00-36.25)	21.00 (15.00-31.00)	22.00 (12.50-32.00)
	Range	3.00-45.00	4.00-45.00	6.00-45.00	5.00-45.00	3.00-45.00	5.00-45.00	5.00-45.00	4.00-45.00	3.00-45.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**850.9** (352.8)	**807.2** (363.8)	**1097.4** (574.5) ** *	**904.0** (465.1)	**814.1** (318.9)	**836.6** (323.4)	**894.5** (382.2)	**850.9** (403.8)	**801.5** (387.2)
	Median (IQR)	806.0 (607.3-1026.3)	736.0 (575.5-1009.5)	983.0 (754.0-1406.0)	802.0 (551.0-1159.0)	780.0 (613.0-968.5)	795.5 (595.5-1038.5)	818.0 (634.0-1098.0)	774.0 (579.5-1035.5)	780.0 (533.5-948.0)
	Range	221.0-2500.0	148.0-2500.0	288.0-2500.0	148.0-2500.0	221.0-2500.0	368.0-1550.0	245.0-2500.0	148.0-2500.0	221.0-2500.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**10.12** (4.06)	**10.27** (2.93)	**8.91** (2.62) *	**9.72** (3.50)	**10.18** (3.28)	**11.17** (4.09)	**9.41** (2.79)	**10.41** (3.97)	**10.37** (3.10)
	Median (IQR)	8.95 (7.63-11.82)	9.69 (8.11-12.01)	8.51 (7.58-9.72)	8.87 (7.57-11.14)	9.41 (8.04-11.81)	9.99 (8.43-12.82)	8.83 (7.59-10.45)	9.56 (7.94-11.83)	9.68 (7.85-13.20)
	Range	4.76-34.13	5.62-19.53	4.57-16.29	4.57-34.13	4.76-24.47	6.07-24.57	4.57-18.58	4.66-34.13	4.76-17.81
Cysteine (µmol/L)	$\bar{x}$ (SD)	**262.9** (39.7)	**263.1** (33.4)	**247.2** (30.2)	**261.4** (38.4)	**260.8** (34.0)	**264.1** (37.8)	**261.1** (33.1)	**262.5** (40.5)	**259.1** (30.4)
	Median (IQR)	257.3 (238.4-284.6)	261.7 (239.1-280.1)	251.8 (229.8-267.7)	256.9 (235.2-283.2)	258.3 (239.1-280.5)	265.5 (244.2-283.2)	257.3 (236.9-279.4)	259.2 (235.1-286.4)	255.0 (240.5-275.1)
	Range	191.6-436.4	180.9-365.8	185.8-307.9	180.9-436.4	191.6-400.8	193.1-385.3	186.1-400.8	180.9-436.4	204.2-344.6
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**25.61** (5.09)	**24.29** (4.79)	**25.80** (4.92)	**25.41** (5.08)	**24.75** (4.88)	**24.66** (4.94)	**25.10** (5.33)	**24.89** (4.71)	**25.30** (4.99)
	Median (IQR)	25.25 (21.95-29.01)	23.99 (21.09-26.49)	26.09 (21.50-29.17)	24.65 (21.56-28.65)	24.60 (21.26-27.36)	24.04 (21.28-26.88)	24.07 (22.09-26.55)	24.76 (21.15-28.43)	25.71 (21.51-28.86)
	Range	15.68-40.81	15.74-46.75	16.93-35.21	16.07-46.75	15.68-40.81	16.03-35.04	15.74-46.75	15.68-36.08	16.03-36.42
Glutathione (µmol/L)	$\bar{x}$ (SD)	**10.91** (4.44)	**10.53** (3.91)	**10.51** (3.81)	**10.94** (4.32)	**10.27** (3.92)	**11.66** (4.16)	**10.76** (3.89)	**10.74** (4.54)	**10.50** (3.52)
	Median (IQR)	10.11 (8.12-12.58)	10.18 (8.04-12.27)	10.05 (7.99-12.52)	10.41 (8.28-12.55)	9.42 (7.75-12.26)	10.73 (9.07-13.41)	10.37 (8.28-12.34)	9.92 (7.90-12.77)	10.24 (8.05-11.81)
	Range	3.17-35.20	4.09-38.14	5.13-21.29	3.50-38.14	3.17-35.20	4.99-21.58	5.25-35.20	3.17-38.14	4.09-21.29

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data log_{10} transformed * Blue asterisk (s) denotes all data log_{10} transformed with outliers removed.

Note: For 677C>T MTHFR significance is achieved with native (untransformed) data for red cell folate both with*** and without** outliers.
For 1298A>C MTHFR significance is achieved with native (untransformed) data for serum vitamin B_{12} both with* and without* outliers.

Table 4-7: All data; B-vitamin/thiol related nutritional genetic data by genotype (2 of 4)

Indices		2756A>G MTR			66A>G MTRR			1947G>A COMT		
		Wt (n=190)	Het (n=114)	Rec (n=18)	Wt (n=52)	Het (n=164)	Rec (n=106)	Wt (n=88)	Het (n=141)	Rec (n=91)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**257.6** (129.6)	**299.1** (191.3)	**294.2** (171.0)	**295.9** (168.8)	**271.0** (158.7)	**268.9** (149.5)	**256.7** (128.8)	**284.1** (159.6)	**273.7** (178.2)
	Median (IQR)	230.0 (174.3-298.0)	244.0 (196.0-321.5)	220.5 (176.3-366.0)	260.0 (187.3-349.3)	231.0 (178.8-314.8)	224.0 (181.0-290.3)	227.0 (165.5-308.8)	249.0 (198.0-327.0)	221.0 (174.5-291.0)
	Range	64.0-1100.0	74.0-1100.0	138.0-778.0	95.0-1100.0	64.0-1100.0	81.0-854.0	81.0-719.0	64.0-1100.0	89.0-1100.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**23.61** (11.81)	**23.71** (11.79)	**25.22** (12.63)	**24.00** (12.40)	**24.65** (11.94)	**22.19** (11.27)	**21.86** (11.74)	**24.99** (11.88)	**23.27** (11.54)
	Median (IQR)	22.00 (14.00-31.00)	20.00 (14.25-33.00)	22.50 (14.00-36.50)	22.00 (12.75-33.00)	22.00 (15.00-34.25)	20.50 (14.00-27.75)	19.00 (14.00-26.00)	23.00 (15.00-33.00)	21.00 (13.00-32.50)
	Range	3.00-45.00	4.00-45.00	5.00-45.00	5.00-45.00	4.00-45.00	3.00-45.00	4.00-45.00	3.00-45.00	5.00-45.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**869.4** (410.2)	**811.9** (332.3)	**1009.5** (531.0)	**908.7** (471.3)	**868.9** (400.3)	**812.4** (336.2)	**856.5** (411.1)	**872.3** (389.3)	**832.2** (389.9)
	Median (IQR)	802.0 (591.8-1088.3)	754.5 (608.3-955.8)	959.5 (633.5-1292.3)	801.0 (613.8-1029.8)	815.0 (575.5-1095.0)	771.0 (597.0-1006.0)	784.5 (601.3-1050.8)	804.0 (613.0-1073.0)	769.0 (536.0-990.0)
	Range	148.0-2500.0	256.0-2057.0	368.0-2500.0	245.0-2500.0	148.0-2500.0	221.0-2057.0	148.0-2500.0	221.0-2500.0	245.0-2500.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**10.13** (3.24)	**10.03** (3.92)	**9.57** (2.98)	**9.68** (3.07)	**10.18** (3.69)	**10.08** (3.33)	**10.00** (3.47)	**10.42** (3.89)	**9.64** (2.69)
	Median (IQR)	9.37 (7.79-11.88)	9.06 (7.70-11.30)	8.79 (8.04-11.57)	8.50 (7.52-11.74)	9.09 (7.84-11.94)	9.48 (7.89-11.09)	9.06 (7.82-10.81)	9.37 (7.86-11.90)	9.20 (7.74-11.44)
	Range	4.66-24.47	4.57-34.13	4.89-15.80	5.56-18.74	4.66-34.13	4.57-24.57	4.57-24.47	4.89-34.13	4.66-18.11
Cysteine (µmol/L)	$\bar{x}$ (SD)	**261.1** (35.0)	**260.0** (38.1)	**273.1** (38.8)	**258.2** (30.7)	**257.9** (36.1)	**268.2** (38.4)	**254.0** (33.5)	**267.6** (39.7)	**259.5** (31.9) *
	Median (IQR)	258.5 (238.7-280.3)	255.1 (234.4-280.9)	266.5 (254.6-307.3)	253.7 (237.3-272.4)	254.4 (233.9-279.1)	265.9 (244.0-286.3)	249.5 (230.5-274.9)	263.1 (239.7-287.1)	255.0 (239.8-279.7)
	Range	185.8-400.8	180.9-436.4	193.1-333.8	205.3-325.1	180.9-436.4	193.1-400.8	185.8-337.3	191.6-436.4	180.9-343.3
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**24.69** (4.71)	**25.52** (5.16)	**25.64** (6.28)	**26.02** (6.17)	**24.70** (4.55)	**25.08** (4.93)	**24.36** (5.04)	**25.26** (5.25)	**25.30** (4.47)
	Median (IQR)	24.50 (21.48-27.77)	24.64 (21.88-28.49)	26.60 (20.30-29.54)	25.01 (22.06-29.12)	24.09 (21.15-27.94)	24.58 (21.50-27.83)	24.06 (20.54-27.16)	24.68 (21.49-27.85)	24.98 (22.32-28.36)
	Range	15.74-46.75	16.03-40.81	15.68-37.15	16.94-46.75	15.74-35.47	15.68-39.09	15.74-38.47	15.68-46.75	16.03-36.42
Glutathione (µmol/L)	$\bar{x}$ (SD)	**10.63** (4.14)	**10.90** (4.07)	**10.15** (4.70)	**11.02** (4.75)	**10.76** (4.46)	**10.44** (3.23)	**9.58** (2.76)	**10.87** (4.16)	**11.42** (4.95) *
	Median (IQR)	9.95 (8.06-12.35)	10.45 (8.28-12.67)	8.55 (7.09-11.74)	10.36 (7.75-12.68)	10.00 (8.05-12.41)	10.01 (8.28-12.34)	9.42 (7.50-11.20)	10.27 (8.25-12.70)	10.44 (8.25-12.83)
	Range	4.09-38.14	3.50-35.20	3.17-23.46	4.38-35.20	3.17-38.14	3.50-23.46	3.50-20.89	3.17-35.20	5.36-38.14

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 1947G>A COMT significance is achieved with native (untransformed) data for cysteine* and glutathione**.

Table 4-8: All data; B-vitamin/thiol related nutritional genetic data by genotype (3 of 4)

Indices		3'UTR 6bp del TS			2R > 3R TSER			1420C>T SHMT		
		Wt (n=152)	Het (n=136)	Rec (n=34)	Wt (n=87)	Het (n=174)	Rec (n=61)	Wt (n=166)	Het (n=133)	Rec (n=23)
Serum Vitamin B_{12} (pmol/L)	$\bar{X}$ (SD)	**270.5** (146.2)	**280.8** (177.8)	**265.3** (114.8)	**269.2** (135.7)	**276.1** (169.4)	**276.6** (151.9)	**273.0** (156.3)	**281.4** (165.4)	**243.4** (110.1)
	Median (IQR)	238.5 (181.0-307.3)	228.5 (179.8-333.0)	245.5 (188.0-288.0)	240.0 (184.0-299.5)	228.0 (179.3-318.0)	247.0 (181.0-308.0)	233.5 (178.5-317.0)	238.0 (188.0-314.0)	215.0 (173.5-291.0)
	Range	89.0-1100.0	64.0-1100.0	74.0-554.0	74.0-778.0	64.0-1100.0	117.0-1100.0	64.0-1100.0	81.0-1100.0	102.0-544.0
Serum Folate (nmol/L)	$\bar{X}$ (SD)	**24.24** (11.92)	**23.11** (11.96)	**24.00** (10.95)	**22.51** (11.93)	**24.35** (11.90)	**23.74** (11.46)	**23.66** (11.97)	**24.20** (12.22)	**21.61** (7.85)
	Median (IQR)	21.50 (15.00-31.25)	20.50 (13.75-32.25)	23.00 (16.25-32.50)	20.00 (13.50-30.00)	23.00 (14.00-33.00)	22.00 (14.00-31.00)	22.00 (14.00-33.00)	21.00 (14.00-33.00)	20.00 (15.50-26.50)
	Range	4.00-45.00	3.00-45.00	5.00-45.00	3.00-45.00	4.00-45.00	4.00-45.00	4.00-45.00	3.00-45.00	12.00-40.00
Red Cell Folate (nmol/L)	$\bar{X}$ (SD)	**835.2** (366.3)	**886.5** (445.3)	**833.4** (272.6)	**824.1** (341.8)	**868.5** (411.1)	**870.6** (415.3)	**846.5** (383.8)	**887.2** (422.3)	**754.3** (259.9)
	Median (IQR)	793.0 (569.8-1007.8)	790.5 (597.8-1125.0)	784.0 (608.3-1017.5)	785.0 (594.5-1028.0)	784.0 (602.0-1087.0)	816.0 (606.5-1022.5)	788.5 (590.8-1042.5)	798.0 (613.0-1092.0)	749.0 (568.5-840.5)
	Range	148.0-2057.0	221.0-2500.0	418.0-1396.0	221.0-1686.0	148.0-2500.0	254.0-2500.0	245.0-2500.0	148.0-2500.0	421.0-1650.0
Homocysteine (µmol/L)	$\bar{X}$ (SD)	**9.91** (3.14)	**10.11** (3.29)	**10.54** (5.24)	**9.96** (3.16)	**10.10** (3.78)	**10.11** (3.00)	**9.99** (3.75)	**10.33** (3.23)	**9.05** (2.58)
	Median (IQR)	9.06 (7.80-11.30)	9.51 (7.68-11.94)	9.17 (7.88-10.48)	9.19 (7.76-11.55)	9.18 (7.76-11.52)	9.54 (7.80-11.92)	9.01 (7.65-11.64)	9.70 (8.07-12.00)	8.47 (7.36-10.11)
	Range	4.57-24.57	5.20-24.47	5.56-34.13	4.76-19.53	4.66-34.13	4.57-18.74	4.66-34.13	4.57-24.47	5.68-15.80
Cysteine (µmol/L)	$\bar{X}$ (SD)	**259.4** (36.7)	**261.1** (34.3)	**271.0** (41.3)	**261.6** (35.7)	**262.4** (36.8)	**258.1** (36.2)	**260.9** (37.9)	**263.3** (36.1)	**253.3** (22.7)
	Median (IQR)	258.6 (234.7-278.2)	254.8 (239.1-282.5)	271.0 (242.8-286.3)	257.3 (234.8-286.1)	258.8 (239.9-281.8)	254.2 (235.0-278.0)	254.8 (234.5-280.3)	259.3 (241.4-285.2)	256.0 (237.5-269.7)
	Range	180.9-400.8	186.1-365.8	207.5-436.4	191.6-343.3	180.9-436.4	200.7-400.8	191.6-436.4	180.9-400.8	213.6-297.8
Cysteinyl-glycine (µmol/L)	$\bar{X}$ (SD)	**24.92** (4.82)	**25.20** (5.26)	**24.95** (4.53)	**24.68** (5.11)	**24.95** (4.77)	**25.81** (5.32)	**25.12** (4.68)	**24.71** (5.15)	**26.38** (5.87)
	Median (IQR)	24.66 (21.76-27.57)	24.52 (21.23-28.77)	24.39 (21.58-26.54)	23.47 (21.04-27.21)	24.67 (21.51-27.96)	25.20 (22.63-28.98)	25.02 (21.58-28.39)	24.47 (21.45-26.92)	24.76 (22.84-29.90)
	Range	15.68-46.75	16.07-40.81	17.72-36.08	16.07-46.75	15.74-39.09	15.68-40.54	15.68-40.54	15.74-46.75	16.93-40.81
Glutathione (µmol/L)	$\bar{X}$ (SD)	**10.89** (4.47)	**10.50** (3.95)	**10.66** (3.30)	**10.27** (2.94)	**10.85** (4.27)	**10.87** (5.12)	**10.91** (4.31)	**10.40** (4.09)	**10.90** (3.11)
	Median (IQR)	10.29 (8.01-12.67)	9.61 (8.11-11.93)	10.18 (8.31-12.38)	10.22 (7.96-12.28)	10.21 (8.27-12.58)	9.65 (7.76-12.40)	10.41 (8.20-12.67)	9.92 (7.58-12.24)	9.65 (8.66-12.75)
	Range	3.17-38.14	3.50-35.20	5.93-20.99	5.25-18.72	3.50-35.20	3.17-38.14	4.09-38.14	3.17-35.20	7.14-18.30

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data log_{10} transformed * Blue asterisk (s) denotes all data log_{10} transformed with outliers removed.

Table 4-9: All data; B-vitamin/thiol related nutritional genetic data by genotype (4 of 4)

Indices		80G>A RFC			1561C>T GCPII		
		Wt (n=100)	Het (n=147)	Rec (n=75)	Wt (n=291)	Het (n=31)	Rec (n=0)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**282.7** (168.8)	**272.6** (161.5)	**266.5** (132.3)	**275.1** (158.1)	**267.6** (151.2)	**0.0** (0.0)
	Median (IQR)	229.0 (192.5-318.5)	231.0 (179.5-309.5)	239.0 (177.5-322.5)	238.0 (182.0-314.0)	224.0 (161.5-315.0)	0.0 (0.0-0.0)
	Range	78.0-1100.0	64.0-1100.0	95.0-702.0	64.0-1100.0	106.0-719.0	0.0-0.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**22.17** (11.85)	**24.27** (11.75)	**24.77** (11.85)	**23.52** (11.91)	**25.77** (10.90)	**0.00** (0.00)
	Median (IQR)	19.00 (13.00-30.00)	23.00 (14.50-33.00)	22.00 (14.50-33.00)	21.00 (14.00-31.50)	24.00 (18.00-35.50)	0.00 (0.00-0.00)
	Range	3.00-45.00	4.00-45.00	7.00-45.00	3.00-45.00	7.00-45.00	0.00-0.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**896.2** (447.6)	**843.5** (361.9)	**829.9** (376.7)	**852.1** (395.0)	**900.5** (384.1)	**0.0** (0.0)
	Median (IQR)	802.0 (610.3-1104.0)	787.0 (602.8-1036.3)	782.5 (569.8-1015.3)	785.0 (598.0-1048.0)	844.0 (598.0-1061.5)	0.0 (0.0-0.0)
	Range	148.0-2500.0	245.0-2500.0	254.0-2500.0	148.0-2500.0	378.0-1726.0	0.0-0.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**10.36** (3.93)	**10.00** (3.50)	**9.79** (2.71)	**10.11** (3.51)	**9.64** (3.12)	**0.00** (0.00)
	Median (IQR)	9.39 (7.82-12.05)	9.15 (7.74-11.35)	9.22 (7.87-11.15)	9.21 (7.81-11.81)	8.86 (7.66-11.09)	0.00 (0.00-0.00)
	Range	5.48-34.13	4.57-24.57	4.66-18.46	4.57-34.13	4.89-18.11	0.00-0.00
Cysteine (µmol/L)	$\bar{x}$ (SD)	**264.4** (37.2)	**259.0** (33.5)	**261.8** (40.4)	**261.4** (36.5)	**260.6** (34.6)	**0.0** (0.0)
	Median (IQR)	264.5 (237.0-285.6)	256.0 (237.1-278.7)	254.6 (236.9-283.7)	258.7 (237.0-281.7)	257.3 (234.6-277.7)	0.0 (0.0-0.0)
	Range	186.1-436.4	180.9-385.3	191.6-400.8	180.9-436.4	191.6-333.8	0.0-0.0
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**25.04** (4.48)	**25.11** (5.38)	**24.89** (4.83)	**25.09** (4.89)	**24.53** (5.76)	**0.00** (0.00)
	Median (IQR)	24.20 (21.50-27.56)	24.69 (21.37-28.51)	24.59 (21.59-27.89)	24.69 (21.77-27.81)	22.66 (20.18-29.07)	0.00 (0.00-0.00)
	Range	17.59-40.54	15.68-46.75	15.74-37.15	15.74-46.75	15.68-38.07	0.00-0.00
Glutathione (µmol/L)	$\bar{x}$ (SD)	**10.43** (3.46)	**10.89** (4.40)	**10.68** (4.46)	**10.60** (3.79)	**11.67** (6.61)	**0.00** (0.00)
	Median (IQR)	10.25 (7.77-12.05)	10.18 (8.10-12.73)	9.78 (8.59-11.83)	10.24 (8.09-12.51)	9.31 (8.21-12.39)	0.00 (0.00-0.00)
	Range	4.09-21.59	3.17-35.20	4.38-38.14	3.17-38.14	5.13-35.20	0.00-0.00

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 1561C>T GCPII an unpaired T-test was performed in place of ANOVA given no recessive genotype was recorded.

The outcomes of the one-way ANOVA's displayed above show that for the 677C>T MTHFR gene, variability exists between levels of red cell folate and between the levels of homocysteine. For the 1298A>C MTHFR SNP, serum vitamin B_{12} levels vary and for 1947G>A COMT genotype, both cysteine and glutathione levels show significant variation. Since the AD phenotype may be modified by B-vitamin-related genetics, impact of each of the eleven genotypes is displayed in the table below. For comparison, the prevalence is displayed alongside the control group (data from chapter 3). The table displays the genotype prevalence (%), allele number (frequency) and carriage of mutant allele for each of the phenotypes.

Table 4-10: Alzheimer's disease phenotype; genotype prevalence, allele number and carriage of mutant allele

SNP	PHENOTYPE	GENOTYPE Prevalence (%)			ALLELE Number (frequency)		Carriage of mutant allele (%)
		Wildtype	Heterozygote	Recessive	Wildtype	Mutant	
677C>T MTHFR	Control	107 (47)	96 (42)	26 (11)	310 (0.68)	148 (0.32)	53
	Alzheimer's disease	38 (41)	48 (51)	7 (8)	124 (0.67)	62 (0.33)	59
1298A>C MTHFR	Control	106 (46)	105 (46)	18 (8)	317 (0.69)	141 (0.31)	54
	Alzheimer's disease	40 (43)	42 (45)	11 (12)	122 (0.66)	64 (0.34)	57
19bp del DHFR	Control	77 (34)	107 (47)	45 (20)	261 (0.57)	197 (0.43)	67
	Alzheimer's disease	33 (35)	46 (50)	14 (15)	112 (0.60)	74 (0.40)	65
2756A>G MTR	Control	126 (55)	88 (38)	15 (7)	340 (0.74)	118 (0.26)	45
	Alzheimer's disease	64 (69)	26 (28)	3 (3)	154 (0.83)	32 (0.17)	31
66A>G MTRR	Control	39 (17)	116 (51)	74 (32)	194 (0.42)	264 (0.58)	83
	Alzheimer's disease	13 (14)	48 (52)	32 (34)	74 (0.40)	112 (0.60)	86
80G>A RFC	Control	69 (30)	104 (45)	56 (24)	242 (0.53)	216 (0.47)	69
	Alzheimer's disease	31 (33)	43 (46)	19 (21)	105 (0.56)	81 (0.44)	67
1561C>T GCPII	Control	210 (92)	19 (8)	0 (0)	439 (0.96)	19 (0.04)	8
	Alzheimer's disease	81 (87)	12 (13)	0 (0)	174 (0.94)	12 (0.06)	13
1420C>T SHMT	Control	115 (50)	94 (41)	20 (9)	324 (0.71)	134 (0.29)	50
	Alzheimer's disease	51 (55)	39 (42)	3 (3)	141 (0.76)	45 (0.24)	45
1947G>A COMT	Control	60 (26)	101 (44)	66 (29)	221 (0.49)	233 (0.51)	73
	Alzheimer's disease	28 (30)	40 (43)	25 (27)	96 (0.52)	90 (0.48)	70
3'URT 6bp del TS	Control	105 (46)	100 (44)	24 (10)	310 (0.68)	148 (0.32)	54
	Alzheimer's disease	47 (50)	36 (39)	10 (11)	130 (0.70)	56 (0.30)	50
2R > 3R TSER	Control	60 (26)	126 (55)	43 (19)	242 (0.53)	216 (0.47)	74
	Alzheimer's disease	27 (29)	48 (52)	18 (19)	102 (0.55)	84 (0.45)	71

Using the data presented above, an odds ratio and 95% confidence interval was calculated in order to assess the degree and significance of risk of an individual SNP for AD. A 2x2 table was then used to carry out a chi-square test and derive a *p* value. This was achieved using the Yates correction, which provides a stricter analysis for small samples. The table below displays the outcomes of all tests for each of the eleven B-vitamin related genetic variants examined.

Table 4-11: Alzheimer's disease phenotype; odds ratio and 95% CI along with chi-square test *p* value

SNP	Odds Ratio	95% Confidence Intervals	*p*	χ^2
677C>T MTHFR	1.05	0.73 - 1.50	0.8750	0.025
1298A>C MTHFR	1.18	0.82 - 1.69	0.4231	0.642
19bp del DHFR	0.88	0.62 -1.24	0.5067	0.441
2756A>G MTR	0.60	0.39 - 0.92	0.0260 *	4.956
66A>G MTRR	1.11	0.79 - 1.57	0.6085	0.262
80G>A RFC	0.86	0.61 - 1.22	0.4554	0.557
1561C>T GCPII	1.59	0.76 - 3.35	0.3010	1.070
1420C>T SHMT	0.77	0.52 - 1.14	0.2289	1.447
1947G>A COMT	0.89	0.63 - 1.25	0.5571	0.345
3'UTR 6bp del TS	0.90	0.62 - 1.31	0.6512	0.204
2R > 3R TSER	0.92	0.66 - 1.30	0.7084	0.140

* Asterisk denotes significance

The 2756A>G MTR SNP indicates a positive protective relationship for Alzheimer's dementia. No other SNP showed a significant relationship with this phenotype.

4.2.5. Alzheimer's disease phenotype; B-vitamin/thiol related nutritional genetic data organised by genotype

Descriptive data organised by genotype is displayed in the next four tables for each B-vitamin and thiol related parameter examined. Data relates to dementia cases only. The control cohort table is not repeated below as it has already been shown in chapter 3 (pages 139-142). In addition to the descriptive data, a one way ANOVA has been performed in order to investigate whether genetic factors influenced variability in blood indices. As alluded to earlier, the data set contains a number of extreme data points as a likely consequence of pharmacologic intervention, and therefore the ANOVA's were performed both with and without these extreme values. Similarly, the result of ANOVA based on $\log_{10}$ transformed (normalised) data is given in the following eight tables, and significance is denoted by either a red asterisk(s) (all $\log_{10}$ transformed values) or a blue asterisk(s) ($\log_{10}$ transformed values with outliers removed).

Table 4-12: Alzheimer's disease cases; B-vitamin/thiol related nutritional genetic data by genotype (1 of 4)

Indices		677C>T MTHFR			1298A>C MTHFR			19bp del DHFR		
		Wt (n=38)	Het (n=48)	Rec (n=7)	Wt (n=40)	Het (n=42)	Rec (n=11)	Wt (n=33)	Het (n=46)	Rec (n=14)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**227.4** (98.0)	**279.8** (158.9)	**453.3** (316.4) **	**303.6** (180.7)	**265.3** (158.1)	**178.0** (36.4) * *	**297.4** (156.1)	**238.0** (114.6)	**320.4** (273.7)
	Median (IQR)	205.5 (167.0-254.3)	225.5 (174.0-300.0)	352.0 (265.0-506.5)	254.5 (201.3-352.3)	216.5 (170.3-304.0)	193.0 (158.5-206.0)	260.0 (179.0-381.0)	212.0 (170.0-267.8)	225.0 (196.0-255.8)
	Range	108.0-517.0	100.0-778.0	178.0-1100.0	132.0-1100.0	100.0-778.0	108.0-217.0	108.0-719.0	100.0-610.0	137.0-1100.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**21.66** (11.67)	**22.06** (12.51)	**22.29** (12.72)	**24.75** (12.87)	**20.67** (11.92)	**16.36** (5.99)	**24.70** (12.85)	**20.28** (11.17)	**20.71** (12.62)
	Median (IQR)	18.00 (13.25-27.75)	19.00 (12.75-31.00)	19.00 (13.50-27.50)	19.50 (15.75-34.75)	17.00 (12.00-27.75)	17.00 (13.50-20.00)	20.00 (14.00-34.00)	17.50 (12.25-26.75)	17.00 (13.00-28.75)
	Range	5.00-45.00	4.00-45.00	10.00-45.00	5.00-45.00	4.00-45.00	5.00-26.00	5.00-45.00	5.00-45.00	4.00-45.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**809.2** (422.7)	**836.6** (401.5)	**1486.3** (781.0) **	**988.7** (561.6)	**825.4** (408.1)	**658.8** (245.1)	**966.4** (515.6)	**826.5** (420.7)	**813.8** (541.1)
	Median (IQR)	775.0 (538.5-941.5)	767.0 (619.5-1049.0)	1396.0 (948.0-2059.0)	867.0 (581.0-1242.0)	780.5 (621.3-904.8)	609.0 (488.5-783.0)	959.0 (522.0-1234.0)	771.0 (619.0-926.0)	779.0 (483.8-887.3)
	Range	295.0-2500.0	148.0-2057.0	494.0-2500.0	148.0-2500.0	295.0-2500.0	368.0-1124.0	245.0-2500.0	148.0-2500.0	270.0-2500.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**12.67** (5.75)	**11.20** (3.34)	**9.10** (2.25)	**11.21** (4.74)	**11.72** (4.08)	**12.95** (5.34)	**9.81** (3.18)	**13.00** (5.21)	**11.52** (3.14) **
	Median (IQR)	11.40 (8.21-14.07)	10.55 (8.37-13.08)	8.86 (8.05-9.87)	10.38 (8.33-12.89)	10.72 (8.33-14.01)	12.09 (8.39-15.70)	8.86 (7.48-12.00)	11.76 (9.62-15.24)	10.76 (9.35-13.46)
	Range	6.44-34.13	6.58-19.53	5.91-13.10	5.91-34.13	6.44-24.47	7.48-24.57	5.91-18.11	7.36-34.13	7.47-17.81
Cysteine (µmol/L)	$\bar{x}$ (SD)	**274.9** (53.7)	**268.5** (34.2)	**240.1** (26.9)	**271.0** (44.0)	**265.7** (42.3)	**274.2** (48.3)	**263.4** (37.5)	**273.7** (49.5)	**266.6** (35.1)
	Median (IQR)	261.3 (246.8-297.6)	262.0 (245.9-284.7)	251.8 (234.7-256.7)	259.3 (246.1-288.8)	257.1 (238.0-282.5)	268.8 (251.1-292.9)	252.5 (246.4-268.8)	272.0 (245.3-300.2)	261.1 (237.6-274.2)
	Range	191.6-436.4	216.6-365.8	185.8-260.1	185.8-436.4	191.6-400.8	193.1-385.3	222.3-400.8	185.8-436.4	227.3-344.6
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**23.81** (4.69)	**22.18** (3.80)	**22.28** (6.44)	**23.46** (4.48)	**21.98** (4.30)	**23.99** (4.44)	**22.82** (3.74)	**22.81** (4.54)	**23.05** (5.72)
	Median (IQR)	23.25 (20.52-26.52)	21.26 (19.36-24.20)	21.45 (17.54-23.66)	22.72 (20.83-25.12)	20.97 (19.15-24.48)	24.93 (21.14-27.62)	22.66 (20.10-25.08)	21.37 (19.66-24.75)	23.07 (17.77-25.17)
	Range	16.03-35.47	16.59-31.29	16.93-35.21	16.07-35.47	16.59-32.78	16.03-29.62	16.07-29.56	16.62-35.47	16.03-35.21
Glutathione (µmol/L)	$\bar{x}$ (SD)	**11.01** (3.79)	**11.63** (5.35)	**12.48** (6.09)	**11.98** (5.94)	**10.39** (3.20)	**13.50** (4.68)	**11.66** (4.02)	**11.37** (5.37)	**11.13** (4.80)
	Median (IQR)	10.33 (8.28-12.36)	10.79 (8.30-13.00)	14.08 (7.34-16.10)	11.03 (8.27-13.66)	9.62 (7.75-12.44)	12.01 (9.71-15.59)	10.79 (8.67-14.54)	10.27 (8.29-12.78)	11.08 (7.25-11.83)
	Range	5.93-21.58	5.10-38.14	5.13-21.29	5.10-38.14	5.93-18.72	8.51-21.58	6.28-21.59	5.10-38.14	5.93-21.29

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 677C>T MTHFR significance is achieved with native (untransformed) data** for both serum vitamin B_{12} and red cell folate.
For 19bp del DHFR significance is achieved with native (untransformed) data** for homocysteine.

Table 4-13: Alzheimer's disease cases; B-vitamin/thiol related nutritional genetic data by genotype (2 of 4)

Indices		2756A>G MTR			66A>G MTRR			1947G>A COMT		
		Wt (n=64)	Het (n=26)	Rec (n=3)	Wt (n=13)	Het (n=48)	Rec (n=32)	Wt (n=28)	Het (n=40)	Rec (n=25)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**258.3** (159.8)	**289.3** (149.4)	**399.0** (328.3)	**394.7** (265.9)	**236.1** (99.1)	**274.5** (168.8) *	**242.6** (134.0)	**277.6** (124.4)	**293.9** (235.0)
	Median (IQR)	224.0 (169.0-281.3)	234.0 (190.0-329.0)	214.0 (209.5-496.0)	308.0 (230.0-517.0)	217.5 (173.8-270.8)	214.5 (170.8-271.8)	200.5 (160.0-281.3)	227.5 (197.5-329.3)	217.0 (151.0-270.0)
	Range	100.0-1100.0	137.0-719.0	205.0-778.0	134.0-1100.0	100.0-530.0	108.0-778.0	100.0-719.0	136.0-610.0	108.0-1100.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**22.14** (12.36)	**22.00** (11.48)	**16.33** (13.32)	**21.69** (15.24)	**22.40** (11.80)	**21.28** (11.39)	**19.61** (12.32)	**24.63** (11.91)	**20.16** (11.59) *
	Median (IQR)	19.00 (13.00-31.00)	19.00 (14.00-28.75)	13.00 (9.00-22.00)	17.00 (10.00-29.00)	19.00 (13.75-31.00)	19.00 (13.00-28.00)	17.00 (12.00-23.00)	24.00 (14.75-31.50)	17.00 (13.00-26.00)
	Range	5.00-45.00	4.00-45.00	5.00-31.00	5.00-45.00	5.00-45.00	4.00-45.00	4.00-45.00	5.00-45.00	5.00-45.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**918.1** (517.7)	**763.4** (336.0)	**929.0** (570.7)	**1230.2** (742.1)	**799.7** (421.6)	**840.5** (350.5)	**866.5** (503.9)	**898.3** (443.1)	**845.0** (509.8)
	Median (IQR)	822.0 (619.5-1108.0)	683.5 (568.5-898.3)	910.0 (639.0-1209.5)	934.0 (725.0-1686.0)	693.0 (508.0-1026.5)	788.5 (637.0-1010.0)	797.0 (594.8-1040.8)	808.5 (618.3-1025.5)	725.0 (493.3-941.8)
	Range	148.0-2500.0	334.0-2057.0	368.0-1509.0	245.0-2500.0	148.0-2500.0	334.0-2057.0	148.0-2500.0	295.0-2500.0	245.0-2500.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**11.69** (3.69)	**11.74** (6.31)	**9.96** (2.31)	**10.29** (3.68)	**11.88** (4.87)	**11.84** (4.27)	**11.53** (4.31)	**12.13** (5.49)	**11.00** (2.67)
	Median (IQR)	11.07 (8.84-13.89)	9.67 (7.84-13.13)	9.96 (8.81-11.12)	9.07 (7.58-12.86)	11.07 (8.42-13.42)	10.68 (8.84-13.89)	9.87 (8.22-15.14)	11.09 (8.20-13.57)	11.11 (9.07-12.82)
	Range	5.91-24.47	6.44-34.13	7.65-12.28	5.91-18.74	6.44-34.13	6.58-24.57	6.58-24.47	5.91-34.13	6.81-18.11
Cysteine (µmol/L)	$\bar{x}$ (SD)	**264.8** (39.2)	**278.1** (49.7)	**279.3** (74.7)	**255.2** (16.7)	**263.9** (43.5)	**282.2** (48.1)	**256.8** (35.3)	**282.1** (52.5)	**261.6** (28.7) *
	Median (IQR)	258.0 (243.9-288.2)	270.0 (250.0-283.8)	320.5 (256.8-322.4)	251.8 (249.7-271.4)	255.8 (236.6-286.9)	271.9 (251.4-303.8)	251.1 (230.0-273.0)	268.8 (251.9-303.8)	253.6 (243.0-277.3)
	Range	185.8-400.8	216.6-436.4	193.1-324.4	216.6-278.0	185.8-436.4	193.1-400.8	185.8-337.3	191.6-436.4	224.2-327.8
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**23.20** (4.53)	**22.37** (4.24)	**19.75** (2.83)	**23.39** (5.18)	**22.27** (4.43)	**23.51** (4.11)	**21.58** (3.70)	**22.88** (4.36)	**24.24** (4.99)
	Median (IQR)	22.64 (19.93-25.63)	20.93 (19.80-24.84)	18.72 (18.15-20.84)	22.51 (21.26-24.96)	21.28 (19.27-24.61)	23.05 (20.69-25.62)	21.38 (18.81-24.17)	22.14 (20.03-24.94)	24.19 (20.78-28.51)
	Range	16.07-35.21	16.03-35.47	17.59-22.96	16.94-35.21	16.03-35.47	16.62-32.78	16.59-31.91	16.07-35.47	16.03-35.21
Glutathione (µmol/L)	$\bar{x}$ (SD)	**11.67** (5.48)	**11.34** (2.61)	**7.45** (0.93)	**11.60** (4.68)	**11.55** (5.76)	**11.21** (3.07)	**10.23** (2.89)	**10.81** (3.31)	**13.81** (7.26) *
	Median (IQR)	10.50 (8.11-14.17)	11.40 (9.23-12.73)	7.08 (6.93-7.79)	10.80 (7.26-14.08)	9.56 (8.10-12.62)	11.05 (8.93-12.94)	10.67 (8.40-12.49)	9.62 (8.21-13.66)	11.85 (9.07-16.20)
	Range	5.10-38.14	7.58-16.77	6.77-8.51	6.28-21.29	5.10-38.14	5.93-18.72	5.10-15.71	6.45-18.72	5.93-38.14

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data log_{10} transformed * Blue asterisk (s) denotes all data log_{10} transformed with outliers removed.

Note: For 66A>G MTRR significance is achieved with native (untransformed) data for serum vitamin B_{12}** and red cell folate*.
For 1947G>A COMT significance is achieved with native (untransformed) data for cysteine*, glutathione* and serum folate*.

