OPTIMISING B VITAMIN STATUS IN OLDER ADULTS: EXPLORING THE ROLE OF ONE-CARBON METABOLITES

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ABSTRACT

Population ageing presents profound challenges for individuals and societies alike, unless countered with strategies which promote 'healthy ageing' over the expected decline of physical, social and cognitive function. B vitamins (folate, riboflavin, B_6, B_{12},) are an attractive target given their implication in several diseases projected to confer an overwhelming burden in ageing societies, including cardiovascular and neurodegenerative diseases. This association is attributed to the role of B vitamins in maintaining one-carbon (1C) metabolism, including homocysteine (Hcy) regulation, DNA synthesis and repair, and methylation reactions. A narrow focus on folate, vitamin B_{12} and Hcy has commanded research attention to date, but the nutrient-function-health relationship concerning B vitamins requires further understanding in light of the complex endogenous and exogenous influences of 1C metabolism in ageing.

The overarching aim of this thesis was to explore how B vitamin status can be optimised and monitored in older adults, using 1C metabolites as measure of functional B vitamin status. A mass spectrometry technique was employed to quantify a comprehensive profile of 1C metabolites, including Hcy alongside those in the central methionine (methionine, *S*-adenosylmethionine, and *S*-adenosylhomocysteine), choline oxidation (choline, betaine, dimethylglycine, glycine), and transsulfuration (cystathionine, cysteine, serine) pathways.

The first objective of this thesis was to describe age-related changes to B vitamin intake and metabolism. This was first achieved by a systematic review in Chapter 3 exploring longitudinal shifts in dietary B vitamin adequacy. Only eight studies (*n*=3119) were included in the final analysis. While evidence for a decline in riboflavin adequacy with age was provided, this study foremost sheds light on the current lack of understanding regarding changes to intake. In Chapter 4, the acute 1C metabolite response to ingestion of a single supplement containing B vitamins alongside a simple meal was evaluated in healthy younger (19-30y, *n*=20) and older (65-76y, *n*=20) adults. Despite divergent baseline metabolite profiles between age groups, the postprandial response was largely similar and again revealed a lack of comparative data in the literature. This research evokes concern around assumptions made concerning B vitamin intake and metabolism in older age, which are the foundations required to inform how and when B vitamin status is optimised.

The second objective was to examine the relationship between B vitamins, 1C metabolites and health, which was evaluated in the context of cardiometabolic and cognitive health in Chapter 5. In a population of healthy older community-dwelling adults (*n*=313, 65-74y), higher glycine concentrations were consistently associated with protective cardiometabolic risk profiles and enhanced cognitive performance. Higher betaine or lower Hcy, dimethylglycine, and cysteine concentrations might also comprise a favourable 1C metabolite profile based on this cohort. However, the relationship between these metabolites and health markers was either less consistent than that of glycine, or dependent on apolipoprotein E genotype, B vitamin intake, or other metabolites. Ultimately, these findings do not point towards a single interpretation of an optimal 1C metabolite profile, and their relevance in light of monitoring habitual dietary intake or interventions seeking to optimise B vitamin status requires further attention.

The final objective was to measure the 1C metabolite response to increased B vitamin intake through interventions already recommended to promote longevity, focusing on higher protein intake. Chapter 6 explores the response of 1C metabolites, cardiometabolic markers, and their co-regulation following six months of resistance training alone or with supplementation in seniors recruited across residential care homes

in Vienna (65-98y, *n*=95). Daily consumption of a protein-based supplement containing moderate quantities of B vitamins (Fortifit; 20.7g protein, 200 µg of folic acid, 3.0 µg of vitamin B_{12}, 750 µg of vitamin B_6) alongside resistance training for six-months did not provide further benefits for 1C regulation compared to resistance training alone or a control group in this population of seniors. Regardless of the intervention, choline concentrations transiently increased, while Hcy, cysteine and methionine concentrations were elevated at the six-month follow-up. These findings reflect the nuanced interpretation required for interpreting shifts in 1C metabolites, and highlight the relevance of competing actions of 1C nutrients such as methionine and B vitamins in determining the Hcy response to intervention, particularly in the very old.

In contrast, Chapter 7 demonstrated that Hcy concentrations declined in community-dwelling older men (>70y) following ten-weeks of intervention with a whole-food diet containing either the current protein recommendations (0.8 g/kg body weight/day; *n*=14) or twice that (*n*=15). This study revealed novel insights into the interplay between choline, betaine, and folate in Hcy remethylation, though it remains difficult to interpret what fluxes in metabolites beyond a decrease in Hcy concentration should be considered favourable. The interactive influence of nutrients within a wider dietary pattern on 1C metabolite status has largely been neglected, and these studies invite future research to address the competing (or synergistic) effect of nutrients like protein and B vitamins on 1C metabolite status. Such research will be instrumental in developing consistent, practical advice for older adults.

This thesis has broadened the scope of research pertaining to B vitamin and 1C metabolite status, and brings healthy ageing principles to the forefront. B vitamins have an irrefutable role in maintaining health, but the true challenge will be ensuring their integration within the rapid evolution of nutrition research which is progressing beyond a nutrient-level focus. While a broader profile of metabolites has undoubtedly added complexity to interpreting the findings presented here, this approach does pave the way for future research. Ultimately, the nuanced interpretation of a 1C metabolite profile compared to health status or in response to intervention points towards the need for a personalised approach to optimising B vitamin status. Future research should also consider more sensitive biomarkers of disease progression or ageing, such as the use of novel epigenetic clocks, to understand the true value of B vitamins in promoting a healthy ageing phenotype. However, this thesis foremost calls for better understanding of shifts in nutrient status which are attributable to ageing – this is a necessary foundation from which we can direct recommendations that will have extensive health benefits for ageing populations.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

1C	One-carbon
2RDA	Twice the recommended daily allowance
5-MTHF	5-methyltetrahydrofolate
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
BHMT	Betaine-homocysteine methyltransferase
BMI	Body mass index
CBS	Cystathionine β-synthase
CGL	Cystathionine γ-lyase
COMPASS	Computerised mental performance assessment system
CT	Cognitive training
DMG	Dimethylglycine
EAR	Estimated average requirement
EGRac	Erythrocyte glutathione reductase activation coefficient
GFR	Glomerular filtration rate
GNMT	Glycine *N*-methyltransferase
Hcy	Homocysteine
HDL	High density lipoprotein
HOMA-IR	Homeostatic model assessment of insulin resistance
LDL	Low density lipoprotein
MCI	Mild cognitive impairment
MMA	Methylmalonic acid
MoCA	Montreal cognitive assessment
MTHFR	Methylenetetrahydrofolate reductase
MVM	Multivitamin and mineral
PBMC	Peripheral blood mononuclear cell
PEMT	Phosphatidylethanolamine methyltranfserase
PLP	Pyridoxal 5’ phosphate
PN	Personalised nutrition
QC	Quality control
RDA	Recommended daily allowance
RDI	Recommended daily intake
RT	Resistance training
RTS	Resistance training with supplementation
SAH	*S*-adenosylhomocysteine
SAM	*S*-adenosylmethionine
SHMT	Serine-homocysteine methyltransferase
SD	Standard deviation
TMAO	Trimethylamine N-oxide
UHPLC-MS/MS	Ultra-high performance liquid chromatography with tandem mass spectrometry
VLDL	Very low density lipoprotein

INTRODUCTION

1

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 OVERVIEW

The importance of maintaining health and preventing functional decline in older years has been critically highlighted during the course of 2020, bringing discourse on 'healthy ageing' to the forefront at the onset of the World Health Organization's "Decade of Healthy ageing (2020 – 2030)"[1].

Adequate nutrition is a pivotal factor in the ageing trajectory. Within the broader scope of nutrition, there is particular interest on the protective role of metabolically-related B vitamins (folate, riboflavin, B_6, B_{12}) due to their concerted regulation of the pathways comprising one-carbon (1C) metabolism. B vitamins and 1C metabolites have been extensively associated with diseases predominant in ageing societies, yet understanding of how B vitamin intake might be optimised to promote healthy ageing remains incomplete.

1.2 AGEING, NUTRITION AND HEALTH

The United Nations describes global population ageing as a "human success story"[2]. Most people born today can expect to live well beyond their 60's owing to remarkable advancements in public health, medicine, and economic and social development. Extending longevity was the impetus for driving total population growth, and a later decline in fertility has triggered successive shifts in the age structure of populations[2]. Loss of function is the cardinal feature of ageing, which combined with increasing chronic disease incidence has wide-reaching impacts for most aspects of society[3].