Table 4-14: Alzheimer's disease cases; B-vitamin/thiol related nutritional genetic data by genotype (3 of 4)

Indices		3'UTR 6bp del TS			2R > 3R TSER			1420C>T SHMT		
		Wt (n=47)	Het (n=36)	Rec (n=10)	Wt (n=27)	Het (n=48)	Rec (n=18)	Wt (n=51)	Het (n=39)	Rec (n=3)
Serum Vitamin B_{12} (pmol/L)	$\bar{X}$ (SD)	**259.5** (142.2)	**297.1** (200.4)	**235.5** (89.2)	**289.7** (187.9)	**242.7** (104.8)	**320.9** (233.2)	**242.0** (126.9)	**311.8** (200.1)	**248.3** (85.7)
	Median (IQR)	217.0 (169.5-291.5)	230.0 (174.0-347.3)	218.0 (184.3-258.3)	206.0 (168.0-322.0)	224.0 (177.3-276.0)	244.0 (187.0-403.8)	212.0 (158.5-279.0)	244.0 (181.5-366.5)	261.0 (209.0-294.0)
	Range	108.0-778.0	100.0-1100.0	138.0-457.0	108.0-778.0	100.0-610.0	126.0-1100.0	100.0-719.0	122.0-1100.0	157.0-327.0
Serum Folate (nmol/L)	$\bar{X}$ (SD)	**22.51** (12.06)	**21.17** (12.10)	**21.80** (12.99)	**21.89** (11.40)	**20.60** (11.84)	**25.44** (13.51)	**21.96** (12.34)	**22.03** (12.09)	**19.67** (9.81)
	Median (IQR)	19.00 (13.50-30.00)	16.50 (12.00-28.50)	19.50 (14.00-30.50)	19.00 (14.50-28.00)	17.00 (12.75-29.00)	21.50 (16.75-35.50)	19.00 (12.50-29.00)	18.00 (13.00-30.50)	14.00 (14.00-22.50)
	Range	5.00-45.00	4.00-45.00	5.00-44.00	5.00-45.00	4.00-45.00	7.00-45.00	4.00-45.00	5.00-45.00	14.00-31.00
Red Cell Folate (nmol/L)	$\bar{X}$ (SD)	**819.0** (402.6)	**962.1** (585.1)	**816.4** (300.1)	**855.7** (366.6)	**840.1** (486.0)	**1002.7** (591.5)	**842.8** (407.8)	**935.0** (560.2)	**622.3** (198.5)
	Median (IQR)	770.5 (566.5-955.8)	830.0 (597.5-1100.0)	762.0 (609.5-979.8)	815.0 (625.0-1028.0)	746.0 (549.5-940.3)	802.0 (613.0-1234.0)	784.0 (613.0-1016.8)	785.0 (611.0-1122.0)	522.0 (508.0-686.5)
	Range	148.0-2057.0	295.0-2500.0	418.0-1396.0	245.0-1686.0	148.0-2500.0	387.0-2500.0	245.0-2500.0	148.0-2500.0	494.0-851.0
Homocysteine (µmol/L)	$\bar{X}$ (SD)	**11.45** (3.76)	**11.32** (4.27)	**13.75** (7.70)	**10.79** (3.82)	**12.07** (5.32)	**11.79** (2.79)	**11.51** (4.94)	**12.06** (4.01)	**8.51** (1.47)
	Median (IQR)	10.79 (8.40-13.22)	10.78 (7.80-13.45)	11.08 (9.35-14.51)	9.22 (7.98-12.91)	10.80 (8.40-13.66)	11.59 (10.07-13.46)	10.32 (8.26-13.23)	11.26 (8.82-13.66)	7.87 (7.67-9.03)
	Range	6.81-24.57	5.91-24.47	8.86-34.13	6.44-19.53	5.91-34.13	7.72-18.74	5.91-34.13	7.02-24.47	7.47-10.20
Cysteine (µmol/L)	$\bar{X}$ (SD)	**266.8** (42.4)	**265.4** (38.1)	**292.0** (61.3)	**267.5** (36.4)	**269.6** (48.0)	**269.5** (42.3)	**267.8** (44.9)	**272.6** (42.7)	**243.2** (20.0)
	Median (IQR)	257.2 (243.6-287.2)	259.5 (248.5-282.0)	285.1 (256.5-305.9)	256.6 (243.0-301.7)	261.6 (245.8-288.5)	257.0 (245.4-273.3)	259.2 (240.0-288.6)	261.5 (250.9-289.2)	245.1 (233.7-253.6)
	Range	185.8-400.8	191.6-365.8	227.3-436.4	191.6-337.3	185.8-436.4	224.2-400.8	191.6-436.4	185.8-400.8	222.3-262.1
Cysteinyl-glycine (µmol/L)	$\bar{X}$ (SD)	**23.22** (4.44)	**21.81** (4.15)	**24.89** (4.80)	**22.05** (3.43)	**22.32** (4.39)	**25.47** (5.05) *	**23.41** (4.17)	**22.48** (4.70)	**18.12** (1.85)
	Median (IQR)	22.51 (19.93-25.87)	20.93 (19.31-23.78)	24.21 (22.12-26.17)	21.45 (20.18-23.67)	21.49 (19.24-24.78)	26.47 (22.85-29.07)	22.96 (20.60-25.17)	21.50 (19.09-25.22)	17.17 (17.05-18.71)
	Range	16.03-32.78	16.07-35.21	17.72-35.47	16.07-29.26	16.03-35.47	16.74-35.21	16.03-35.47	16.07-35.21	16.93-20.25
Glutathione (µmol/L)	$\bar{X}$ (SD)	**11.41** (5.61)	**11.10** (3.68)	**12.80** (4.35)	**11.10** (3.47)	**10.88** (3.93)	**13.45** (7.62)	**11.79** (5.43)	**11.20** (4.00)	**8.61** (1.28)
	Median (IQR)	10.56 (7.98-12.71)	9.54 (8.35-13.14)	11.72 (10.04-15.76)	10.56 (8.66-13.54)	9.87 (8.27-12.62)	11.77 (8.69-15.26)	10.80 (8.39-12.78)	10.92 (7.83-14.03)	9.21 (8.17-9.35)
	Range	5.10-38.14	6.79-21.58	5.93-20.99	5.93-18.72	5.10-21.59	6.71-38.14	5.13-38.14	5.10-21.29	7.14-9.49

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 2R > 3R TSER significance is achieved with native (untransformed) data for cysteinyl-glycine*.

Table 4-15: Alzheimer's disease cases; B-vitamin/thiol related nutritional genetic data by genotype (4 of 4)

Indices		80G>A RFC			1561C>T GCPII		
		Wt (n=31)	Het (n=43)	Rec (n=19)	Wt (n=81)	Het (n=12)	Rec (n=0)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**338.7** (231.0)	**232.0** (101.8)	**251.2** (105.4) *	**268.5** (157.2)	**291.5** (206.2)	**0.0** (0.0)
	Median (IQR)	236.0 (182.5-433.5)	207.0 (170.0-271.0)	230.0 (185.5-259.0)	224.0 (177.0-292.0)	210.0 (170.5-305.0)	0.0 (0.0-0.0)
	Range	108.0-1100.0	100.0-606.0	108.0-530.0	100.0-1100.0	108.0-719.0	0.0-0.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**21.94** (12.63)	**20.35** (11.46)	**25.42** (12.34)	**21.86** (12.41)	**22.25** (9.73)	**0.00** (0.00)
	Median (IQR)	19.00 (13.00-29.00)	18.00 (11.50-27.50)	19.00 (14.50-31.00)	18.00 (13.00-31.00)	20.50 (13.75-26.50)	0.00 (0.00-0.00)
	Range	5.00-45.00	4.00-45.00	12.00-45.00	4.00-45.00	12.00-45.00	0.00-0.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**1028.5** (585.7)	**798.6** (415.3)	**791.8** (333.9)	**867.4** (489.9)	**923.6** (374.4)	**0.0** (0.0)
	Median (IQR)	822.0 (681.5-1184.5)	767.0 (526.5-940.0)	683.0 (524.8-1016.8)	780.5 (554.0-1012.3)	827.0 (662.3-1076.8)	0.0 (0.0-0.0)
	Range	148.0-2500.0	245.0-2500.0	396.0-1489.0	148.0-2500.0	479.0-1686.0	0.0-0.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**268.2** (39.8)	**271.1** (42.9)	**265.5** (51.5)	**11.74** (4.62)	**11.03** (3.82)	**0.00** (0.00)
	Median (IQR)	259.8 (249.4-280.7)	268.8 (246.2-298.1)	252.6 (239.7-283.0)	10.93 (8.40-13.48)	10.07 (8.18-12.95)	0.00 (0.00-0.00)
	Range	223.4-436.4	185.8-385.3	191.6-400.8	5.91-34.13	6.58-18.11	0.00-0.00
Cysteine (µmol/L)	$\bar{x}$ (SD)	**11.68** (5.60)	**11.91** (4.38)	**10.99** (2.50)	**271.0** (44.5)	**255.0** (33.1)	**0.0** (0.0)
	Median (IQR)	10.27 (8.07-13.95)	11.54 (8.47-13.95)	10.93 (9.52-11.97)	260.1 (246.0-289.2)	251.7 (232.4-280.0)	0.0 (0.0-0.0)
	Range	5.91-34.13	7.36-24.57	7.47-18.46	185.8-436.4	191.6-299.6	0.0-0.0
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**24.04** (4.50)	**22.11** (4.19)	**22.58** (4.64)	**22.83** (4.39)	**22.98** (4.89)	**0.00** (0.00)
	Median (IQR)	23.14 (21.04-25.98)	21.45 (18.80-24.99)	21.26 (19.73-24.94)	22.51 (19.63-25.08)	21.36 (19.77-24.16)	0.00 (0.00-0.00)
	Range	17.59-35.47	16.03-31.91	16.74-31.29	16.03-35.47	17.62-32.78	0.00-0.00
Glutathione (µmol/L)	$\bar{x}$ (SD)	**11.68** (4.15)	**11.08** (4.17)	**11.86** (6.91)	**11.68** (4.87)	**9.80** (4.06)	**0.00** (0.00)
	Median (IQR)	10.92 (9.02-13.80)	10.44 (7.96-13.12)	9.60 (8.78-11.77)	11.24 (8.28-13.92)	9.26 (8.17-10.36)	0.00 (0.00-0.00)
	Range	5.10-21.59	5.13-21.49	6.71-38.14	5.10-38.14	5.13-21.58	0.00-0.00

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 1561C>T GCPII an unpaired T-test was performed in place of ANOVA given no recessive genotype was recorded.
For 80G>A RFC significance is achieved with native (untransformed) data* for serum vitamin B_{12} and without outliers*.

The tables displayed above show that serum vitamin B_{12} levels vary significantly as a consequence of 677C>T MTHFR, 1298A>C MTHFR, 66A>G MTRR, and 80G>A RFC genotypes. Red cell folate levels varied as a consequence of the 677C>T MTHFR polymorphism, and serum folate varied within the 1947G>A COMT genotype. For transsulphuration pathway thiols, homocysteine levels varied according to the 19bp DHFR deletion gene, while the TS SNP (2R > 3R TSER) influenced cysteinyl-glycine levels. Both cysteine and glutathione varied significantly within the 1947G>A COMT SNP.

4.2.6. Analysis of combined nutritional biochemistry and genetic data to establish any relationship to Alzheimer's dementia

This analysis of the data draws upon both the cohort of Alzheimer's dementia patients and the health retirement village control cohort (see chapter 3, page 129). As previously conducted (chapter 3), linear models have been examined using ordinal logistic regression analysis with the Wald χ^2 one-step linear approximation to the likelihood ratio test used to determine a significant *p* value. These models have been performed based on an *a priori* understanding of possible biochemical, genetic and physiologic relationships. Ordinal logistic regression has been perform to include the following factors: age, BMI, gender, dietary folate intake, serum B_{12}, serum folate, red cell folate, cysteine, homocysteine, cysteinyl-glycine, glutathione, eleven B-vitamin related genotypes. Data presented is native and contains all data points (black text); $\log_{10}$ transformed data has had extreme data points removed (blue text). The results for an examination of all variables are presented below.

Table 4-16: Ordinal logistic regression; model for all genetic, metabolic and physiologic variables

	Native data			Transformed data		
Variable	**R^2**	**Chi-square**	***p* value**	**R^2**	**Chi-square**	***p* value**
Serum Vitamin B_{12} (pmol/L)	0.4620		-	0.2859	4.25	0.0393*
Red Cell Folate (nmol/L)		8.83	0.0030**		5.16	0.0230*
Homocysteine (µmol/L)		14.40	0.0001****		16.39	<0.0001****
Cysteinyl-glycine (µmol/L)		29.06	< 0.0001****		32.53	<0.0001****
Glutathione (µmol/L)		6.38	0.0115*		7.31	0.0069**
Total Dietary Folic acid (µg/day)		5.65	0.0175*		4.85	0.0276*
2756A>G MTR			0.0668 ‡		6.27	0.0433*

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡ denotes approaching significance
Black = Native data including all data points. Blue = $\log^{10}$ transformed data with extreme data points removed.

As can be seen in table 4-16, the results for homocysteine and cysteinyl-glycine are highly significant, and strongly predict AD. This supports the t-tests performed above (figure 4-1). In addition, the 2756A>G MTR SNP shows a significant protective relationship with Alzheimer's dementia, and this supports the odds ratio and chi-square tests. Given this interesting outcome, the next model examined only the eleven genotypes independent of any other parameter. Ordinal logistic regression was repeated and the outcome is displayed below.

Table 4-17: Ordinal logistic regression; model for gene variants only

	Native data			Transformed data		
Variable	**R^2**	**Chi-square**	***p* value**	**R^2**	**Chi-square**	***p* value**
2756A>G MTR	0.0512	6.34	0.0419*	0.0512	6.34	0.0419*

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡denotes approaching significance
Black = Native data including all data points. Blue = log^{10} transformed data with extreme data points removed.

As shown in both ordinal logistic models, the 2756A>G MTR polymorphism indicates a positive (protective) relationship against AD. The next linear model examined non-B vitamin related 'population' indices – age, and gender. As with previous outcomes, no significance was detected, although age approached significance.

Ordinal logistic regression analysis of all B-vitamin metabolites and thiols was performed next (excluded gene variants and any fundamental population indices). The table below shows the outcome of ordinal logistic regression analysis involving the following parameters; serum vitamin B_{12}, serum folate, red cell folate, cysteine, homocysteine, cysteinyl-glycine and glutathione.

Table 4-18: Ordinal logistic regression; model for all B-vitamin related blood metabolites and thiols combined

	Native data			Transformed data		
Variable	**R^2**	**Chi-square**	***p* value**	**R^2**	**Chi-square**	***p* value**
Red Cell Folate (nmol/L)	0.2056	5.26	0.0219*	0.2106		-
Homocysteine (µmol/L)		17.86	<0.0001****		21.27	<0.0001****
Cysteinyl-glycine (µmol/L)		33.90	<0.0001****		37.30	<0.0001****
Glutathione (µmol/L)		9.89	0.0017**		10.03	0.0015**

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡denotes approaching significance
Black = Native data including all data points. Blue = log^{10} transformed data with extreme data points removed.

Clearly, when the parameters are examined independently of genetics and population descriptive indices, both homocysteine and cysteinyl-glycine show a very strong relationship with AD. As indicated in the histogram above (figure 4-1), AD subjects have higher homocysteine levels and lower cysteinyl-glycine levels than controls,

which is consistent with current research. Glutathione also shares a strong relationship with this type of dementia. However, the histogram above indicates higher levels of glutathione with the AD cohort, which conflicts with McCaddon *et al*. [361], but perhaps this indicates increased tissue damage [955, 956].

To further explore this interesting thiol relationship, the subsequent ordinal logistic regression analysis performed included only the transsulphuration metabolites. Outcomes are displayed in table 4-19 below.

Table 4-19: Ordinal logistic regression; model for thiol transsulphuration pathway metabolites

	Native data			Transformed data		
Variable	**R^2**	**Chi-square**	***p* value**	**R^2**	**Chi-square**	***p* value**
Homocysteine (µmol/L)	0.1878	21.25	<0.0001****	0.1885	23.37	<0.0001****
Cysteinyl-glycine (µmol/L)		32.71	<0.0001****		34.99	<0.0001****
Glutathione (µmol/L)		13.02	0.0003***		13.12	0.0003***

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡denotes approaching significance
Black = Native data including all data points. Blue = log^{10} transformed data with extreme data points removed.

Table 4-19 shows that with the removal of the B-vitamin metabolites, the relationship between AD and homocysteine, cysteinyl-glycine and glutathione remains intact and highly significant. For completion, the table below describes B-vitamin related blood metabolites only. The following parameters; serum vitamin B_{12}, serum folate and red cell folate have been used in the ordinal logistic regression analysis, and outcomes are displayed below.

Table 4-20: Ordinal logistic regression; model for B-vitamin related blood metabolites

	Native data			Transformed data		
Variable	**R^2**	**Chi-square**	***p* value**	**R^2**	**Chi-square**	***p* value**
Red Cell Folate (nmol/L)	0.0188	3.99	0.0458*	0.0181		-
Serum Folate (nmol/L)		6.46	0.0110*		6.69	0.0097**

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡denotes approaching significance
Black = Native data including all data points. Blue = log^{10} transformed data with extreme data points removed.

The three parameters used in this model contain information with a large range of magnitude due to the data set outliers. Red cell folate appears to have a significant relationship with AD, although this association is not sustained when the data is transformed and the individuals with pharmacological intervention are removed (outliers). Serum folate however does show significance whether data is native or transformed, or outliers are included or excluded.

Given the relationship between serum folate and Alzheimer's dementia, the next two models displayed below are of interest, and include the dietary folates (synthetic, natural & total) as parameters. Ordinal logistic regression was performed for total dietary folate intake, which includes 5-methy-lH_4folic acid combined with any sources of pteroylmonoglutamic acid in the diet, and the model outcome is display in table 4-21 below. For completion, ordinal logistic regression was performed for total natural folic acid (5-methyl-H_4folic acid) and total synthetic folic acid (supplemental pteroylmonoglutamic acid); outcomes are displayed in table 4-22 below.

Table 4-21: Ordinal logistic regression; model for total dietary folic acid

	Native data			Transformed data		
Variable	**R^2**	**Chi-square**	***p* value**	**R^2**	**Chi-square**	***p* value**
Total Dietary Folic Acid (µg/day)	0.0241	7.40	0.0065**	0.0231	7.75	0.0054**

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡denotes approaching significance
Black = Native data including all data points. Blue = log^{10} transformed data with extreme data points removed.

Table 4-22: Ordinal Logistic regression; model for total natural and synthetic folic acid intakes

	Native data			Transformed data		
Variable	**R^2**	**Chi-square**	***p* value**	**R^2**	**Chi-square**	***p* value**
Total Natural Folic Acid (µg/day)	0.0445	13.16	0.0003***	0.0581	15.75	<0.0001****

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡denotes approaching significance
Black = Native data including all data points. Blue = log^{10} transformed data with extreme data points removed.

Both total dietary folic acid intake and total natural folic acid intake significantly predict risk for AD, with lower levels of both parameters observed in the AD cohort compared with controls. Interestingly, the synthetic pteroylmonoglutamic acid form of folate does not exhibit a significant relationship with Alzheimer's dementia.

Summary of results for B-vitamin nutritional genetics in the elderly; risk for Alzheimer's disease

Data published in this chapter indicates that several parameters predict risk for AD; the most notable of these is the 2756A>G MTR polymorphism, which affords a positive (protective) relationship towards AD.

Data analysis clearly shows that the transsulphuration metabolites homocysteine, cysteinyl-glycine and glutathione are highly significant factors in risk for AD. Increased homocysteine and glutathione levels were observed, along with lower cysteinyl-glycine levels in AD subjects.

Other notable associations exist for both total dietary folic acid intake and total natural folic acid intake, where lower levels of these dietary markers significantly predict AD. Interestingly, the synthetic pteroylmonoglutamic acid form of folate does not exhibit a significant relationship with Alzheimer's dementia.

CHAPTER 5

B-VITAMIN RELATED NUTRITIONAL GENETICS AND OCCURRENCE OF ADENOMATOUS POLYPS - A MAJOR ANTECEDENT OF COLORECTAL CANCER

5. Overview

As reviewed in the introductory chapter of this thesis, folate is a necessary micronutrient required by humans, which must be obtained from the diet. It is essential for transferring single carbon units for important biochemical reactions such as the biosynthesis of methionine, thymidylate, purines, and glycine and in the metabolism of serine, formate, and histidine. Inadequate folate availability can result in changes not only to DNA methylation, but also synthesis and repair; these changes are strongly linked to the occurrence of malignant transformation and therefore carcinogenesis [792]. In addition to this, there is now the very recently understood paradoxical phenomenon in which an overly abundant supply of folate may lead to an increased risk of developing and progressing cancer, specifically in an individual who has existing foci of precancerous or cancerous cells [167, 266, 267].

Colorectal cancer is a major health concern in Australia, and is a serious degenerative disease with high morbidity and mortality [9]. It is widely accepted that colorectal cancer arises from colorectal adenomatous polyps, and that an individual's risk of developing these is influenced by inappropriate nutrition. Vegetables, particularly green leafy and cruciferous vegetables, are a major source of folate as well as other important micronutrients and phytoprotectants. While many plant components have recognised anticancer properties, many studies suggest that both folate intake and folate status are also important, and may be inversely associated with neoplastic risk [165-168, 957]. Common polymorphisms of the genes responsible for folate metabolism have also been associated with colorectal neoplasia, providing further evidence for a causal relationship between folate and neoplasia [497, 506, 580]. Therefore, the final study presented in this thesis, will examine a population over 40 years of age who have undergone screening for adenomatous polyps. This study will again examine folate related nutritional biochemistry and genetic data to establish any relationship to pre-malignant changes that are known to lead on to cancer. Also, a closer look at the relationship of synthetic folic acid will be conducted given recent data on the harmful effect it may have in the colonic mucosa [167, 170, 266, 267, 849].

5.1. Study Design

5.1.1. Ethics approval

Approval for this study was obtained from the University of Newcastle Human Research Ethics Committee (H-429-0407) and the Northern Sydney Central Coast

Health Committee (0702/005C). Informed written consent was obtained prior to the volunteers being recruited into the study

5.1.2. Recruitment and clinical assessment

This study is part of a larger project, which is investigating bitter taste phenotype, dietary pattern, and nutritional genetics in the aetiopathology of colonic adenomatous polyps. Participants were chosen based on selection criteria from the gastroenterology practice of Dr Martin Veysey, and who were undergoing colonoscopy as a screening protocol for colonic pathology. Potential participant's details were entered into a password-protected database; then mail outs were conducted. The information pack sent out contained; a covering letter, an information statement, a consent form and a self-addressed reply-paid envelope. Patients who returned the consent form were contacted by telephone to arrange a time to attend a clinic.

The clinics were held weekly at the Gosford Hospital, NSW and were conducted from May 2007 to September 2009. Data collection was multifaceted and included information on patient demographics, venepuncture, and a FFQ. Specifically information regarding a participant's age, medical and surgical history, colonoscopy history, polyp status (if known) and medications were recorded in the patient's research study files. Participants were required to perform a sensory test for bitterness perception, however, the data collected from this assessment has not be reported on in this thesis.

In total, two hundred and three participants were recruited; subjects were between 40 and 89 years of age at time of colonoscopy. Subjects were mentally competent and not highly dependent on medical care and did not have known cancer or an adenomatous polyp diagnosis prior to the date of colonoscopy. For statistical analysis, these participants were separated into three phenotypic groups of interest. Firstly, participants with no polyps of any kind (controls), participants with polyps that have an adenoma histology (adenomatous polyp) only and the third group containing participants whose colonoscopy resulted in a polyp diagnosis which included both adenomatous and non-adenomatous/hyperplastic polyps.

5.1.3. Food frequency questionnaire

Volunteers were required to undergo an interviewer administered FFQ during the clinical assessment. Estimated total dietary folic acid, total natural, and synthetic folic acid intake was determined using Foodworks™. For further details on the

questionnaire and dietary estimation, see section 2.4 on page 124. Dietary assessment of this population took place during the phasing in of mandatory fortification of wheat flour with pteroylmonoglutamic acid. This program officially commenced on the 19th September 2009, all participants however, were recruited prior to this date.

5.1.4. Non-clinical measurements

During the clinical assessment approximately 30mls of blood was collected from each participant (for further information see section 2.1.1 on page 107). The blood was separated accordingly and distributed to the Molecular Nutrition PC2 Laboratory, University of Newcastle, Ourimbah and ICPMR at Westmead Hospital Sydney. Using the appropriately designated blood, the following methods were undertaken:

- Blood levels of red cell folate, serum folate and vitamin B_{12}, were measured using an automated Access Immunoassay System. For detailed information regarding the automated access immunoassay system, see chapter 2 section 2.1.2 on page 108. As with the previous studies reported, the results for these tests included outliers, statistical analysis has been performed to both include and excluded where appropriate.

- Homocysteine, cysteine, cysteinyl-glycine, and glutathione concentrations were determined by HPLC with florescence detection using SBD-F. The full description of the methods employed and the validation process is described in section 2.2 on page 109. Despite 203 participants being recruited over a two year period, logistical reasons have resulted in thiol analysis only being completed for 113 participants.

- For gene polymorphism detection, ten SNPs were analysed on this study population based on methodologies described in section 2.3 beginning on page 114. Note, unlike the previous studies the 1947G>A COMT SNP was not completed for this population cohort. Like the transsulphuration pathway thiols above, genetic data is presented for 113 participants only.

5.1.5. Statistical analysis

Linear models utilising ordinal logistic regression analysis with the Wald χ^2 one-step linear approximation to the likelihood ratio test have been used to determine whether any variable significantly predicts clinical phenotype. As with previous chapters, some of the B-vitamin concentrations were exceptionally high which is indicative of

pharmacologic intervention. Due to this, the data was examined in two ways, as the complete data set in an unmodified 'native' format, and then analysis was performed after $\log_{10}$ transformation with all extreme data points removed. The results displayed below report outcomes according to both approaches. A standard least squares regression model has also been used for an integrated analysis of folic acid examining both type and level of the vitamer. Descriptive statistics have been calculated with data tabulated and presented as appropriate. The utilisation of one-way ANOVA, unpaired t-test and odds ratio analysis have also been employed were appropriate. A full description of all statistical methods employed for the entire thesis can be found in section 2.5, page 128.

5.2. Results

5.2.1. Descriptive statistics

The data presented in this section examines nutritional genetics in relation to the aetiopathology of colonic adenomas. The section begins with a simple table illustrating the age distribution of the study population using basic descriptive statistics.

Table 5-1: Descriptive data based on age (years)

	All subjects	Male	Female
n	203	87	116
$\bar{x}$	63	65	61
SD	11	12	10
Median	64	65	63
IQR	56-72	58-75	55-69
Range	40-89	40-89	40-82

n=number, $\bar{x}$ =mean, SD=standard deviation, IQR=interquartile range.

5.2.2. B-vitamin metabolites and related indices

The following three tables (table 5-2, table 5-3, table 5-4) contain complete descriptive statistics for all non-genetic nutritional and blood indices and present the data for all subjects, males and females respectively.

Table 5-2: Data for all subjects (adenomatous and non-adenomatous polyps and controls); blood metabolites and related indices

Indices	$\bar{x}$ (SD)	Median (IQR)	Range
Serum Vitamin B_{12} (pmol/L)	280.1 (148.5)	251.0 (195.0-321.0)	91.0-1100.0
Serum Folate (nmol/L)	21.66 (12.01)	18.00 (12.00-30.00)	4.00-45.00
Red Cell Folate (nmol/L)	983.4 (520.4)	868.5 (589.3-1178)	184.0-2500.0
Homocysteine (µmol/L)	10.10 (2.85)	9.81 (7.83-11.75)	5.60-20.88
Cysteine (µmol/L)	264.5 (40.4)	259.5 (238.1-292.9)	173.0-374.5
Cysteinyl-glycine (µmol/L)	22.78 (4.73)	22.41 (19.99-25.28)	9.20-43.91
Glutathione (µmol/L)	11.66 (4.09)	10.66 (9.41-12.95)	4.18-32.43
Total Dietary Folic Acid (µg/day)	470.5 (243.6)	406.6 (326.6-573.8)	80.2-2348.3
Total Synthetic Folic Acid (µg/day)	147.6 (210.3)	91.3 (13.3-198.2)	0-2062.0
Total Natural Folic Acid (µg/day)	322.9 (114.9)	318.6 (240.7-388.2)	79.7-930.3

Note: Data for transsulphuration pathway related thiols n=113, all other variables n=203

Table 5-3: Data for male subjects (adenomatous and non-adenomatous polyps and controls); blood metabolites and related indices

Indices	$\bar{x}$ (SD)	Median (IQR)	Range
Serum Vitamin B_{12} (pmol/L)	282.9 (136.0)	247.5 (195.5-331.5)	96.0-841.0
Serum Folate (nmol/L)	21.66 (11.88)	18.00 (13.00-27.00)	4.00-45.00
Red Cell Folate (nmol/L)	1000.1 (500.4)	918.5 (646.5-1180.0)	223.0-2500.0
Homocysteine (µmol/L)	10.78 (2.74)	11.12 (8.67-12.41)	5.85-17.55
Cysteine (µmol/L)	266.9 (38.0)	260.9 (240.5-289.7)	194.6-346.1
Cysteinyl-glycine (µmol/L)	22.90 (4.62)	23.46 (20.12-26.70)	9.20-30.90
Glutathione (µmol/L)	11.72 (4.05)	10.70 (9.38-13.16)	4.18-21.83
Total Dietary Folic Acid (µg/day)	492.8 (286.0)	417.0 (349.8-575.6)	164.5-2348.3
Total Synthetic Folic Acid (µg/day)	149.1 (258.2)	74.5 (11.9-171.2)	0.0-2062.0
Total Natural Folic Acid (µg/day)	343.7 (124.1)	336.6 (258.2-398.3)	162.5-930.3

Note: Data for transsulphuration pathway related thiols n=51, all other variables n=87

Table 5-4: Data for female subjects (adenomatous and non-adenomatous polyps and controls); blood metabolites and related indices

Indices	$\bar{x}$ (SD)	Median (IQR)	Range
Serum Vitamin B_{12} (pmol/L)	277.9 (157.7)	252.0 (192.0-301.5)	91.0-1100.0
Serum Folate (nmol/L)	21.65 (12.17)	18.00 (11.00-31.00)	4.00-45.00
Red Cell Folate (nmol/L)	970.8 (536.9)	798.5 (578.5-1174.3)	184.0-2500.0
Homocysteine (µmol/L)	9.55 (2.83)	9.04 (7.37-10.66)	5.60-20.88
Cysteine (µmol/L)	262.5 (42.4)	257.6 (237.1-294.8)	173.0-374.5
Cysteinyl-glycine (µmol/L)	22.69 (4.85)	21.73 (19.87-24.49)	16.38-43.91
Glutathione (µmol/L)	11.61 (4.15)	10.60 (9.43-12.56)	6.36-32.43
Total Dietary Folic Acid (µg/day)	453.9 (206.6)	396.2 (308.5-568.5)	80.2-1028.3
Total Synthetic Folic Acid (µg/day)	146.4 (167.4)	99.9 (14.5-245.9)	0-821.4
Total Natural Folic Acid (µg/day)	307.5 (105.4)	302.3 (221.3-376.1)	79.7-649.4

Note: Data for transsulphuration pathway related thiols n=62, all other variables n=116

5.2.3. B-vitamin related genetics – prevalence

The table below outlines the prevalence (n) and percentage for each genotype examined in the population (adenomatous and non-adenomatous polyps and controls), along with allele number and frequency.

Table 5-5: Complete genetic data for the adenomatous and non-adenomatous polyp and control cohorts; genotype prevalence and allele number

SNP	GENOTYPE Prevalence (%)			ALLELE Number (frequency)	
	Wildtype	Heterozygote	Recessive	Wildtype	Mutant
677C>T MTHFR	59 (52)	45 (40)	9 (8)	163 (0.72)	63 (0.28)
1298A>C MTHFR	55 (49)	48 (42)	10 (9)	158 (0.70)	68 (0.30)
19bp del DHFR	37 (33)	51 (45)	25 (22)	125 (0.55)	101 (0.45)
2756A>G MTR	77 (68)	33 (29)	3 (3)	187 (0.83)	39 (0.17)
66A>G MTRR	24 (21)	54 (48)	35 (31)	102 (0.45)	124 (0.55)
80G>A RFC	43 (38)	46 (41)	24 (21)	132 (0.58)	94 (0.42)
1561C>T GCPII	99 (88)	14 (12)	0 (0)	212 (0.94)	14 (0.06)
1420C>T SHMT	61 (54)	40 (35)	12 (11)	162 (0.72)	64 (0.28)
3'UTR 6bp del TS	52 (46)	50 (44)	11 (10)	154 (0.68)	72 (0.32)
2R > 3R TSER	36 (32)	56 (50)	21 (19)	128 (0.57)	98 (0.43)

5.2.4. B-vitamin/thiol related nutritional genetics organised by genotype for all subjects (adenomatous and non-adenomatous polyps and controls)

Complete descriptive data (independent of phenotype) is display in the next four tables for each B-vitamin or thiol related parameter examined - each is categorised by genotype. In order to investigate whether genetic factors can influence variability in blood indices, a one-way ANOVA was performed. All subject data was examined twice, since the data set contains a number of extreme data points (a likely consequence of pharmacologic intervention), and therefore the ANOVA's were performed both with and without these extreme points. The result of ANOVA based on $\log_{10}$ transformed (normalised) data is given in the following four tables, and significance is denoted by either a red asterisk(s) (all $\log_{10}$ transformed values) or a blue asterisk(s) ($\log_{10}$ transformed values with outliers removed).

Note: significant statistical outcomes for untransformed data are displayed as footnotes in each table.

Table 5-6: All data (adenomatous and non-adenomatous polyps and controls); B-vitamin/thiol related nutritional genetic data by genotype (1 of 4)

Indices		677C>T MTHFR			1298A>C MTHFR			19bp del DHFR		
		Wt (n=59)	Het (n=45)	Rec (n=9)	Wt (n=55)	Het (n=48)	Rec (n=10)	Wt (n=37)	Het (n=51)	Rec (n=25)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**269.2** (118.3)	**309.9** (192.5)	**297.4** (173.7)	**289.6** (152.6)	**288.6** (169.1)	**272.6** (116.2)	**315.9** (152.5)	**274.6** (182.5)	**272.6** (86.3)
	Median (IQR)	250.0 (194.5-324.0)	259.0 (195.0-329.0)	267.0 (224.0-320.0)	254.0 (195.0-326.0)	261.0 (205.8-320.3)	230.0 (181.3-357.5)	284.0 (205.0-349.0)	228.0 (186.5-289.5)	254.0 (208.0-321.0)
	Range	91.0-798.0	105.0-1100.0	121.0-730.0	105.0-841.0	91.0-1100.0	154.0-501.0	128.0-798.0	91.0-1100.0	122.0-495.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**22.03** (12.42)	**22**.73 (12.73)	**25.89** (14.85)	**24.60** (12.86)	**18.73** (11.01)	**30.40** (14.11) ** *	**24.43** (13.04)	**21.24** (12.43)	**22.76** (12.74)
	Median (IQR)	18.00 (13.00-30.50)	20.00 (12.00-34.00)	23.00 (12.00-42.00)	20.00 (13.50-36.50)	15.00 (10.00-24.75)	31.00 (22.75-44.25)	21.00 (14.00-35.00)	17.00 (12.00-29.00)	22.00 (13.00-31.00)
	Range	5.00-45.00	4.00-45.00	9.00-45.00	4.00-45.00	4.00-45.00	9.00-45.00	6.00-45.00	4.00-45.00	5.00-45.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**799.6** (430.7)	**796.9** (433.3)	**1131.0** (501.9) *	**869.1** (401.9)	**697.2** (373.0)	**1194.9** (701.7) ** **	**896.8** (495.3)	**779.0** (428.7)	**812.2** (388.8)
	Median (IQR)	635.0 (500.5-1003.5)	755.0 (505.0-947.0)	998.0 (736.0-1459.0)	793.0 (560.5-1041.5)	601.0 (480.0-806.5)	1055.0 (655.0-1650.5)	811.0 (532.0-1043.0)	691.0 (489.0-930.0)	645.0 (520.0-986.0)
	Range	357.0-2500.0	184.0-2500.0	540.0-2082.0	223.0-2082.0	184.0-2500.0	413.0-2500.0	433.0-2500.0	184.0-2082.0	374.0-1794.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**10.18** (3.19)	**9.99** (2.55)	**10.13** (1.84)	**9.82** (2.54)	**10.49** (3.27)	**9.75** (2.16)	**10.27** (2.65)	**10.40** (3.11)	**9.24** (2.50)
	Median (IQR)	9.55 (7.69-11.79)	9.85 (7.72-11.84)	10.59 (8.89-11.38)	9.48 (7.86-11.36)	9.98 (7.59-12.16)	9.45 (8.30-10.91)	9.84 (8.30-11.73)	10.19 (8.13-12.14)	8.87 (7.28-10.76)
	Range	5.60-20.88	6.14-15.43	6.37-12.24	5.85-15.56	5.60-20.88	7.06-14.32	6.57-15.56	5.60-20.88	5.85-14.68
Cysteine (µmol/L)	$\bar{x}$ (SD)	**262.8** (43.9)	**268.0** (36.9)	**258.5** (34.7)	**269.4** (40.3)	**263.2** (42.4)	**244.0** (23.2)	**277.2** (37.7)	**257.5** (39.8)	**259.7** (42.3) ‡
	Median (IQR)	258.8 (234.0-293.1)	265.6 (240.1-291.4)	256.9 (233.8-265.1)	262.1 (240.2-295.3)	260.5 (235.8-294.0)	252.3 (227.2-261.6)	267.2 (244.1-303.5)	257.0 (231.2-283.5)	251.7 (239.5-287.6)
	Range	176.2-374.5	173.0-361.6	217.0-321.2	202.8-374.5	173.0-346.1	203.3-267.2	221.6-361.6	173.0-338.0	176.2-374.5
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**22.70** (3.68)	**22.97** (6.00)	**22.43** (4.22)	**22.49** (4.19)	**22.95** (5.44)	**23.56** (4.02)	**23.74** (5.40)	**22.45** (4.45)	**22.03** (4.12)
	Median (IQR)	22.47 (20.16-25.29)	21.96 (19.83-25.32)	22.38 (19.72-24.54)	21.96 (20.02-24.91)	22.48 (19.89-25.28)	24.27 (20.38-26.50)	23.25 (20.43-26.85)	22.45 (20.02-24.73)	20.82 (19.47-24.45)
	Range	16.27-30.14	9.20-43.91	17.33-29.98	12.05-32.04	9.20-43.91	16.92-29.98	12.05-43.91	9.20-36.55	16.27-30.05
Glutathione (µmol/L)	$\bar{x}$ (SD)	**11.56** (4.86)	**11.85** (3.23)	**11.34** (2.18)	**11.62** (3.21)	**12.08** (5.07)	**9.82** (2.58)	**11.98** (4.61)	**11.70** (3.82)	**11.11** (3.92)
	Median (IQR)	10.52 (9.09-12.19)	11.32 (9.53-14.01)	11.25 (10.13-11.72)	11.12 (9.97-13.06)	10.55 (9.38-13.61)	9.46 (8.03-10.30)	10.91 (9.20-13.96)	10.68 (9.87-11.91)	10.21 (8.84-12.63)
	Range	5.19-32.43	4.18-19.30	8.84-16.13	5.19-24.73	4.18-32.43	7.30-16.13	5.82-32.43	4.18-24.73	5.19-21.83

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 677C>T MTHFR significance is achieved with native (untransformed) data for red cell folate*.
For 1298A>C MTHFR significance is achieved with native (untransformed) data for serum folate ** * and red cell folate ** ** both with and excluding outliers.
For 19bp del DHFR ‡ denotes approaching significance using transformed data for cysteine.