Interest on the topic of 'healthy ageing' has piqued, now recognised in the World Health Organization's call for a 'Decade of Healthy Ageing'[1]. It is of considerable public health interest to develop recommendations and policy which aims to slow, delay, or even reverse the ageing process, ensuring that declining functional capacity does not prevail over added years of life[4]. To this end, nutrition has emerged as a powerful modifiable determinant of health in older age[5].

1.2.1 POPULATION AGEING

1.2.1.1 Demographics of population ageing

Virtually all developing and developed countries are experiencing a profound demographic shift, with growth in both the size and proportion of older persons. In 2019, it was estimated that 9% of the global population were aged 65 years or older, which is projected to almost double by 2050, reaching 16% of the population[6]. The most dramatic change is in the 'very old' population. Between 1990 and 2019, the number of persons aged 80 years or older tripled, growing from 54 million to 143 million, and this is projected to triple again by 2050[2].

New Zealand follows the same trend as most developed nations, and significant population ageing is expected to continue for the coming decades. Life expectancy in New Zealand is rising. Men and women born today are expected to live 10.5 and 7.7 years longer, respectively, compared to those born in the 1970s[7]. At the same time, the proportion of older adults is also rising. Those aged 65 years or older comprised 15% of the population in 2016, which is projected to increase to 21-26% of the population in 2043, and up to 24-33% of the New Zealand population by 2068[7]. While there is a temporary 'bulge' as the baby boomer generation reaches

retirement, ageing in New Zealand will be sustained by declining fertility and mortality rates and characterised by 'ageing of the aged'. Following global trends, the population of individuals aged 80 years or older is growing the fastest. There were approximately 84,000 individuals aged 85 years or older in 2016, only 2% of the population, which is expected to increase four-fold by 2068[7]. Although most older adults are currently New Zealand European, the burgeoning ageing population will become more culturally diverse as the elderly Māori population is expanding more rapidly than other groups[8].

1.2.1.1.1 Demographics of ageing and challenges for dietary intake

Certain demographic changes which occur with advancing age create challenges in older people's ability to access and consume nutritious, quality food[9]. For instance, reduced income with the onset of retirement, compounded by rising health-care costs, can restrict the ability of older adults to purchase fresh, nutritious food. Similarly, financial insecurity can limit one's access to social networks, leading to compromised food and nutrient intake[10]. Living arrangements may be considerably altered for older adults which can either positively (living somewhere that provides appropriate support) or adversely (living alone) impact dietary intake[9]. These demographic changes collectively contribute to an increased risk of food insecurity in older adults[9].

1.2.1.2 Health burden of population ageing

Ageing is a complex process characterised by the lifelong accumulation of damage at molecular, cellular, and tissue levels, which leads to progressively impaired physical function, frailty, disability, and disease[11,12]. Globally, chronic diseases have replaced communicable diseases as the leading cause of mortality[13]. There is a dramatically increased chronic disease prevalence in ageing populations, including neurological, metabolic, cardiovascular, and musculoskeletal disorders. Cardiovascular diseases, including ischaemic heart diseases and cerebrovascular diseases, are the leading cause of mortality in developed and developing countries alike. Cancers, dementia, and diabetes mellitus further contribute to a significant proportion of mortality in developed countries[13].

In New Zealand, the presence of risk factors for chronic disease increases with age, including high blood pressure, elevated cholesterol, and reduced physical activity, while the proportion of individuals who report being in good health concurrently declines[14]. It comes as no surprise then that older adults in New Zealand are over-represented in the prevalence of chronic disease[14] and multimorbidity[15]. Notably, 40% of the burden of disease in New Zealand is carried by those aged 65 years or older, and this is dominated by chronic conditions[16]. In addition to mortality from chronic disease, perhaps most importantly is the profound impact that the prolonged nature of many of these conditions will have on disability and diminished quality of life for ageing individuals and their families[13,17]. Indeed, the likelihood of having a disability increases markedly with advancing age, as does the severity of disability[18].

1.2.1.3 Societal burden of population ageing

A major concern with ageing is the escalating demand for disability and healthcare services[19]. Older adults in New Zealand have higher hospitalisation rates and utilise greater public health expenditure for general practice, pharmaceutical, laboratory, and disability support services than younger and middle-aged adults. The cost of an average person aged 65 years or older to the New Zealand public health system is approximately five-fold their younger counterparts[20]. In 2016, it was estimated that 42% of total healthcare services were used by older adults, despite comprising only 15% of the population[20], and projected analyses suggest that publicly funded health spending would rise from 6.5% of GDP in 2001 to 8.4% in 2051[21].

Ageing populations are typically associated with a declining workforce, and increasing expenditure on government pensions and social care will further increase the societal burden of ageing. Indeed, the old-age dependency ratio is projected to steeply rise from 27 older persons per 100 persons of working age in 2019 to 42 per 100 by 2050 in Australia and New Zealand[2]. There are striking differences in levels of employment according to age, which is the lowest amongst older people and declines rapidly with age. As levels of income are closely related to paid employment, older adults have lower average income than the working-age population, which comes with increasing demand on government expenditure for social support.

1.2.2 HEALTHY POPULATION AGEING

Despite ageing often being seen as synonymous with frailty, there is considerable variation in each individual's ageing process due to varied genetic and environmental exposures[22]. While both endogenous and exogenous factors contribute to the ageing process, only about one-third of the variation in longevity is suggested to be heritable, with most inter-individual variation due to stochastic and environmental factors[23]. Although many individuals do reach older age in good health, every individual faces the potential consequences associated with ageing owing to the collection of molecular and cellular damage.

Strategies must shift to promoting healthy over usual ageing, given the overwhelming health and societal burden that comes with the expected decline of physical, social and cognitive functioning with age[24,25]. This concept of ageing well can be traced back to Renaissance texts[26], with a modern interpretation pioneered by Rowe and Kahn in the late 20th century[24], who argued that old age should not equate to loss and disability. Their multidimensional model of successful ageing included engagement in life, avoiding disease, and maintaining high cognitive and physical functioning. The term 'successful' has since been met with resistance and 'healthy ageing' is perhaps a more appropriate definition, which the World Health Organisation defines as the "process of developing and maintaining functional ability that enables wellbeing in older age"[27].

Evidence describing whether the rapid increase in life expectancy is paralleled by the same increase in health-span, the period of life spent in good health, free from chronic disease and disabilities of ageing remains unclear[28–32]. It is of considerable public health interest to develop recommendations and policies which promote healthy ageing and ensure that declining functional capacity does not prevail over these added years of life. To this end, nutrition has a critical influence on the health trajectory of ageing societies[31].

1.2.3 ROLE OF NUTRITION IN THE AGEING TRAJECTORY

Remarkable advances have been made in the field of nutrition and health over the past century. In 1913, Casimir Funk proposed the existence of a 'vital amine' in food, which was isolated and chemically defined in 1926. Less than 100 years later, nutrition science has reached a critical point where the role of diet in complex diseases is at the foreground[33]. It is now indisputable that the impact of nutrition on health spans the life-course from the 'first 1000 days', through to modifying the ageing trajectory in older years[23,34,35].

Over 80 years ago now, rodent studies showed that dietary restriction led to increased lifespan[36], providing the first irrefutable evidence for the influence that nutrition has on the trajectory of ageing[5]. Decades later, there is now substantial evidence for the role of broader dietary aspects in preventing age-related diseases and mortality[5,37], even reversing disease diagnosis[38], and contributing to people continuing to live in the community with independence, psychological well-being and good self-rated health[39].

It has become clear that for dietary recommendations to effectively support health in advancing age, they must aim to counter the accumulation of damage to cellular macromolecules which characterises the ageing process[5]. It is promising to know that the ageing trajectory is plastic and responds to dietary modification, likely attributed to the collective action of dietary factors, including energy, individual nutrients, and non-nutrient bioactive constituents[5]. However, dietary advice for older adults remains inconsistent and divergent, which perhaps reflects the historically reductionist approach of investigating diet and health rather than seeking to maximise health benefits[33]. For example, expert consensus statements recommend higher protein diets for older adults to prevent the onset of sarcopenia[40]. In contrast, evidence is accumulating for the benefits of older adults adopting a Mediterranean-style diet, which does not typically align with higher protein intake. A Mediterranean dietary pattern is now associated with a substantial reduction in the risk of several age-related conditions including cardiovascular and neurodegenerative diseases[41], and even enhanced longevity[42–44].