Table 5-7: All data (adenomatous and non-adenomatous polyps and controls); B-vitamin/thiol related nutritional genetic data by genotype (2 of 4)

Indices		2756A>G MTR			66A>G MTRR			1947G>A COMT		
		Wt (n=77)	Het (n=33)	Rec (n=3)	Wt (n=24)	Het (n=54)	Rec (n=35)	Wt	Het	Rec
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**277.5** (145.4)	**310.1** (184.1)	**303.3** (68.4)	**279.4** (162.8)	**286.8** (148.4)	**294.7** (166.8)			
	Median (IQR)	252.0 (194.0-318.0)	254.0 (199.0-339.0)	270.0 (264.0-326.0)	242.5 (192.8-292.3)	252.0 (199.8-328.8)	266.0 (182.5-334.5)			
	Range	91.0-841.0	121.0-1100.0	258.0-382.0	91.0-798.0	128.0-1100.0	105.0-841.0			
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**21.03** (12.17)	**25.82** (13.06)	**28.33** (17.56)	**20.21** (11.88)	**22.63** (12.20)	**24.26** (13.93)			
	Median (IQR)	18.00 (12.00-29.00)	23.00 (14.00-37.00)	30.00 (20.00-37.50)	15.50 (11.00-25.00)	18.50 (13.00-30.75)	20.00 (13.00-35.50)			
	Range	4.00-45.00	9.00-45.00	10.00-45.00	6.00-45.00	5.00-45.00	4.00-45.00			
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**800.5** (453.9)	**850.1** (315.4)	**1173.3** (1156.8)	**815.8** (388.0)	**797.9** (314.5)	**872.9** (621.3)	NOT APPLICABLE		
	Median (IQR)	635.0 (496.0-957.0)	793.0 (558.0-1007.0)	645.0 (510.0-1572.5)	791.5 (510.0-956.8)	749.5 (551.8-997.5)	622.0 (477.5-1080.0)			
	Range	184.0-2500.0	433.0-1794.0	375.0-2500.0	357.0-1991.0	361.0-1794.0	184.0-2500.0			
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**10.20** (2.93)	**9.94** (2.76)	**9.37** (1.94)	**10.47** (3.46)	**9.94** (2.72)	**10.10** (2.63)			
	Median (IQR)	9.88 (7.72-12.00)	9.84 (8.12-11.07)	8.69 (8.27-10.12)	10.12 (8.01-11.51)	9.51 (7.67-11.80)	9.34 (7.92-12.03)			
	Range	5.60-20.88	6.14-17.55	7.85-11.56	5.85-20.88	5.60-15.56	6.84-15.42			
Cysteine (µmol/L)	$\bar{x}$ (SD)	**264.1** (42.0)	**266.3** (38.6)	**255.2** (13.3)	**264.3** (33.3)	**267.1** (45.6)	**260.5** (36.6)			
	Median (IQR)	264.2 (233.4-294.9)	257.1 (240.8-286.6)	251.7 (247.9-260.8)	259.6 (241.5-285.8)	262.2 (235.0-303.0)	254.6 (239.7-283.3)			
	Range	173.0-361.6	202.8-374.5	244.1-269.9	212.6-361.6	176.2-374.5	173.0-346.1			
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**22.65** (5.15)	**23.31** (3.70)	**20.56** (3.81)	**22.69** (3.63)	**22.67** (4.62)	**23.03** (5.63)			
	Median (IQR)	21.93 (19.92-25.07)	23.45 (20.31-25.73)	18.95 (18.39-21.93)	23.42 (20.73-24.80)	22.66 (20.04-25.55)	21.00 (19.92-25.18)			
	Range	9.20-43.91	16.27-30.14	17.83-24.91	15.58-28.66	9.20-36.55	16.40-43.91			
Glutathione (µmol/L)	$\bar{x}$ (SD)	**11.80** (4.52)	**11.50** (3.12)	**9.81** (1.11)	**12.44** (5.01)	**11.48** (4.25)	**11.40** (3.03)			
	Median (IQR)	10.63 (9.36-13.18)	10.70 (9.46-12.41)	9.56 (9.20-10.29)	10.70 (10.32-13.04)	10.57 (9.03-12.39)	10.78 (9.55-13.16)			
	Range	4.18-32.43	5.19-20.88	8.84-11.02	6.59-32.43	4.18-24.73	6.36-18.96			

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Table 5-8: All data (adenomatous and non-adenomatous polyps and controls); B-vitamin/thiol related nutritional genetic data by genotype (3 of 4)

Indices		3'UTR 6bp del TS			2R > 3R TSER			1420C>T SHMT		
		Wt (n=52)	Het (n=50)	Rec (n=11)	Wt (n=36)	Het (n=56)	Rec (n=21)	Wt (n=61)	Het (n=40)	Rec (n=12)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**286.8** (139.9)	**302.5** (178.2)	**224.6** (107.6)	**326.3** (184.2)	**261.3** (139.3)	**291.9** (137.7)	**285.0** (172.8)	**290.9** (132.5)	**290.8** (150.3)
	Median (IQR)	262.0 (204.5-322.8)	258.0 (197.3-330.8)	206.0 (161.0-267.0)	272.0 (213.3-356.8)	225.5 (186.8-286.5)	270.0 (207.0-321.0)	249.0 (198.0-288.0)	270.5 (193.8-332.3)	253.5 (179.3-347.8)
	Range	122.0-841.0	121.0-1100.0	91.0-459.0	122.0-1100.0	91.0-841.0	122.0-730.0	91.0-1100.0	105.0-798.0	128.0-569.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**24.04** (13.13)	**22.48** (12.60)	**16.55** (9.36)	**24.28** (12.83)	**19.68** (11.53)	**27.62** (13.75) *	**20.69** (12.85)	**24.28** (12.35)	**26.92** (11.84) *
	Median (IQR)	20.50 (13.00-32.25)	19.00 (12.25-31.75)	14.00 (11.00-18.00)	20.50 (13.00-34.75)	16.50 (10.00-29.25)	25.00 (15.00-45.00)	14.00 (10.00-30.00)	20.00 (15.00-32.25)	23.50 (18.75-37.75)
	Range	4.00-45.00	4.00-45.00	7.00-37.00	4.00-45.00	4.00-45.00	10.00-45.00	4.00-45.00	4.00-45.00	11.00-45.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**904.1** (536.7)	**793.1** (349.2)	**594.9** (180.1)	**835.9** (445.6)	**748.4** (390.0)	**1010.2** (528.2) *	**793.3** (448.4)	**843.2** (449.3)	**924.7** (406.6)
	Median (IQR)	742.0 (533.5-1074.0)	747.5 (533.8-1015.3)	505.0 (474.5-738.0)	728.5 (534.3-1073.3)	621.0 (497.0-902.5)	847.0 (645.0-1303.0)	622.0 (497.0-986.0)	764.5 (503.0-992.0)	818.5 (661.0-1065.5)
	Range	223.0-2500.0	184.0-1991.0	357.0-885.0	184.0-2500.0	223.0-2500.0	375.0-2082.0	223.0-2500.0	184.0-2500.0	511.0-1999.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**9.62** (2.32)	**10.12** (2.90)	**12.49** (4.03) *	**10.46** (2.54)	**10.20** (3.21)	**9.22** (2.20)	**10.29** (2.92)	**10.10** (2.88)	**9.12** (2.31)
	Median (IQR)	9.14 (7.59-11.30)	9.81 (7.86-11.54)	11.90 (10.80-14.50)	10.19 (8.34-12.04)	9.19 (7.56-11.98)	8.91 (7.28-11.26)	10.06 (8.04-12.07)	10.27 (7.43-11.78)	8.55 (8.09-9.32)
	Range	5.60-15.42	5.85-17.55	6.43-20.88	6.14-15.31	5.60-20.88	5.95-14.09	5.60-20.88	5.85-17.55	6.59-15.31
Cysteine (µmol/L)	$\bar{x}$ (SD)	**265.1** (37.2)	**261.1** (44.8)	**278.0** (32.2)	**271.4** (48.1)	**257.3** (36.4)	**271.4** (33.8)	**261.4** (41.5)	**268.5** (40.1)	**267.2** (37.3)
	Median (IQR)	263.0 (240.0-290.9)	257.0 (233.8-288.2)	288.3 (244.4-296.4)	259.2 (237.3-308.2)	258.2 (235.3-284.3)	264.4 (251.7-289.4)	252.9 (239.5-287.1)	264.4 (236.0-291.7)	268.6 (256.6-296.0)
	Range	186.3-346.1	173.0-374.5	237.6-327.8	173.0-374.5	176.2-346.1	217.0-338.0	176.2-374.5	173.0-343.3	202.8-325.3
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**23.22** (5.90)	**22.57** (3.32)	**21.55** (3.92)	**22.65** (5.43)	**23.33** (3.91)	**21.59** (5.37)	**22.73** (4.11)	**23.12** (5.76)	**21.98** (4.21)
	Median (IQR)	21.58 (19.91-26.80)	22.61 (20.18-24.52)	22.65 (18.42-24.57)	21.95 (19.72-24.42)	23.20 (20.75-26.26)	20.40 (18.32-23.64)	22.38 (19.95-25.51)	23.62 (20.14-25.26)	20.58 (19.34-24.28)
	Range	9.20-43.91	16.40-30.14	15.58-26.91	15.58-43.91	12.05-30.14	9.20-32.04	15.58-36.55	9.20-43.91	16.87-30.90
Glutathione (µmol/L)	$\bar{x}$ (SD)	**11.60** (4.10)	**12.17** (4.31)	**9.38** (1.66)	**11.10** (3.27)	**12.53** (4.88)	**10.34** (2.34)	**11.41** (3.46)	**12.05** (5.00)	**11.67** (4.02)
	Median (IQR)	10.78 (9.24-13.48)	10.71 (9.84-13.06)	9.34 (8.65-10.35)	10.26 (9.24-12.74)	10.70 (9.49-13.69)	10.87 (9.27-11.40)	10.67 (9.41-13.17)	10.70 (9.68-12.19)	9.95 (9.25-13.02)
	Range	4.18-21.83	7.72-32.43	6.59-11.95	5.19-19.57	5.82-32.43	4.18-14.54	5.19-21.83	4.18-32.43	6.93-19.57

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 3'UTR 6bp TS deletion significance is achieved with native (untransformed) data for homocysteine*.
For 2R > 3R TSER significance is achieved with native (untransformed) data for both serum folate* and red cell folate*.
For 1420C>T SHMT significance was detected for serum folate* when native (untransformed) data was used and outliers were removed.

Table 5-9: All data (adenomatous and non-adenomatous polyps and controls); B-vitamin/thiol related nutritional genetic data by genotype (4 of 4)

Indices		80G>A RFC			1561C>T GCPII		
		Wt (n=43)	Het (n=46)	Rec (n=24)	Wt (n=99)	Het (n=14)	Rec (n=0)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**299.2** (154.9)	**279.9** (130.9)	**282.1** (201.9)	**286.3** (154.8)	**297.4** (170.3)	**0.0** (0.0)
	Median (IQR)	264.0 (195.0-330.0)	256.5 (203.0-326.0)	224.0 (191.5-295.5)	255.0 (196.0-322.0)	268.5 (181.0-331.5)	0.0 (0.0-0.0)
	Range	122.0-841.0	122.0-798.0	91.0-1100.0	91.0-1100.0	105.0-798.0	0.0-0.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**24.05** (14.22)	**20.37** (10.89)	**24.38** (12.74)	**23.16** (12.98)	**18.79** (9.64)	**0.00** (0.00)
	Median (IQR)	20.00 (12.50-38.50)	17.00 (13.00-24.50)	23.00 (11.75-34.25)	19.00 (12.50-33.00)	15.00 (12.50-26.75)	0.00 (0.00-0.00)
	Range	4.00-45.00	7.00-45.00	5.00-45.00	4.00-45.00	7.00-40.00	0.00-0.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**903.6** (515.4)	**750.3** (366.4)	**826.9** (428.9)	**827.6** (421.9)	**806.1** (588.3)	**0.0** (0.0)
	Median (IQR)	847.0 (545.0-1041.5)	628.5 (507.3-884.0)	785.0 (490.8-1025.5)	740.0 (529.5-1003.5)	545.0 (480.3-795.0)	0.0 (0.0-0.0)
	Range	184.0-2500.0	342.0-1999.0	357.0-1820.0	184.0-2500.0	361.0-2500.0	0.0-0.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**9.79** (2.77)	**10.43** (2.60)	**10.01** (3.47)	**10.11** (2.86)	**10.05** (2.87)	**0.00** (0.00)
	Median (IQR)	9.03 (7.56-11.34)	9.88 (8.67-11.94)	9.85 (7.05-11.13)	9.84 (7.90-11.65)	9.79 (7.85-11.85)	0.00 (0.00-0.00)
	Range	5.60-15.56	6.32-17.55	6.14-20.88	5.60-20.88	6.37-15.56	0.00-0.00
Cysteine (µmol/L)	$\bar{x}$ (SD)	**262.9** (43.4)	**268.9** (35.5)	**258.6** (44.5)	**262.9** (38.8)	**276.5** (51.3)	**0.0** (0.0)
	Median (IQR)	258.8 (230.2-292.0)	264.3 (245.3-294.1)	254.1 (236.7-290.2)	258.8 (237.9-289.0)	286.6 (240.2-310.9)	0.0 (0.0-0.0)
	Range	173.0-346.1	203.3-361.6	176.2-374.5	173.0-361.6	194.6-374.5	0.0-0.0
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**23.93** (5.06)	**22.02** (4.95)	**22.18** (3.11)	**22.80** (4.91)	**22.67** (3.11)	**0.00** (0.00)
	Median (IQR)	23.16 (20.47-26.81)	21.55 (19.08-24.75)	21.81 (20.45-24.40)	22.44 (19.98-25.29)	21.24 (20.15-24.91)	0.00 (0.00-0.00)
	Range	16.38-43.91	9.20-36.55	16.27-27.90	9.20-43.91	18.70-27.73	0.00-0.00
Glutathione (µmol/L)	$\bar{x}$ (SD)	**11.78** (3.01)	**11.53** (5.04)	**11.68** (3.89)	**11.47** (3.57)	**13.14** (6.92)	**0.00** (0.00)
	Median (IQR)	10.71 (9.71-13.17)	10.41 (8.86-12.56)	10.55 (9.64-12.91)	10.67 (9.41-12.76)	10.61 (8.97-13.96)	0.00 (0.00-0.00)
	Range	6.59-20.88	4.18-32.43	5.19-21.83	4.18-24.73	7.37-32.43	0.00-0.00

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

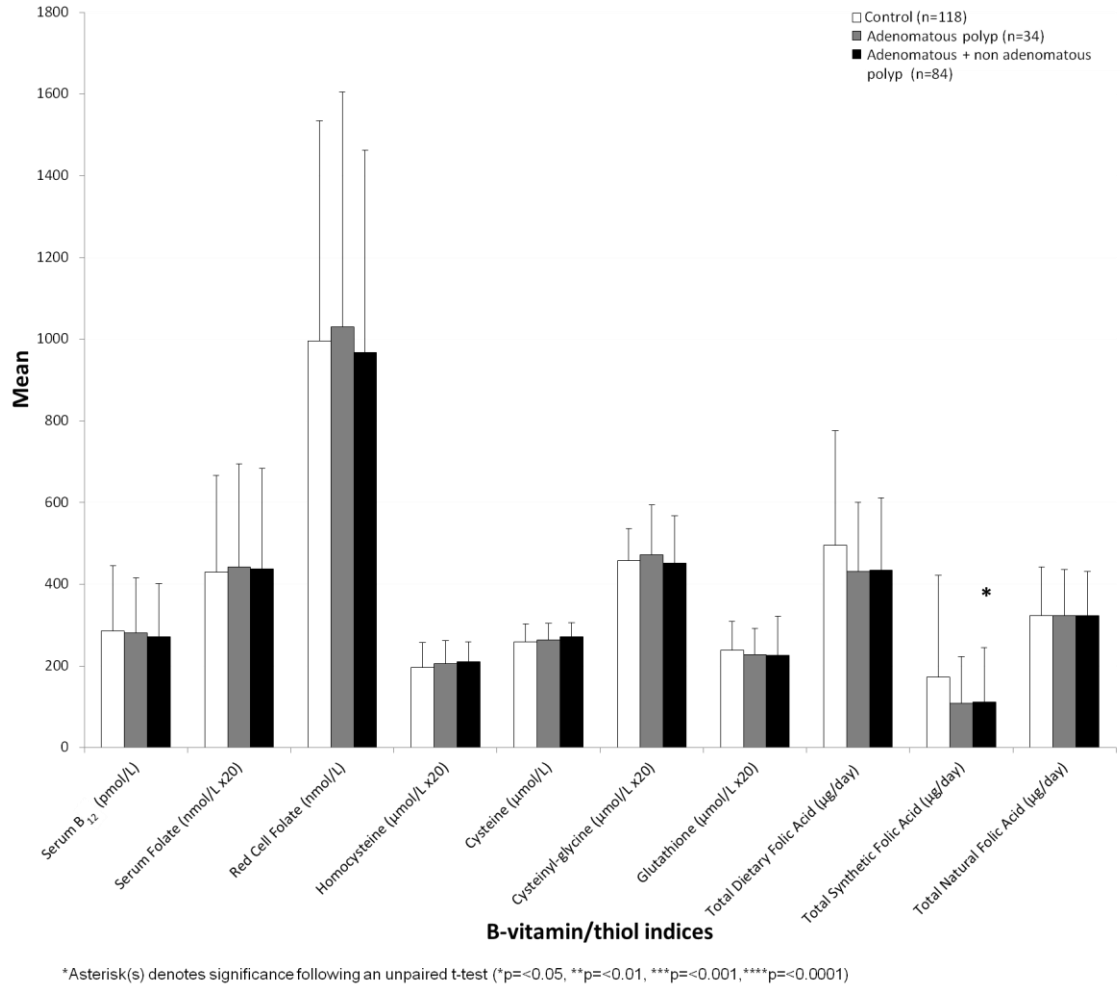
One-way ANOVA results displayed in the above tables shows that for red cell folate there is significant variability in blood metabolites within the 677C>T and 1298A>C MTHFR genotypes and within the variant forms of the 2R > 3R TSER gene. Serum folate level also shows evidence of variation between genotypes for the 2R > 3R TSER gene, 1298A>C MTHFR and the 1420C>T SHMT genes. Finally, significant variation was also detected for homocysteine level between 3'UTR 6 bp deletion TS genotypes.

5.2.5. Adenomatous polyps – phenotype specific analysis

Following the outcomes for the complete data set described above, the next section analysed the data by the three phenotypic groups of interest. Firstly, participants with no polyps of any kind (controls), then participants with an adenomatous polyp histology only, and the third group contained participants whose colonoscopy resulted in a polyp diagnosis which included both adenomatous and non-adenomatous (hyperplastic) polyps.

These clinical phenotypes are examined below, and compare B-vitamin and related thiols in the adenomatous polyp phenotype, adenomatous plus non-adenomatous polyp phenotype and controls. The histogram below (figure 5-1) displays both the mean and standard deviation (errors bars) for each variable within the three phenotypes.

The means for each variable have been compared between phenotypes using an unpaired t-test. This was conducted on both native and $\log_{10}$ transformed data, and was performed both with and without outliers; an asterisk(s) denotes significance level. Synthetic folic acid values were not $\log_{10}$ transformed as there were a number of zero values in the data set, and so only native data was examined for this parameter. The t-test indicates that subjects with a polyp have a lower intake of synthetic folic acid (*p=0.042*).



*Asterisk(s) denotes significance following an unpaired t-test (*p=<0.05, **p=<0.01, ***p=<0.001,****p=<0.0001)

Figure 5-1: Mean and standard deviation values for B-vitamin/thiol measurements comparing controls with subjects who have a polyp (adenomatous and adenomatous plus non-adenomatous)

B-vitamin related genetics are considered important in the aetiology of adenomatous polyps. Therefore, each of the ten genotypes examined for this cohort are displayed below (table 5-10). Genotype prevalence (%), allele number (frequency) and carriage of mutant allele are all presented.

Table 5-10: Clinical phenotype; genotype prevalence, allele number and carriage of mutant allele

SNP	PHENOTYPE	GENOTYPE Prevalence (%)			ALLELE Number (frequency)		Carriage of mutant allele (%)
		Wildtype	Heterozygote	Recessive	Wildtype	Mutant	
677C>T MTHFR	Control	32 (49)	28 (43)	5 (8)	92 (0.71)	38 (0.29)	51
	Adenomatous polyp	13 (54)	8 (33)	3 (13)	34 (0.71)	14 (0.29)	46
	Adenomatous polyp + non-adenomatous polyp	27 (56)	17 (35)	4 (8)	71 (0.74)	25 (0.26)	44
1298A>C MTHFR	Control	28 (43)	31 (48)	6 (9)	87 (0.67)	43 (0.33)	57
	Adenomatous polyp	13 (54)	8 (33)	3 (13)	34 (0.71)	14 (0.29)	46
	Adenomatous polyp + non-adenomatous polyp	27 (56)	17 (35)	4 (8)	71 (0.74)	25 (0.26)	44
19bp del DHFR	Control	20 (31)	32 (49)	13 (20)	72 (0.55)	58 (0.45)	69
	Adenomatous polyp	10 (42)	8 (33)	6 (25)	28 (0.58)	20 (0.42)	58
	Adenomatous polyp + non-adenomatous polyp	17 (35)	19 (40)	12 (25)	53 (0.55)	43 (0.45)	65
2756A>G MTR	Control	45 (69)	18 (28)	2 (3)	108 (0.83)	22 (0.17)	31
	Adenomatous polyp	18 (75)	5 (21)	1 (4)	41 (0.85)	7 (0.15)	25
	Adenomatous polyp + non-adenomatous polyp	32 (67)	15 (31)	1 (2)	79 (0.82)	17 (0.18)	33
66A>G MTRR	Control	18 (28)	30 (46)	17 (26)	66 (0.51)	64 (0.49)	72
	Adenomatous polyp	2 (8)	10 (42)	12 (50)	14 (0.29)	34 (0.71)	92
	Adenomatous polyp + non-adenomatous polyp	6 (13)	24 (50)	18 (38)	36 (0.38)	60 (0.63)	88
G80G>A RFC	Control	27 (42)	24 (37)	14 (22)	78 (0.60)	52 (0.40)	58
	Adenomatous polyp	10 (42)	9 (38)	5 (21)	29 (0.60)	19 (0.40)	58
	Adenomatous polyp + non-adenomatous polyp	16 (33)	22 (46)	10 (21)	54 (0.56)	42 (0.44)	67
1561C>T GCPII	Control	59 (91)	6 (9)	0 (0)	124 (0.95)	6 (0.05)	9
	Adenomatous polyp	18 (75)	6 (25)	0 (0)	42 (0.88)	6 (0.13)	25
	Adenomatous polyp + non-adenomatous polyp	40 (83)	8 (17)	0 (0)	88 (0.92)	8 (0.08)	17
1420C>T SHMT	Control	39 (60)	21 (32)	5 (8)	99 (0.76)	31 (0.24)	40
	Adenomatous polyp	13 (54)	8 (33)	3 (13)	34 (0.71)	14 (0.29)	46
	Adenomatous polyp + non-adenomatous polyp	22 (46)	19 (39)	7 (15)	63 (0.66)	33 (0.34)	54
3'URT 6bp del TS	Control	31 (48)	30 (46)	4 (6)	92 (0.71)	38 (0.29)	52
	Adenomatous polyp	8 (33)	10 (42)	6 (25)	26 (0.54)	22 (0.46)	67
	Adenomatous polyp + non-adenomatous polyp	21 (44)	20 (42)	7 (15)	62 (0.65)	34 (0.35)	56
2R > 3R TSER	Control	20 (31)	31 (48)	14 (22)	71 (0.55)	59 (0.45)	69
	Adenomatous polyp	9 (38)	13 (54)	2 (8)	31 (0.65)	17 (0.35)	63
	Adenomatous polyp + non-adenomatous polyp	16 (33)	25 (52)	7 (15)	57 (0.59)	39 (0.41)	67

An odds ratios and the associated 95% confidence interval was calculated in order to assess the degree and significance of risk of an individual SNP for occurrence of adenomatous or adenomatous and non-adenomatous polyps. A 2x2 table was used to carry out a chi-square test and derive a *p* value. This was achieved using the Yates correction, which provides a stricter analysis for small samples. The table below displays the outcomes for all these tests for each of the ten B-vitamin related genetic variants examined.

Table 5-11: Clinical phenotype; odds ratio and 95% CI along with chi-square test *p* value

SNP	Classification	Odds Ratio	95% Confidence Intervals	*p*	χ^2
677C>T MTHFR	Adenomatous polyp	1.00	0.48 - 2.06	0.9933	0.000
	Adenomatous polyp + non-adenomatous polyp	0.85	0.47 - 1.54	0.7051	0.143
1298A>C MTHFR	Adenomatous polyp	0.83	0.40 - 1.71	0.7526	0.099
	Adenomatous polyp + non-adenomatous polyp	0.71	0.40 - 1.28	0.3206	0.986
19bp del DHFR	Adenomatous polyp	0.89	0.45 - 1.73	0.8558	0.033
	Adenomatous polyp + non-adenomatous polyp	1.01	0.59 - 1.71	0.9790	0.001
2756A>G MTR	Adenomatous polyp	0.84	0.33 - 2.11	0.8836	0.021
	Adenomatous polyp + non-adenomatous polyp	1.06	0.53 - 2.12	0.8773	0.024
66A>G MTRR	Adenomatous polyp	2.50	1.23 - 5.10	0.0163 *	5.767
	Adenomatous polyp + non-adenomatous polyp	1.72	1.00 - 2.94	0.0648 ‡	3.409
80G>A RFC	Adenomatous polyp	0.98	0.50 - 1.93	0.9598	0.003
	Adenomatous polyp + non-adenomatous polyp	1.17	0.68 - 1.99	0.6680	0.184
1561C>T GCPII	Adenomatous polyp	2.95	0.90 - 9.65	0.1273	2.326
	Adenomatous polyp + non-adenomatous polyp	1.88	0.63 - 5.61	0.3859	0.752
1420C>T SHMT	Adenomatous polyp	1.31	0.63 - 2.76	0.5958	0.281
	Adenomatous polyp + non-adenomatous polyp	1.67	0.93 - 3.00	0.1125	2.519
3'UTR 6bp del TS	Adenomatous polyp	2.05	1.04 - 4.05	0.0573 ‡	3.613
	Adenomatous polyp + non-adenomatous polyp	1.33	0.76 - 2.33	0.3997	0.709
2R > 3R TSER	Adenomatous polyp	0.66	0.33 - 1.31	0.3066	1.045
	Adenomatous polyp + non-adenomatous polyp	0.82	0.48 - 1.40	0.5633	0.334

* denotes significance

‡ denotes approaching significance

The 66A>G MTRR SNP contributes a positive risk for the occurrence of an adenomatous polyp. The same SNP approaches significance as a risk factor when occurrence of a non-adenomatous polyp is included in the analysis. The 3'UTR 6bp del TS SNP also approaches significance as a positive risk for the occurrence of an adenomatous polyp.

Given the focus of folate status on nutritional genetics and chronic disease described throughout this thesis, the next set of analyses delineate the entire group based on whether they belong to a high folate status group or low folate status group. This was conducted using the most robust parameter; red cell folate level. The median red cell folate level was obtained (868.5nmol/L) and the data was spilt into above and below

the median. The next series of tables and graphs describes results conducted on each group, beginning with low folate status. Featured below is a histogram (figure 5-2) showing both the mean and standard deviation (error bars) for each variable in the low folate status group (below median red cell folate).

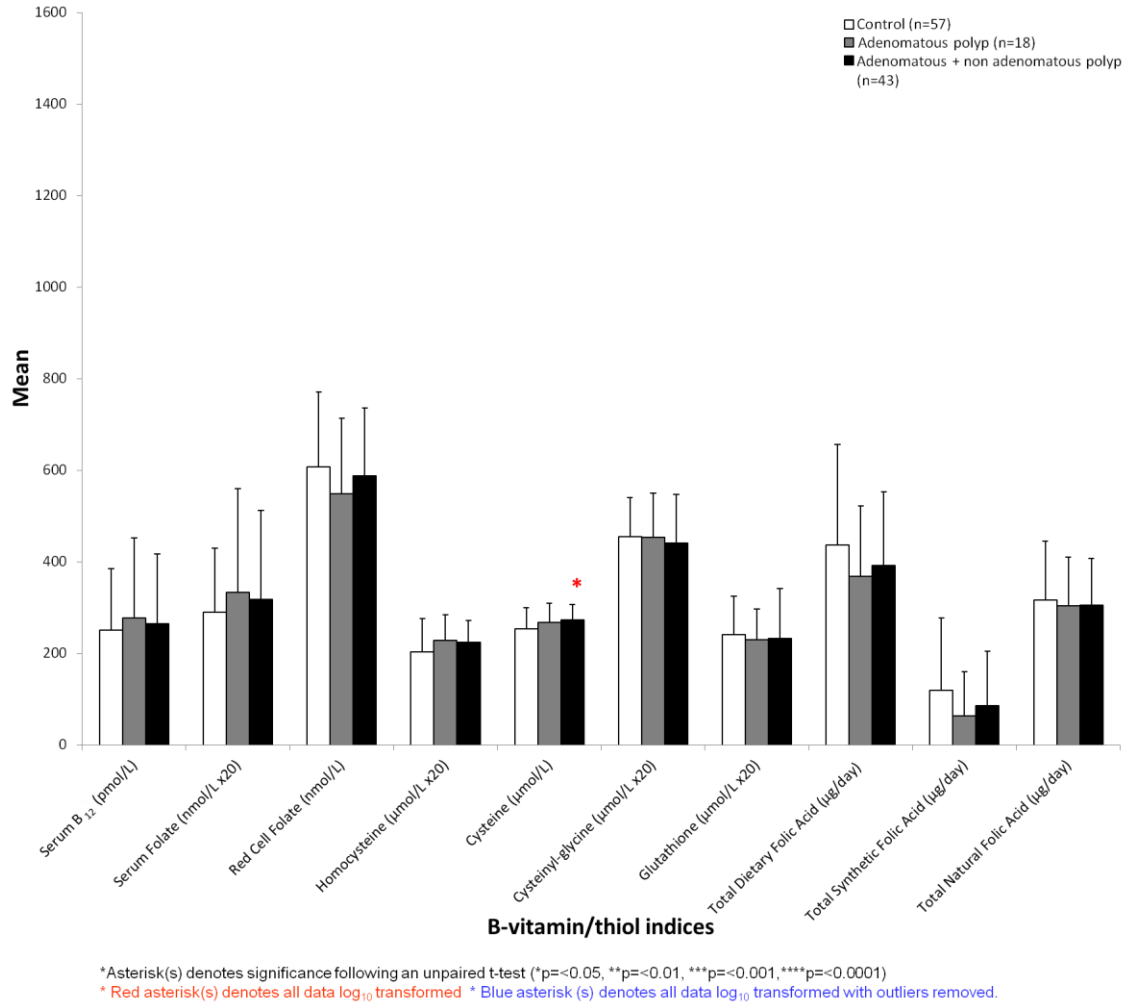


Figure 5-2: Low folate status (below median red cell folate); mean and standard deviation values for B-vitamin/thiol measurements comparing controls with subjects who have a polyp (adenomatous and adenomatous plus non-adenomatous)

The means for each variable have been compared using an unpaired t-test by comparing control with adenomatous polyps, and control with adenomatous polyps and non-adenomatous polyps. An asterisk denotes significance level, the only significant outcome was found to be cysteine levels, which varied between control and adenomatous plus non-adenomatous polyp group.

The adenomatous polyp phenotype is likely to be aetiologically linked to B-vitamin related genetics. Therefore, for those individuals with a red cell folate status below the median value, each of the ten genotypes that have been examined has also been

grouped into controls, adenomatous polyp and adenomatous plus non-adenomatous polyp phenotypes. These are shown below (table 5-12) for low folate status and display genotype prevalence (%), allele number (frequency) and carriage of mutant allele for each phenotype.

Table 5-12: Clinical phenotype; genotype prevalence, allele number and carriage of mutant allele for individuals with a low folate status (below median red cell folate)

SNP	PHENOTYPE	GENOTYPE Prevalence (%)			ALLELE Number (frequency)		Carriage of mutant allele (%)
		Wildtype	Heterozygote	Recessive	Wildtype	Mutant	
677C>T MTHFR	Control	23 (58)	16 (40)	1 (3)	62 (0.78)	18 (0.22)	43
	Adenomatous polyp	8 (50)	7 (44)	1 (6)	23 (0.72)	9 (0.28)	50
	Adenomatous polyp + non-adenomatous polyp	17 (53)	13 (41)	2 (6)	47 (0.73)	17 (0.27)	47
1298A>C MTHFR	Control	14 (35)	24 (60)	2 (5)	52 (0.65)	28 (0.35)	65
	Adenomatous polyp	10 (62)	6 (38)	0 (0)	26 (0.81)	6 (0.19)	38
	Adenomatous polyp + non-adenomatous polyp	17 (53)	14 (44)	1 (3)	48 (0.75)	16 (0.25)	47
19bp del DHFR	Control	10 (25)	22 (55)	8 (20)	42 (0.53)	38 (0.47)	75
	Adenomatous polyp	6 (38)	6 (37)	4 (25)	18 (0.56)	14 (0.43)	63
	Adenomatous polyp + non-adenomatous polyp	10 (31)	13 (41)	9 (28)	33 (0.52)	31 (0.48)	69
2756A>G MTR	Control	29 (73)	10 (25)	1 (3)	68 (0.85)	12 (0.15)	28
	Adenomatous polyp	12 (75)	3 (19)	1 (6)	27 (0.84)	5 (0.16)	25
	Adenomatous polyp + non-adenomatous polyp	23 (72)	8 (25)	1 (3)	54 (0.84)	10 (0.16)	28
66A>G MTRR	Control	8 (20)	23 (57)	9 (23)	39 (0.49)	41 (0.51)	80
	Adenomatous polyp	1 (6)	5 (31)	10 (63)	7 (0.22)	25 (0.78)	94
	Adenomatous polyp + non-adenomatous polyp	4 (13)	14 (43)	14 (43)	22 (0.34)	42 (0.66)	88
80G>A RFC	Control	15 (38)	15 (38)	10 (25)	45 (0.56)	35 (0.44)	63
	Adenomatous polyp	6 (38)	7 (44)	3 (18)	19 (0.60)	13 (0.40)	63
	Adenomatous polyp + non-adenomatous polyp	8 (25)	18 (56)	6 (19)	34 (0.53)	30 (0.47)	75
1561C>T GCPII	Control	35 (88)	5 (13)	0 (0)	75 (0.94)	5 (0.06)	13
	Adenomatous polyp	12 (75)	4 (25)	0 (0)	28 (0.88)	4 (0.12)	25
	Adenomatous polyp + non-adenomatous polyp	26 (81)	6 (19)	0 (0)	58 (0.91)	6 (0.09)	19
1420C>T SHMT	Control	24 (60)	12 (30)	4 (10)	60 (0.75)	20 (0.25)	40
	Adenomatous polyp	11 (69)	5 (31)	0 (0)	27 (0.84)	5 (0.16)	31
	Adenomatous polyp + non-adenomatous polyp	17 (53)	13 (41)	2 (6)	47 (0.73)	17 (0.27)	47
3'URT 6bp del TS	Control	17 (43)	19 (48)	4 (10)	53 (0.66)	27 (0.34)	58
	Adenomatous polyp	5 (31)	5 (31)	6 (38)	15 (0.47)	17 (0.53)	69
	Adenomatous polyp + non-adenomatous polyp	14 (44)	12 (38)	6 (19)	40 (0.62)	24 (0.38)	56
2R > 3R TSER	Control	11 (28)	22 (55)	7 (18)	44 (0.55)	36 (0.45)	73
	Adenomatous polyp	6 (38)	9 (56)	1 (6)	21 (0.66)	11 (0.34)	63
	Adenomatous polyp + non-adenomatous polyp	11 (34)	17 (53)	4 (13)	39 (0.61)	25 (0.39)	66

By examining the number of alleles for each SNP, an odds ratios and the associated 95% confidence interval was calculated in order to assess the degree and significance of risk for the two clinical phenotypes within the low folate status group. As previously described, a 2x2 table was used to carry out a chi-square test and derive a *p* value. Where a frequency of less than 5 a fishers exact test was used in place of chi-square. The table below displays the outcomes for the low folate status group (below median red cell folate) for each of the ten B-vitamin related genetic variants examined.

Table 5-13: Clinical phenotype; odds ratio and 95% CI along with chi-square test *p* value for individuals with a low folate status (below median red cell folate)

SNP	Classification	Odds Ratio	95% Confidence Intervals	*p*	χ^2
677C>T MTHFR	Adenomatous polyp	1.35	0.53 - 3.42	0.7008	0.148
	Adenomatous polyp + non-adenomatous polyp	1.25	0.58 - 2.67	0.7119	0.136
1298A>C MTHFR	Adenomatous polyp	0.43	0.16 - 1.16	0.1437	2.138
	Adenomatous polyp + non-adenomatous polyp	0.62	0.30 - 1.28	0.2660	1.238
19bp del DHFR	Adenomatous polyp	0.86	0.38 - 1.96	0.8809	0.022
	Adenomatous polyp + non-adenomatous polyp	1.04	0.54 - 2.00	0.9109	0.013
2756A>G MTR	Adenomatous polyp	1.05	0.34 - 3.26	1.0000 ^	-
	Adenomatous polyp + non-adenomatous polyp	1.05	0.42 - 2.61	0.9175	0.011
66A>G MTRR	Adenomatous polyp	3.40	1.32 - 8.75	0.0164 *	5.756
	Adenomatous polyp + non-adenomatous polyp	1.82	0.92 - 3.57	0.1176	2.449
80G>A RFC	Adenomatous polyp	0.88	0.38 - 2.02	0.9278	0.008
	Adenomatous polyp + non-adenomatous polyp	1.13	0.59 - 2.20	0.8368	0.042
1561C>T GCPII	Adenomatous polyp	2.14	0.54 - 8.56	0.2731 ^	-
	Adenomatous polyp + non-adenomatous polyp	1.55	0.45 - 5.34	0.5390 ^	-
1420C>T SHMT	Adenomatous polyp	0.56	0.19 - 1.64	0.3262 ^	-
	Adenomatous polyp + non-adenomatous polyp	1.09	0.51 - 2.30	0.9830	0.000
3'UTR 6bp del TS	Adenomatous polyp	2.22	0.97 - 5.13	0.0925	2.831
	Adenomatous polyp + non-adenomatous polyp	1.18	0.59 - 2.34	0.7701	0.085
2R > 3R TSER	Adenomatous polyp	0.64	0.27 - 1.50	0.4137	0.668
	Adenomatous polyp + non-adenomatous polyp	0.78	0.40 - 1.53	0.5845	0.299

* denotes significance

^ The *p* value was derived from a fishers-exact test, this was performed in place of chi-square test due to the low frequency of mutant alleles

For the low folate status group, the 66A>G MTRR SNP contributes a positive risk towards the occurrence of an adenomatous polyp.

To complement the above findings for the low folate status group (below median red cell folate) the next set of data displayed features results for the high folate status group (at or above the median red cell folate value of 868.5nmol/L).

Firstly, displayed below is a histogram showing both the mean and standard deviation (error bars) for each variable in the high folate status group. As in the previous sections, the difference between the B-vitamin and related thiol indices for clinical phenotype has been examined using an unpaired t-test. No significance between any of the groups was achieved.

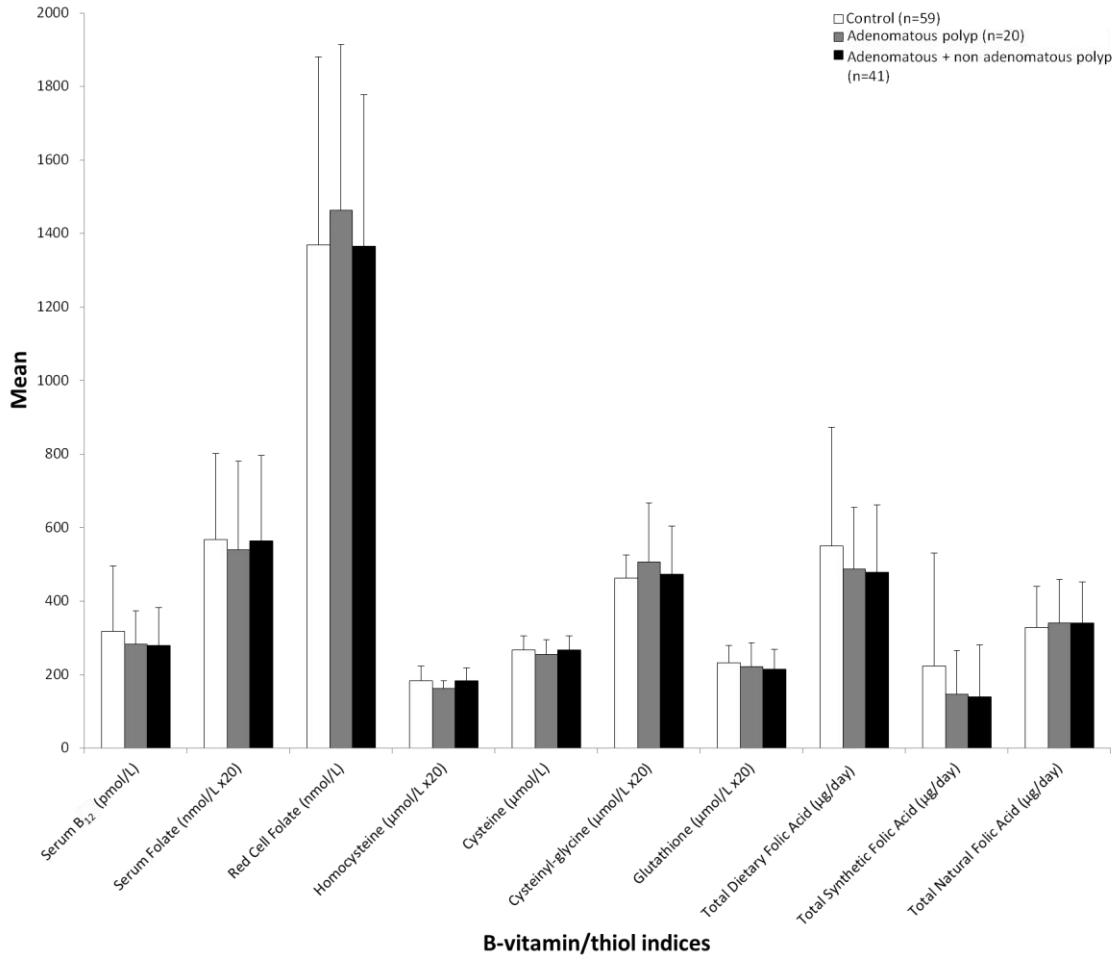


Figure 5-3: High folate status (above median red cell folate); mean and standard deviation values for B-vitamin/thiol measurements comparing controls with subjects who have a polyp (adenomatous and adenomatous plus non-adenomatous)

As previously mentioned, each of the ten genotypes that have been examined are grouped into no polyps (controls), adenomatous polyps and adenomatous plus non-adenomatous polyps. Therefore, the table below displays genotype prevalence (%), allele number (frequency) and carriage of mutant allele for subjects with a high folate status (at or above the median red cell folate value of 868.5nmol/L).