Meanwhile, micronutrient recommendations largely still reflect the mid-20th century focus on preventing deficiency diseases. It is intriguing that research attention is shifting towards wider dietary patterns to support health when nutrient needs to enhance or optimise healthy ageing remains poorly understood. Indeed, micronutrients play a critical role in modifying the molecular changes characteristic of ageing, such as telomere damage, DNA instability, and epigenetic alterations[45], and likely underpin the success of dietary patterns outlined above. Evidence regarding the consequences of suboptimal micronutrient intake is only emerging, where it is proposed that even modest shortages of micronutrients may accelerate age-related diseases[46]. In this context, there is growing attention on the protective role of metabolically-related B vitamins (folate, riboflavin, vitamins B_6 and B_{12}, herein termed 'B vitamins') in ageing research.

1.3 B VITAMINS AND HEALTHY AGEING

Enormous progress has been made in understanding the role of B vitamins in health since their discovery less than a century ago. In 1926, it was found that daily consumption of half a pound of liver could prevent pernicious anaemia[47], which led to the isolation of vitamin B_{12} as 'small red needles' from liver extracts in 1948[48]. Similarly, the discovery of folic acid eventuated in modern-day Mumbai in 1928 during the search to cure the common occurrence of macrocytic anaemia in pregnant women[49]. Around the same time, riboflavin, originally called 'vitamin G' was identified and shown to treat cheilosis[49], and vitamin B_6 was discovered as a compound which cured rat acrodynia[50].

The role of B vitamins in preventing such classical deficiency diseases is now well established, though not the concern of this review or subsequent research presented in this thesis. Though it is pertinent to appreciate the history and rapid advances of micronutrient research in order to understand more recent shifts in focus. In a short time frame, attention has progressed from single nutrients in disease treatment, to acknowledging the interaction of vitamins in maintaining health throughout the life-course. This observation is exemplified in the journey of folic acid research - from discovering its crucial role in neural tube defect prevention, to the adoption of fortification policies around the world. Now, research suggests that 'too much of a good thing' likely exists, with concerns of fortification masking vitamin B_{12} deficiency in older adults and exacerbating neurological symptoms[51].

Most important is the emerging link between marginal B vitamin deficiencies in diseases predominant in ageing societies, ranging from cardiovascular diseases, to neurodegenerative disorders, certain cancers, and osteoporosis[52]. This interest in B vitamins stems from their inter-connected role in maintaining one-carbon metabolism, a network of reactions involving the transfer of 1C units, which will be discussed in further detail later in this review. What is important to note here is that the metabolism of these B vitamins is inextricably linked through their role in balancing 1C reactions which are implicated in disease processes, including homocysteine (Hcy) regulation, DNA synthesis and repair, and methylation reactions. For instance, folate and vitamin B_{12} metabolism is connected by their concerted remethylation of Hcy to methionine, and in the case of vitamin B_{12} deficiency folate essentially becomes trapped and its role in DNA synthesis/repair is compromised[53]. While the primary research focus to date has largely been on the co-regulation of Hcy by folate and vitamin B_{12}, vitamin B_6 and riboflavin are also required for 1C metabolism, and even a marginal deficiency in any of these nutrients may disrupt the complex regulatory network that maintains 1C metabolism.

1.3.1 SPECIAL CONSIDERATIONS FOR OLDER ADULTS

1.3.1.1 Changes to nutrient intake – the 'anorexia of ageing'

Nutritional adequacy is critical for healthy ageing, yet becomes increasingly difficult for older adults to achieve. Bodily functions progressively decline with age, which causes deterioration of the physiological mechanisms controlling food intake, nutrient absorption and utilisation[54]. The ageing process involves several small changes in taste and smell due to a decline in olfactory receptors[55], altered gastric fundal compliance, and subtle neuroendocrine changes to both orexigenic and anorexigenic hormones, as well as altered autonomic nervous system feedback[56,57]. These changes collectively result in older adults consuming smaller meals and fewer snacks, feeling fuller between meals, eating more slowly, and experiencing more rapid satiation[54,58].

The physiological loss of appetite and reduced energy intake is termed the 'anorexia of ageing'[54], and is compounded by psychological, social, and physical changes which occur more frequently with advancing age. For example, older adults are more likely to encounter challenges with swallowing and chewing such as poor dentition, or neurological disorders and physical disabilities which limit mobility[59]. Declining gastric acid secretion is also prevalent amongst older adults, predisposing individuals to stomach and proximal small bowel bacterial overgrowth[60], which is associated with reduced micronutrient intake[61]. Beyond these more insidious changes to appetite and energy intake, ageing is associated with profound changes to an individual's psychosocial and environmental status. These changes may include isolation, loneliness, depression or anxiety, bereavement, and financial difficulties, all of which can significantly impact one's dietary intake[62].