Table 5-14: Clinical phenotype; genotype prevalence, allele number and carriage of mutant allele for individuals with a high folate status (above median red cell folate)

SNP	PHENOTYPE	GENOTYPE Prevalence (%)			ALLELE Number (frequency)		Carriage of mutant allele (%)
		Wildtype	Heterozygote	Recessive	Wildtype	Mutant	
677C>T MTHFR	Control	9 (36)	12 (48)	4 (16)	30 (0.60)	20 (0.40)	64
	Adenomatous polyp	5 (63)	1 (13)	2 (25)	11 (0.69)	5 (0.31)	38
	Adenomatous polyp + non-adenomatous polyp	10 (63)	4 (25)	2 (13)	24 (0.75)	8 (0.25)	38
1298A>C MTHFR	Control	14 (56)	7 (28)	4 (16)	35 (0.70)	15 (0.30)	44
	Adenomatous polyp	3 (38)	2 (25)	3 (38)	8 (0.50)	8 (0.50)	63
	Adenomatous polyp + non-adenomatous polyp	10 (63)	3 (19)	3 (19)	23 (0.72)	9 (0.28)	38
19bp del DHFR	Control	10 (40)	10 (40)	5 (20)	30 (0.60)	20 (0.40)	60
	Adenomatous polyp	4 (50)	2 (25)	2 (25)	10 (0.63)	6 (0.38)	50
	Adenomatous polyp + non-adenomatous polyp	7 (44)	6 (38)	3 (19)	20 (0.63)	12 (0.38)	56
2756A>G MTR	Control	16 (64)	8 (32)	1 (4)	40 (0.80)	10 (0.20)	36
	Adenomatous polyp	6 (75)	2 (25)	0 (0)	14 (0.88)	2 (0.13)	25
	Adenomatous polyp + non-adenomatous polyp	9 (56)	7 (44)	0 (0)	25 (0.78)	7 (0.22)	44
66A>G MTRR	Control	10 (40)	7 (28)	8 (32)	27 (0.54)	23 (0.46)	60
	Adenomatous polyp	1 (12)	5 (63)	2 (25)	7 (0.44)	9 (0.56)	88
	Adenomatous polyp + non-adenomatous polyp	2 (13)	10 (62)	4 (25)	14 (0.44)	18 (0.56)	88
80G>A RFC	Control	12 (48)	9 (36)	4 (16)	33 (0.66)	17 (0.34)	52
	Adenomatous polyp	4 (50)	2 (25)	2 (25)	10 (0.63)	6 (0.38)	50
	Adenomatous polyp + non-adenomatous polyp	8 (50)	4 (25)	4 (25)	20 (0.63)	12 (0.38)	50
1561C>T GCPII	Control	24 (96)	1 (4)	0 (0)	49 (0.98)	1 (0.02)	4
	Adenomatous polyp	6 (75)	2 (25)	0 (0)	14 (0.88)	2 (0.13)	25
	Adenomatous polyp + non-adenomatous polyp	14 (88)	2 (13)	0 (0)	30 (0.94)	2 (0.06)	13
1420C>T SHMT	Control	15 (60)	9 (36)	1 (4)	39 (0.78)	11 (0.22)	40
	Adenomatous polyp	2 (25)	3 (38)	3 (38)	7 (0.44)	9 (0.56)	75
	Adenomatous polyp + non-adenomatous polyp	5 (31)	6 (38)	5 (31)	16 (0.50)	16 (0.50)	69
3'URT 6bp del TS	Control	14 (56)	11 (44)	0 (0)	39 (0.78)	11 (0.22)	44
	Adenomatous polyp	3 (38)	5 (62)	0 (20)	11 (0.69)	5 (0.31)	63
	Adenomatous polyp + non-adenomatous polyp	7 (44)	8 (50)	1 (6)	22 (0.69)	10 (0.31)	56
2R > 3R TSER	Control	9 (36)	9 (36)	7 (28)	27 (0.54)	23 (0.46)	64
	Adenomatous polyp	3 (38)	4 (50)	1 (13)	10 (0.63)	6 (0.38)	63
	Adenomatous polyp + non-adenomatous polyp	5 (31)	8 (50)	3 (19)	18 (0.56)	14 (0.44)	69

As with the low folate status group, an odds ratio and associated 95% confidence interval was calculated for this high folate status group. Additionally, a chi-square test and *p* value was also ascertained. The table below displays the outcomes of these contingency tests for the high folate status group (at or above median red cell folate) for each of the ten B-vitamin related genetic variants studied.

Table 5-15: Clinical phenotype; odds ratio and 95% CI along with chi-square test *p* value for individuals with a high folate status (above median red cell folate)

SNP	Classification	Odds Ratio	95% Confidence Intervals	*p*	χ^2
677C>T MTHFR	Adenomatous polyp	0.68	0.21 - 2.26	0.5710 ^	-
	Adenomatous polyp + non-adenomatous polyp	0.50	0.19 - 1.33	0.2466	1.342
1298A>C MTHFR	Adenomatous polyp	2.33	0.74 - 7.38	0.2461	1.345
	Adenomatous polyp + non-adenomatous polyp	0.91	0.34 - 2.43	0.8556	0.033
19bp del DHFR	Adenomatous polyp	0.90	0.28 - 2.87	0.8586	0.032
	Adenomatous polyp + non-adenomatous polyp	0.90	0.36 - 2.24	0.8209	0.051
2756A>G MTR	Adenomatous polyp	0.57	0.11 - 2.93	0.7150 ^	-
	Adenomatous polyp + non-adenomatous polyp	1.12	0.38 - 3.32	0.8381	0.042
66A>G MTRR	Adenomatous polyp	1.51	0.49 - 4.69	0.6696	0.182
	Adenomatous polyp + non-adenomatous polyp	1.51	0.62 - 3.69	0.4970	0.461
80G>A RFC	Adenomatous polyp	1.16	0.36 - 3.75	0.7982	0.065
	Adenomatous polyp + non-adenomatous polyp	1.16	0.46 - 2.94	0.9310	0.008
1561C>T GCPII	Adenomatous polyp	7.00	0.59 - 83.00	0.1434 ^	-
	Adenomatous polyp + non-adenomatous polyp	3.27	0.28 - 37.60	0.5574 ^	-
1420C>T SHMT	Adenomatous polyp	4.56	1.38 - 15.03	0.0225 *	5.208
	Adenomatous polyp + non-adenomatous polyp	3.55	1.35 - 9.29	0.0168 *	5.717
3'UTR 6bp del TS	Adenomatous polyp	1.61	0.46 - 5.63	0.5094 ^	-
	Adenomatous polyp + non-adenomatous polyp	1.61	0.59 - 4.39	0.4985	0.458
2R > 3R TSER	Adenomatous polyp	0.70	0.22 - 2.24	0.7589	0.094
	Adenomatous polyp + non-adenomatous polyp	0.91	0.37 - 2.23	0.8417	0.040

* denotes significance

^ The *p* value was derived from a fishers-exact test, this was performed in place of chi-square test due to the low frequency of mutant alleles

The major finding from this sub analysis was that for the high folate status group, the 1420C>T SHMT SNP contributes a positive risk towards both adenomatous polyps and adenomatous plus non-adenomatous polyps group. The expression product for this SHMT SNP is the main entry point for one-carbon units into one-carbon metabolism, and hence SHMT represents a significant locus for onward metabolism directed towards methionine and DNA biosynthesis.

5.2.6. B-vitamin/thiol related nutritional genetics organised by clinical phenotype

All descriptive data for each of the clinical phenotypes is displayed in the next twelve tables and describes each of the B-vitamin and thiol related parameters examined - each is categorised by genotype. The tables begin with the control group (no polyp), followed by the adenomatous polyp group and finally the adenomatous plus non-adenomatous polyp group. In order to investigate whether genetic factors can influence variability in blood indices, a one-way ANOVA was conducted. As previously described all data was examined twice, both with and without outliers, and on both $\log_{10}$ transformed and native data. A significant result is denoted by either a red asterisk(s) (all $\log_{10}$ transformed values) or a blue asterisk(s) ($\log_{10}$ transformed values with outliers removed). *Note: Statistical results for the untransformed data are displayed as footnotes in each table*.

Significant outcomes following one way-ANOVA are overlayed on the tables below, as indicated by red boxes and asterisks(s) for levels of significance. Subjects with no polyps (controls) demonstrated variability within red cell folate level for the 1298A>C MTHFR gene variant. However, in the adenomatous polyp group and the adenomatous polyp plus non-adenomatous polyp group, red cell folate levels showed significant variation according to 677C>T MTHFR genotype. For the control group, the 3'UTR 6 bp deletion TS SNP had serum vitamin B_{12} and homocysteine levels which varied by genotype and for the 2R > 3R TSER gene, serum folate and cysteine levels exhibited variability. In addition to this, variability was also seen in cysteine levels for adenomatous polyps by 1561C>T GCPII genotype and with the adenomatous plus non-adenomatous polyp group by the 1298A>C MTHFR and 80G>A RFC genotypes.

Table 5-16: Controls; B-vitamin/thiol related nutritional genetic data by genotype (1 of 4)

Indices		677C>T MTHFR			1298A>C MTHFR			19bp del DHFR		
		Wt (n=32)	Het (n=28)	Rec (n=5)	Wt (n=28)	Het (n=31)	Rec (n=6)	Wt (n=20)	Het (n=32)	Rec (n=13)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**264.0** (84.0)	**312.9** (195.3)	**335.8** (269.9)	**293.2** (147.1)	**285.6** (172.4)	**300.2** (120.9)	**306.4** (124.4)	**283.9** (191.4)	**280.8** (97.0)
	Median (IQR)	260.0 (216.0-318.8)	264.0 (205.0-323.0)	246.0 (199.0-382.8)	261.5 (198.8-321.5)	264.0 (212.0-316.5)	259.5 (227.5-357.5)	278.5 (228.0-341.5)	249.5 (197.3-275.3)	276.0 (207.0-323.0)
	Range	91.0-501.0	128.0-1100.0	121.0-730.0	121.0-730.0	91.0-1100.0	174.0-501.0	128.0-673.0	91.0-1100.0	154.0-495.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**19.88** (11.12)	**23.10** (12.68)	**29.75** (16.92)	**23.93** (12.64)	**18.68** (10.89)	**29.33** (13.98)	**23.65** (12.42)	**21.25** (12.87)	**20.92** (11.04)
	Median (IQR)	16.50 (12.75-26.00)	20.00 (12.00-34.00)	32.50 (19.50-42.75)	19.50 (12.75-32.00)	16.00 (10.00-26.50)	27.50 (22.75-41.25)	23.00 (11.75-31.75)	17.00 (12.00-29.00)	22.00 (13.00-24.00)
	Range	5.00-45.00	4.00-45.00	9.00-45.00	10.00-45.00	4.00-45.00	9.00-45.00	6.00-45.00	4.00-45.00	5.00-42.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**784.1** (458.4)	**834.4** (360.5)	**1208.3** (646.6)	**907.6** (405.0)	**682.4** (275.4)	**1259.0** (824.0) * *	**892.0** (451.1)	**807.3** (473.6)	**803.8** (306.3)
	Median (IQR)	656.5 (496.8-909.0)	777.0 (581.0-990.0)	1105.5 (883.5-1430.3)	868.0 (592.8-1054.5)	622.0 (495.0-811.5)	1055.0 (659.5-1766.3)	850.0 (570.0-1054.5)	686.0 (528.5-945.8)	769.0 (511.0-986.0)
	Range	357.0-2500.0	184.0-1999.0	540.0-2082.0	440.0-2082.0	184.0-1420.0	413.0-2500.0	440.0-2500.0	184.0-2082.0	405.0-1420.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**9.61** (3.64)	**9.82** (2.63)	**11.34** (0.83)	**9.45** (2.59)	**9.98** (3.67)	**10.58** (2.28)	**9.78** (2.60)	**10.33** (3.55)	**8.57** (2.43)
	Median (IQR)	8.09 (7.22-11.45)	9.78 (7.55-11.56)	11.43 (11.10-11.68)	8.95 (7.42-11.32)	8.86 (7.31-11.98)	10.66 (9.06-11.26)	9.43 (7.72-11.27)	9.82 (7.42-12.29)	7.47 (7.05-9.59)
	Range	5.60-20.88	6.14-15.43	10.24-12.24	5.85-15.43	5.60-20.88	7.85-14.32	6.57-15.42	5.60-20.88	5.85-13.47
Cysteine (µmol/L)	$\bar{x}$ (SD)	**249.8** (44.1)	**268.2** (40.7)	**275.5** (44.9)	**265.6** (42.7)	**257.7** (46.4)	**241.2** (20.1)	**270.0** (41.1)	**255.4** (43.8)	**253.9** (44.9)
	Median (IQR)	245.0 (212.4-270.7)	270.8 (236.7-295.4)	279.6 (248.2-306.8)	261.2 (233.8-297.1)	254.1 (231.8-293.1)	246.8 (227.2-253.7)	255.9 (240.6-296.0)	255.6 (225.7-287.2)	245.8 (216.5-289.4)
	Range	176.2-346.1	173.0-361.6	221.6-321.2	202.8-361.6	173.0-346.1	212.6-264.4	221.6-361.6	173.0-338.0	176.2-326.6
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**22.10** (3.52)	**23.61** (4.16)	**24.16** (4.36)	**22.78** (3.72)	**22.84** (4.01)	**23.74** (4.65)	**23.65** (3.56)	**22.66** (4.05)	**22.35** (4.12)
	Median (IQR)	22.13 (20.06-23.83)	23.17 (20.90-24.80)	23.46 (21.72-25.90)	22.41 (20.51-24.56)	22.49 (20.47-24.29)	24.27 (21.09-26.29)	23.00 (21.22-25.37)	22.83 (20.04-24.05)	20.90 (19.81-24.35)
	Range	16.38-30.05	17.93-36.55	19.72-29.98	16.38-32.04	16.87-36.55	16.92-29.98	18.47-32.04	16.38-36.55	16.87-30.05
Glutathione (µmol/L)	$\bar{x}$ (SD)	**11.41** (4.07)	**12.39** (3.07)	**12.39** (2.57)	**12.28** (3.54)	**11.78** (3.75)	**10.82** (2.90)	**12.27** (2.96)	**11.83** (3.90)	**11.55** (3.78)
	Median (IQR)	10.55 (9.52-11.64)	11.36 (9.76-14.54)	11.59 (11.16-12.82)	11.32 (10.50-13.28)	10.61 (9.50-13.07)	10.13 (9.62-11.16)	12.20 (9.73-14.44)	10.79 (10.41-11.74)	10.27 (9.27-12.63)
	Range	6.36-24.73	7.53-19.30	10.27-16.13	6.59-24.73	6.36-21.83	7.55-16.13	7.55-18.22	6.36-24.73	7.72-21.83

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 677C>T MTHFR significance is achieved with native (untransformed) data for red cell folate*.
For 1298A>C MTHFR significance is achieved with native (untransformed) data for red cell folate ** *.

Table 5-17: Controls; B-vitamin/thiol related nutritional genetic data by genotype (2 of 4)

Indices		2756A>G MTR			66A>G MTRR			1947G>A COMT		
		Wt (n=45)	Het (n=18)	Rec (n=2)	Wt (n=18)	Het (n=30)	Rec (n=17)	Wt	Het	Rec
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**271.5** (122.9)	**333.6** (220.5)	**320.0** (87.7)	**262.1** (135.2)	**288.8** (176.4)	**322.5** (138.1)			
	Median (IQR)	264.0 (205.0-286.0)	285.0 (207.5-336.3)	320.0 (289.0-351.0)	226.0 (195.3-283.5)	253.5 (199.8-312.8)	284.0 (264.0-339.0)			
	Range	91.0-730.0	121.0-1100.0	258.0-382.0	91.0-673.0	128.0-1100.0	122.0-730.0			
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**21.36** (12.69)	**23.56** (11.52)	**20.00** (14.14)	**20.50** (11.64)	**20.73** (11.62)	**25.53** (13.91)			
	Median (IQR)	18.00 (12.00-30.00)	22.00 (14.25-31.00)	20.00 (15.00-25.00)	16.50 (11.25-29.00)	18.00 (12.25-27.75)	25.00 (13.00-35.00)			
	Range	4.00-45.00	9.00-45.00	10.00-30.00	6.00-45.00	5.00-45.00	4.00-45.00			
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**802.7** (427.2)	**840.2** (240.9)	**1437.5** (1502.6)	**856.8** (395.6)	**721.6** (251.1)	**1003.1** (646.3)	NOT APPLICABLE		
	Median (IQR)	691.0 (496.0-986.0)	790.0 (689.3-996.0)	1437.5 (906.3-1968.8)	891.0 (596.5-976.3)	705.5 (500.5-838.3)	811.0 (540.0-1175.0)			
	Range	184.0-2082.0	511.0-1420.0	375.0-2500.0	357.0-1991.0	361.0-1303.0	184.0-2500.0			
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**9.79** (3.14)	**9.86** (3.23)	**9.71** (2.62)	**10.69** (3.96)	**9.54** (2.88)	**9.35** (2.39)			
	Median (IQR)	8.87 (7.40-11.89)	9.81 (7.35-11.20)	9.71 (8.78-10.63)	10.75 (7.54-11.81)	8.87 (7.34-11.71)	8.66 (7.43-10.29)			
	Range	5.60-20.88	6.14-17.55	7.85-11.56	5.85-20.88	5.60-15.43	6.84-15.42			
Cysteine (µmol/L)	$\bar{x}$ (SD)	**260.1** (46.5)	**258.5** (37.0)	**257.0** (18.3)	**261.2** (34.3)	**257.0** (46.9)	**262.5** (46.5)			
	Median (IQR)	258.2 (221.8-293.4)	244.2 (234.5-279.6)	257.0 (250.5-263.5)	259.6 (240.7-280.3)	247.7 (216.6-297.1)	255.1 (233.4-302.0)			
	Range	173.0-361.6	202.8-338.0	244.1-269.9	212.6-361.6	176.2-338.0	173.0-346.1			
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**22.91** (4.15)	**22.98** (3.35)	**21.93** (4.22)	**23.02** (3.19)	**22.79** (3.99)	**22.97** (4.56)			
	Median (IQR)	22.44 (20.43-24.63)	23.32 (21.01-24.49)	21.93 (20.44-23.42)	23.71 (21.70-24.73)	22.31 (20.45-24.12)	21.81 (19.72-24.91)			
	Range	16.38-36.55	16.87-29.98	18.95-24.91	16.38-28.66	16.87-36.55	16.83-32.04			
Glutathione (µmol/L)	$\bar{x}$ (SD)	**11.91** (3.95)	**12.08** (2.68)	**10.29** (1.03)	**11.94** (2.92)	**12.24** (4.35)	**11.28** (2.64)			
	Median (IQR)	10.87 (9.62-13.21)	11.03 (10.22-14.13)	10.29 (9.93-10.66)	10.86 (10.48-13.35)	10.77 (9.50-13.07)	10.91 (9.56-13.12)			
	Range	6.36-24.73	8.86-17.90	9.56-11.02	6.59-19.30	7.53-24.73	6.36-16.13			

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Table 5-18: Controls; B-vitamin/thiol related nutritional genetic data by genotype (3 of 4)

Indices		3'UTR 6bp del TS			2R > 3R TSER			1420C>T SHMT		
		Wt (n=31)	Het (n=30)	Rec (n=4)	Wt (n=20)	Het (n=31)	Rec (n=14)	Wt (n=39)	Het (n=21)	Rec (n=5)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**285.9** (122.3)	**312.0** (186.4)	**160.5** (62.5) * *	**346.1** (223.5)	**245.3** (69.2)	**309.9** (158.5)	**300.1** (183.7)	**276.5** (83.9)	**270.4** (170.9)
	Median (IQR)	266.0 (216.0-307.5)	264.5 (207.5-326.0)	161.0 (118.8-202.8)	272.0 (209.8-374.0)	249.0 (205.0-280.0)	271.0 (214.0-326.3)	258.0 (207.0-301.5)	265.0 (235.0-328.0)	207.0 (187.0-267.0)
	Range	122.0-730.0	121.0-1100.0	91.0-229.0	128.0-1100.0	91.0-408.0	122.0-730.0	91.0-1100.0	121.0-464.0	128.0-563.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**23.97** (12.55)	**21.10** (12.14)	**12.25** (5.56)	**24.25** (12.07)	**17.48** (10.08)	**28.43** (13.83) * *	**20.33** (12.34)	**24.43** (12.10)	**23.80** (12.76)
	Median (IQR)	22.00 (13.00-31.00)	18.50 (12.25-23.75)	11.00 (9.25-14.00)	21.50 (15.25-31.75)	14.00 (10.00-23.00)	24.50 (17.50-45.00)	14.00 (10.00-30.00)	21.00 (16.00-31.00)	20.00 (19.00-24.00)
	Range	6.00-45.00	4.00-45.00	7.00-20.00	4.00-45.00	5.00-42.00	10.00-45.00	5.00-45.00	4.00-45.00	11.00-45.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**916.9** (506.5)	**786.4** (353.9)	**527.0** (141.8)	**797.8** (316.7)	**758.7** (400.4)	**1046.1** (588.3)	**823.3** (444.6)	**823.1** (389.5)	**945.6** (598.1)
	Median (IQR)	768.0 (560.0-1080.0)	768.0 (543.5-981.3)	534.0 (445.5-615.5)	768.5 (579.5-1049.3)	691.0 (507.5-912.0)	902.0 (636.8-1250.3)	744.0 (521.5-1030.5)	811.0 (557.0-990.0)	767.0 (683.0-768.0)
	Range	361.0-2500.0	184.0-1991.0	357.0-683.0	184.0-1420.0	357.0-2500.0	375.0-2082.0	357.0-2500.0	184.0-1991.0	511.0-1999.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**9.19** (2.29)	**9.87** (3.15)	**14.10** (5.38) *	**10.20** (2.44)	**9.89** (3.73)	**9.06** (2.44)	**10.06** (3.15)	**9.40** (3.00)	**9.57** (3.70)
	Median (IQR)	8.86 (7.43-11.28)	9.85 (7.35-11.54)	13.69 (11.09-16.70)	10.06 (8.05-11.88)	8.13 (7.35-11.98)	8.39 (7.20-11.30)	9.59 (7.57-11.98)	8.87 (7.28-11.32)	7.47 (7.17-11.29)
	Range	5.60-15.42	5.85-17.55	8.15-20.88	6.14-15.31	5.60-20.88	5.95-14.09	5.60-20.88	5.85-17.55	6.59-15.31
Cysteine (µmol/L)	$\bar{x}$ (SD)	**266.6** (42.5)	**250.7** (44.7)	**271.9** (27.9)	**269.7** (49.4)	**244.9** (38.5)	**277.6** (33.6) *	**257.8** (45.4)	**262.0** (41.3)	**262.9** (39.6)
	Median (IQR)	270.8 (236.7-298.7)	241.7 (223.9-268.6)	274.7 (252.6-294.0)	267.4 (235.9-308.2)	241.8 (214.1-267.5)	271.5 (254.4-298.9)	248.9 (230.2-290.0)	264.3 (233.8-289.4)	273.0 (245.8-295.4)
	Range	186.3-346.1	173.0-361.6	240.3-297.6	173.0-361.6	176.2-346.1	230.3-338.0	176.2-361.6	173.0-326.6	202.8-297.6
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**23.83** (4.46)	**22.16** (3.13)	**21.23** (3.25)	**22.63** (4.46)	**23.08** (2.90)	**22.88** (5.08)	**22.91** (3.88)	**22.99** (3.78)	**22.44** (5.25)
	Median (IQR)	22.49 (20.48-25.21)	22.61 (19.79-23.74)	22.65 (20.94-22.94)	22.58 (19.72-24.42)	22.83 (21.40-24.67)	21.01 (19.14-25.80)	22.79 (20.20-24.85)	22.44 (20.79-24.54)	20.74 (20.43-23.25)
	Range	17.93-36.55	16.83-28.66	16.38-23.25	16.38-36.55	16.83-29.98	16.87-32.04	16.38-36.55	16.92-32.04	16.87-30.90
Glutathione (µmol/L)	$\bar{x}$ (SD)	**12.22** (3.92)	**11.97** (3.25)	**8.97** (1.71)	**11.17** (2.92)	**12.95** (4.19)	**10.65** (2.13)	**12.04** (3.66)	**11.79** (3.48)	**11.32** (3.88)
	Median (IQR)	11.28 (9.51-13.73)	10.94 (10.32-12.76)	9.37 (8.37-9.96)	10.46 (9.53-12.74)	11.35 (10.41-14.17)	10.89 (9.52-11.39)	11.02 (9.71-13.73)	10.70 (10.46-12.35)	9.76 (9.27-10.14)
	Range	6.36-21.83	7.72-24.73	6.59-10.55	6.59-18.22	7.72-24.73	6.36-14.54	6.36-21.83	8.31-24.73	9.19-18.22

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 3'UTR 6bp TS deletion significance is achieved with native (untransformed) data for homocysteine**.
For 2R > 3R TSER significance is achieved with native (untransformed) data for serum folate*, red cell folate* and cysteine*.

Table 5-19: Controls; B-vitamin/thiol related nutritional genetic data by genotype (4 of 4)

Indices		80G>A RFC			1561C>T GCPII		
		Wt (n=43)	Het (n=46)	Rec (n=24)	Wt (n=99)	Het (n=14)	Rec (n=0)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**286.9** (119.9)	**274.7** (124.5)	**323.3** (247.8)	**290.7** (161.0)	**285.0** (96.1)	**0.0** (0.0)
	Median (IQR)	264.0 (206.0-309.0)	261.5 (211.0-319.3)	271.0 (208.5-333.0)	264.0 (205.0-319.5)	268.5 (234.0-358.5)	0.0 (0.0-0.0)
	Range	184.0-730.0	122.0-673.0	91.0-1100.0	91.0-1100.0	154.0-408.0	0.0-0.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**23.37** (13.91)	**20.83** (11.02)	**21.00** (11.43)	**22.22** (12.51)	**19.00** (9.90)	**0.00** (0.00)
	Median (IQR)	20.00 (12.50-33.50)	17.00 (12.75-26.00)	22.50 (10.00-30.75)	19.00 (12.00-31.00)	19.50 (10.75-27.50)	0.00 (0.00-0.00)
	Range	4.00-45.00	10.00-45.00	5.00-38.00	4.00-45.00	7.00-30.00	0.00-0.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**877.1** (488.8)	**845.7** (420.7)	**724.6** (344.5)	**823.9** (390.8)	**918.7** (792.4)	**0.0** (0.0)
	Median (IQR)	767.0 (560.0-1004.0)	741.5 (603.3-965.3)	658.0 (428.5-951.5)	767.0 (539.5-1008.0)	682.0 (511.3-795.0)	0.0 (0.0-0.0)
	Range	184.0-2500.0	375.0-1999.0	357.0-1420.0	184.0-2082.0	361.0-2500.0	0.0-0.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**9.26** (2.79)	**10.28** (2.82)	**10.05** (4.10)	**9.87** (3.19)	**9.17** (2.25)	**0.00** (0.00)
	Median (IQR)	8.12 (7.38-11.17)	10.02 (7.52-11.88)	8.49 (7.02-11.92)	9.06 (7.38-11.52)	8.36 (7.47-11.10)	0.00 (0.00-0.00)
	Range	5.60-15.43	6.32-17.55	6.14-20.88	5.60-20.88	7.05-12.07	0.00-0.00
Cysteine (µmol/L)	$\bar{x}$ (SD)	**260.7** (46.8)	**267.6** (40.6)	**243.7** (38.2)	**259.8** (43.1)	**257.6** (46.8)	**0.0** (0.0)
	Median (IQR)	245.8 (230.2-295.7)	267.2 (240.2-296.0)	239.7 (217.8-257.9)	255.1 (233.6-291.1)	250.4 (233.6-284.2)	0.0 (0.0-0.0)
	Range	173.0-346.1	204.2-361.6	176.2-309.7	173.0-361.6	194.6-326.6	0.0-0.0
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**22.94** (4.16)	**23.13** (4.11)	**22.44** (3.12)	**23.07** (3.99)	**21.20** (2.27)	**0.00** (0.00)
	Median (IQR)	22.17 (20.34-25.72)	23.23 (20.38-24.33)	22.65 (21.04-24.12)	22.79 (20.64-24.71)	20.48 (19.89-22.33)	0.00 (0.00-0.00)
	Range	16.38-32.04	16.83-36.55	16.92-27.90	16.38-36.55	18.70-24.91	0.00-0.00
Glutathione (µmol/L)	$\bar{x}$ (SD)	**11.51** (2.54)	**11.93** (4.13)	**12.64** (4.32)	**11.95** (3.46)	**11.50** (4.86)	**0.00** (0.00)
	Median (IQR)	10.70 (9.71-13.17)	11.15 (9.62-12.68)	10.61 (10.32-14.15)	11.00 (9.93-13.34)	9.92 (9.12-10.53)	0.00 (0.00-0.00)
	Range	6.59-17.90	6.36-24.73	7.72-21.83	6.36-24.73	8.31-21.27	0.00-0.00

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Table 5-20: Adenomatous polyp; B-vitamin/thiol related nutritional genetic data by genotype (1 of 4)

Indices		677C>T MTHFR			1298A>C MTHFR			19bp del DHFR		
		Wt (n=13)	Het (n=8)	Rec (n=3)	Wt (n=13)	Het (n=8)	Rec (n=3)	Wt (n=10)	Het (n=8)	Rec (n=6)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**241.5** (98.0)	**385.1** (243.1)	**271.7** (48.5)	**316.9** (191.0)	**270.4** (146.5)	**250.7** (127.4)	**288.9** (129.7)	**311.0** (249.4)	**276.3** (96.1)
	Median (IQR)	203.0 (165.0-330.0)	358.0 (212.0-488.3)	270.0 (247.0-295.5)	270.0 (195.0-400.0)	260.5 (173.0-309.0)	203.0 (178.5-299.0)	267.0 (197.0-331.5)	207.0 (144.5-411.0)	279.5 (249.0-313.0)
	Range	122.0-400.0	105.0-841.0	224.0-321.0	105.0-841.0	122.0-576.0	154.0-395.0	154.0-576.0	105.0-841.0	122.0-414.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**24.08** (14.51)	**21.00** (15.82)	**30.33** (13.65)	**22.54** (13.11)	**20.00** (16.01)	**39.67** (6.81) ‡	**25.40** (15.46)	**18.25** (11.37)	**28.67** (16.55)
	Median (IQR)	18.00 (14.00-40.00)	14.50 (9.25-36.25)	28.00 (23.00-36.50)	18.00 (14.00-36.00)	13.50 (8.50-26.25)	42.00 (37.00-43.50)	17.00 (14.00-41.50)	17.00 (9.75-23.00)	32.00 (15.25-42.75)
	Range	7.00-45.00	4.00-45.00	18.00-45.00	4.00-45.00	7.00-45.00	32.00-45.00	7.00-45.00	4.00-37.00	7.00-45.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**841.0** (436.0)	**726.0** (735.2)	**1226.3** (506.8) *	**753.7** (413.3)	**832.5** (747.0)	**1320.7** (438.2) ‡	**992.5** (680.7)	**682.0** (424.5)	**839.8** (503.0)
	Median (IQR)	635.0 (532.0-1142.0)	501.0 (411.0-583.8)	1459.0 (1052.0-1517.0)	635.0 (514.0-848.0)	489.5 (438.5-763.3)	1142.0 (1071.0-1481.0)	741.5 (511.8-1132.8)	528.5 (415.5-880.3)	601.5 (525.0-1191.0)
	Range	374.0-1820.0	223.0-2500.0	645.0-1575.0	223.0-1575.0	374.0-2500.0	1000.0-1820.0	434.0-2500.0	223.0-1459.0	374.0-1575.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**10.73** (2.77)	**10.45** (3.15)	**7.98** (1.40)	**9.94** (2.97)	**11.71** (2.43)	**7.88** (0.71)	**10.80** (3.04)	**10.20** (2.58)	**9.52** (2.99)
	Median (IQR)	10.11 (8.40-11.73)	11.16 (7.72-13.04)	8.69 (7.53-8.79)	8.94 (8.07-11.88)	11.23 (9.97-13.98)	8.30 (7.68-8.30)	10.08 (8.36-13.24)	10.11 (8.59-11.81)	9.12 (7.49-10.46)
	Range	7.06-15.56	6.43-14.03	6.37-8.89	6.37-15.56	8.35-14.75	7.06-8.30	7.06-15.56	6.43-14.03	6.37-14.68
Cysteine (µmol/L)	$\bar{x}$ (SD)	**275.5** (47.8)	**252.5** (21.8)	**241.9** (21.7)	**275.7** (45.9)	**254.4** (29.2)	**243.5** (35.1)	**277.4** (33.5)	**239.9** (23.8)	**270.3** (56.0)
	Median (IQR)	267.2 (251.1-294.7)	240.0 (238.5-265.6)	251.7 (234.4-254.3)	260.5 (248.7-302.4)	245.5 (228.4-286.8)	260.1 (231.7-263.7)	276.9 (254.4-293.6)	237.6 (225.1-260.5)	251.4 (242.4-278.7)
	Range	203.3-374.5	229.4-290.1	217.0-256.9	217.0-374.5	224.9-290.1	203.3-267.2	229.4-327.8	203.3-267.1	217.0-374.5
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**23.80** (3.42)	**24.13** (10.13)	**21.59** (4.75)	**23.44** (4.35)	**24.30** (9.02)	**22.49** (3.80)	**24.92** (7.88)	**22.69** (4.49)	**22.51** (4.58)
	Median (IQR)	23.94 (20.72-26.85)	19.95 (17.16-27.59)	20.00 (18.92-23.46)	24.47 (19.99-27.09)	21.17 (19.42-26.32)	20.76 (20.31-23.80)	24.47 (20.13-27.40)	22.14 (19.93-25.26)	20.45 (20.01-25.37)
	Range	19.85-29.47	15.58-43.91	17.83-26.92	16.40-29.93	15.58-43.91	19.85-26.85	15.58-43.91	16.40-29.93	17.83-29.47
Glutathione (µmol/L)	$\bar{x}$ (SD)	**11.10** (3.52)	**12.54** (3.18)	**9.83** (1.26)	**11.90** (2.94)	**11.65** (3.86)	**8.53** (1.09)	**10.49** (3.31)	**12.56** (3.91)	**11.45** (2.07)
	Median (IQR)	10.21 (8.55-13.17)	11.40 (10.06-14.93)	9.41 (9.12-10.33)	11.32 (9.27-13.90)	10.38 (9.41-12.55)	8.92 (8.11-9.14)	9.28 (8.52-12.25)	11.40 (10.03-14.30)	10.90 (10.30-13.23)
	Range	7.30-18.96	9.20-17.20	8.84-11.25	8.52-17.20	7.37-18.96	7.30-9.36	7.30-15.97	8.92-18.96	8.84-13.96

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data log_{10} transformed * Blue asterisk (s) denotes all data log_{10} transformed with outliers removed.

Note: For 677C>T MTHFR significance is achieved with native (untransformed) data for red cell folate*.
For 1298A>C MTHFR significance is achieved with native (untransformed) data for serum folate * and red cell folate* when outliers are excluded, ‡ denotes approaching significance for log10 transformed data with outliers removed .

Table 5-21: Adenomatous polyp; B-vitamin/thiol related nutritional genetic data by genotype (2 of 4)

Indices		2756A>G MTR			66A>G MTRR			1947G>A COMT		
		Wt (n=18)	Het (n=5)	Rec (n=1)	Wt (n=2)	Het (n=10)	Rec (n=12)	Wt	Het	Rec
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**293.9** (185.7)	**294.8** (112.7)	**270.0** (N/A)	**295.5** (9.2)	**271.9** (101.2)	**310.4** (221.0)			
	Median (IQR)	228.0 (171.3-379.3)	289.0 (242.0-330.0)	270.0 (270.0-270.0)	295.5 (292.3-298.8)	237.0 (202.3-329.3)	236.5 (144.5-403.5)			
	Range	105.0-841.0	154.0-459.0	270.0-270.0	289.0-302.0	154.0-459.0	105.0-841.0			
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**21.00** (13.36)	**29.80** (16.08)	**45.00** (N/A)	**26.00** (26.87)	**25.10** (12.05)	**22.42** (15.85)			
	Median (IQR)	17.00 (10.75-31.00)	37.00 (14.00-42.00)	45.00 (45.00-45.00)	26.00 (16.50-35.50)	23.00 (14.50-35.75)	17.00 (9.75-38.25)			
	Range	4.00-45.00	11.00-45.00	45.00-45.00	7.00-45.00	11.00-42.00	4.00-45.00			
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**865.4** (620.9)	**839.6** (362.4)	**645.0** (N/A)	**903.5** (664.0)	**958.9** (380.2)	**752.0** (683.9) ‡	NOT APPLICABLE		
	Median (IQR)	546.0 (454.3-1168.3)	793.0 (558.0-1000.0)	645.0 (645.0-645.0)	903.5 (668.8-1138.3)	924.0 (616.8-1168.3)	505.5 (423.5-637.5)			
	Range	223.0-2500.0	474.0-1373.0	645.0-645.0	434.0-1373.0	505.0-1575.0	223.0-2500.0			
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**10.48** (2.87)	**9.96** (3.12)	**8.69** (N/A)	**10.36** (1.14)	**9.85** (3.05)	**10.67** (2.92)			
	Median (IQR)	10.11 (8.35-12.32)	9.55 (8.30-10.76)	8.69 (8.69-8.69)	10.36 (9.95-10.76)	8.64 (8.30-11.49)	10.11 (8.52-13.18)			
	Range	6.37-15.56	6.43-14.75	8.69-8.69	9.55-11.16	6.37-15.56	7.06-14.75			
Cysteine (µmol/L)	$\bar{x}$ (SD)	**257.4** (35.7)	**289.3** (52.0)	**251.7** (N/A)	**263.8** (33.7)	**278.7** (53.9)	**250.9** (19.7)			
	Median (IQR)	252.5 (229.4-267.2)	286.6 (260.1-287.6)	251.7 (251.7-251.7)	263.8 (251.9-275.7)	275.1 (242.4-317.7)	251.7 (234.5-265.6)			
	Range	203.3-327.8	237.6-374.5	251.7-251.7	240.0-287.6	203.3-374.5	224.9-286.6			
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**24.26** (6.74)	**22.58** (3.23)	**17.83** (N/A)	**17.88** (3.26)	**23.42** (3.51)	**24.83** (7.82)			
	Median (IQR)	23.94 (19.95-27.59)	20.72 (20.19-25.25)	17.83 (17.83-17.83)	17.88 (16.73-19.04)	24.47 (20.18-26.45)	22.14 (19.93-28.53)			
	Range	15.58-43.91	19.92-26.85	17.83-17.83	15.58-20.19	17.92-27.73	16.40-43.91			
Glutathione (µmol/L)	$\bar{x}$ (SD)	**11.77** (3.48)	**10.53** (2.44)	**8.84** (N/A)	**10.02** (0.76)	**10.65** (2.52)	**12.27** (3.93)			
	Median (IQR)	10.65 (9.20-13.89)	10.55 (9.36-11.40)	8.84 (8.84-8.84)	10.02 (9.75-10.29)	9.39 (8.99-11.36)	11.41 (9.52-14.93)			
	Range	7.30-18.96	7.37-13.96	8.84-8.84	9.48-10.55	8.52-15.96	7.30-18.96			

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 66A>G MTRR ‡ denotes approaching significance for log10 transformed data with outliers removed.

Table 5-22: Adenomatous polyp; B-vitamin/thiol related nutritional genetic data by genotype (3 of 4)

Indices		3'UTR 6bp del TS			2R > 3R TSER			1420C>T SHMT		
		Wt (n=8)	Het (n=10)	Rec (n=6)	Wt (n=9)	Het (n=13)	Rec (n=2)	Wt (n=13)	Het (n=8)	Rec (n=3)
Serum Vitamin B_{12} (pmol/L)	$\bar{X}$ (SD)	**367.1** (239.1)	**247.5** (102.7)	**270.5** (122.2)	**332.2** (133.6)	**265.7** (197.9)	**295.5** (36.1)	**291.3** (191.2)	**295.9** (154.8)	**293.7** (125.0)
	Median (IQR)	295.5 (199.8-454.5)	233.0 (156.8-321.3)	267.0 (204.3-323.0)	330.0 (242.0-395.0)	195.0 (154.0-289.0)	295.5 (282.8-308.3)	242.0 (165.0-330.0)	260.5 (193.8-355.5)	332.0 (243.0-363.5)
	Range	122.0-841.0	122.0-400.0	105.0-459.0	122.0-576.0	105.0-841.0	270.0-321.0	122.0-841.0	105.0-576.0	154.0-395.0
Serum Folate (nmol/L)	$\bar{X}$ (SD)	**27.38** (18.15)	**25.00** (13.45)	**17.17** (10.26)	**24.33** (14.25)	**21.54** (14.95)	**36.50** (12.02)	**19.15** (14.00)	**26.13** (14.66)	**38.00** (5.29) ‡
	Median (IQR)	32.00 (8.50-45.00)	19.00 (15.00-38.00)	15.00 (13.25-16.00)	20.00 (13.00-37.00)	16.00 (10.00-36.00)	36.50 (32.25-40.75)	14.00 (10.00-20.00)	22.00 (15.25-39.00)	40.00 (36.00-41.00)
	Range	4.00-45.00	10.00-45.00	7.00-37.00	7.00-45.00	4.00-45.00	28.00-45.00	4.00-45.00	9.00-45.00	32.00-42.00
Red Cell Folate (nmol/L)	$\bar{X}$ (SD)	**1011.4** (838.7)	**877.8** (398.1)	**591.8** (179.7)	**904.8** (660.8)	**773.6** (496.9)	**1110.0** (657.6)	**659.2** (456.7)	**1066.4** (713.4)	**1106.3** (93.7)
	Median (IQR)	579.5 (423.5-1636.3)	817.5 (558.5-1168.3)	501.0 (479.8-721.0)	560.0 (505.0-1142.0)	532.0 (440.0-1000.0)	1110.0 (877.5-1342.5)	532.0 (434.0-635.0)	820.5 (503.0-1423.5)	1142.0 (1071.0-1159.5)
	Range	223.0-2500.0	342.0-1459.0	434.0-848.0	434.0-2500.0	223.0-1820.0	645.0-1575.0	223.0-1820.0	440.0-2500.0	1000.0-1177.0
Homocysteine (µmol/L)	$\bar{X}$ (SD)	**9.48** (2.97)	**10.29** (2.53)	**11.56** (3.22)	**10.22** (2.72)	**10.79** (2.92)	**7.53** (1.64)	**11.08** (2.95)	**9.64** (2.79)	**8.33** (0.06)
	Median (IQR)	8.52 (7.08-11.56)	9.28 (8.52-10.60)	11.73 (11.16-13.75)	10.11 (8.35-11.16)	10.43 (8.74-12.75)	7.53 (6.95-8.11)	10.76 (8.89-14.03)	9.55 (7.39-11.52)	8.30 (8.30-8.35)
	Range	6.37-14.68	8.30-15.56	6.43-14.75	6.43-14.75	7.06-15.56	6.37-8.69	7.06-15.56	6.37-13.75	8.30-8.40
Cysteine (µmol/L)	$\bar{X}$ (SD)	**243.6** (18.9)	**274.4** (49.2)	**276.4** (38.0)	**268.0** (55.6)	**266.2** (26.9)	**234.4** (24.6)	**267.0** (37.4)	**259.3** (42.3)	**262.9** (61.1)
	Median (IQR)	245.3 (228.4-255.6)	262.2 (253.6-293.0)	286.6 (240.0-290.1)	240.0 (229.4-290.1)	262.2 (252.1-272.3)	234.4 (225.7-243.1)	256.9 (251.1-267.2)	237.6 (227.4-288.9)	260.1 (231.7-292.7)
	Range	217.0-267.2	203.3-374.5	237.6-327.8	203.3-374.5	225.4-327.8	217.0-251.7	224.9-374.5	217.0-327.8	203.3-325.3
Cysteinyl-glycine (µmol/L)	$\bar{X}$ (SD)	**26.76** (8.25)	**22.54** (3.85)	**20.73** (4.30)	**23.25** (8.28)	**24.10** (4.45)	**22.38** (6.43)	**22.16** (4.90)	**26.35** (8.39)	**23.54** (3.52)
	Median (IQR)	26.09 (20.56-29.59)	21.43 (20.05-26.12)	19.92 (17.92-25.00)	20.72 (19.85-23.94)	25.13 (20.14-27.62)	22.38 (20.11-24.65)	20.72 (19.92-27.59)	25.25 (22.60-26.09)	23.94 (21.89-25.39)
	Range	17.83-43.91	16.40-27.73	15.58-25.25	15.58-43.91	16.40-29.93	17.83-26.92	15.58-29.93	17.92-43.91	19.85-26.85
Glutathione (µmol/L)	$\bar{X}$ (SD)	**12.13** (3.88)	**11.85** (3.08)	**9.20** (1.47)	**11.52** (3.14)	**11.48** (3.62)	**10.04** (1.70)	**10.88** (2.94)	**12.27** (3.80)	**11.42** (3.94)
	Median (IQR)	10.95 (9.87-14.41)	10.98 (9.38-13.76)	9.20 (8.55-9.48)	11.40 (9.20-13.96)	10.38 (9.16-13.35)	10.04 (9.44-10.64)	10.21 (8.84-13.17)	11.25 (9.88-13.68)	9.36 (9.14-12.66)
	Range	7.30-18.96	8.52-17.20	7.37-11.40	7.37-15.97	7.30-18.96	8.84-11.25	7.30-17.20	8.55-18.96	8.92-15.96

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data log_{10} transformed * Blue asterisk (s) denotes all data log_{10} transformed with outliers removed.

Note: For 1420C>T SHMT ‡ denotes approaching significance for log10 transformed data with outliers removed, significance was achieved on native data*.

Table 5-23: Adenomatous polyp; B-vitamin/thiol related nutritional genetic data by genotype (4 of 4)

Indices		80G>A RFC			1561C>T GCPII		
		Wt (n=10)	Het (n=9)	Rec (n=5)	Wt (n=18)	Het (n=6)	Rec (n=0)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**301.8** (234.8)	**302.1** (90.9)	**259.6** (130.4)	**307.8** (184.0)	**249.2** (96.4)	**0.0** (0.0)
	Median (IQR)	192.5 (156.8-329.3)	302.0 (232.0-395.0)	242.0 (203.0-289.0)	251.0 (191.3-398.8)	281.5 (184.3-327.8)	0.0 (0.0-0.0)
	Range	122.0-841.0	152.0-414.0	105.0-459.0	122.0-841.0	105.0-332.0	0.0-0.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**22.50** (15.31)	**21.44** (13.08)	**30.80** (16.22)	**24.94** (15.57)	**20.50** (11.24)	**0.00** (0.00)
	Median (IQR)	18.00 (10.25-37.00)	18.00 (13.00-32.00)	37.00 (16.00-45.00)	19.00 (10.75-40.75)	15.00 (14.00-25.00)	0.00 (0.00-0.00)
	Range	4.00-45.00	7.00-45.00	11.00-45.00	4.00-45.00	11.00-40.00	0.00-0.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**922.9** (691.0)	**683.3** (370.6)	**1008.2** (570.6)	**867.1** (595.9)	**802.2** (462.9)	**0.0** (0.0)
	Median (IQR)	704.0 (463.0-1132.8)	514.0 (474.0-645.0)	793.0 (558.0-1373.0)	640.0 (456.3-1106.5)	545.0 (505.8-1022.3)	0.0 (0.0-0.0)
	Range	223.0-2500.0	342.0-1459.0	497.0-1820.0	223.0-2500.0	474.0-1575.0	0.0-0.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**10.71** (2.97)	**10.63** (2.87)	**8.45** (2.05)	**10.04** (2.50)	**11.17** (3.97)	**0.00** (0.00)
	Median (IQR)	10.71 (8.36-12.18)	9.00 (8.69-13.75)	8.31 (6.90-9.86)	9.28 (8.31-11.63)	10.76 (8.40-14.75)	0.00 (0.00-0.00)
	Range	6.37-15.56	7.09-14.75	6.43-10.76	6.43-14.68	6.37-15.56	0.00-0.00
Cysteine (µmol/L)	$\bar{x}$ (SD)	**262.3** (41.3)	**253.9** (26.1)	**291.7** (58.9)	**254.2** (28.6)	**299.6** (57.6)	**0.0** (0.0) *
	Median (IQR)	255.6 (226.4-287.8)	252.5 (240.0-264.2)	277.4 (259.8-309.4)	252.1 (238.1-266.4)	294.7 (286.6-325.3)	0.0 (0.0-0.0)
	Range	217.0-327.8	203.3-290.1	237.6-374.5	203.3-327.8	217.0-374.5	0.0-0.0
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**28.12** (6.04)	**19.45** (3.47)	**21.73** (2.36) ***	**23.55** (6.71)	**23.84** (3.53)	**0.00** (0.00)
	Median (IQR)	26.88 (25.07-29.04)	19.85 (17.83-19.95)	20.74 (20.59-21.89)	21.45 (19.87-26.45)	23.94 (20.72-26.92)	0.00 (0.00-0.00)
	Range	22.14-43.91	15.58-27.59	20.19-25.25	15.58-43.91	19.92-27.73	0.00-0.00
Glutathione (µmol/L)	$\bar{x}$ (SD)	**12.08** (3.60)	**10.83** (3.19)	**10.80** (2.75)	**11.36** (3.23)	**11.41** (3.60)	**0.00** (0.00)
	Median (IQR)	10.95 (9.58-14.82)	9.41 (8.92-13.17)	10.97 (9.74-12.04)	10.38 (9.24-12.73)	11.25 (8.52-13.96)	0.00 (0.00-0.00)
	Range	8.52-18.96	7.37-17.20	7.30-13.96	7.30-18.96	7.37-15.96	0.00-0.00

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 80G>A RFC significance is achieved with native (untransformed) data for cysteinyl-glycine***.
For 1561C>T GCPII significance is achieved with native (untransformed) data for cysteine *.

Table 5-24: Adenomatous plus non-adenomatous polyp; B-vitamin/thiol related nutritional genetic data by genotype (1 of 4)

Indices		677C>T MTHFR			1298A>C MTHFR			19bp del DHFR		
		Wt (n=27)	Het (n=17)	Rec (n=4)	Wt (n=27)	Het (n=17)	Rec (n=4)	Wt (n=17)	Het (n=19)	Rec (n=12)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**275.5** (150.7)	**298.4** (189.1)	**283.8** (46.4)	**285.9** (160.9)	**294.2** (168.0)	**231.3** (111.0)	**327.2** (183.6)	**258.8** (170.4)	**263.8** (76.4)
	Median (IQR)	226.0 (182.5-331.0)	254.0 (160.0-330.0)	295.0 (258.5-320.3)	242.0 (174.0-330.5)	252.0 (190.0-330.0)	188.0 (168.3-251.0)	302.0 (183.0-400.0)	206.0 (156.0-305.5)	253.0 (232.0-297.0)
	Range	122.0-798.0	105.0-841.0	224.0-321.0	105.0-841.0	122.0-798.0	154.0-395.0	154.0-798.0	105.0-841.0	122.0-414.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**24.59** (13.58)	**21.47** (13.00)	**25.50** (14.75)	**25.30** (13.28)	**18.82** (11.56)	**32.00** (16.31)	**25.35** (14.06)	**21.21** (12.00)	**24.75** (14.59)
	Median (IQR)	18.00 (14.00-38.50)	16.00 (12.00-31.00)	23.00 (16.25-32.25)	23.00 (15.00-37.00)	14.00 (13.00-20.00)	37.00 (26.25-42.75)	18.00 (14.00-40.00)	18.00 (12.00-28.50)	20.50 (13.25-38.25)
	Range	7.00-45.00	4.00-45.00	11.00-45.00	4.00-45.00	7.00-45.00	9.00-45.00	7.00-45.00	4.00-45.00	7.00-45.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**818.0** (403.2)	**740.8** (533.0)	**1103.8** (481.0) *	**829.1** (402.3)	**724.2** (515.0)	**1098.8** (570.1)	**902.5** (557.0)	**731.3** (347.0)	**821.3** (476.7)
	Median (IQR)	635.0 (538.5-1023.5)	520.0 (481.0-805.0)	1097.5 (713.3-1488.0)	740.0 (526.0-1023.5)	550.0 (480.0-702.0)	1071.0 (858.3-1311.5)	740.0 (505.0-1040.0)	702.0 (480.5-880.0)	584.0 (538.8-941.8)
	Range	374.0-1820.0	223.0-2500.0	645.0-1575.0	223.0-1794.0	374.0-2500.0	433.0-1820.0	433.0-2500.0	223.0-1459.0	374.0-1794.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**10.86** (2.47)	**10.38** (2.37)	**8.63** (1.74)	**10.21** (2.48)	**11.42** (2.19)	**8.52** (1.39)	**10.85** (2.67)	**10.54** (2.21)	**9.97** (2.46)
	Median (IQR)	10.11 (8.91-12.70)	10.16 (8.77-11.95)	8.79 (8.11-9.31)	10.02 (8.69-11.49)	11.16 (9.79-13.75)	8.30 (7.99-8.83)	9.98 (8.70-13.75)	10.32 (8.89-11.69)	9.81 (8.57-10.87)
	Range	7.06-15.56	6.43-14.12	6.37-10.59	6.37-15.56	8.35-14.89	7.06-10.41	7.06-15.56	6.43-14.89	6.37-14.68
Cysteine (µmol/L)	$\bar{x}$ (SD)	**278.1** (39.2)	**265.5** (29.5)	**247.7** (21.2)	**273.5** (37.9)	**273.3** (33.0)	**248.2** (30.1) *	**285.6** (32.6)	**261.2** (32.5)	**266.0** (40.3)
	Median (IQR)	267.2 (253.3-303.3)	258.0 (240.1-286.1)	254.3 (243.1-258.9)	262.1 (246.5-293.1)	273.3 (251.1-295.8)	261.1 (245.9-263.4)	286.6 (262.1-310.9)	259.3 (240.6-266.6)	252.9 (244.6-276.9)
	Range	203.3-374.5	229.4-324.7	217.0-265.1	217.0-374.5	224.9-324.7	203.3-267.2	229.4-343.3	203.3-324.7	217.0-374.5
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**23.41** (3.81)	**21.83** (8.29)	**20.52** (4.42)	**22.18** (4.70)	**23.15** (7.54)	**23.30** (3.50)	**23.86** (7.11)	**22.09** (5.20)	**21.68** (4.27)
	Median (IQR)	23.94 (20.18-26.81)	20.23 (17.45-25.92)	18.92 (17.71-21.73)	20.98 (19.96-25.19)	22.14 (18.36-26.91)	23.25 (20.53-26.01)	23.94 (19.92-27.00)	20.82 (20.02-25.31)	20.45 (18.23-25.03)
	Range	16.27-30.14	9.20-43.91	17.33-26.92	12.05-30.14	9.20-43.91	19.85-26.85	12.05-43.91	9.20-30.14	16.27-29.47
Glutathione (µmol/L)	$\bar{x}$ (SD)	**11.75** (5.72)	**10.94** (3.30)	**9.91** (1.04)	**10.91** (2.70)	**12.64** (6.98)	**8.33** (0.97)	**11.65** (6.10)	**11.46** (3.75)	**10.63** (4.17)
	Median (IQR)	10.21 (8.53-12.60)	11.02 (9.37-12.36)	9.77 (9.27-10.41)	10.68 (9.41-12.27)	10.21 (9.20-15.97)	8.33 (7.62-9.03)	9.48 (8.52-12.41)	10.51 (9.41-11.95)	10.06 (8.36-11.91)
	Range	5.19-32.43	4.18-17.20	8.84-11.25	5.19-17.20	4.18-32.43	7.30-9.36	5.82-32.43	4.18-19.57	5.19-20.88

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 1298A>C MTHFR significance is achieved with native (untransformed) data for red cell folate*.

Table 5-25: Adenomatous plus non-adenomatous polyp; B-vitamin/thiol related nutritional genetic data by genotype (2 of 4)

Indices		2756A>G MTR			66A>G MTRR			1947G>A COMT		
		Wt (n=32)	Het (n=15)	Rec (n=1)	Wt (n=6)	Het (n=24)	Rec (n=18)	Wt	Het	Rec
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**285.8** (174.0)	**281.9** (129.7)	**270.0** (N/A)	**331.3** (235.5)	**284.4** (107.3)	**268.4** (190.1)			
	Median (IQR)	236.0 (172.5-329.3)	242.0 (186.0-343.5)	270.0 (270.0-270.0)	271.5 (205.3-298.8)	247.0 (203.8-330.5)	196.5 (144.5-315.0)			
	Range	105.0-841.0	131.0-569.0	270.0-270.0	156.0-798.0	154.0-569.0	105.0-841.0			
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**20.56** (11.58)	**28.53** (14.63)	**45.00** (N/A)	**19.33** (13.66)	**25.00** (12.73)	**23.06** (14.24)			
	Median (IQR)	18.00 (12.75-27.25)	31.00 (14.00-43.50)	45.00 (45.00-45.00)	15.00 (11.75-21.25)	20.50 (14.00-37.00)	18.00 (12.50-33.75)			
	Range	4.00-45.00	9.00-45.00	45.00-45.00	7.00-45.00	9.00-45.00	4.00-45.00			
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**797.5** (496.1)	**861.9** (395.8)	**645.0** (N/A)	**692.7** (369.1)	**893.2** (362.5)	**750.0** (588.1) *	NOT APPLICABLE		
	Median (IQR)	602.5 (493.0-873.0)	793.0 (535.0-1023.5)	645.0 (645.0-645.0)	500.0 (480.0-781.8)	801.5 (587.0-1065.5)	529.5 (448.5-687.8)			
	Range	223.0-2500.0	433.0-1794.0	645.0-645.0	434.0-1373.0	433.0-1794.0	223.0-2500.0			
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**10.79** (2.54)	**10.04** (2.18)	**8.69** (N/A)	**9.83** (0.97)	**10.43** (2.48)	**10.85** (2.71)			
	Median (IQR)	10.11 (8.90-13.00)	10.10 (8.50-10.59)	8.69 (8.69-8.69)	9.88 (9.61-10.20)	10.25 (8.63-11.75)	10.11 (8.91-13.67)			
	Range	6.37-15.56	6.43-14.75	8.69-8.69	8.21-11.16	6.37-15.56	7.06-14.89			
Cysteine (µmol/L)	$\bar{x}$ (SD)	**269.8** (34.4)	**275.6** (39.8)	**251.7** (N/A)	**273.4** (31.2)	**279.7** (41.4)	**258.5** (24.4)			
	Median (IQR)	265.1 (245.6-295.3)	261.8 (249.5-287.1)	251.7 (251.7-251.7)	270.9 (246.7-299.6)	264.6 (256.2-311.5)	252.9 (240.2-267.2)			
	Range	203.3-327.8	237.6-374.5	251.7-251.7	240.0-310.9	203.3-374.5	224.9-317.8			
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**22.26** (6.39)	**23.69** (4.16)	**17.83** (N/A)	**21.71** (4.94)	**22.52** (5.38)	**23.10** (6.68)			
	Median (IQR)	20.76 (18.14-26.11)	23.62 (20.25-26.81)	17.83 (17.83-17.83)	20.97 (18.40-25.69)	24.20 (19.48-26.79)	20.40 (19.95-25.27)			
	Range	9.20-43.91	16.27-30.14	17.83-17.83	15.58-27.94	9.20-30.14	16.40-43.91			
Glutathione (µmol/L)	$\bar{x}$ (SD)	**11.64** (5.30)	**10.81** (3.56)	**8.84** (N/A)	**13.95** (9.11)	**10.52** (4.01)	**11.52** (3.46)			
	Median (IQR)	10.21 (9.06-12.76)	10.55 (9.31-11.99)	8.84 (8.84-8.84)	10.23 (9.59-11.67)	9.42 (8.54-11.97)	10.65 (9.41-13.17)			
	Range	4.18-32.43	5.19-20.88	8.84-8.84	9.26-32.43	4.18-20.88	6.93-18.96			

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Table 5-26: Adenomatous plus non-adenomatous polyp; B-vitamin/thiol related nutritional genetic data by genotype (3 of 4)

Indices		3'UTR 6bp del TS			2R > 3R TSER			1420C>T SHMT		
		Wt (n=21)	Het (n=20)	Rec (n=7)	Wt (n=16)	Het (n=25)	Rec (n=7)	Wt (n=22)	Het (n=19)	Rec (n=7)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**288.1** (165.8)	**288.3** (168.9)	**261.3** (114.2)	**301.5** (121.2)	**281.2** (194.2)	**255.9** (80.5)	**258.1** (151.9)	**306.8** (172.3)	**305.3** (146.0)
	Median (IQR)	252.0 (189.0-329.0)	234.0 (171.0-347.8)	232.0 (200.5-316.0)	272.5 (226.0-347.8)	203.0 (165.0-329.0)	270.0 (209.5-320.5)	216.0 (175.5-267.3)	289.0 (189.5-343.5)	291.0 (198.0-363.5)
	Range	122.0-841.0	122.0-798.0	105.0-459.0	122.0-576.0	105.0-841.0	131.0-330.0	122.0-841.0	105.0-798.0	154.0-569.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**24.14** (14.25)	**24.55** (13.31)	**19.00** (10.55)	**24.31** (14.12)	**22.40** (12.79)	**26.00** (14.54)	**21.32** (13.98)	**24.11** (12.96)	**29.14** (11.61) *
	Median (IQR)	18.00 (14.00-36.00)	19.00 (13.50-37.75)	16.00 (13.50-23.00)	18.00 (12.75-37.75)	18.00 (14.00-31.00)	26.00 (13.50-36.50)	16.00 (11.00-30.75)	18.00 (14.50-32.50)	32.00 (20.50-38.50)
	Range	4.00-45.00	9.00-45.00	7.00-37.00	7.00-45.00	4.00-45.00	11.00-45.00	4.00-45.00	9.00-45.00	12.00-42.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**885.3** (590.9)	**803.3** (350.9)	**633.7** (197.9)	**883.5** (576.2)	**735.5** (384.5)	**938.4** (413.7)	**740.1** (460.7)	**865.4** (517.5)	**909.7** (253.1)
	Median (IQR)	645.0 (514.0-875.0)	668.5 (529.0-1065.5)	505.0 (485.5-820.5)	602.5 (516.3-1150.8)	563.0 (480.0-885.0)	805.0 (690.5-1163.5)	559.0 (475.8-783.5)	736.0 (501.0-941.0)	1000.0 (732.0-1091.0)
	Range	223.0-2500.0	342.0-1459.0	434.0-885.0	434.0-2500.0	223.0-1820.0	481.0-1575.0	223.0-1820.0	421.0-2500.0	545.0-1177.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**10.26** (2.26)	**10.49** (2.49)	**11.42** (2.90)	**10.77** (2.72)	**10.61** (2.39)	**9.54** (1.75)	**10.71** (2.49)	**10.92** (2.57)	**8.80** (0.60)
	Median (IQR)	10.10 (8.91-11.30)	9.67 (8.63-11.49)	11.45 (10.80-13.24)	10.19 (8.38-13.78)	10.02 (8.97-11.88)	10.06 (8.80-10.48)	10.10 (8.93-12.03)	10.89 (9.61-13.21)	8.70 (8.35-8.96)
	Range	6.37-14.68	8.18-15.56	6.43-14.75	6.43-14.89	7.06-15.56	6.37-11.82	7.06-15.56	6.37-14.89	8.30-9.98
Cysteine (µmol/L)	$\bar{x}$ (SD)	**263.0** (28.5)	**276.7** (41.4)	**282.1** (36.7)	**273.5** (47.9)	**273.3** (26.5)	**259.0** (33.1)	**267.7** (33.7)	**275.9** (38.4)	**270.3** (38.4)
	Median (IQR)	258.8 (244.7-273.3)	263.3 (253.7-298.8)	288.3 (251.6-305.6)	259.2 (239.4-301.3)	265.6 (256.2-290.0)	252.9 (246.0-263.4)	255.5 (246.3-281.7)	274.9 (245.1-305.7)	264.2 (261.3-288.4)
	Range	217.0-324.7	203.3-374.5	237.6-327.8	203.3-374.5	225.4-327.8	217.0-324.7	224.9-374.5	217.0-343.3	203.3-325.3
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**22.32** (7.58)	**23.20** (3.57)	**21.76** (4.60)	**22.66** (6.61)	**23.66** (4.97)	**19.00** (5.34)	**22.41** (4.58)	**23.27** (7.56)	**21.65** (3.72)
	Median (IQR)	20.40 (17.80-26.92)	22.88 (20.19-26.01)	22.46 (18.42-25.19)	21.24 (19.48-24.27)	25.13 (20.13-27.14)	20.07 (17.58-20.82)	21.00 (19.92-26.61)	24.31 (20.08-26.89)	20.31 (18.82-24.63)
	Range	9.20-43.91	16.40-30.14	15.58-26.91	15.58-43.91	12.05-30.14	9.20-26.92	15.58-29.93	9.20-43.91	17.48-26.85
Glutathione (µmol/L)	$\bar{x}$ (SD)	**10.68** (4.27)	**12.48** (5.63)	**9.66** (1.73)	**11.01** (3.77)	**11.99** (5.69)	**9.73** (2.77)	**10.29** (2.81)	**12.34** (6.44)	**11.93** (4.41)
	Median (IQR)	10.21 (7.30-12.35)	10.46 (9.40-13.37)	9.34 (8.71-10.92)	9.86 (9.13-12.51)	10.46 (9.05-12.60)	10.13 (9.12-11.60)	9.70 (8.60-12.25)	10.46 (9.30-12.01)	10.71 (9.14-14.00)
	Range	4.18-20.88	7.73-32.43	7.37-11.95	5.19-19.57	5.82-32.43	4.18-12.35	5.19-17.20	4.18-32.43	6.93-19.57

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data log_{10} transformed * Blue asterisk (s) denotes all data log_{10} transformed with outliers removed.

Note: For 1420C>T SHMT significance is achieved with native (untransformed) data for serum folate*.

Table 5-27: Adenomatous plus non-adenomatous polyp; B-vitamin/thiol related nutritional genetic data by genotype (4 of 4)

Indices		80G>A RFC			1561C>T GCPII		
		Wt (n=16)	Het (n=22)	Rec (n=10)	Wt (n=40)	Het (n=8)	Rec (n=0)
Serum Vitamin B_{12} (pmol/L)	$\bar{x}$ (SD)	**319.9** (203.9)	**285.5** (140.2)	**224.4** (95.6)	**279.8** (146.8)	**306.6** (216.9)	**0.0** (0.0)
	Median (IQR)	289.5 (162.3-384.0)	253.0 (197.8-329.8)	204.5 (180.5-233.5)	236.0 (187.5-329.3)	281.5 (163.8-330.5)	0.0 (0.0-0.0)
	Range	122.0-841.0	142.0-798.0	105.0-459.0	122.0-841.0	105.0-798.0	0.0-0.0
Serum Folate (nmol/L)	$\bar{x}$ (SD)	**25.19** (15.13)	**19.86** (10.98)	**29.10** (13.56)	**24.55** (13.70)	**18.63** (10.13)	**0.00** (0.00)
	Median (IQR)	23.00 (13.25-40.50)	17.00 (13.25-21.75)	28.50 (17.75-43.00)	19.00 (13.75-37.00)	14.00 (13.50-19.00)	0.00 (0.00-0.00)
	Range	4.00-45.00	7.00-45.00	11.00-45.00	4.00-45.00	11.00-40.00	0.00-0.00
Red Cell Folate (nmol/L)	$\bar{x}$ (SD)	**948.4** (571.2)	**646.3** (268.5)	**970.1** (509.5)	**833.0** (469.2)	**721.8** (418.6)	**0.0** (0.0)
	Median (IQR)	861.5 (545.5-1205.8)	554.0 (480.0-716.3)	795.5 (594.0-1251.0)	719.0 (518.5-1001.8)	514.5 (480.8-712.8)	0.0 (0.0-0.0)
	Range	223.0-2500.0	342.0-1459.0	481.0-1820.0	223.0-2500.0	474.0-1575.0	0.0-0.0
Homocysteine (µmol/L)	$\bar{x}$ (SD)	**10.69** (2.56)	**10.60** (2.39)	**9.95** (2.41)	**10.45** (2.28)	**10.81** (3.30)	**0.00** (0.00)
	Median (IQR)	10.48 (8.63-11.88)	9.88 (8.84-13.21)	10.06 (9.55-10.68)	10.10 (8.79-11.75)	10.06 (9.09-12.76)	0.00 (0.00-0.00)
	Range	6.37-15.56	7.09-14.75	6.43-14.89	6.43-14.89	6.37-15.56	0.00-0.00
Cysteine (µmol/L)	$\bar{x}$ (SD)	**266.7** (38.0)	**270.3** (29.7)	**281.6** (45.8)	**267.5** (31.2)	**292.7** (52.6)	**0.0** (0.0)
	Median (IQR)	260.9 (245.7-274.0)	264.2 (251.9-289.7)	267.2 (244.7-310.8)	261.9 (249.5-287.8)	294.7 (263.4-318.1)	0.0 (0.0-0.0)
	Range	217.0-343.3	203.3-324.7	237.6-374.5	203.3-343.3	217.0-374.5	0.0-0.0
Cysteinyl-glycine (µmol/L)	$\bar{x}$ (SD)	**25.60** (6.06)	**20.81** (5.58)	**21.76** (3.23) *	**22.40** (6.06)	**23.92** (3.33)	**0.00** (0.00)
	Median (IQR)	25.13 (21.70-27.12)	19.93 (17.81-25.63)	20.76 (20.19-24.45)	20.58 (18.25-25.99)	23.94 (20.98-26.96)	0.00 (0.00-0.00)
	Range	17.33-43.91	9.20-30.14	16.27-26.91	9.20-43.91	19.92-27.73	0.00-0.00
Glutathione (µmol/L)	$\bar{x}$ (SD)	**12.25** (3.71)	**11.10** (5.95)	**10.19** (2.69)	**10.75** (3.66)	**14.55** (8.42)	**0.00** (0.00)
	Median (IQR)	10.98 (9.95-13.01)	9.42 (8.01-12.32)	10.55 (9.21-11.95)	10.02 (9.13-11.97)	12.35 (9.88-14.96)	0.00 (0.00-0.00)
	Range	8.52-20.88	4.18-32.43	5.19-13.96	4.18-20.88	7.37-32.43	0.00-0.00

* Asterisk(s) denotes significance following one-way ANOVA (* p=<0.05, ** p=<0.01, *** p=<0.001) * Red asterisk(s) denotes all data $\log_{10}$ transformed * Blue asterisk (s) denotes all data $\log_{10}$ transformed with outliers removed.

Note: For 80G>A RFC significance is achieved with native (untransformed) data for cysteinyl-glycine*.

5.2.7. Analysis of combined nutritional biochemistry and genetic data to establish any relationship to adenomatous polyps and non-adenomatous polyps

Linear models utilising ordinal logistic regression analysis with the Wald χ^2 one-step linear approximation to the likelihood ratio test have been used to determine whether any variable significantly predicts clinical phenotype. These models have been performed based on an *a priori* understanding of possible biochemical, genetic and physiologic relationships. Ordinal logistic regression has been performed taking account of the following factors: age, gender, dietary folic acid intake, serum B_{12}, serum folate, red cell folate, cysteine, homocysteine, cysteinyl-glycine, glutathione, and ten B-vitamin related genotypes. The values presented in the following tables are based on native data and contain all data points (black text); $\log_{10}$ transformed data has had extreme data points removed (blue text).

As previously was the case, data has been examined in two ways; firstly comparing control subjects with individuals who have an adenomatous polyp, and secondly controls versus participants with any type of polyp, i.e. adenomatous and non-adenomatous polyps. The results following examination of all variables within the adenomatous polyp group are presented below.

Table 5-28: Ordinal logistic regression; model for adenomatous polyp - all genetic, metabolic and physiologic variables

	Native data			Transformed data		
Variable	**R^2**	**Chi-square**	***p* value**	**R^2**	**Chi-square**	***p* value**
Total Dietary Folic Acid (µg/day)	0.3555	3.53	0.0602 ‡	0.5564		-
677C>T MTHFR		5.67	0.0586 ‡			-
19bp del DHFR		6.14	0.0463*			-
66A>G MTRR		6.90	0.0316*			-
1561C>T GCPII			-		4.29	0.0382*
3'UTR 6bp del TS		7.69	0.0214*		5.73	0.0568 ‡

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡ denotes approaching significance
Black = Native data including all data points. Blue = $\log^{10}$ transformed data with extreme data points removed.

As can be seen in the table above, of the many polymorphisms examined some show significance, although 1561C>T GCPII is the only parameter that is significant when the $\log_{10}$ transformed data with outliers are removed. Given these outcomes, the next model to be examined excluded all metabolic variables, leaving only the gene polymorphisms. Outcomes are shown in table 5-29 below.

Table 5-29: Ordinal logistic regression; model for adenomatous polyp - gene variants only

	Native data			Transformed data		
Variable	**R^2**	**Chi-square**	***p* value**	**R^2**	**Chi-square**	***p* value**
66A>G MTRR	0.2744	8.40	0.0149*	0.2744	8.40	0.0149*
3'UTR 6bp del TS		9.83	0.0073**		9.83	0.0073**

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡denotes approaching significance
Black = Native data including all data points. Blue = log^{10} transformed data with extreme data points removed.

In the above ordinal logistic model, 66A>G MTRR and 3'UTR 6bp deletion TS genes predict adenomatous polyp. These outcomes are supported by the odds ratio and chi-square outcomes displayed in table 5-11. Subsequent models examined different permutations (non-genetic) involving; population variables – age and gender, blood metabolites and thiols. These were examined in a range of ways, and finally included dietary folic acid (natural and synthetic forms) in the analysis. None of these models resulted in any statistically significant outcomes.

Similarly, analysis was also conducted on data from individuals who had any type of polyp i.e. adenomas and non-adenomatous. The next data set displayed therefore represents all polyps, and as previously mentioned, the models have been performed based on an *a priori* understanding of possible biochemical, genetic and physiologic relationships. The results for an examination of all variables for the all polyp group (adenomatous and non-adenomatous polyps) compared to no polyp (controls) are presented below.

Table 5-30: Ordinal logistic regression; model for adenomatous polyp plus non-adenomatous polyp - all genetic, metabolic and physiologic variables

	Native data			Transformed data		
Variable	**R^2**	**Chi-square**	***p* value**	**R^2**	**Chi-square**	***p* value**
Gender	0.3118	4.93	0.0264*	0.3748	8.43	0.0037**
Age (yrs)		10.20	0.0014**		8.82	0.0030**
Red Cell Folate (nmol/L)			-		5.04	0.0248*
Total Dietary Folic Acid (µg/day)		5.55	0.0184*			-
677C>T MTHFR			-		7.11	0.0286*
66A>G MTRR		6.06	0.0482*			-

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡denotes approaching significance
Black = Native data including all data points. Blue = log^{10} transformed data with extreme data points removed.

As can be seen in the table above, age and gender significantly predict having a polyp, and is consistent with the literature. Red cell folate and the 677C>T MTHFR SNP also predict the presence of polyps. Following the same approach as previously, the model was repeated to only include the ten folate polymorphisms examined. As with the

previous findings in the adenomatous polyp only group 66A>G MTRR is again flagged as a predictor of polyps of any kind, see table below.

Table 5-31: Ordinal logistic regression; model for adenomatous polyp plus non-adenomatous polyp - gene variants only

	Native data			Transformed data		
Variable	**R^2**	**Chi-square**	***p* value**	**R^2**	**Chi-square**	***p* value**
66A>G MTRR	0.1367	6.22	0.0444*	0.1367	6.22	0.0444*

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡denotes approaching significance
Black = Native data including all data points. Blue = log^{10} transformed data with extreme data points removed.

The next model examined population based variables: age and gender. The outcomes are display in the table below.

Table 5-32: Ordinal logistic regression; model for adenomatous polyp plus non-adenomatous polyp – basic population information

	Native data			Transformed data		
Variable	**R^2**	**Chi-square**	***p* value**	**R^2**	**Chi-square**	***p* value**
Age (yrs)	0.0698	12.30	0.0005***	0.0685	11.79	0.0006***

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡denotes approaching significance
Black = Native data including all data points. Blue = log^{10} transformed data with extreme data points removed.

When examined independently (i.e. no genetics or B-vitamins/thiols) age strongly predicts the development of a polyp, this is consistent with the previous model (table 5-30) above and with other published research that demonstrates increasing age is linked to polyp development [152, 153, 958, 959].

The following regression models examined blood metabolites and thiols; these were analysed both combined and individually, and no significance was detected. The examination of dietary folate intake either as 5-methyl-H_4folic acid or as pteroylmonoglutamic acid, or as the two combined, resulted in no relevant outcomes.

Given the findings described above for the entire population, further examination is warranted for the low (below median red cell folate) and high (at or above median red cell folate) folate status group. Again, ordinal logistical regression modelling has been applied to the data set beginning with the below median red cell folate group. The *a priori* basis for categorising variables has been applied as previously, beginning with the ten folate related gene variants.

Table 5-33: Ordinal logistic regression; model for below median red cell folate status – adenomatous polyp - gene variants only

	Native data			Transformed data		
Variable	**R^2**	**Chi-square**	***p* value**	**R^2**	**Chi-square**	***p* value**
66A>G MTRR	0.5799	7.29	0.0261*	0.5799	7.29	0.0261*
3'UTR 6bp del TS		6.86	0.0324*		6.86	0.0324*

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡denotes approaching significance
Black = Native data including all data points. Blue = log^{10} transformed data with extreme data points removed.

The outcomes from this model are identical to the results from the entire population (table 5-29). The 66A>G MTRR and 3'UTR 6bp deletion TS genes are again significant in relation to the occurrence of an adenomatous polyp. The next model performed was in relation to population based indices. Age and gender have been examined for the low folate status group; outcomes are displayed in the table below.

Table 5-34: Ordinal logistic regression; model for below median red cell folate status – adenomatous polyp – basic population information

	Native data			Transformed data		
Variable	**R^2**	**Chi-square**	***p* value**	**R^2**	**Chi-square**	***p* value**
Gender	0.2177	10.55	0.0012**	0.2232	10.62	0.0011*
Age (yrs)		4.49	0.0340*		4.65	0.0310*

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡denotes approaching significance
Black = Native data including all data points. Blue = log^{10} transformed data with extreme data points removed.

The table above (table 5-34) shows that in individuals with a low folate status, gender along with age are important in the development of adenomatous polyps. In keeping with the *a priori* philosophy established earlier, the next analysis included the transsulphuration pathway thiols and blood metabolites, the outcomes are displayed in table 5-35 below.

Table 5-35: Ordinal logistic regression; model for below median red cell folate status – adenomatous polyp – thiols and blood metabolites only

	Native data			Transformed data		
Variable	**R^2**	**Chi-square**	***p* value**	**R^2**	**Chi-square**	***p* value**
Serum Folate (nmol/L)	0.1021	4.08	0.0435*			-

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡denotes approaching significance
Black = Native data including all data points. Blue = log^{10} transformed data with extreme data points removed.

Serum folate acts as a significant predictor of adenomatous polyp within the native data. However, once normalised with outliers removed, this relationship is not maintained. The same model was repeated without the transsulphuration pathway thiols and the outcomes are displayed below in table 5-36.

Table 5-36: Ordinal logistic regression; model for below median red cell folate status – adenomatous polyp – blood metabolites only

	Native data			Transformed data		
Variable	**R^2**	**Chi-square**	***p* value**	**R^2**	**Chi-square**	***p* value**
Red Cell Folate (nmol/L)	0.0578	3.96	0.0465*	0.0586	4.64	0.0312*

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡denotes approaching significance
Black = Native data including all data points. Blue = log^{10} transformed data with extreme data points removed.

Red cell folate level appears to significantly predict risk for an adenomatous polyp when examined in participants with a low folate status. The same *a priori* method for organising ordinal logistic regression modelling was repeated for individuals at or above the median red cell folate value. Examination of this high folate status group resulted in no significant outcomes for any of the parameters examined.