1.3.1.2 Gastrointestinal dysfunction

As reviewed by Milan and Cameron-Smith[63], the ageing process is accompanied by impaired macronutrient digestion and metabolism, which is compounded by the presence of underlying comorbidities. Comparatively, the impact of age on micronutrient digestion, absorption and metabolism remains poorly understood.

It was assumed for many years that gastrointestinal structural integrity and function declines with age, leading to reduced micronutrient absorption. Early studies reported broader[64,65] and shorter[65–67] villi with ageing, yet there is remarkably little evidence to date demonstrating structural or functional changes in the small bowel mucosa that are attributable to the normal ageing process in humans[63,68,69]. Rather, the presence of structural abnormalities of the small intestine is likely secondary to gastrointestinal complications rather than the process of ageing *per se*[70,71]. For example, atrophic gastritis is not a product of ageing, but does occur with increasing prevalence in older age[71]. Atrophic gastritis is characterised by mucosal atrophy and reduced gastric acid secretion, leading to decreased acid-pepsin digestion in the stomach and elevated proximal small bowel pH, which impacts vitamin B_{12} and folic acid bioavailability amongst others where absorption occurs within a defined pH range[72,73]. Atrophic gastritis also promotes small intestinal bacterial overgrowth and *Helicobacter Pylori* infection, which may further impair micronutrient absorption[61,74].

Following B vitamin absorption, little is known about age-related changes to metabolism of vitamins into active vitamer forms, yet evidence suggests the presence of some degree of impairment in the case of vitamin B_6 metabolism[75,76] and perhaps for riboflavin[77,78]. Metabolic alterations of B vitamins remain inadequately understood in ageing populations and requires careful consideration in the wider context of absorptive dynamics to better direct nutrient recommendations for older adults.

1.3.2 B VITAMIN RECOMMENDATIONS FOR OLDER ADULTS

Older adults are not simply an 'aged' version of younger adults, and rather have distinct metabolic characteristics, though the extent to which this impacts B vitamin absorptive dynamics is unclear[79]. Regardless, changes to dietary intake and nutrient absorption occur with age, indicating that B vitamin requirements are likely unique as well. Remarkably, these are poorly characterised and nutrient recommendations for older adults are largely extrapolated from young or middle-aged populations[80], including the joint Australian and New Zealand reference values [81]. A classic example of the issues this approach entails lies in the same reference values used for vitamin B_{12} intakes across all age groups, despite the fact that vitamin B_{12} bioavailability from food sources is decreased for a substantial proportion of older adults with atrophic gastritis. Older adults may therefore require higher intakes of vitamin B_{12} rich or fortified foods, or even depend more on supplemental intakes, but this is not reflected in nutrient reference values[71].

Defining appropriate nutrient recommendations for older adults is a challenging task, driven in part by the vast heterogeneity characterising ageing populations, with variation in age-related functional changes and diverse nutritional needs[82]. Nutritional guidelines for older people can address this age-related heterogeneity by categorising older people and their nutritional needs according to their functional ability and illness, such as in the Finnish guidelines[83]. Further complicating this is the limited agreement of outcome measures useful in older populations to derive markers of nutritional adequacy, and particularly so for biomarkers of healthy ageing[84,85]. The observation that the relationship between biomarkers and health outcomes can differ considerably even within an older population. For instance, high blood pressure has been associated with greater risk of cognitive impairment in those aged 65-74 years, but with better cognitive performance in those over 85 years[86]. It is important to note here that the applicability of dietary reference values for Māori and other minority ethnic populations is uncertain and must be addressed when considering the task of optimizing nutrient recommendations for ageing populations in the New Zealand context.