These same models were repeated for data from individuals in the adenomatous polyp plus non-adenomatous polyp group (all polyps). They were delineated according to low and high folate groups. Some marginal significance was uncovered in the low folate group, although as it was fairly limited, it is not included in this thesis. Again, in the high folate group no important statistical outcomes were found to exist.

5.2.8. Integrated analysis of dietary folic acid (type and level of vitamer), folic acid cellular status and risk for colonic adenomatous polyp

In order to further examine the various relationships influenced by dietary folic acid, two histograms, one for each of the folate status groups (below median red cell folate and at or above median red cell folate) are displayed below. The histograms represent the mean and standard error for both natural (5-methyl-H_4folic acid) and synthetic (pteroylmonoglutamic acid) forms of folic acid in controls and adenomatous polyp patients.

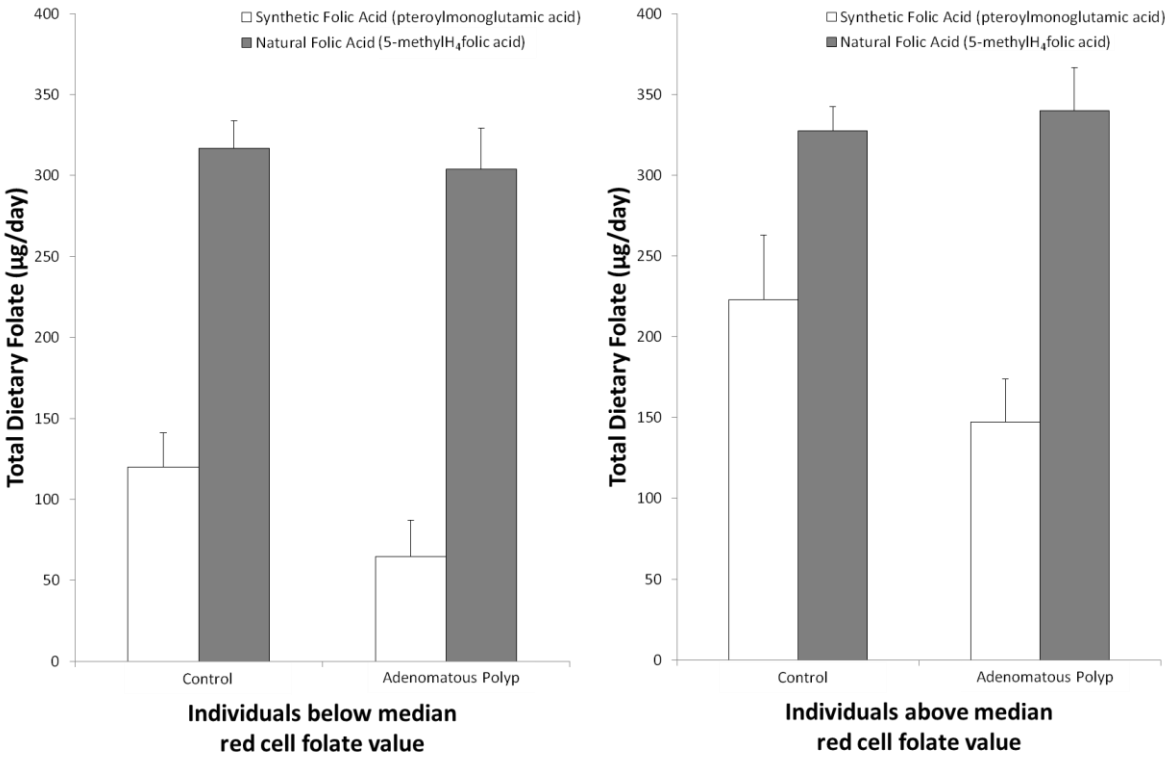


Figure 5-4: Mean folic acid intake (5-methyl-H_4folic acid and pteroylmonoglutamic acid) for control and adenomatous polyp patients by median red cell folate value.

The consumption of these two folyl vitamers in the diet was examined using a standard least squares regression model to see how each one predicts red cell folate status. This potential association was explored in i) all subjects, ii) subjects where red cell folate status was below the population median value and iii) subjects whose red cell folate status was at or above the population median value.

Table 5-37: Analysis using a standard least squares model to examine the relationship between dietary vitamers of folic acid and red cell folate in all subjects

		Native data		
Variable	**R^2**	**Estimate**	**T value**	***p* value**
Total Dietary Folic Acid (µg/day)	0.0929	0.651	4.52	<0.0001****

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001)

Table 5-38: Analysis using a standard least squares model to examine the relationship between dietary vitamers of folic acid and red cell folate for individuals below the population median value for red cell folate status

		Native data		
Variable	**R^2**	**Estimate**	**T value**	***p* value**
Total Natural Folic Acid (µg/day)	0.1116	0.409	3.26	0.0019**
Total Synthetic Folic Acid (µg/day)				-

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001)

Table 5-39: Analysis using a standard least squares model to examine the relationship between dietary vitamers of folic acid and red cell folate for individuals at or above the population median value for red cell folate status.

		Native data		
Variable	**R^2**	**Estimate**	**T value**	***p* value**
Total Natural Folic Acid (µg/day)				-
Total Synthetic Folic Acid (µg/day)	0.0579	0.442	2.44	0.0165*

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001)

The above tables show that, using standard least squares regression, total dietary folic acid profoundly predicts red cell folate level. In the low folate status group (table 5-38) intakes of natural folic acid (5-methyl-H_4folic acid) predict red cell folate status. However, this contrasts with the high folate status group where the synthetic form of folic acid (pteroylmonoglutamic acid) predicts red cell folate level.

Given this interesting relationship between dietary folic acid intake and serum red cell folate level, the next figure (displayed below) shows the difference between controls and adenomatous polyp cases for red cell folate level. Each histogram represents the mean and standard error (error bars) for above and below median red cell folate, respectively, for the entire population.

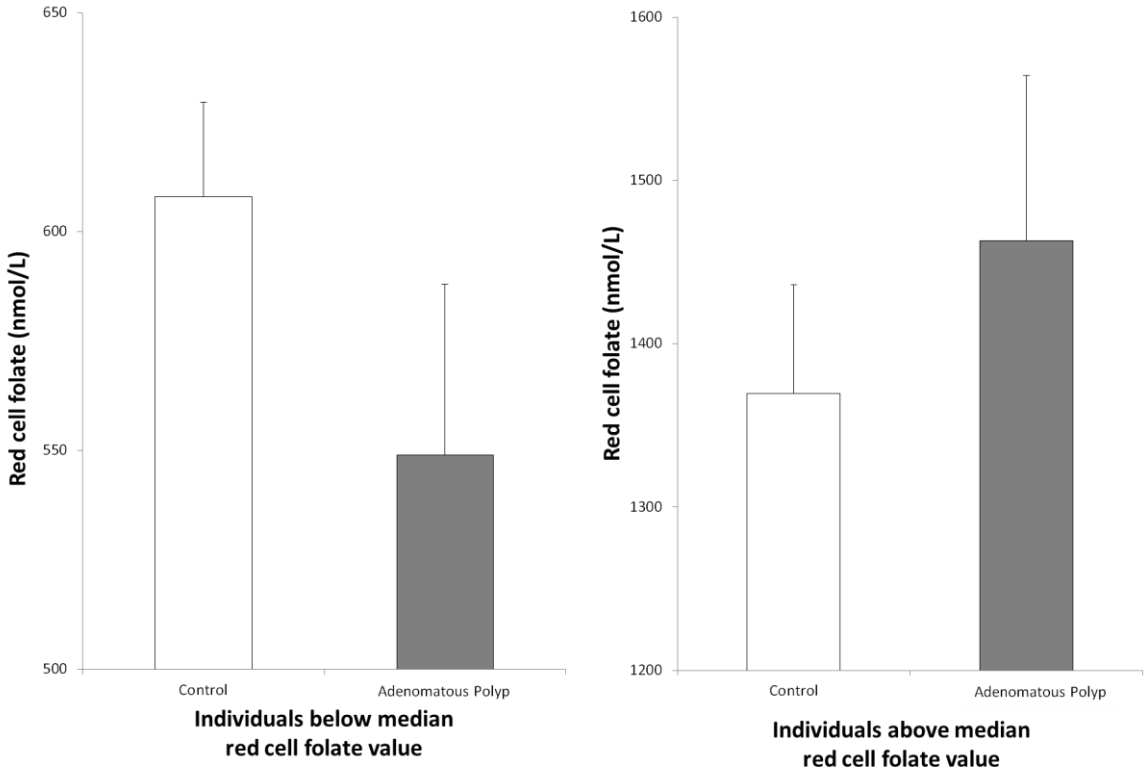


Figure 5-5: Mean red cell folate determined for control individuals and adenomatous polyp patients delineated by whether they are below or above the overall population median red cell folate value.

An unpaired t-test was performed on the data represented in the histograms displayed above (figure 5-5); individuals below the median red cell folate value with no polyps were compared against individuals below the median red cell folate value with an adenomatous polyp. The outcome indicated no significant difference between the control and adenomatous polyp subjects. The t-test was repeated for subjects above the median red cell folate value (controls vs. adenomas polyp) and again no significant difference was found to exist.

In order to further explore the relationship between red cell folate level and risk of adenomatous polyp; additional ordinal logistic regression models were performed. This time the models examined only blood folate parameters (serum folate and red cell folate) in relation to adenomatous polyp. The first model displayed below is for the low folate status group (below median red cell folate).

Table 5-40: Ordinal logistic regression; risk for adenomatous polyps below median red cell folate value – examination of all blood folate parameters

	Native data			Transformed data		
Variable	**R^2**	**Chi-square**	***p* value**	**R^2**	**Chi-square**	***p* value**
Red Cell Folate (nmol/L)	0.0529	3.91	0.0480*	0.0548	4.54	0.0331*

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡denotes approaching significance
Black = Native data including all data points. Blue = log^{10} transformed data with extreme data points removed.

As displayed in table 5-40 above, red cell folate levels predict the occurrence of an adenomatous polyp in the low folate status group (below the median red cell folate value). The same model was repeated for the above median red cell folate subjects; the outcomes show that red cell folate level is approaching significance for predicting the occurrence of an adenomatous polyp (*p=0.0507, table not included*). Finally, the above two models have been repeated to include gender with the blood folates.

Table 5-41: Ordinal logistic regression; risk for adenomatous polyps below median red cell folate value – examination of blood folate parameters and gender

	Native data			Transformed data		
Variable	**R^2**	**Chi-square**	***p* value**	**R^2**	**Chi-square**	***p* value**
Serum Folate (nmol/L)	0.2602	6.45	0.0111*	0.2395	4.49	0.0341*
Red Cell Folate (nmol/L)		5.57	0.0183*		6.09	0.0136*
Gender		14.09	0.0002***		13.36	0.0003***

Asterisk(s) denotes significance (p=<0.05,** p=<0.01,*** p=<0.001,**** p=<0.0001) ‡denotes approaching significance
Black = Native data including all data points. Blue = log^{10} transformed data with extreme data points removed.

Clearly, when gender is included in the model, it and both folate parameters show a significant relationship with adenomatous polyp occurrence.

The above ordinal logistic regression was repeated, but this time including subjects who were above the median red cell folate value; the model included both folate parameters and gender and no significance was found to exist.

Summary of results for B-vitamin nutritional genetics and occurrence of an adenomatous polyp – a major antecedent of colorectal cancer

Data in this chapter indicates that several parameters predict polyp risk; of the non-B vitamin related 'population' indices, age is a highly significant factor and gender a moderately significant predictor of adenomatous plus non-adenomatous polyps.

Data analysis clearly shows that the 66A>G MTRR and 3'UTR 6bp deletion TS gene variants contribute to risk for adenomatous polyp occurrence, with these genes being an important factor in subjects with low folate intakes. In contrast, the 1420C>T SHMT gene variant contributes a positive risk for occurrence of an adenomatous polyp in subjects with a high folate status (above the median red cell folate value of 868.5 nmol/L).

Other notable associations exist for the types (vitamers) of dietary folic acid consumed, with overall total dietary folic acid intake predicting red cell folate level. However, once split according to median red cell folate value, the below median value subjects had a red cell folate level that was predicted by natural folic acid (5-methyl-H_4folic acid) consumption. But when the above median value subjects were examined, synthetic folic acid (pteroylmonoglutamic acid) consumption predicted red cell folate level.

Subjects with a low folate status (below median red cell folate value) were found to have red cell folate levels that predicted adenomatous polyp occurrence. No similar significance occurred in the high folate (above median red cell folate value) subjects.

CHAPTER 6

DISCUSSION

6. Overview

This discussion will examine and review results for each of the four phenotypes under investigation taking account of the contemporary literature in this field. It will additionally consider study limitations, and give a brief discussion relating to the implications of mandatory folic acid fortification. Finally, gaps in the research and ideas for future endeavour will be explored along with the important role of recent technological advances in the analyses of, and interaction between, genes, nutrients, and disease, providing the appropriate conclusion to this thesis.

6.1. The Role of Folic Acid and Nutritional Genetics in Common Chronic Degenerative Disorders

Folic acid has gained considerable scientific attention in recent years, and compared to most other micronutrients, has been, and continues to be studied comprehensively and across a broad sphere of clinical conditions. Such attention may, in part, be due to the complexities that surround its biochemistry: variation in chemistry can lead to as many as 150 different vitamer forms [377]. Structurally, there can be different oxidation states of the main pteridine ring, there can be a number of different one-carbon adducts and also the number of conjugated glutamic acid residues can differ (see figure 1-3 on page 34) [283]. This variation permits a number of critical cellular processes to occur which are now inexplicably linked to the modulation of a range of both developmental and degenerative disorders.

As already described in the introductory chapter, folic acid is a one-carbon donor that is critical in the synthesis of DNA, methionine, purine and amino acids. One carbon metabolism contains two parallel cytosolic and mitochondrial pathways; these are connected by one-carbon units that are derived from the catabolism of serine, glycine and formate [315]. The sequencing of the human genome has enabled the identification of polymorphic proteins that participate in compartmented one-carbon metabolism. This has enabled a greater understanding of how these polymorphic proteins can modify metabolism and the crucial role that folic acid plays as a mediator, thereby influencing underlying disease risk. Historically, a deficiency of folate manifested as the disease megaloblastic anaemia, but when pharmacological doses were proven to prevent NTDs, and regulate vascular toxic homocysteine, research interest intensified, purporting folic acid to be a nutritional wonder drug – a potential panacea for a great many ills.

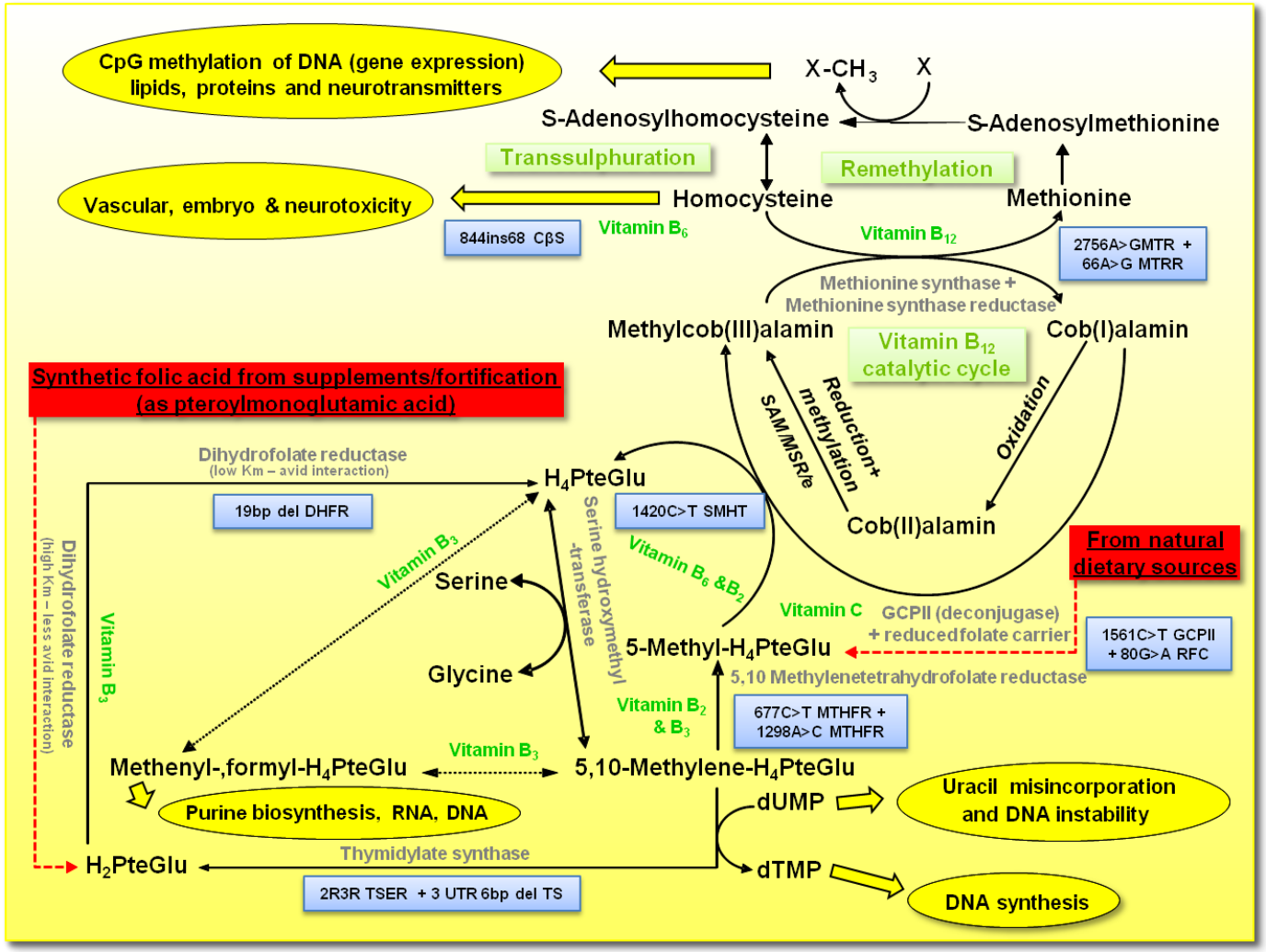


Figure 6-1: Folate biochemistry with key gene-nutrient interactions that can modify clinical phenotype (Figure courtesy of A/Prof M Lucock article Folic acid: Beyond Metabolism [377])

Unfortunately, the past 5 years have seen the tide change somewhat, and several significant publications have reported negative health and metabolic effects, including associations with some common cancers (see section 1.3.4 above for more details). The figure 6-1 above, aims to simplify the complex interactions that involve folic acid. It includes not just a simple annotation of folate biochemistry, but also combines the key folate-related SNPs that influence the phenome. It also includes the consequences of key gene-gene or gene-nutrient interactions that collaborate to yield a wide and seemingly disparate range of high-risk clinical phenotypes. The impact on genomic integrity may be very subtle and even marginal deficiencies or more recently, an altered balance in the forms of folate can change the equilibrium of genomic stability. Folate is not alone in this respect, and the influence of other B-group vitamins also has a significant role in many developmental and degenerative disorders - in the case of vitamins B_6 and B_{12} there are crucially attuned synergies with folate. There is still a lot to learn about folate nutritional genetics and phenomics, particularly in the context of correlates at the end of the human life cycle.

This thesis has focused on degenerative conditions associated with ageing. This is predominately because globally, most western populations are ageing. According to government statistics, a century ago if you were a male you would on average live to see your 55st Birthday [62]. For women it was closer to the 58th year of life [62]. But in just one century, longevity has increased 28 years for males and almost 30 years for females [62]. It was recorded in June 2011 that there were 3.01 million people in Australia over the age of 65; there has been a 14% increase in just the past 5 years alone [62]. It is predicted that by the year 2050, the number of people over 65 will double, but due to declining fertility rates there will be half the number of taxpayers to support this large older population [62]. At those rates, there will be more than 5% of the population over 85; a group for whom the need for services and assistance in health care will generate an exceptional burden for society.

Although modern medicine combined with exceptional healthcare is enabling people to live longer, it is also resulting in an increase in age-related chronic diseases – an obvious, but unfortunate correlate of a longer life. These statistics create a distinct need to better understand the various biologic and environmental determinates of healthy ageing. In particular, what role do chronic diseases play in ageing, and at what point in the lifecycle to they originate. Uncovering common features of disease processes should lead to better treatment and ultimately prevention. A part of this discovery needs to be the recognition that treatments must consider the individual

person, and be specialised to meet specific genetic and environmental risk factors, but also develop into realistic and affordable methods aimed at preventing chronic disease. This is the concept of personalised nutrition, which has the potential to eliminate this enormous burden of age-related morbidity that society endures.

Using an elderly Australian retirement village population (n=233) it was established that age was associated with diastolic blood pressure. This was the case for both recumbent *(p=<0.0001*) and standing (*p=<0.0001*) blood pressure measurements (table 3-23). Typically, a progressive increase in blood pressure during a person's lifespan is observed, until a person reaches around the age of 60, at which point the systolic and diastolic pressures diverge onto different trajectories, with systolic continuing to rise but the diastolic falling [960]. The present results do indicate a decreasing diastolic pressure with age, which is consistent with results from the Framingham Heart Study and other previous cross-sectional studies [960-963]. There are various explanations for this age-related fall in diastolic blood pressure. Some involve a 'burned out' diastolic hypertension or a 'selective survivorship' theory, but the best explanation lies with an increase in large artery stiffness [960, 964-966]. This results in less blood remaining in the aorta at the beginning of the diastole and combined with decreased elasticity, diastolic pressure decreases. Normal ageing is associated with progressive endothelial dysfunction and degeneration of the vasculature; however, falls in diastolic blood pressure are associated with an excess risk of atherosclerosis [967, 968]. It is considered that the decline in diastolic blood pressure seen in the elderly is probably the result rather than the cause of the disease process. A more detailed examination of the potential causes for this within the hypertensive phenotype is examined below.

In addition to the significant findings on age and diastolic blood pressure, data from the same population was used to examine the depression phenotype. It was found that age and depression are closely associated (table 3-40). Although not statistically significant, this association is a well-recognised one [946-948]. Ageing is associated with a decline in cognitive function, and is sometimes confused with dementia; depression can in part be explained by changes in neural plasticity or cellular alterations that directly affect mechanisms of plasticity [969]. These cellular alterations may include increases in homocysteine concentration and decreasing B-vitamin status, which will be elaborated on below.

Finally, another significant age-related finding was found in the study on colorectal polyps. This cohort (n=203) had a lower average age range then the previous cohort,

as the focus was not over 65s but individuals aged 40 and over; a time when colorectal cancer incidence increases. The analysis of this cohort showed age to be a significant factor in the presence of a colorectal polyp of any kind (*p=0.0006*, table 5-32). This is consistent with evidence showing ageing to be the most important risk factor in the development of colorectal neoplasia [152, 153, 958, 959]. The incidence of cancer rises sharply with age and although there are various modifiable risk factors that play a role in colorectal cancer, such as diet and physical activity, age is a consistent non-modifiable risk factor [970]. It can be said that cancer, particularly colon cancer, is a disease of ageing that possibly arises through a cumulative exposure to carcinogens over time [970, 971]. However, ageing *per se* is not a disease process, although changes in physiology that accompany ageing may make people more susceptible to chronic degenerative disease.

Although this thesis reports statistical associations with age and certain of the phenotypes examined, the central purpose was to closely examine gene-nutrient interactions and the impact they have on phenotypic traits. These interactions that centre on folate and its metabolism, include specific candidate polymorphisms, which are often critical for major biological pathways. The key SNPs examined are known to exhibit functional relevance, and this has led to the present examination of them within each of the phenotypes reported here. Before thoroughly exploring these phenotypes, it is worthwhile mentioning that, in order to successfully examine complex biological epidemiological interactions appropriate statistical analysis is required, along with adequate sample size to support evidence for gene-nutrient interactions. The studies presented here are limited in their sample size, and thus the gene-nutrient interactions reported in this thesis would undoubtedly benefit from confirmation in much larger study populations. Further discussion on such limitations is detailed below (page 279).

There were also considerations to be taken account of in the assessment of B-vitamin blood metabolites. As described in biochemical measurements (page 108), the assay of serum folate, red cell folate and vitamin B_{12} all have calibration limits. In our study populations there were several recorded values above the upper limit of measurement. These plateau values are highly indicative of pharmacological intervention; but it would be inappropriate to simply exclude these as outliers because the saturation is still indicative of a high blood metabolite level, which is biologically relevant. For this reason, the data was treated in two ways; the complete data set in an unmodified format referred to as ‘native data’, and a ‘transformed data’ set that had the extreme (or saturated) values removed, and was $\log_{10}$ transformed to achieve normality. All

analyses were performed in this manner. The term pharmacological intervention is reflective of wide scale use of supplements and fortification of food groups, and further discussion on the implications of this is detailed below.

6.1.1. Phenotype I: hypertension

The leading cause of death in Australia is CVD, and one of its many clinical risk factors is hypertension. Defined as a blood pressure of ≥ 140/90 mm Hg, it is a silent risk factor with little symptomology and is often under-diagnosed [119]. Hypertension is consistently associated with increasing rates of CVD; with lower blood pressures, observational studies find lower rates of death, stroke and coronary heart disease [120, 121]. In addition to this, elevated homocysteine level (hyperhomocysteinemia) is an independent risk factor for both hypertension and CVD, as well as stroke and myocardial infarction to name a few [232, 335, 747, 972-975]. Dietary folate, and to a lesser extent vitamins B_{12} and B_6 are determining factors in homocysteine concentration. It is worth noting that two significant groups in society with elevated homocysteine are the elderly and vegans who are both often reported to be at risk of low vitamin B_{12} status.

As previously discussed, variation in gene products that depend on these vitamins (folate, vitamins B_{12} and B_6) can alter homocysteine status and therefore modify risk of disease. Indeed, these SNPs may alter folate status and this might have a direct influence on CVD risk. Alternatively, folate might have a direct effect on blood pressure via mechanisms independent of homocysteine. Folate is known to enhance endothelial nitric oxide which is a marker of cardiovascular health; it also has anti-inflammatory actions via the inhibition of oxygen-derived free radicals such as the superoxide anion and hydrogen peroxide [770, 771, 976, 977]. Additionally, folate can stimulate the regeneration of tetrahydrobiopterin, which is a necessary co-factor for nitric oxide production, and these actions of folate are all independent of its capacity to lower plasma homocysteine levels [761, 771, 978]. Significantly, impaired endothelium-dependent vasodilatation due to reduced nitric oxide production is a key risk factor in hypertension, making folate a critical component in this process [979].

In the study of hypertension in the elderly retirement village population, blood pressure was confirmed on three separate visits in both the recumbent and standing positions, also recorded was a recumbent pulse rate measurement. Each of these measurements was averaged to determine a final blood pressure result. It was determined that of the total population (n=229) 51 participants were classified as having hypertension. The

majority of the analysis for this phenotype was conducted using stepwise linear regression modelling, treating the blood pressure measurements as continuous data. However, with the ability to split the population into normotensive and hypertensive phenotypes, various other categoric statistical analyses were applied to examine potential nutrigenetic correlates.

This study uncovered several genetic relationships to blood pressure. The most significant of these was the 677C>T MTHFR SNP. This SNP was associated with recumbent diastolic blood pressure (*p=0.0139,* table 3-21), although significance was also determined for standing systolic blood pressure in a regression model containing only the eleven genetic parameters (*p=0.0443* table 3-22). As expected, a post hoc test confirmed the difference lies between the CC and TT genotype, suggesting the mutant allele is a potential risk factor for hypertension in the elderly. This is consistent with various meta-analyses that support a role for the TT genotype in CVD risk [475, 476]. Furthermore, the hypertensive subjects had a significantly lower level of serum folate (figure 3-2) when compared to normotensives. Also, serum folate levels in both the whole population (table 3-6) and the hypertensive subjects (table 3-17) were significantly different across each of the genotypes for 677C>T MTHFR, but no significant variation was present among the normotensive subjects (table 3-13). These additional findings suggest that the elderly participants may be hypertensive due to either or both their low serum folate levels and or their 677C>T MTHFR genotype.

However, in terms of red cell folate, which is often considered to be the best measure of overall systemic folate status, the results were inconsistent. There was significant variability between the genotypes for 677C>T MTHFR for the entire population (see table 3-6 on page 139), however, no variability existed when blood pressure was considered. Additionally, the red cell folate levels of TT carriers were significantly higher than the other genotypes, which is contrary to the vast amounts of evidence for this genetic mutation. As described in section 1.2.11, page 54, the 677C>T MTHFR SNP directly affects MTHFR which is the sole producer of 5-methyl-H_4PteGlu. Consistent evidence points towards the fact that this mutation leads to a decreased availability of 5-methyl-H_4PteGlu, which has a considerable effect on the functioning of one-carbon metabolism. Possible reasons for the conflicting findings are that within this population there were disproportionate numbers of people consuming fortified foods and vitamin supplements, which contained high levels of pteroylmonoglutamic acid. This also translated into many people who had red cell folate levels that were outside the upper reference range for the assay. Alternatively, another explanation could be

that the synthetic form of folate causes a differential response in the assay, which does not occur with the natural form of folate. This could account for the high levels of red cell folate within the recessive (TT) genotype group. Certainly, explanations for contradictory findings exist, but assessing them in a comprehensive way is outside the scope of this thesis, although some mention of them is justified.

Given the amount of supporting evidence indicating this folate related genotype increases homocysteine and decreases folate levels, and that folate is associated with tetrahydrobiopterin-related nitric oxide metabolism, which is critical in blood pressure regulation, it is not surprising to find this phenotypic gene-nutrient relationship for hypertension [770, 771, 976, 978, 980]. In addition to this, MTHFR's second common mutation 1298A>C was also associated with recumbent diastolic blood pressure (*p=0.0102,* table 3-21). However, when multiple regression was performed on only the eleven SNPs the association was reduced to a marginal, but non-significant result (*p=0.0504,* table 3-22). Like its better known counterpart, this mutation resembles 677C>T by decreasing MTHFR activity resulting in the same increase of homocysteine and decrease of serum folate levels [469, 523]. However, no significant results were generated from the one-way ANOVA that examined 1298A>C MTHFR and its influence on variability of blood indices such as serum folate, red cell folate, and homocysteine (table 3-13 and table 3-17). Despite this, there have been suggestions that the C allele is associated with early onset coronary artery disease regardless of homocysteine concentration [530], and a small Indian study found the combined 1298 CC and the 677 CT compound genotype were associated with an increased risk of hypertension [981]. In terms of these results it is not clear as to why the significant association with these two MTHFR genetic mutations, and the clinical phenotype of hypertension, did not translate into a marked alteration in blood metabolite levels, particularly for homocysteine.

The next genetic association found to exist for this phenotype was the 19 bp DHFR deletion SNP. This polymorphism was found to be associated with systolic pressure in both the standing and recumbent positions (table 3-21). Since its discovery, the 19 bp del/del genotype has been reported to lead to increased DHFR expression, which ultimately facilitates the remethylation of homocysteine, and correspondingly when compared to wild-type carriers, the del/del is associated with reduced plasma homocysteine concentrations [546]. An explanation for this is unclear as DHFR is remote from homocysteine remethylation, however hypotheses have been put forward [546, 982]. Despite conflicting studies [547], some authors suggest that this deletion

allele with its higher expression of DHFR might predispose one-carbon metabolism to favour DNA synthesis because of ultimately higher levels of 5,10-methylene-H_4PteGlu [548, 549, 880]. If compounded with the 677TT genotype, whose lower enzyme activity leaves more 5,10-methylene-H_4PteGlu in the cell, the compound del/del/TT genotype may assist TS activity which could reduce detrimental uracil misincorporation and therefore avoid DNA instability [880]. This has been hypothesised in the case of acute lymphocytic leukaemia in which Gemmati *et al.* [983] consider the deletion allele to be protective rather than a risk factor. These same factors could also play a role in hypertension, where carriers of the deletion gene might be afforded protection due to a lower risk of hyperhomocysteinemia, and a better folate status. However, alteration in blood metabolite levels was not observed in the elderly population in the present study.

Another genetic association found to exist in this elderly population was in the context of standing systolic blood pressure and 1561C>T GCPII (*p=0.0397*, table 3-22, it should be noted that no recessive TT genotype was detected). It has previously been suggested that this SNP may affect predisposition to CVD, as it has been known to alter red cell folate and plasma folate concentrations [661]. However, conflicting evidence exists, with the Framingham Offspring Study finding the mutation was not a determinant of plasma folate or total homocysteine concentrations [984]. The original author of the first publication, Devlin *et al.* [422] suggested that the allele was significantly associated with lower folate and higher homocysteine levels because the mutation impairs intestinal absorption of dietary folates, resulting in relatively low folate levels and as a consequence, hyperhomocysteinemia. Given these conflicting gene-nutrient findings and the relatively low prevalence (estimated to be less than 2% of the population [422, 642, 653, 661, 663]), the explanation for this association with systolic blood pressure is unclear. The present data would support a prevalence towards the bottom of this range.

Despite these genetic associations which were obtained after treating blood pressure as a continuous variable using multiple regression analysis; examination of the data as a categorical variable (hypertensive/normotensive) resulted in no polymorphism being significantly associated with hypertension. The results of odds ratio analysis (± 95% CIs and Yates corrected chi-square *p* values) demonstrated no associations with this phenotype (table 3-12, page 146). Significance is related to recruitment size and large numbers are often required when examining candidate polymorphic genes. The present study was relatively small (n=229).

In addition to the genetic relationships uncovered, there were also significant associations with the B vitamin and thiol indices. Red cell folate, total dietary folic acid, cysteine and cysteinyl-glycine are all predictive of recumbent diastolic blood pressure (table 3-21). Given that red cell folate represents the best measure of folate status, this finding is consistent with the vast majority of research that demonstrates a role for folate in cardiovascular health and function [985, 986]. The results from a stepwise regression model specifically for blood metabolites showed that red cell folate concentration is associated with both lower diastolic blood pressure (table 3-26, *p=0.0236*) and lower pulse rate (table 3-26, *p=0.0293*).

There are a variety of potential mechanisms in which folate may be an important determinant of hypertension, beyond the already model described genetic interactions. The maintenance of normal blood pressure involves endothelium derived nitric oxide; this assists in vasodilation and is critical in maintaining the elasticity of the vascular wall of arteries [761]. The most prominent mechanism in which folate impacts blood pressure is through the distinct relationship involving the regulation of homocysteine. Homocysteine is known to decrease the bioavailability of nitric oxide and increase production of reactive oxygen species. Reactive oxygen species such as superoxide anions and hydrogen peroxide can lead to oxidation and depletion of tetrahydrobiopterin, which is an essential cofactor in nitric oxide biosynthesis [761]. This homocysteine induced oxidant stress can lead to injury of the endothelium, which alters the elastic properties of the vasculature, affecting vasomotor regulation, and hence leading to increased blood pressure [987].

In addition to lowering homocysteine, folate can directly impact blood pressure via pleiotropic effects. The pteridine ring structure of folate coenzymes is similar to that of tetrahydrobiopterin, and it is thought that when tetrahydrobiopterin is low, H_4PteGlu can serve as a replacement cofactor and bind directly to eNOS [978]. Although this alternate scenario is thought to be somewhat inefficient, it is does stop further oxidation of tetrahydrobiopterin. Another hypothesis is that under oxidative stress, tetrahydrobiopterin is oxidised to its inactive form, dihydrobiopterin. Folate may restore dihydrobiopterin to its reduced form by donating hydrogen/electrons. With tetrahydrobiopterin restored, *eNOS* can be recoupled and production of nitric oxide is normalised [770, 976]. These anti-oxidative effects, which are independent of homocysteine, contribute to repairing *eNOS,* enabling the maintenance of regular blood pressure.

Interestingly, dietary pteroylmonoglutamic acid is fully oxidised, and therefore cannot act as a reducing agent like the natural forms of folate. This is important because in the hypertensive cohort examined, an inverse association between blood pressure and both total dietary folic acid and natural dietary folic acid was uncovered (see table 3-21 *p=0.0139* and table 3-27 *p=0.0485* respectively). However, no association with the synthetic form - pteroylmonoglutamic acid was found. While it is possible to synthesize 5-methyl-H_4PteGlu from pteroylmonoglutamic acid, their distinct chemical properties may result in different effects on the oxidation of tetrahydrobiopterin and the nitric oxide system. Each form of dietary folic acid may therefore differ in their role of regulating blood pressure. Despite the lack of association in this study, there have been some clinical studies that have demonstrated that synthetic folic acid improves endothelial function in vivo [977, 980], and in a large study of over 7,000 nurses, synthetic folic acid appeared to be independently associated with a reduction in risk for hypertension [255]. Dose of synthetic folic acid may also be important, given the findings from a small study of coronary artery disease patients, which found only high doses (5mg/day) and not low doses (400µg/day) was associated with an effect on atrial flow mediated dilation, a marker for endothelial function [988].

As folate coenzymes are responsible for driving synthesis of methyl groups in the remethylation of homocysteine, given the knowledge that homocysteine is an independent risk factor for CVD, including hypertension, an association between blood pressure and homocysteine concentrations might have also been expected in this study, but none was established (table 3-21 and table 3-25). A possible reason for this is that folate levels within the recruited cohort were relatively high, most likely due to increased discretionary intake of supplemental folate. This elevation in folate status has likely masked any association that might have existed between homocysteine and elevated blood pressure. This cohort was recruited prior to the implementation of mandatory fortification, so the extent of masking may now be even greater, any effect homocysteine might have on cardiovascular function may be becoming increasingly difficult to observe. Despite this finding, very clear associations have been demonstrated with other thiol metabolites. In this respect, both cysteine and cysteinyl-glycine are predictive of recumbent diastolic blood pressure (table 3-25). With cysteine, an inverse linear relationship exists, possibly indicating a protective association between cysteine levels and elevated blood pressure. This is perhaps due to cysteine acting as an antioxidant protecting nitric oxide production from the oxidative effects of reactive oxygen species, and therefore preserving its bioavailability [989].

Cysteine is an important antioxidant formed endogenously from the metabolism of methionine. It is a substrate in the biosynthesis of another vital antioxidant, glutathione. Glutathione is a tripeptide containing glutamate and glycine, and was also measured in the sample population although no significant relationship to hypertension was established (table 3-25). Cysteine in combination with glutathione form part of the thiol pool that forms nitrosothiol, this can increase the bioavailability of nitric oxide and potentiate vasorelaxation and anti-inflammatory effects [989, 990]. Like folate, a disruption to cysteine's availability or an increase in thiol demand affects vascular function, which may contribute to the aetiology of hypertension. As an aside, using the current study population, a novel investigation into the effect of hydrogen sulphide and thiol metabolism in hypertension was examined. Hydrogen sulphide is a gaseous signalling molecule, similar to nitric oxide, which can regulate blood flow, arterial diameter and hence pressure, as well as being a vital component of endothelium-derived relaxation factor [991]. It was determined that cysteine was a biological determinant of hydrogen sulphide and therefore could potentially influence the hypertensive phenotype [992]. This provides another example of how cysteine might influence blood pressure regulation, and like folate, could potentially be used as a biological marker relevant to the pathoaetiology of hypertension.

In addition to these findings relating to cysteine, cysteinyl-glycine was also predictive of recumbent diastolic blood pressure. Cysteinyl-glycine is a pro-oxidant thiol generated during the catabolism of glutathione. Unlike cysteine, there was a direct linear relationship to diastolic blood pressure, indicating a potentially harmful association of cysteinyl-glycine levels on blood pressure. The formation of cysteinyl-glycine occurs through the action of γ-glutamyl-transferase, and can be catabolised to cysteine through the action of cysteinyl-dipeptidase activity [339]. Given, cysteinyl-glycine levels are correlated inversely with cysteine, the results uncovered are perhaps not surprising. Interestingly, elevated γ-glutamyl-transferase which assists in the formation of cysteinyl-glycine is a recognised predictor of CVD, partly because it catalyses the cleavage of glutathione [367-369]. It also has a role in the iron-dependent oxidation of LDL within atherosclerotic plaques [372]. Cysteinyl-glycine has been positively associated with myocardial infarction [371], and given its pro-oxidative actions it is not surprising that there is an association with blood pressure.