A final challenge is the paradigm shift from preventing deficiency to interest in nutrient recommendations that can prevent or delay chronic disease states. Nutritional adequacy of individuals is typically based on whether their recommended dietary intake (RDI) is met, or an estimated average requirement (EAR) is used to consider the adequacy of populations. These reference values are determined for levels of nutrients that are required for avoidance of deficiency diseases and sustenance[87] (**Table 1.1**). Although the evidence concerning micronutrient intake and chronic disease risk is extensive, identifying specific levels of optimal nutrient intake is difficult due to methodological inconsistencies. It is promising to see recognition that intake at levels above the RDI likely impacts chronic disease aetiology in the Australia and New Zealand joint dietary guidelines. Alongside other select nutrients, a Suggested Dietary Target has been defined for folate of 300-600 µg/day, which is 100-400 µg/day more than the RDI. This recommendation for increased folate intake stems from evidence on the role of folate in regulating Hcy, reducing cardiovascular and neurological diseases, and preventing DNA damage[87]. Although promising, these guidelines are only available for folate and not for other B vitamins involved in similar disease processes, do not consider evidence specifically for older adults, and are still not widely used as a standard for adequacy.

Table 1.1: Summary of dietary B vitamin recommendations and intake according to the 2008/09 Adult Nutrition Survey in New Zealand adults.

Nutrient	Sex	Age group	EAR	RDI	SDT	Intake		% inadequate intake
						Mean	Median (95% CI)	
Folate, µg/day	Males	19-30	320	400	300-600	NA	NA	NA
		31-50						
		51-70						
		>70						
	Females	19-30	320	400	300-600	NA	NA	NA
		31-50						
		51-70						
		>70						
Riboflavin, mg/day	Males	19-30	1.1	1.3	NA	2.4	2.4 (2.1-2.6)	0.2%
		31-50				2.4	2.3 (2.2-2.4)	1.6%
		51-70				2.2	2.0 (1.9-2.1)	6.6%
		>70	1.3	1.6		2.0	1.8 (1.7-1.9)	18.7%
	Females	19-30	0.9	1.1	NA	1.7	1.6 (1.5-1.8)	8.5%
		31-50				1.8	1.7 (1.6-1.8)	4.2%
		51-70				1.7	1.7 (1.6-1.8)	1.3%
		>70	1.1	1.3		1.6	1.5 (1.5-1.6)	15.4%
Vitamin B_6, mg/day	Males	19-30	1.4	1.7	NA	3.3	2.9 (1.7-4.0)	1.5%
		31-50				2.6	2.4 (1.9-2.9)	0.2%
		51-70				1.9	1.7 (1.6-1.9)	27.5%
		>70				1.6	1.6 (1.5-1.7)	28.8%
	Females	19-30	1.3	1.5	NA	2.3	2.1 (1.6-2.7)	0.8%
		31-50				1.8	1.6 (1.5-1.7)	17.0%
		51-70				1.5	1.4 (1.3-1.5)	36.6%
		>70				1.3	1.3 (1.2-1.4)	53.0%
Vitamin B_{12}, µg/day	Males	19-30	2.0	2.4	NA	5.3	5.2 (4.6-6.2)	0.0%
		31-50				5.2	5.0 (4.2-5.9)	0.2%
		51-70				5.2	4.5 (3.6-5.4)	2.8%
		>70				5.7	4.2 (2.6-5.8)	3.8%
	Females	19-30	2.0	2.4	NA	3.6	3.0 (2.6-3.4)	22.8%
		31-50				3.7	3.3 (2.9-3.6)	16.1%
		51-70				3.5	3.4 (2.8-4.0)	1.1%
		>70				3.2	2.7 (2.2-3.1)	27.0%

Dietary recommendations are listed according to the joint Australia and New Zealand Nutrient Reference Values[87]. For each age group, the intake of B vitamins and the prevalence of dietary inadequacy (calculated by probability analysis) are based on the 2008/09 Adult Nutrition Survey report[88]. Dietary folate intake was not assessed in the Adult Nutrition Survey due to limitations in estimating folate contents of foods from the New Zealand Food Composition Database at the time[89]
Abbreviations: EAR, Estimated average requirement; RDI, recommended dietary intake; SDT, suggested dietary target.

1.3.3 MARKERS OF B VITAMIN STATUS

1.3.3.1 Dietary assessment

Dietary assessment remains the cornerstone of monitoring nutrient status. Ideally, dietary assessment methods should be inexpensive, quick, and provide precise estimates of food and nutrient intake. However, the choice between conventional methods is often a compromise between accuracy, suitability for the population, and burden to the participant and researcher[90]. Methods can broadly be categorised as prospective and retrospective. A seven-day weighed food record is considered the gold standard of dietary assessment but is laborious for participants to complete, and three- or four-day records are the most widely used prospective methodology. Prospective methods are however subject to social bias, where participants might modify their diet for the course of dietary assessment to align with perceived social norms[90]. On the other hand, retrospective methods, such as 24-hour diet recalls and food frequency questionnaires depend more heavily on participants' memory. Further, list-based tools like the food frequency questionnaire require participants to accurately interpret questions on portion size and types of food consumed, while interviewer-based methods like the 24-hour recall rely heavily on the expertise of the research staff[90].