Given the four thiols examined are all metabolically inter-related and that they are all important in free radical formation and the maintenance *eNOS*, the results presented here are perhaps not surprising. The fact that there was no significant association

between homocysteine and blood pressure was not totally unexpected, as there is conflicting evidence for this relationship, and the small population sample size is also a likely factor. Many epidemiologic studies have linked plasma homocysteine concentrations to blood pressure; especially systolic pressure [993-996], but some of these studies examined a much younger population (<50yrs) than the participants in the present investigation. Other studies have reported no statistical significance between the two variables [330, 384, 997], and there is ongoing debate as to whether the increased plasma homocysteine levels in hypertensive people are concomitant rather than a precursor of high blood pressure [998, 999]. Whether hyperhomocysteinemia exhibits a casual or causal link to hypertension needs to be clarified, particularly given the role of folic acid in lowering elevated levels. This may be difficult to prove given the antihypertensive effects of folate, which are independent of the homocysteine lowering effect of this vitamin.

The establishment of a relationship between blood pressure and red cell folate is an important one that is often reported. However, folate shares a close synergistic relationship to vitamin B_{12}, a relationship that affects the utilisation and function of folate in one-carbon metabolism. Vitamin B_{12} is also responsible for the remethylation of homocysteine to methionine. Serum vitamin B_{12} was predictive of systolic blood pressure in this investigation, but this result was much weaker than the other previously discussed results (table 3-21). The results for vitamin B_{12} are affected by elevated values that are at, or approaching the assay calibration limits, and thus indicative of pharmacological intervention which is not uncommon in elderly subjects.

Like red cell folate and cysteine, there was an inverse linear relationship with vitamin B_{12}. This is consistent with the knowledge that low levels of vitamin B_{12} are associated with hyperhomocysteinemia [973, 1000, 1001], and through impaired endothelium-dependent vasodilation is a likely factor in hypertension. Unfortunately, vitamin B_{12} is often overshadowed by folate, and focused research on this key vitamin is limited, particularly in relation to hypertension. It has been suggested that within an environment where mandatory folic acid fortification occurs, vitamin B_{12} becomes a better nutritional determinant of homocysteine levels than serum folate [1001]. There is evidence that this is the case for carotid plaque formation, which is a strong predictor of cardiovascular events [1002]. Certainly, there have been calls for the fortification of enriched cereal grain products to include vitamin B_{12}, [398, 870, 873, 1003] but no action on this has occurred, future research will hopefully examine the role of vitamin B_{12} and vascular health more closely.

Finally, in addition to the association of increasing age with diastolic blood pressure highlighted above, a stepwise regression model also examined using other population-based indices. BMI and gender were examined: BMI was positively associated with diastolic blood pressure, and gender was found to be a factor in pulse rate (see table 3-23, page 158). Such findings are to be expected and consistent with the existing literature, especially for body weight and hypertension. BMI as an indicator of obesity is independently associated with hypertension, CVD, diabetes and total mortality [1004-1007]. As described in the first chapter, obesity is increasing worldwide because developing countries are joining Western Nations in consuming energy-dense, nutrient poor foodstuffs that are typically high in fat and refined carbohydrates. This is exacerbated by the fact that populations are living increasingly sedentary lifestyles.

It is well recognised that the relationship between obesity and hypertension is multifaceted, with impaired endothelial vasodilation and arterial stiffness likely contributors [1008]. Obesity also leads to extracellular volume expansion and the demands for greater blood flow [1009], but body fat distribution, particularly visceral abdominal fat, shares the strongest association with hypertension, mostly likely because of compressed kidneys which can increase renal artery pressure [1010]. In terms of the relationship between resting pulse rate and gender, the association is well known [1011-1015]. The gender difference in the incidence of hypertension and CVD in men and post-menopausal women is much greater than premenopausal women [1016-1018]. This is mostly like due to vascular protective effects of female hormones such as oestrogen and progesterone; oestrogen in particular, can beneficially modify direct functions of the endothelium and vascular smooth muscle [1018].

In summary, the results from this investigation of a hypertensive phenotype and nutritional and genetic factors that underpin the pathoaetiology of this condition clearly show that folate dependent one-carbon metabolism and the related transsulphuration pathway are important with significant associations detected. Most notably the 677C>T MTHFR variant, red cell folate status, total dietary folic acid, cysteine and cysteinyl-glycine seem to be relevant in the hypertensive phenotype.

6.1.2. Phenotype II: depression

As outlined in the introductory chapter, depression is considered to be a leading cause of disability, and in terms of disease burden, is second only to coronary heart disease [62]. Given depression affects a significant number of older people and under diagnosis is prevalent; an examination of this disorder in this NSW elderly retirement

village population is clearly justified. A consensus view now exists that lower folate levels are associated with depression, and this may be due to altered one-carbon metabolism resulting in elevated homocysteine concentrations and/or the role of folate either directly or indirectly in neurotransmitter synthesis [187-192, 785].

In the present study on depression in the elderly NSW retirement village population, participants used the 14 item self-reported HADS to determine current anxiety and depressive symptomatology. Before participating in the wider study individuals were screened for reduced cognitive function using the MMSE (see page 134 for further information). The HADS results were assessed for internal consistency and reliability. Consistent with other research [943], a HADS score of 8 or more on either scale is considered to indicate anxiety or depression. Using this defined cut-off it was determined that of the total population (n=229), 17 individuals were classified as depressed. The statistical analysis for this phenotype was conducted in two ways; the nature of the HADS questionnaire results provided data that was both numeric continuous and categorical. No consistent approach has been taken in the existing literature, and therefore both approaches have been presented here with no assumptions relating to the relative merits of either of the two assessment methods being made. The numeric data was analysed using stepwise multiple regression, and ordinal logistic regression was employed for the categorical data analysis. Additionally, with the ability to divide the population into depressed and control phenotypes, various other statistical analyses could be applied to examine potential nutrigenetic correlates.

Ordinal logistic regression analysis resulted in depression being associated with red cell folate, cysteine, cysteinyl-glycine and the genetic polymorphism 677C>T MTHFR (see table 3-43). These significant outcomes were only indicative of an effect for native data, which was untransformed and retained supraphysiological values from likely pharmacologic intakes. The only significant result maintained once the data was $\log_{10}$ transformed and exclusions were made based on supraphysiologic intake and the consequent outlying values, was cysteinyl-glycine (*p=0.0493*). Additionally, the results of stepwise multiple regression for continuous numeric data also resulted in cysteinyl-glycine being associated with depression (table 3-39, *p=0.0115* and table 3-42, *p=0.0046*). No other genetic, nutrient or thiol association was evident using this approach to the data analysis.

The observed association between 677C>T MTHFR and the depression phenotype has previously been described [189, 1019-1021], and a large meta-analysis containing 10 studies with over 11,000 participants and 1,280 cases of depression support this

association [484]. Unfortunately, the results from the present investigation are not supported by a significant odds ratio analysis, which was employed to detect any potential association between the genetic mutations studied and depression (table 3-30). The number of clinical conditions that the 677C>T MTHFR variant is associated with is now quite large, and it likely modulates disease risk via a range of molecular mechanisms because this mutation results in reduced catalytic activity of the expressed enzyme MTHFR. Obviously, MTHFR is critical in the delivery of one-carbon units for conversion of homocysteine into methionine, as well as for DNA metabolism. Therefore, in the context of this phenotype, it could potentially alter *de novo* methylation, a particularly important component within the pathway for biogenic amine neurotransmitter synthesis. There is considerable evidence suggesting that in depressive disorders there is a dysfunction of amine neurotransmitter systems [192].

Given this polymorphism results in elevated homocysteine, and elevated homocysteine lowers SAM concentrations, which can impair methylation of DNA, myelin, neuronal receptors and neurotransmitters, it's not surprising that there is a likely impact on brain function [192]. Furthermore, as described in the hypertensive phenotype section, homocysteine enhances oxidative stress, as well as contributing to endothelial damage and hence possible cerebral ischemia [769, 789]. Homocysteine is also excitotoxic; in the brain it can either act as an agonist at glutamate receptors, or it blocks the glycine site of the NMDA receptor complex [192, 790]. This modulation of NMDA receptor activation has led homocysteine to be considered as having a role in the pathophysiology of depression. Despite a likely influence of 677C>T MTHFR on homocysteine concentrations, independently it was not a predictor of depression, however the particularly small sample size may account for this lack of association.

In addition to the association between 677C>T MTHFR and depression, some other SNPs have also been implicated as having a role in depression. This study examined a common mutation in COMT, which as previously discussed (page 71) is a methylation enzyme that catalyses biosynthesis of endogenous catecholamines such as dopamine, epinephrine, and norepinephrine [664]. No significant association with depression symptomology was established, despite variable effects described by others in the literature which demonstrate this SNP (1947G>A) is associated with a range of psychiatric disorders, including depression [666, 715-717]. However, other conditions such as schizophrenia, bipolar disorder, substance dependence and ADHD are also linked to this polymorphism [683-685, 696, 700, 701]. Also, interestingly, another study of 900 people found the 1561C>T GCPII polymorphism may be associated with risk of

depressive symptoms [1022], but no such association was demonstrated in this investigation. Again, a small population size certainly may account for these inconsistent findings, but certainly in the case of the 1561C>T GCPII polymorphism results have been inconsistent [1023].

Beyond the genetic association alluded to above, the major finding for the depression phenotype was a clear relationship between the depression index score and cysteinyl-glycine levels. The result was present when the data was analysed as both categorical and numeric data (table 3-39 and table 3-43), the latter model indicating a negative association. Additionally, significant variation between controls and depressed individuals was also evident (figure 3-3, page 162). As mentioned earlier, cysteinyl-glycine is a dipeptide that is generated from glutathione by the action of γ–glutamyl transpeptidase [338]. It can also supply cysteine and glycine used by neurons and can act as an intermediary for neuronal glutathione synthesis [1024, 1025]. Despite this role, there is little research on cysteinyl-glycine in relation to neurobiology; a recent study using an animal model demonstrated decreasing spontaneous activity and increasing sedation with the administration of cysteinyl-glycine [374]. The relationship to glutathione is also key given that along with homocysteine, these thiols are recognised ligands for the NMDA subclass of glutamate receptor [363, 375]. Disturbance to these receptors and to glutathione metabolism itself has been associated with depression, and severe suicidal tendencies [1026]. Figure 6-2 below depicts how folate and transsulphuration products might act as ligands for the NMDA subtype of glutamate receptor and hence influence mood.

Even though cysteinyl-glycine is a component of glutathione metabolism, and there is growing evidence that deficiencies in brain glutathione metabolism appear to be connected with the pathogenesis of psychiatric disorders, the examination of glutathione yielded no significant findings with depression in this study. While complex, there is a clear metabolic link between folate-mediated one-carbon metabolism and brain function. This may be relevant in the depression phenotype, and larger studies that specifically examine the disposition of cysteinyl-glycine are certainly warranted.

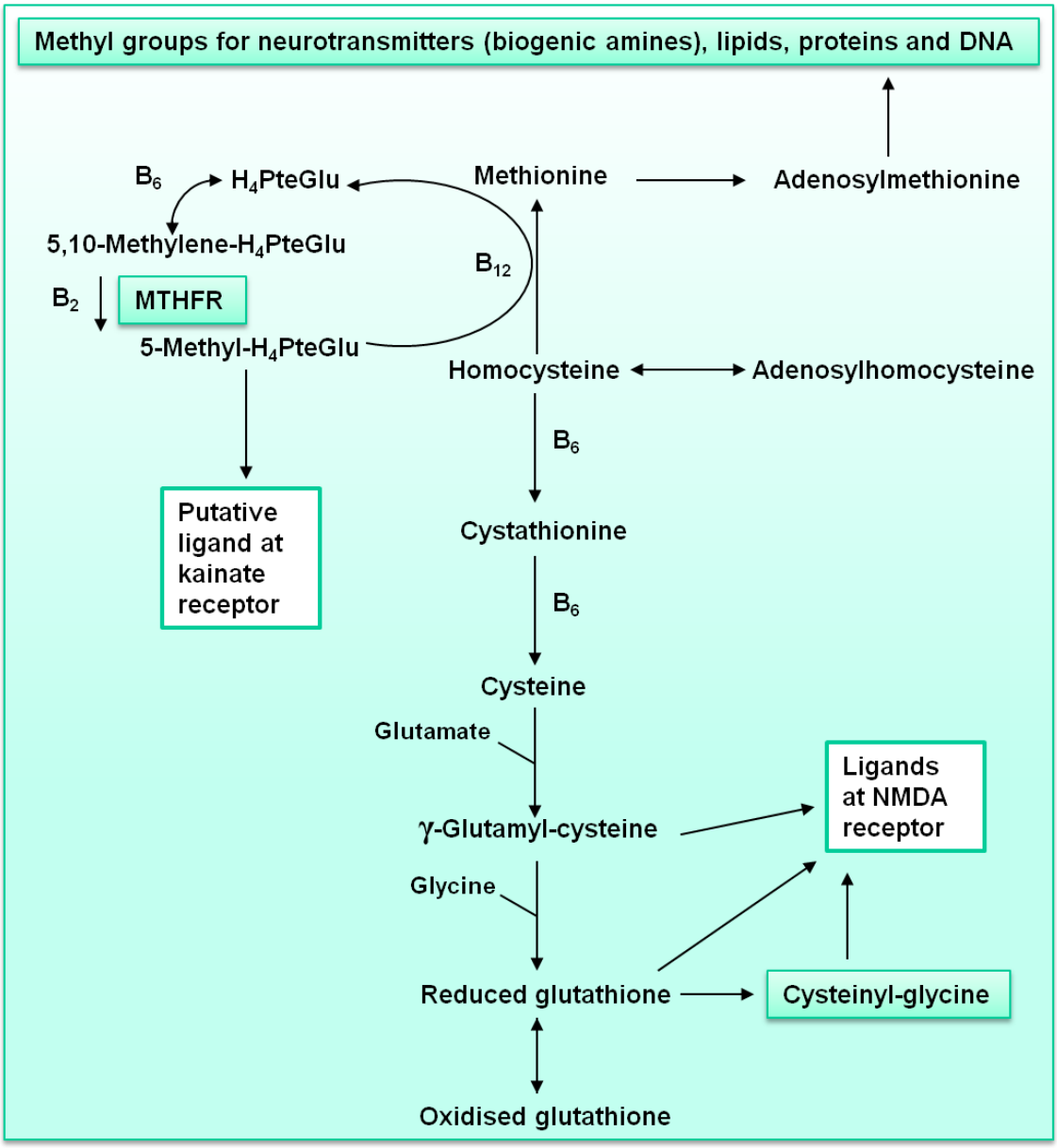


Figure 6-2: Folate and thiol metabolism in neurochemistry (Figure courtesy of A/Prof M Lucock [375])

It has often been reported that individuals suffering from depression have reduced folate levels [187, 190, 191, 1027]. The histogram on page 162 shows that for this study, red cell folate levels in depressed individuals were significantly lower than in non-depressed subjects (unpaired t-test). This is in addition to the ordinal logistic regression analysis that indicates an association between red cell folate and depression. Obviously, a low folate status is associated with elevated homocysteine and an impaired ratio of the two has been associated with low levels of the serotonin metabolite hydroxyindoleacetic acid in the cerebrospinal fluid [785, 810, 1028]. Also, homovanillic acid, an indicator of dopamine turnover and 3-methoxy-4-hydroxyphenylglycol, an indicator of noradrenaline turnover have been shown to be

significantly lower in depressed individuals with altered folate/homocysteine levels [192, 785]. Additionally, as discussed in the section on the hypertensive phenotype, folate is essential for the regeneration of tetrahydrobiopterin. This reduced biopterin is, a cofactor involved in the formation of noradrenaline, serotonin and dopamine [192]. Decreased levels/reduced availability of tetrahydrobiopterin, has been found in depression patients compared to healthy controls [1029]. Finally, in the brain, SAM acts as the main methyl donor for methylation reactions that include neurotransmitter synthesis; SAM has been shown in numerous studies to have antidepressant activity [1030-1032]. Furthermore, SAM levels in cerebrospinal fluid have been shown to be significantly reduced in a cohort of depression patients when compared to a neurological control group [1033].

This association between low folate and depression suggests a potential role for folate in the treatment of depressive disorders. Weaker treatment response to antidepressants has been observed in patients with low folate status [187, 1034, 1035], and there are several studies that support a role for folic acid supplementation in enhancing response to antidepressant medications [193-195, 1036]. It is important to be aware that the association presented here, like other evidence, is observational, and does not constitute proof of an association between folate and depression. Folate deficiency may produce depressive symptoms, but equally, poor nutrition as a result of depression may cause a decrease in folate levels. Research to prove that low folate levels in depression are the direct result of poor diet have been inadequate, this is in part due to difficulties in obtaining accurate dietary records among people with depressive symptoms [259]. It is likely that depression has a multifactorial aetiology, with a decreased appetite resulting in decreased absorption, and as a consequence changes in the utilisation of folate in one-carbon metabolism. The net result of this folate depletion will be an eventual deleterious effect on the central nervous system.

In summary, folate status, homocysteine levels, one-carbon metabolism related genetic polymorphisms, and the status of other related B-vitamins, are commonly involved in processes that are important for central nervous system function. Homocysteine may have a direct excitotoxic effect via the NMDA glutamate receptor, cause oxidative stress in brain and cerebrospinal fluid, and may also inhibit methylation processes in the central nervous system. The strong association demonstrated between cysteinyl-glycine and depression in this study is novel, particularly given the link to altered spontaneous activity and sedation in an animal model and the role in NMDA glutamate receptors. Red cell folate and the 677C>T MTHFR SNP seem to be relevant in this

depression phenotype, which is supported by previous research, however confirmation in larger populations is warranted.

6.1.3. Phenotype III: Alzheimer's dementia

In Australia, AD currently affects 1 in 25 people aged over 60 [127]. The 'baby boomer' generation gives Australia an ageing demographic profile that, in the coming decades, will result in a huge impact on healthcare systems, particularly in terms of direct cost and in effective and appropriate care. Predictions are that in just 8 years there will be over 400,000 people over 65 suffering from AD [144] and in 10 years it will overtake CVD as the fastest growing source of major disease burden [144]. For a physical disease which attacks the brain resulting in impaired memory, thinking and behaviour, AD is often considered to be an unnatural part of ageing. However, there are some interesting hypothesis that see AD as a natural consequence of human biology [950]. Despite research, there is currently no cure for AD and few treatment options. It has been reported that individuals with AD often have low blood levels of vitamin B_{12} and folate [131, 132] and high levels of homocysteine [128, 133]. In fact, increased plasma homocysteine level is now considered to be a strong independent risk factor for the development of dementia and AD [133]. Additionally, very recent research has demonstrated that a combination of folic acid, vitamin B_{12} and B_6 can slow the atrophy in specific regions of the brain, which is a key component associated with cognitive decline and the overall AD process [952].

The fourth chapter of this dissertation explores a small cohort of AD patients. This examination, like the previous two, involves nutritional and genetic factors that might underpin the pathoaetiology of the condition. A total of 99 participants were recruited (6 excluded) who were defined as having AD using the NINCDS-ADRDA criteria. These participants (n=93) were compared to the previous cohort of elderly retirement village residents (n=229), whose selection was based on their MMSE score which reflected the specified threshold for cognitive function. The analysis of the data was performed using ordinal logistic regression with the Wald χ^2 one-step linear approximation to the likelihood ratio test used to determine significance (p=<0.05). As previous, analysis was performed both as native and transformed data sets.

The results from table 4-16 clearly show that there are significant associations detected that involve folate dependent one-carbon metabolism and the related transsulphuration pathway. The only genetic association found out of the eleven SNPs examined, was

the 2756A>G MTR (table 4-17, *p=0.0419*). This finding is supported by an odds ratio analysis (OR=0.60:95%CI;0.39-0.92) in which a significant *p* value (*p=0.0260*) was found and indicates a positive protective relationship to AD (table 4-11). Methionine synthase, as discussed on page 62, is a critical enzyme that catalyses the methylation of homocysteine to methionine, and the 2756A>G polymorphisms is known to have a modulating effect on homocysteine levels, although, this was not demonstrated in the present AD study cohort. Currently, research seems to indicate lower homocysteine concentrations occur with this variant [452, 499, 568-571], but other results have demonstrated no such effect [419, 567, 572-574]. Similarly, folate and vitamin B_{12} levels so far appear not to be reported as being influenced by this variant [452, 574].

Previous research on this gene variant has also found an association with AD. In 2003, a study of 166 controls and 172 AD patients determined the AA genotype as being an independent risk factor for the development of the disease [578]. They found this to be the case for all age-at-onset subgroups which included both people under 60 and over 80 years of age [577]. Additionally, a second study with similar numbers found the AA genotype was associated with a risk for severe dementia [564]. They also determined a gene-gene interaction for this SNP and the ε4 allele of APO-E in which the risk of Alzheimer's increased by 3.3 fold with carriage of both genotypes [564]. Another study also found a weak and non-significant association between 2756AA and AD in a Chinese population [1037]. Although weak, an interesting trend was still detected, which in a larger study might have the power to show a significant effect. To date, there appears to be limited evidence published for this association. So far research suggests that the AA genotype appears to increase risk for AD. This is consistent with the evidence presented here that the G allele is protective (OR=0.60, *p=0.0260*), supporting the body of current evidence.

Consideration as to why this association exists has centred on the effect of oxidative stress, in particular, the effect on MTR activity. Oxidative stress in brain tissue could deactivate cob(I)alamin, the active form of vitamin B_{12}. This would lead to reduced MTR activity, resulting in diminished homocysteine transformation into methionine, and hence to an accumulation of homocysteine. In the context of this genetic mutation, the presence of the AA genotype may lead to a greater decline in enzyme activity as opposed to the AG or GG genotypes, and this may explain why there is a greater proportion of G alleles in the non-affected group. Additionally, the reduced enzyme activity associated with carriage of the A allele could lead to DNA hypomethylation. This is important because methylation levels are critical in gene function, and the GG

genotype might therefore modify AD development by changing the activity of key genes. Additional studies with much larger patient and control populations could help to further explain the observed association between the 2756A>G MTR and AD.

Among the non-genetic findings were highly significant results for the transsulphuration pathway thiols. As displayed in table 4-16 (page 198), highly significant results for homocysteine (*p=<0.0001*), cysteinyl-glycine (*p=<0.0001*) and glutathione (*p=<0.0069*) indicate a strong association between these metabolites and AD. When ordinal logistic models were repeated to only include the four thiols examined, the results for homocysteine and cysteinyl-glycine were maintained, and in the case of glutathione were strengthened (*p=<0.0003,* table 4-19). In the depression phenotype, homocysteine was discussed as having a direct excitotoxic effect on the central nervous system, through either an effect via the NMDA subtype of glutamate receptor, oxidative stress or through inhibition of methylation in the brain and cerebrospinal fluid. It is the same metabolic actions that have linked homocysteine to impaired cognitive function and AD. In fact, homocysteine and deficiency of B vitamins was thought to play a role in psychogeriatric populations long before the now well established connection to cardiovascular health [1038, 1039]. A prospective study of over a 1000 people from the Framingham Study cohort found that hyperhomocysteinemia was a strong independent risk factor for dementia and AD [133]. The findings included a graded association, in which a 5µmol/L increment in plasma homocysteine increased risk by 40% [133]. Also a doubling in the risk of either dementia or AD was seen in subjects with homocysteine levels in the highest age-specific quartile [133].

These findings have been supported by similar studies which all strongly connect hyperhomocysteinemia and AD [130, 132, 361, 782, 1040]. A longitudinal study (The Nun Study) found that dementia is worse in the presence of brain infarcts, suggesting that hyperhomocysteinemia could be an important contributor to vascular changes [331, 1041]. Furthermore, other research has shown homocysteine to be excitotoxic to cortical neurones [789]. As described previously, homocysteine could also contribute to neuronal apoptosis via depleted SAM concentrations [1042]. Some AD research has also focused on amyloid-β peptide, which when accumulated, forms senile plaques, a hallmark of AD and one that is suspected of contributing to the progression of the disease by inducing oxidative stress. A recent animal model study found that homocysteine induces apoptosis in hippocampal neurons and increased amyloid-β peptide induced death; when the presence of the two were combined, irreversible DNA damage was observed [1042].

Hyperhomocysteinemia should not be considered as an isolated factor in relation to chronic neurodegeneration; in this study lower cysteinyl-glycine levels and higher glutathione levels were also significantly associated with Alzheimer's dementia (table 4-19, figure 4-1). Glutathione is a transsulphuration product of homocysteine, and the dipeptide cysteinyl-glycine is a component of glutathione metabolism. As an endogenous antioxidant, glutathione is the predominant defence against reactive oxygen species and is an excellent scavenger of lipid peroxidation. The loss of glutathione and therefore oxidative damage has been shown to lead to apoptotic cell death [1043]. Glutathione has been observed to be lower in AD patients [361], however this was not reflected in this study, as higher levels were seen (figure 4-1, page 186). One study of total brain glutathione levels in AD subjects, found that levels appeared to be unaffected [1044], whereas some have suggested a compensatory increase of glutathione may indicate increased tissue damage and hence severe AD [955, 956]. As with depression (see page 261), excitotoxicity via excess NMDA receptor activation could also occur in the AD brain and lead to neurodegeneration, although currently this is considered to be a secondary event to oxidative stress [1045]. As previously mentioned, there is a limited amount of research on cysteinyl-glycine's role in brain function and the pathogenesis of psychiatric disorders. Consistent with this study, one small study has found reduced cysteinyl-glycine and glutathione levels in AD patients [373]. However, with a sample population of fewer than 100, larger studies are needed for conformation.

These thiol species; homocysteine, cysteine, cysteinyl-glycine and glutathione interact with each other through a complex range of redox and disulphide exchange reactions. While there is a clear metabolic link to AD, considerably more research is required to understand their role in brain function, and in the case of glutathione, its potential use as a therapeutic agent in preventing oxidative stress also needs to be clarified.

Finally, given the interactive nature of amino-thiols with B-group vitamins, and the strong reported association between AD and elevated homocysteine levels, many research studies have also concurrently examined the role of folate in the development of this condition. Out of the ten predominant studies investigating folate status in relation to AD and dementia [130, 133, 135-142], six have linked either prevalence or incidence of AD to a low folate status [130, 136, 138-140, 142]. The large Framingham Study cohort (discussed previously for its strong results for homocysteine levels) failed to link a low folate status to the development of this condition [133]. It has been suggested that the failure to demonstrate a role for folate has been complicated by the

establishment of government mandated folic acid fortification of foods during the 8 year follow-up period for this study [133] and the lack of significance for other studies may also be a reflection of this change in folate status across populations.

In this phenotypic study, results from ordinal logistic regression analysis show red cell folate (*p=0.0230*), serum vitamin B_{12} *(p=0.0393)* and total dietary folic acid (*p=0.0276*) are associated with AD (table 4-16). When analyses were repeated to include just the measured B-vitamin indices, significance for red cell folate was only achieved for native data, however, serum folate demonstrated significance for both data forms (native *p=0.0110* and transformed *p=0.0097*, table 4-20). Interestingly, additional analyses of total dietary folate status found that when dietary folate was broken into total natural (5-methyl-H_4folic acid) and synthetic folic acid (pteroylmonoglutamic acid), results for natural folic acid were highly significant (*p=<0.0001*, table 4-22). However, there was no significance established for synthetic pteroylmonoglutamic acid. These findings are also illustrated in the histogram (figure 4-1, page 186), which demonstrates the AD cohort have both lower total dietary folic acid and total natural folic acid intakes.

Despite these significant findings, it is important to highlight some of the limitations, which include the small population size and the difficulty in accurately obtaining nutrient intakes from people with impaired cognitive function. However, the findings presented here highlight the importance of considering 5-methyl-H_4folic acid and pteroylmonoglutamic acid as separate sources of dietary folic acid. As discussed earlier (section 1.3.4, page 88), there are a variety of possible adverse effects due to mandatory fortification programs; central is the potential harm to older populations. This is particularly important because of work conducted by Morris *et al.* [137], which like the large Framingham Study [133] failed to find any association between baseline folate intake and subsequent AD diagnosis. Importantly, and somewhat unexpectedly, an additional study found that the rate of cognitive decline among the cohort increased significantly with increasing folate intake. Interestingly, participants that used supplements providing between 401 to 1,200µg/day folic acid declined more rapidly over the median 5.5 years of follow-up than non-supplement users [866].

One theory is that higher intakes cause synthetic folic acid to become an inhibitor of DHFR's ability to reduce pteroylmonoglutamate and subsequently form 5-methyl-H_4PteGlu [169, 226]. This results in impairment of MTR activity. Therefore, a high folate intake may exacerbate neurological syndromes associated with vitamin B_{12} deficiency, which can result in neurologic damage that is characterised by memory loss, and ultimately may be implicated in the development of AD. This idea is supported by

further work conducted by Morris *et al.* [784] who later demonstrated significantly reduced cognitive function in subjects with high folate status (>59 nmol/l), which was paired with low vitamin B_{12} (<148pmol/L). In contrast, normal vitamin B_{12} levels plus a high folate status was significantly protective of cognitive decline [784]. Further work to uncover the role of not only vitamin B_{12} but the impact of the pteroylmonoglutamate form of folate on the development of cognitive function is required.

Certainly, epidemiological evidence indicates that derangement of folate-mediated one-carbon metabolism has a detrimental effect on the proper functioning of the central nervous systems. The evidence provided here demonstrates that impairment of MTR activity through carriage of the 2756A>G polymorphism may be protective in the risk for AD. However, confirmation of this phenomenon in larger populations is warranted. In addition to this, the findings presented here supports current conclusions that homocysteine concentrations play an important role in the development of AD. Research conducted by others that found high-dose vitamin supplementation reduces homocysteine levels in patients with AD [1046] requires further evaluation, especially considering the impact of the pteroylmonoglutamate form of folate and the effect of vitamin B_{12}. This is imperative, because the findings presented here found that only lower total natural dietary folic acid was associated with AD, not synthetic pteroylmonoglutamic acid. This dichotomy requires further explanation. The significant association between AD and glutathione and cysteinyl-glycine also warrants further investigation.

6.1.4. Phenotype IV: colorectal adenomatous polyps

One of the principal roles of folate is to serve as a methyl donor for biological methylations. The vitamin is also critical for the *de novo* biosynthesis of purines and thymidylate [792]. It is through these roles that the cell depends on folate to maintain normal patterns of synthesis, repair, and methylation of its DNA. Inadequate folate availability can result in changes not only to DNA methylation, but also synthesis and repair; these changes are strongly linked to the occurrence of malignant transformation and hence carcinogenesis [792]. As mentioned in the earlier literature review, despite having a lower incidence a few hundred years ago, industrialised nations are now finding cancer to be the second leading cause of death behind CVD. In Australia, colorectal cancer is the third leading cause of cancer deaths, and given its location within the large intestine, this disease has strong links to nutrition, diet and physical activity [9].

Colorectal cancer is often preceded by adenomatous polyps, which may become invasive cancer if left undetected. These adenomatous polyps share identical genetic mutations to those found in colorectal cancer. Results from epidemiologic studies involving both colorectal adenomatous polyps and colorectal cancer indicate a significant inverse association between folate status and colorectal neoplasia; with a high folate status being consistently associated with decreased risk [165-168]. Complicating this story is the paradoxical phenomenon in which an overly abundant supply of folate may lead to an increased risk of developing and progressing cancer in an individual who has existing foci of precancerous or cancerous cells [167, 266, 267] (previously discussed in section 1.3.4 on page 88).

Unlike previous chapters, the research presented in this final chapter (chapter 5) involved a discrete study cohort. It is part of a larger study investigating bitter taste phenotype, dietary patterns, and nutritional genetics in the aetiopathology of colonic adenomatous polyps. For this study, a total of 203 participants were recruited aged between 40 and 89 years, with all participants having undergone a colonoscopy to determine the presence of colorectal polyps. For ease of data analysis, the cohort was assessed as either having; no evidence of a polyp, as having an adenomatous polyp or secondary sub-group which contained subjects with any kind of colorectal polyp (both adenomatous and non-adenomatous/hyperplastic polyps). The analysis of the data was performed using ordinal logistic regression with the Wald χ^2 one-step linear approximation to the likelihood ratio test used to determine significance (p=<0.05). A further integrated analysis of dietary folic acid vitamer and level was also examined using standard least squares regression. Like previous data sets, analyses were performed both as native and transformed data sets.

As already discussed, age was significantly associated with the presence of a non-specific colorectal polyp diagnosis (*p=0.0006*, table 5-32), but not with an adenomatous polyp alone. However, when participants were separated based on median red cell folate status, age was significantly associated with adenomatous polyps (*p=0.0310*) for subjects with a low folate status (at or >868.5 nmol/L) (table 5-34, page 240). These findings are consistent with previous research linking age to not only adenomatous polyp development, but also to colorectal cancer in general [152, 153, 958, 959]. In addition to these findings, and again consistent with established work, gender was also associated with a non-specific colorectal polyp diagnosis (*p=0.0030*, table 5-30). Positive associations between male gender and an increased risk of colorectal polyps

and cancer have been reported, and male gender is now considered to be a risk factor for colorectal cancer development [153, 1047-1049].

From the various statistical models performed in this study, several genetic associations to the presence of colorectal adenomatous polyps were uncovered. The first was the MTRR gene polymorphism 66A>G; this gene was associated with adenomatous polyp occurrence (*p=0.0149*, table 5-29), and also for all polyp occurrence *(p=0.0444*, table 5-31). This association was supported by odds ratio analysis (OR=2.50:95%CI;1.23-5.10) in which a significant chi square p value for adenomatous polyps was found (*p=0.0163*). The result of this analysis for polyps *per se* only approached significance (see table 5-11). In addition to this, for subjects below the median red cell folate level, the 66A>G MTRR SNP was also significantly associated with adenomatous polyp occurrence. Both ordinal logistic regression (*p=0.0261*, table 5-33) and odds ratio analysis with chi square test p value (OR=3.40:95%CI;1.32-8.75, *p=0.0164*, table 5-13) support this finding .

The results from the present study show that the 66A>G MTRR SNP contributed a positive risk towards the occurrence of adenomatous polyps. This finding is consistent with a small Japanese study conducted by Matsuo *et al.* [562] in which carriage of the G allele was shown to triple the risk of colorectal cancer with an odds ratio of 2.77. This association however, has not been consistently reported, with several studies reporting no significance or weak associations to colorectal cancer [497, 561, 579, 581, 1050]. Hubner *et al*. [1051] studied the role of folate and aspirin in colorectal adenoma recurrence after a previous diagnosis. They found a significantly reduced risk of adenoma recurrence in patients heterozygous for the MTRR A66G polymorphism [1051]. In fact, carriage of either one or two G alleles resulted in a significantly reduced relative risk of adenoma recurrence [1051]. This contrasts with the current findings, but the authors suggest that the protective effect they observed for MTRR 66A>G may be because of a differential effect on the risk for colorectal adenoma development and recurrence [1051].

The contrasting results may stem from confusion surrounding the designation of the mutant allele when first discovered, given the almost identical allele frequency (see page 63 for more details). Certainly, the functional effects of this variant require further characterisation. It has been shown to influence plasma homocysteine levels [583, 584], however in this cohort it was not observed (see table 5-7), and carriers of the GG genotype have been reported to be at an increased risk for NTD [420, 576] for which low folate levels are central. It is known that MTRR activates MTR by reducing

cob(II)alamin and the G allele is considered to decrease the enzyme activity which may culminate in hypomethylation of DNA, resulting in a possible predisposition to colorectal cancer development [420, 562].

In relation to other genetic variants associated with folate-mediated one-carbon metabolism, the well-studied MTHFR 677C>T polymorphism has demonstrated a fairly consistent reduction in risk for colorectal cancer [495-498, 510]. However, in relation to risk of adenomatous polyp, results have been non-significant [499, 502, 506, 510]. An alteration in the thymidylate pool associated with MTHFR activity is suggested as the underlying mechanism in which the 677TT genotype confers protection. Some research work has considered the 677TT genotype in relation to an increased risk of colorectal cancer when folate status is poor [499]. In fact, Ulrich *et al.* [505] reported that a low intake of folate, vitamin B_{12}, vitamin B_6 and methionine, combined with the 677TT genotype was associated with an increased risk of colorectal adenomatous polyps. Conversely, high intakes of these vitamins and the 677TT carriage tended to be associated with lower risk [505]. However, analysis performed in this dissertation only found an association between MTHFR 677C>T and risk for colorectal polyp, i.e. both adenomatous and non-adenomatous polyps (*p=0.0268*, table 5-30), but not for adenomatous polyps individually, and not within individuals who have either a high or low folate status. Like the hypertensive cohort discussed above, red cell folate levels were actually higher in TT carriers than the other genotypes (see table 5-6 page 210). This is contrary to the vast amounts of research that has demonstrated how this SNP impairs folate-mediated one-carbon metabolism. As described above (page 252), exposure to synthetic pteroylmonoglutamic acid may be causing a differential response in the red cell folate assay, which may be affecting individuals who are 677 TT carries more so than the other genotypes. However, there may be other explanations for these contradicting results, which are unclear at this point. Certainly, the small population size is a potential confounder (see study limitations below).

The SHMT 1420C>T polymorphism was another variant examined in relation to colorectal adenomatous polyp. No ordinal logistical regression models associated this gene variant with adenomatous polyp occurrence, although when the population was stratified into above and below median red cell folate, the odds ratio for 1420C>T was significant for both adenomatous polyps (OR=4.56:95%CI;1.38-15.03, *p=0.0225*) and polyps of any kind (OR=3.55:95%CI;1.35-9.29, *p=0.0168*). These findings contrast with a study in which a borderline significant decreased risk of colon cancer was observed with the TT genotype when compared to the CC genotype [1052]. Other research has

found no association [1053], and certainly with the small population in this study, the results require conformation in a much larger population, but are certainly worth highlighting in this discussion.

The final genetic association uncovered was between colorectal adenomatous polyp occurrence and a genetic mutation within TS. As described earlier, TS catalyses the intracellular conversion of dUMP to dTMP using 5,10-methylene-H_4PteGlu as the carbon donor. The 3'UTR 6bp deletion polymorphism has not been as extensively studied compare to the 2R>3R repeat/insertion TSER gene, however it has been associated with reduced mRNA stability and translation in colorectal tumours [424, 629, 636]. In this study, the 3'UTR 6bp deletion was associated with adenomatous polyp occurrence (*p=0.0073*, table 5-29). It was also significant for subjects with an adenomatous polyp and who were below the median folate status value (*p=0.0324*, table 5-33). Odds ratio analysis for adenomatous polyp occurrence however, was not significant (OR=2.05:95%CI; 1.04-4.05, *p=0.0573*, table 5-11), but indicates an association for increased risk with this SNP.

Two reports investigating this genetic mutation found no influence on colorectal cancer risk and survival [580, 615]. One of those also reported the variant did not modify plasma folate and total homocysteine levels [615]. However, red cell folate has been reported to be higher in del6/del6, and correspondingly, homocysteine concentration was lower compared with the other genotypes [631]. Table 5-8 on page 212 shows findings that are broadly equivalent to this study, with significant variability existing between genotypes for homocysteine. Similarly, a possible trend may exist towards higher red cell folate levels in the wild type (del6/del6) carriers, although this doesn't achieve significance. This gene variant has also been investigated for lung and gastric cancers, non-Hodgkin lymphoma, and squamous cell carcinoma of the head and neck with various reported outcomes; most favouring the ins6/ins6 genotype with increased risk of cancers and short survival times [606, 618, 634, 635, 1054]. The other TS SNP 2R>3R TSER was not found to be significantly related to adenomatous polyps in this examination, but some published research has suggested that the 2R/2R genotype is associated with a decreased risk of colorectal neoplasia [607, 1055]. Certainly, the functional role of the genetic mutations of TS requires further characterisation. The central role of TS is to convert dUMP to dTMP; without adequate folate, uracil misincorporation occurs resulting in increased risk for DNA strand breaks. There is high demand for nucleotide availability for DNA synthesis and repair in colonic cells and thus it has been suggested that pyrimidine synthesis is a biological mechanisms

that links one-carbon metabolism to colorectal carcinogenesis [460, 580]. Larger, systematic studies are required to confirm the various genetic associations presented here.

Outside the significant results for the genetic and population indices described, there was no significant association between adenomatous and colonic polyps with respect to any of the four thiols examined. In the case of homocysteine, a polyp prevention study involving the effect of mandatory folate fortification found that among non-users of multivitamins, individuals with the highest homocysteine levels versus the lowest homocysteine levels had statistically higher odds of colorectal adenoma recurrence [1056]. The authors report that despite mandatory fortification improving baseline plasma folate levels, no reduction in homocysteine concentrations resulted [1056], which is consistent with other reports [843, 1057]. It is quite likely that homocysteine levels may be relevant given that this thiol acts as a methyl group acceptor in the *de novo* synthesis of methionine, and hence may be an important factor in maintaining the epigenome, a phenomenon closely associated with colorectal cancer aetiology [319, 792]. Certainly, homocysteine has been under-investigated as there is very little data on its relationship to colorectal neoplasia. Further investigation is required to better understand it's role in the development and reoccurrence of the disease.

As mentioned before, epidemiologic studies indicate an inverse relationship between folate status and colorectal neoplasia. There has been a recent flurry of publications discussing the vitamer type, level, and timing of dietary/supplemented folate in relation to the development of neoplastic changes in the colon. Statistical models in this study showed that red cell folate levels are associated with the presence of a colorectal polyp (hyperplastic and adenomatous polyps, *p=0.0248,* table 5-30). However, this outcome was not seen in the adenomatous polyp only group. Further analysis for subjects separated by folate status (based above or below median red cell folate level) found that for subjects in the high folate status group, folate parameters such as serum folate and red cell folate levels did not predict risk for adenomatous polyps (see pages 221 and 241). However, in the low status group, red cell folate level was a predictor of adenomatous polyps (*p=0.0331*, table 5-40). When the statistical model was repeated to include both folate parameters and gender all three (serum folate, red cell folate, and gender) showed a significant risk for adenomatous polyp (for *p* values see table 5-41, page 245).

In addition to this, a standard least squares model was performed to see whether dietary forms of folic acid would predict red cell folate status. Not unsurprisingly, for all subjects, total dietary folic acid profoundly predicts red cell folate level (*p=<0.0001*,

table 5-37). However, when the same analysis was applied to subjects below the population median value for red cell folate status, it was found that only total natural folic acid (5-methyl-H_4folic acid) predicted the red cell folate level (*p=0.0019*,

table 5-38). In addition, the same model was repeated for subjects above median red cell folate. Here the result was very different with total synthetic folic acid (pteroylmonoglutamic acid) predicting red cell folate level (*p=0.0165*, table 5-39). No association with total natural folic acid was evident in this high status population. These findings were further elaborated in Lucock *et al.* [376], in which dietary pteroylmonoglutamic acid was examined to see whether 14 folate-related SNPs could alter its association with red cell folate levels. Pteroylmonoglutamic acid appeared to be profoundly predictive of red cell folate status at higher levels, to the extent that it seems to mask the subtlety of any genetic effects [376].

Finally, despite the lack of any significant difference, the two histograms displayed on page 244 clearly indicate that in the low folate status population, the average red cell folate is higher in the control group when compared to the adenomatous polyp group. Interestingly, in the high folate status population (at or above median red cell folate level), the average red cell folate level is highest for the adenomatous polyp population. Unfortunately, statistical significance is not evident despite this effect, but larger numbers recruited into further studies may change this outcome.

Since the introduction of mandatory folate fortification programs, the role of folate in its various dietary forms, as well as blood indices of folate status have been closely examined. Many reports now label folate as having a dual role in the development of carcinogenesis [169, 849, 855, 856]. This is largely due to both human and animal cell studies that have shown that both the level and timing of folate are critical because it may either protect against the initiation of cancer, or alternatively it may facilitate growth of pre-neoplastic cells [167, 266, 267]. This concept is difficult to explain in the case of colorectal cancer and its antecedent, adenomatous polyps because epidemiological evidence supports higher levels of folate having a protective risk from the disease. However, a trial for the use of folic acid supplementation in the prevention of adenomas found after a 6 year follow-up period, that supplemented participants had a 67% increase in the risk of advanced highly malignant lesions [170]. The authors

conclude that the supplemented participants may have promoted the growth of previously undiagnosed or missed adenomas from previous colonoscopies [170]. Given this recent research, the role of folate based on vitamer form, timing, and dose requires further examination and prospective clinical research. Particularly, considering the adoption of mandatory fortification programs in many countries including Australia.

Overall, instability in folate-mediated one-carbon metabolism has a detrimental effect on DNA synthesis and therefore influences the development of colorectal neoplasia. Age and gender both seem to be relevant in the adenomatous polyp phenotype. Additionally, both the MTRR 66A>G gene and the 3'UTR 6bp deletion TS gene have been positively associated with risk for adenomatous polyp occurrence. And finally, red cell folate level was associated with colorectal polyps (adenomatous and hyperplastic polyps), and when separated according to median red cell folate status, subjects below the median had red cell folate levels that were also associated with the presence of an adenomatous polyp. The population size limits the power of this study, and certainly further research is required to fully comprehend the entire story that exists with respect to folate and colorectal cancer formation and progression.

6.2. Limitations of this Research

The observational studies presented in this thesis have detected significant associations between genes, diet and nutritional biochemistry, and their impact on some common chronic degenerative disorders. Whilst these findings are associative they do not represent causal relationships. This approach is the dominant form used in all nutrigenetic and nutrigenomic research. This is predominantly because human intervention studies are difficult to conduct and can be costly. Because all chronic diseases are in one way or another influenced by a variable and/or individual specific diet, gene-nutrient-disease interactions are hard to establish unless the diet and genotype are controlled for in study design, i.e. having the same diet with different genotypes. Nevertheless, this research does provide substantial evidence, and combined with similar studies and *in vivo* animal studies that assess mechanistic and interventional evidence, large swathes of information are beginning to paint a clearer picture about chronic disease development.

This style of research has its limitations. In particular, very large numbers are often required to have valid statistical power. In order to provide stable estimates for gene-nutrient interactions, so that findings can be attributed to biological relationships that generate meaningful epidemiological data, both appropriate statistical methods and

sample size are required. This lack of statistical power plagues many studies of this kind. Obviously, larger association studies with greater power provide greater creditability and ultimately may be more appropriate in directing potential ideas of personalised nutrition as a preventive measure. Other study designs such as intervention studies, which involve the examination of particularly important genetic profiles, may also have hugely beneficial effects. In addition, different study designs that involve the use of site directed mutagenesis in animal models could examine specific genetic profiles to see how they behave with the aim of then looking at humans in the same way.

In the phenotypic studies presented in this dissertation, the population sizes of a little over 200 people are certainly low. Although there is evidence throughout for a statistically significant relationship between a response variable and a predictor variables(s) of less than *p=0.05*, the R^2 values reported in each table are generally low. Around 10% or less of the variation in the response variable throughout this body of work can generally be explained by the regression model. This is dependent on the nature of the relationship examined and it can increase to over 55% or more in some analyses (table 5-36, page 241).

Sources of variability such as uncharacterised independent variables that also have an effect on the dependent variable could be a contributing factor where a low R^2 value is obtained. For example, the relationship between intake of dietary folate and red cell folate status has high significance (*p=<0.0001*,

table 5-37, page 242), however, around only 9% of the variation is explained by the model. This variability is hardly surprising given the use of FFQ's which characterise highly labile nutrients that are unpredictable between similar food sources [376]. Despite this, in the case of the association between low red cell folate status and the MTRR 66A>G and TS 3'UTR 6bp deletion genes and adenomatous polyps, 58% of the variation is explained by the regression models. Findings such as these are important major outcomes of this thesis.

Other limitations are potential heterogeneity within cohorts. This may not be a major issue in the present study though. For each of the four phenotypes examined, most of the participants recruited resided in and around the Central Coast, NSW Australia. Subjects were also predominantly Caucasian and aged on average between 63-79 years old depending on the cohort. Quite often observational gene-nutrient interactions such as the ones discussed here are difficult to replicate. In fact, consistent

reproducibility in the literature is something that continues to be lacking in this field of research. One of the predominant reasons for this is simply that most chronic degenerative disease is not caused by mutations in a single gene, but is due to complex interactions among variants of many genes. The studies performed in this dissertation largely focus on specific “candidate polymorphisms”; these candidate genes were selected because they are related to proteins that appear critical in the biological pathway, and that have previously demonstrated functional relevance. This approach however, is limited, as it not only doesn’t account for other genetic variants within folate-mediated one-carbon metabolism, it also doesn’t account for other SNPs within the same gene being examined.

These limitations therefore mean that the candidate polymorphism does not become ‘causal’ as there may be linkage disequilibrium among the genes and there are so many other variables that may have caused the observed association. One of the ways to overcome this limitation is to investigate gene-wide haplotypes, and methods that combine genes and incorporate them into epidemiological data analysis. An example of this is there now appears to be at least three important genetic SNPs within the TS gene that influence gene expression and/or the expressed protein’s stability [424, 425, 604]. It is now recommended that further studies explore diplotype for any effect on the functional protein [1058]. This can also be applied to other functional proteins within folate metabolism such as MTHFR gene variants.

Finally, as mentioned above, one of the main potential sources of variability in this study surrounds the estimation of nutrient intake. FFQ’s are convenient tools for assessing dietary intake. However, they do not always necessarily reflect nutrient status. Accurately representing the amount and type of food, as well as the differences in food composition data, can result in imprecise estimations. The validity of the results may be subject to variability and not translate into accurate intakes and therefore nutrient exposure may predict nutrient status with less precision than one might hope for. However, out of the many micronutrients surveyed some of the strongest evidence for the validity of this method of estimation occurs for folate, with the results for other B-group vitamins being, perhaps, slightly less accurate [1059].

There is however, potential misclassification where supplemental pteroylmonoglutamic acid is concerned because it can have a 1.7 fold greater bioavailability than natural dietary folate sources, and the simple addition of both sources may not accurately reflect status [1060]. Certainly, inexact dietary estimation could have potentially influenced the observed associations seen in this thesis, and is a very significant

limitation noted in this body of work. Nevertheless, FFQ's are the standard tool for dietary assessment in human nutrigenetic studies and are considered a valid measurement of folate intake. Quantifying food intake is challenging and ideally a more reliable measurement tool for assessing nutrient intake is needed in coming years to avoid misclassifications and increase accuracy of results.

The association studies discussed throughout this thesis do provide valuable information despite the various limitations described. Nutrigenomic studies like these can help clarify the biological mechanisms within a complex pathway such as in folate-mediated one-carbon metabolism. This type of research does link biological pathways to disease end points, but it also helps us understand whether genetically susceptible individuals and sub-populations could benefit from either higher or lower intakes of specific nutrients with the overall aim of preventing chronic disease development.

6.3. Ramifications of Mandatory Folic Acid Fortification

Chronic degenerative diseases are often preventable. The goal of modern medical and nutritional research is to best figure out not only what components make them preventable, but also how to best improve the health of an individual already afflicted by disease. The advent of the human genome project shifted the way we apply preventive medicines, instead of viewing whole populations, today nutrigenomic research lets us assess nutritional and genomic health on an individual basis. Although this thesis has focused on a single nutrient, folate, the ideal focus would be on the impact of multiple nutritional imbalances, which may include both deficiency and excess states.

During the past 20 years, clear clinical and epidemiological evidence has supported the role of folate in protecting against the development of many diseases. With this knowledge it can be argued that the best way to apply this to whole populations is through government legislation. This means that once intervention has occurred, little choice exists as to whether individuals are exposed to folate or not, and therefore such decisions must be anchored securely in robust scientific evidence. Recent publications suggest a lack of consensus still exists [171, 856, 872, 1056]. There is a perception that food and nutrition researchers sometimes resist population wide mandatory prevention programmes due to the simple fact that eating adequate amounts of natural foods is better than modification of the food supply with synthetic alternatives.

However, there are equally compelling arguments that the most effective method of prevention is to limit individual choice in order to protect vulnerable groups, and therefore it is best achieved by government action. Public health policies need to be based on a thorough and critical review of the scientific evidence, but also consider the financial cost, political risk and public acceptance, which may make it a difficult decision. In 2009, the decision to mandatorily fortify wheat flour with folic acid was made and implemented in Australia. Meanwhile, other countries such as New Zealand, the UK and most of mainland Europe are so far less convinced about the robustness of the scientific research. Australia's decision has generally been widely accepted by the scientific community and falls into line around 10 years after the US, Canada and others which have adopted such programs.

Outlined in the literature review section of this thesis are many of the concerns that surround this decision. There is, in the author's opinion, insufficient data in humans to assess the long-term effects of exposure to synthetic folic acid. While a significant amount of this evidence was reviewed during the decision making process for mandatory fortification, some countries felt that it would be best to adopt a 'wait and see' approach. The longest held concern is that of the effect on older populations. The debate has been contextualised as 'fortification benefits babies but may harm grandparents'. But such thoughts were outweighed by the feeling that any risk began at exposure levels of more than 5000µg per day [831]. The masking of the haematological symptoms of vitamin B_{12} deficiency is arguably one of the biggest concerns with mandatory folic acid fortification. High rates of vitamin B_{12} malabsorption exist in elderly populations and there is now evidence that points to harmful effects through a more rapid cognitive decline where there is high folate status combined with low vitamin B_{12} levels [784].

In addition to this, there is concern that long term exposure to synthetic folic acid can lead to its appearance in the circulation in an unmetabolised form, for which the consequences are yet to be fully established. In the liver, synthetic folic acid is converted by the enzyme DHFR, and recent research has uncovered low DHFR activity, which is slow and rate limiting, and thus has a poor ability to reduce synthetic folic acid [827]. Regular daily intake of synthetic folic acid could saturate the preliminary liver folate-monoglutamate pool and result in unmetabolised folic acid in the blood system [313, 314, 855, 919]. A notable study examining this found the threshold intake of 266 µg folic acid per meal was the point at which unaltered folic acid appeared in the serum [314]. Whilst Australia's fortification level of 120µg per 100g

flour is lower than many other countries, there is still concern, particularly when it comes on top of voluntary fortified breakfast cereals and the unregulated consumption of over the counter vitamin supplements that contain folic acid. In addition, a large and recent report has linked use of dietary vitamins and mineral supplements to increased total mortality risk [851]. This association includes a slight risk for folic acid use, with the negative effect observed most profoundly post-fortification [851].

The other area of concern for mandatory fortification is the recent development of the effect folate has on cancer, particularly colorectal cancer. This stems from two key areas of research. The first being that despite epidemiological evidence of an inverse relationship to colorectal cancer, it is now considered that folate has a 'dual role', in that it can prevent the establishment of neoplastic cells, but once these cells are established folate can enhance growth and progression of mutated cells [167, 170, 266, 267, 849]. The second key piece of research is that in countries which have fortified grain sources with folic acid there has been an immediate reversing in the downward trends for colorectal cancer [265, 854], with reports estimating trends exceeding the pre-fortification incidence. It has been suggested that fortification could be partially responsible for an estimated 15,000 additional cases per year [169]. Increased colorectal cancer screening does not appear to be accountable for this change in trend [169].

Additionally, exposure to synthetic folic acid has been associated with an increased risk of both breast [268] and prostate cancers [270, 1061]. This would also fit with the previous idea that folate can facilitate the growth and progression of cancer cells. Australia will need to closely examine future changes to cancer rates and determine whether any increase is in fact due to the mandatory program implemented in 2009. Even so, these reports prompted many people to seriously question the appropriateness of fortification programs, with such serious consequences and major concerns for future generations being brought into question [169, 171, 855].

The major ramification of folic acid fortification is that it may become increasingly difficult to establish statistically significant effects due to folate dependent genes. Earlier, it has been discussed that the failure to link the role of folate to CVD, particularly for the causality between the 677TT MTHFR genotype and elevated homocysteine levels may have been hampered by the implementation of mandatory fortification [476, 972]. Also, the large Framingham Study cohorts have failed to link low folate to AD; the authors suggest this is due to the establishment of government mandated fortification during the 8 year follow-up period of the study [133]. Although

there are other examples showing similar effects of adding folic acid to the food supply, it is likely that population based exposure is hindering scientific research on related genes and metabolites, and the consequences of this are unknown.

There is no doubt that mandatory fortification programs succeed, data from the US has seen a doubling of median serum folate levels in non-pregnant women of reproductive age since the program began [1062, 1063]. Additionally, NTD rates have been reported to be reduced by between 30-70% depending on the country of implementation [298, 891, 1057, 1064, 1065]. There are limited reported findings of the effect that mandatory folic acid fortification has had on the Australian population to date, but certainly there needs to be continued research that identifies any as yet uncharacterised effects caused by a high intake of the vitamin.

6.4. Future Undertakings

Thesis specific future research should include further detailed studies examining how the different forms of folate, both natural and synthetic, are absorbed and utilised in the human body. In addition to this, further work on the upper tolerable limits of folic acid and the impact of excess folate intakes in humans is needed. In relation to nutrient interactions, further work that examines the impact upon folate of other nutrients such as other B-group vitamins, especially vitamin B_2, B_6 and B_{12}, but also the influence of vitamins E, D and C and minerals zinc and iron needs much greater attention. One of the clear thesis highlights was the significant association between cysteinyl-glycine and both depression and AD. Whilst homocysteine has received a lot of focused attention, other parts of the transsulphuration pathway have hardly been reviewed. Cysteine, cysteinyl-glycine and glutathione may all have a much bigger impact on folate and its metabolism, especially in relation to neurobiology than has previously been considered.

Although this dissertation has focused on degenerative disorders associated with folate-mediated one-carbon metabolism, folate also has a significant role in key developmental disorders. Despite significant evidence for preventing NTD and other birth defects, it appears that folate is not often considered as having a role in other childhood related illness and disease. One area that desperately needs more attention is folate's possible role in autism and other development conditions. As discussed earlier, folate deficiency can be manifested as damage to nervous tissues. It has been speculated that just like too little, too much folic acid could also result in nervous tissue destruction [1066]. Associations between the increasing rates of autism and the

increasing dose of folic acid prescribed in prenatal and paediatric vitamins is being investigated, but certainly future research in this area is necessary.

Within the limitations of the work conducted for this thesis, the idea that statistical power is something that needs to be addressed in nutrigenetic studies was discussed. One way of overcoming this limitation has been the development of a mathematical model of folate metabolism. Developed by Reed *et al.* [1067] this model incorporates standard biochemical kinetics for folate metabolism allowing for simulations of the system to be performed. It also allows for variables to be modified and predicts the effect on the pathway. Mathematical models can be especially useful in identifying specific interactions that could be investigated *in vivo*, whilst generating hypotheses for experimental testing [1067]. These models have been particularly useful in providing preliminary predictions on the effects of genetic polymorphisms on disease risk biomarkers and have generated some consistent findings with previously published data [1068, 1069]. Certainly, this form of statistical modelling can assist in predicting effects of variations in nutritional and genetic factors on mechanisms relevant to disease risk. Investment in this kind of research tool would be very useful.

Another area of weakness in nutritional based research is the lack of accuracy when quantifying dietary intakes. The reliability of FFQ's as a measurement tool could be improved in the future. Quantifying food intake is challenging, however, potential use of computer-administered questionaries delivered on touch-screen tablet computers could improve the accuracy of information obtained from participants [1070]. Future technology could also be used to collect metrics about real time food consumption which may improve the accuracy of FFQ's and enhance the accuracy of estimation of dietary nutrient intake [1070].

The final area for future research centres on rapid technological changes in assessing genetic data. Genome-wide association studies (GWAS) have created the ability to assay up to a million SNPs for a relatively low cost and in a short period of time [1071]. This technology is much more efficient than selecting single candidate genes as was performed in this dissertation. However, considerably more research is needed in this area to fully analyse and comprehend information from such a wide investigation of the genome in a focused and useful way. Identification of mutations, genes and pathways that are involved in particular diseases could offer potential for improved diagnosis and disease prevention [1072]. Ultimately, technological advancement of this nature will be a powerful tool for better understanding the common causes of human disease [1073].

In addition to this type of technological advancement, in 2012, an American company Life Technologies™ released a printer size system that could map a person's entire genome for $1,000 USD in the space of a few hours [1074, 1075]. Every year that passes the cost and speed of obtaining genomic data is decreasing. Years ago, it cost hundreds of millions of dollars, and took many months. Although, it is likely to be many years before such technology can be used more routinely, the future possibilities that such applications bring to nutrigenomic assessment on an individual basis, the potential to help tailor appropriate nutritional (dietary and supplemental) intake to an individual's genomic status are enormous in terms of the ultimate goal of preventing common chronic degenerative diseases.

6.5. Conclusion

In summary, folate-mediated one-carbon metabolism is a fascinating yet complex biological system, which represents a key locus in the development of a vast array of chronic degenerative diseases. Folate is one of the most intensely researched vitamins due to its role in carrying one-carbon units for the synthesis of DNA thymine, methionine, purine, and amino acids. There are many polymorphic genes encoding folate dependent enzymes, which can modify gene regulation and expression. Epidemiological evidence indicates that derangement of folate-mediated one-carbon metabolism has a detrimental effect on the proper functioning of many biological systems, including the vascular system, the central nervous system, and mutations leading to cancer of bodily organ systems such as the colon.

With this in mind, the goal of this dissertation was to examine nutrient-gene interactions among some of the commoner degenerative disorders within our contemporary ageing population. The results from examining a hypertensive phenotype, which is a pathologic entity and risk factor of CVD, uncovered several significant associations. The 677C>T MTHFR variant was found to be associated with hypertension. Furthermore, both red cell folate status and its predictor, total dietary folic acid intakes were also significantly associated with hypertension. The related transsulphuration metabolites cysteine and cysteinyl-glycine were similarly associated with this vascular phenotype, but no significance for the well-characterised homocysteine and CVD connection were uncovered. A second phenotype, depression, was examined in the context of folate and its related genes; a novel link between cysteinyl-glycine was established. In addition, red cell folate and the 677C>T MTHFR gene also appeared to have significant relevance within this phenotype.

AD is a huge burden within our ageing population, given the effect folate-mediated one-carbon metabolism has on the proper functioning of the central nervous and cardiovascular systems, it is perhaps unsurprising that folate plays a role in the pathoaetiology of this disease. This phenotype was examined for nutrient-gene interactions and the findings showed carriage of 2756A>G MTR may be considered protective against AD, although a small population size warrants further examination using a larger cohort. The transsulphuration pathway thiols, homocysteine, cysteinyl-glycine, and glutathione were all associated with this disorder. As was total dietary folic acid intake, of which the natural 5-methyl-H_4folic acid form appeared to be associated with the phenotype, but not the synthetic pteroylmonoglutamate form.

The final phenotype examined involved subjects with a diagnosed adenomatous polyp. The role of folate-related nutrient-gene interactions may be important in the pathology of this potential precursor of colorectal cancer. Results showed that both the 66A>G MTRR gene and the 3'UTR 6bp deletion TS gene contributed positively towards the risk for adenomatous polyp occurrence. Also, red cell folate levels were associated with adenomatous and non-adenomatous polyp presence, and when separated according to the median red cell folate value, those in the lower red cell folate category were more likely to have an adenomatous polyp diagnosis.

Ideally, the findings presented in this thesis would benefit from confirmation in larger populations, possibly involving GWAS. There is still much more to be learned about these particular clinical correlates that impact the later phases of the human life cycle. The aetiology of human disease is complex. Increasingly in the context of folate, and with mandatory fortification in place, it centres on the nature verse nurture debate. Molecular processes and particular gene mutations that can predispose an individual to any given disease needs to be understood. Inevitably, whether early pathology eventually manifests into a chronic degenerative disease depends entirely on a complex interaction between the genome, nutritional and other environmental factors. In the future, the concept of personalised nutrition may address many of these complex issues.

Postscript: the author notes that after preparing this dissertation it was too late to incorporate the very recent publication from *Vollset et al*. [1076] published in the Lancet January 25, 2013, regarding folic acid supplementation and cancer incidence. This very large meta-analysis over 50,000 individuals, comprising 13 randomised trails found that folic acid supplementation did not substantially increase nor did it decrease the incidence of site-specific cancers during the first 5 years of treatment. This included no effect of supplementation on colorectal, prostate, lung, breast, or any other specific site cancer. There was however findings that the point estimate for overall cancer incidence was slightly increased [1076].

The researchers note that this finding is compatible with a chance effect, and the risk did not appear to change with increased daily dose of folic acid or with length of treatment [1076]. It is important to note that while the supplemental dose of folic acid used in the studies was about ten times higher than the fortification levels, there still may be an increased risk to individuals who are exposed not only to mandatory fortification but who combined this exposure with supplemental use [1077]. It is also likely that the effects of folic acid supplementation on site specific cancers; whether they are harmful or beneficial could emerge among the participant's years after the study has ended [1077].

This null finding is contradictory to the research reviewed in section 1.3.4 page 88, but nevertheless it is a significant piece of research in this field and worth highlighting.

CHAPTER 7

REFERENCES

&

APPENDICES

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7.2 Appendix 1: Food Frequency Questionnaire

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Survey Instructions Id No…….. Study No……..

- This questionnaire is to be administered as a **interview**
- Serving sizes are either specified in the question or a model of the serving is shown.
- If a **single response** is specified, reply with the type of food you **most frequently** eat.
- These are the following definitions of the answers

"Daily" If you eat the food once or more times a day, and please specify how many times a day
"Weekly" If you eat the food between once and six times a week, and please specify how many times in a week you would eat the food.
"Monthly" If you eat the food once to three times a month (a month being four weeks), and please specify how many times
"Yearly" If you eat the food less than once a month and specify how many times a year.
"Never" If you never eat the food.

- **NB: Record the <u>frequency</u> *daily*, *weekly*, *monthly and yearly* as a <u>number per</u> that period in the space provided**.
- **Record *never* as just a tick in the space provided.**

1a. How often do you have the following serves of milk including non-dairy milks? ***For example: a glass of milk <u>3 times</u> daily, or milk in tea and coffee <u>2 times</u> weekly.***

SEE PHOTO 1

Food	**Daily**	**Weekly**	**Monthly**	**Yearly**	**Never**
Glass of milk (1 cup or 250ml)					
Milk on cereal (1/2cup or 125mls)					
Milk in tea and coffee (20ml)					
Milk in cooking (1/2 cup or 125mls)					

If "Never" for all, skip to 2

1b. What type of milk do you <u>usually</u> have? *(single response)*

Full Cream		Life	
Skim		Pura Start	
Shape		Pura No Fat	
Lite White		Soy Milk	
Farmers Best Polyunsaturated		Yakult	
Farmers Best Omega-3		Other ……	

2. How often do you eat *cheese* (like cheddar, edam or other hard cheese, cream cheese or cheese like camembert) or *cream* (full cream, lite cream, whipped cream)?

SEE PHOTO 2

Food	Daily	Weekly	Monthly	Yearly	Never	Type/Brand
Block Full Fat Cheese						
Slice Full Fat Cheese						
Block Low Fat Cheese						
Slice Low Fat Cheese						
Deli Cheese (e.g. Brie)						
Cream Cheese						
Cream Full Fat						
Cream Fat Reduced						
Whipped Cream						
Other:						

3. How often do you eat a tub (150g) of yoghurt or other dairy desserts (not including ice cream)?

Food	Daily	Weekly	Monthly	Yearly	Never	Type/ Brand
Yogurt						
Yogo						
Fruche						
Custard						
Rice Pudding						
Other:						

4. How often do you have a serving of the following spreads or oils, including in cooking? Please specify brands. (A 'serving' is defined as 1 teaspoon for spreads and a 2 teaspoons for oil)

SEE PHOTO 3

Spread/Oil	Daily	Weekly	Monthly	Yearly	Never	Type/ Brand
Margarine						
Butter						
Cholesterol-lowering Margarine						Logicol, Pro-active, other:
Olive Oil spread						
Cooking oil						
Salad dressing oil						
Other……						

5a. How often would you eat a 125g serve of the following red meats? *(serving size indicated as the palm of the hand)*

SEE PHOTO 4

Meat	Daily	Weekly	Monthly	Yearly	Never
Beef or lamb					
Pork					
Mince					
Sausages					
Other:					

If "Never", skip to 6a.

5b. How is your meat usually cooked? *(circle the response number)*

Fried or Stir-Fried with fat (5)
Fried or Stir-Fried without fat (4)
Stewed or goulash (3)
Grilled or roasted with oil or fat (2)
Grilled or roasted without oil or fat (1)

6a. How often do you eat a 125g serving of poultry including chicken, turkey duck and quail?

SEE PHOTO 5

Daily	Weekly	Monthly	Yearly	Never

If "Never", skip to 7a.

6b. How much of the skin on your chicken do you eat? *(circle the response number)*

Most or all of the skin (2)
Some of the skin (1)
None (0)

7a. How often do you eat a piece of fish or tin of fish (95g)?

	Daily	Weekly	Monthly	Yearly	Never
Fish (fresh or frozen)					
Fish (Tinned)					

***If "Never", skip to 7c*.**

7b. What sort of fish do you eat most frequently? ……

7c. How often do you eat a serving of seafood such as prawns, oysters and shellfish?

Food	Daily	Weekly	Monthly	Yearly	Never
Prawns					
Oysters					
Shellfish					
Other					

8a. How often do you eat a serve of sandwich meats or deli meats for example ham, bacon, salami or chicken loaf?

Food	Daily	Weekly	Monthly	Yearly	Never

8b. Do you usually eat the omega-3 type processed meats? *(circle the response number)*

(1) Yes (2) No

9a. How often do you eat an egg (cooked in any way) or in cooking (e.g. cake)?

Daily	Weekly	Monthly	Yearly	Never

If "Never", skip to 10a

9b. Do you usually eat the omega-3 eggs? *(circle the response number)*

(1) Yes (2) No

10. How often do you do you eat a serve bread? (A serve is 2 slices of bread, 1 roll, 1 English muffin, 2 crisp bread biscuits, lavash bread etc)

And, what type do you usually eat?

Food	Daily	Weekly	Monthly	Yearly	Never	Type/ Brand
White (no additions)						
Wholemeal						
Multi-grain						
High Fibre White						
Omega-3						
Linseed and Soy						
Rye						
Sourdough						
Soy Bread						
Raisin Bread						
Other:						

11. How often do you eat a cup of cereal or 2 Weetbix or ½ cup of cooked porridge or 1/3 cup of oat based muesli?

Brand Type	Daily	Weekly	Monthly	Yearly	Never
Weetbix					
Just Right					
Sultana Bran					
Weeties					
Nutrigrain					

Special K					
Lite Start					
Cornflakes					
Coco Pops					
Rice Bubbles					
Soytana					
Muesli (brand:……...)					
Porridge					
Other:					

12. How often do you eat a cup of the following cooked foods?

Food	Daily	Weekly	Monthly	Yearly	Never	Type/ Brand
Pasta						
Rice						
Noodles Type:						
Other:						

13a. What types of fruit or berries do you usually eat (**fresh, dried, cooked or canned**) and how often would you have a serve of them? ***(Serve = medium size piece of fruit or a handful of dried fruit or berries.)***

Fruit	Daily	Weekly	Monthly	Yearly	Never
Apple					
Banana					
Citrus: Orange					
Mandarin					
Lemon					
Grapefruit					
Citrus, Other:					
Grapes/sultanas					
Kiwi Fruit					
Melons: Watermelon					
Rockmelon					
Honeydew					
Melon, Other:					
Pineapple					
Stone Fruit: Apricot					

Mango					
Nectarine					
Peach					
Plum					
Avocado-*guacamole*					
Stone Fruit, Other:					
Berries	**Daily**	**Weekly**	**Monthly**	**Yearly**	**Never**
Blueberries					
Raspberries					
Strawberries					
Other:					

13b. What types of fruit juice do you usually drink and how often do you have a glass (250 ml) of these fruit juices?

Fruit Juice	Daily	Weekly	Monthly	Yearly	Never	Type/ Brand
Apple						
Apple and other (e.g. apple and mango, apple and blackcurrant)						
Cranberry or combination						
Orange						
Pineapple						
Juice, Other:						

13c. Are any of the fruit juices you drink fortified with vitamins? If yes, can you name the type of vitamin?

……

14. How often do you have 1 medium sized potato including mashed, boiled or roasted, not including hot chips (or similar) and potato crisps?

Type	Daily	Weekly	Monthly	Yearly	Never
mashed					
boiled					
roasted/baked in oil or fat					
jacket roasted					

15a. How often do you have the following tomato products?

Food	Daily	Weekly	Monthly	Yearly	Never
Fresh Tomato (one tomato)					
Tomato sauce (1 tbs)					
Tomato paste (1 tbs)					
Fried in oil or butter (one tomato)					
Tomato juice (1 250ml cup)					
Tomato pasta sauce (1/2 cup, 125ml)					
Tomato Soup (1 cup, 250ml)					
Canned tomatoes					
Other:					

15b. How often do you have a serve of other vegetables– ***fresh vegetables including on sandwiches (FV), cooked (C,) frozen (Fr, salad including on sandwiches(S) or soup (Sp)?***
(A serve is ½ cup of cooked vegetables, 1 cup of raw vegetables, salad vegetables or soup).

Vegetable	Variety (FV, C, Fr,S or Sp)	Daily	Weekly	Monthly	Yearly	Never
Alfalfa Sprouts						
Asparagus						
Broccoli						
Brussel Sprouts						
Cabbage						
Capsicum						
Carrot						
Cauliflower						
Celery						
Corn						
Cucumber						
Green Beans						
Lettuce						
Onion						
Peas						
Pumpkin						
Shallots, leaks, green onions, etc.						
Mixed frozen **(FR)** Vegetables Type?						
Mixed Salad **(S)** VegetablesType?						
Mixed Soup **(Sp)** Vegetable Type ***or Meat (if not covered)***						
Other:						

15c. How often would you consume a teaspoon of chopped herbs, fresh or dried? e.g. Basil, chives, coriander, dill, parsley, thyme, oregano, other.

Herb	Variety	Daily	Weekly	Monthly	Yearly	Never
Basil						
Chives						
Coriander						
Dill						
Garlic						
Ginger						
Oregano						
Parsley						
Rosemary						
Thyme						
Other:						

11. How often do you eat a serving of nuts or nut spreads? (A serve is a small handful of nuts or a tablespoon of spreads)

Nuts and Nut Products	Daily	Weekly	Monthly	Yearly	Never
Almonds					
Beer Nuts					
Brazil Nut					
Cashews					
Hazelnuts					
Macadamias					
Peanuts					
Pecans					
Walnuts					
Other Nuts:					
Peanut Butter					
Satay Sauce					
Nutella					
Other Nut products:					

17a. How often do you eat a ½ cup of beans, lentils etc?
(Green Beans should be under other vegetables.- 15c)

Foods	Daily	Weekly	Monthly	Yearly	Never
Baked Beans					
Broad Beans					
Chickpeas					
Hummus					
Kidney Beans					
Lentils					
Mung Beans					
Refried Beans (Mexican)					
Three (3) Bean Mix					
Other:					

17b. How often would you eat soy foods and products?

Soy Food	Brand	Daily	Weekly	Monthly	Yearly	Never
Soybeans (1/2 cup, 125ml)						
Soymilk (1 cup, 250ml)						
Soy ice cream (1 cup)						
Soy custard (1/2 cup, 125ml)						
Nut meal						
Soy bread (Two slices)						
Soy yoghurt (150g tub)						
Soy bacon or sausages (2 rashes or 2 sausages)						
Tofu						
TVP						
Other:						

TVP **=** texturized vegetable protein

18. How often do you eat the following foods and what type or brand?

(F*or example, McDonalds: a large quarter pounder meal deal.)*

Food	Type/ Brand	Daily	Weekly	Monthly	Yearly	Never
Ice Cream, Sorbet, Frozen Yoghurt :						
Chocolate, Chocolate Biscuits, chocolate bars						
Lollies, candies						
Fast Foods of cooked at home (Hamburgers e.g. McDonalds, Fried						

Chicken e.g KFC, Pizza etc.						
Take-away eg (Chinese, Thai, Kebabs)						
Hot Chips and other fried potato products						
Potato crisps or corn chips:						
Hot Dogs, pies, sausage rolls, pasties						
Pastries, Cakes, Sweet Biscuits, Croissants						
Cream sauces, gravy, cheese sauces						
Soft drinks, cordial including energy drinks *(ask if diet or not)*						

19. How often do you drink alcohol and in what type of drinks?

Drink	**Brand**	**Daily**	**Weekly**	**Monthly**	**Yearly**	**Never**
Beer (schooner, can, stubbie 375ml)						
Beer light strength (375ml)						
White Wine (glass, 150ml)						
Red Wine (glass, 125ml)						
Spirits (30ml nip)						
Mixed bottle drinks eg Vodka Cruisers (bottle, 275ml)						
Liqueurs (30mL nip)						
Port (glass, 100mL)						`
Other						

20a. How often would you have a vitamin, mineral or other supplements (tablets, capsules, drinks or injection)

Supplement	Brand	Daily	Weekly	Monthly	Yearly	Never
Multi vitamin /mineral						
Vitamin C						
Vitamin E						
Vitamin B Complex						
Berocca						
Folate						
Vitamin B12						
Vitamin D						
Vitamin A						
Iron						
Protein						
Fish oil						
Sustagen products						
Other:						

If they do not take any supplements, please skip to 21.

20b. Which of these supplements would you have taken in the last two weeks and how often? (R*ecord these supplements including type and brand)*

……..…
……
……
……

20. How often do you eat a serve (serve is 1 teaspoon) of:

Food	Daily	Weekly	Monthly	Yearly	Never
Vegemite					
Honey					
Jam					
Other spreads:					
Sugar (in cereal, coffee or tea)					

21. How often do you have a cup (250ml) of tea?

Drink	Daily	Weekly	Monthly	Yearly	Never
Green Tea					
Normal (Black tea)					
Oolong					
Other tea: e.g. Jasmine, Chamomile					
Coffee (caffeinated)					

Coffee (decaf.)					

22. Are there any foods that you feel play an important part in your diet which have not been mentioned in this interview and how often do you eat them?

Food (description and serving size)	**Frequency**

23. Have you ever had a change in your sense of taste, other when you have had a cold/flu or allergy symptoms? (*circle <u>one only</u>*)

(1) Yes (2) No (3) Unsure

If you answered "No", please skip to 27.

24. If you don't have a normal sense of taste, please mark the box which most applies to you and write down your age when you first noticed this difference in your sense of taste. (*circle <u>one only and</u> write down age if appropriate*)

(1) I have never had a sense of taste

(2) I lost my sense of taste at age_____

(3) I noticed an increased sensitivity to taste at age _____

(4) I noticed a decreased sensitivity to taste at age _____

25. Was there an illness (other than cold / flu) or an injury around the time you first noticed this difference in your sense of taste? (*circle <u>one only</u>*)

(1) Yes (please specify what happened) _____
(2) No
(3) Unsure

26. Did you change medications around this time? (*circle <u>one only</u>*)

(1) Yes (around this time did you start a new medicine or stop one?)
_____
(2) No
(3) Unsure

27. Do you eat certain foods because they have a specific health benefit or are thought to be healthy? *(circle response number)*

If there is any indication at all that they are eating a certain food or certain foods because of a perceived health benefit or are avoiding a certain food or certain foods because of a perceived health risk then tick yes.

(1) Yes (2) No

When the questionnaire is complete please check that all questions have been answered.

"Thank you very much for your time and cooperation."

7.3 Appendix 2: Hospital Anxiety and Depression Scale

Date: _____
Study No:___ ___ ___ ___

We are aware that emotions play an important part in most illnesses.
This questionnaire is designed to help us to know how you feel.
Read each item and underline the reply which comes closest to how you have been feeling in **the past week**.
Don't take too long over your replies; your immediate reaction to each item will probably be more accurate than a long thought out response.

I feel tense or 'wound up':
- Most of the time
- A lot of the time
- From time to time, occasionally
- Not at all

I still enjoy the things I used to enjoy:
- Definitely as much
- Not quite as much
- Only a little
- Hardly at all

I get a sort of frightened feeling as if something awful is about to happen:
- Very definitely and quite badly
- Yes, but not too badly
- A little, but it doesn't worry me
- Not at all

I can laugh and see the funny side of things:
- As much as I always could
- Not quite so much now
- Definitely not so much now
- Not at all

Worrying thoughts go through my mind:
- A great deal of the time
- A lot of the time
- From time to time but not too often
- Only occasionally

I feel cheerful:
- Not at all
- Not often
- Sometimes
- Most of the time

I can sit at ease and feel relaxed:

Definitely
Usually
Not often
Not at all

I feel as if I am slowed down:

Nearly all the time
Very often
Sometimes
Not at all

I get a sort of frightened feeling like 'butterflies' in the stomach:

Not at all
Occasionally
Quite often
Very often

I have lost interest in my appearance:

Definitely
I don't take so much care as I should
I may not take quite as much care
I take just as much care as ever

I feel restless as if I have to be on the move:

Very much indeed
Quite a lot
Not very much
Not at all

I look forward with enjoyment to things:

As much as ever I did
Rather less than I used to
Definitely less than I used to
Hardly at all

I get sudden feelings of panic:

Very often indeed
Quite often
Not very often
Not at all

I can enjoy a good book or radio or TV programme:

Often
Sometimes
Not often
Very seldom

Now check you have answered all questions. Thank you