Further concerns arise on the topic of dietary assessment in older adults, specifically[91]. The onset of cognitive decline with impaired memory, attention, and communication skills in older adults creates difficulties in capturing an accurate picture of dietary intake, particularly in retrospective methods. Memory can start to decline in mid-life, and this may be an issue even in healthy older adults[92]. Physical limitations, such as visual impairment, hearing problems, and difficulties writing, may also be an issue across dietary assessment methodologies. There is further difficulty in recalling or recording intake if individuals have little or no involvement in purchasing and preparing food, which occurs more frequently with age, and particularly in settings where additional care is required[91–93]. Older adults also tend to have different eating behaviours to younger counterparts which needs to be taken into account, including higher consumption of ethnic foods, smaller portion sizes, and a more traditional, organised meal pattern[94]. Despite these potential complications, there is little evidence that healthy community-dwelling older adults have greater difficulties in providing an accurate account of dietary intake than younger adults[90,92,93,95]. In saying this, care needs to be taken to choose a dietary assessment method that is both appropriate for the research question and also considers the cognitive skills and functional status of the study population[92].

Beyond those issues in actual dietary assessment, the conversion of food to nutrient intake relies on the accuracy and completeness of food composition tables, how robust the dietary assessment software is, and the skill of the researcher. A particular concern for measuring B vitamin intake is how accurately databases account for food fortification, particularly voluntary fortification from industry. For instance, dietary folate intake was omitted from the New Zealand Adult Nutrition Survey as data in the food composition tables is largely based on manufacturer's claims which may not reflect the true analytical value[89], and was considered unreliable[89]. Progressive changes to fortification policy and how quickly food composition tables keep up will significantly impact measures of nutrient intake. Other factors which will complicate estimates of B vitamin intake as a marker of status include expression of genetic variants that modify nutrient absorption and metabolism, for example carrying the methylene tetrahydrofolate reductase (MTHFR) C677T polymorphism[96–98], and microbial production of B vitamins in the large intestine[99,100].

1.3.3.2 Biochemical status

Several direct or functional markers are available to measure B vitamin status, each with their own advantages and limitations (**Table 1.2**). Direct markers are more commonly used for several reasons, including cost, availability of equipment, ability to reflect recent dietary intake or stores, and having established cut-off values applicable to clinical and research settings. However, the greatest limitation of direct markers is the typically poor reflection of functional status, though this does not hold true for all direct markers. For example, plasma pyridoxal 5' phosphate (PLP) is the most widely used marker of vitamin B_6 status and reflects the active circulating form of vitamin B_6[101]. In comparison, serum vitamin B_{12}, also widely used in both research and clinical settings, reflects the total circulating concentration of vitamin B_{12}, of which only 20% is metabolically active[102]. Holo-transcobalamin is considered a more robust direct marker of vitamin B_{12} status, as it reflects the biologically active fraction of vitamin B_{12}. However, the assay is not as widely available and does not have defined cut-off values to suggest inadequacy[102]. Functional markers are promising in their potential to reflect the link between B vitamin status and health, but those currently available are not without limitations. For example, plasma Hcy is considered a functional biomarker of folate or vitamin B_{12} status, but it lacks specificity as concentrations can accumulate with deficiency of other B vitamins[103–105]. Comparatively, methylmalonic acid (MMA) is a highly specific and sensitive biomarker of vitamin B_{12} status. However the use of MMA is somewhat limited in older adults, as MMA is elevated with renal dysfunction, and its analysis is costly and requires more specialist equipment[106].

The use of single biomarkers and inconsistent application of cut-off points to define deficiency has created difficulties in interpreting B vitamin status in both the clinical and research setting, particularly for folate and vitamin B_{12}[107,108]. This issue is of particular concern amongst older adults, who are subject to the confounding influences of renal impairment, comorbidities, and polypharmacy, which impact the use and interpretation of both direct and functional markers of status[109]. Moving forward, combined biomarkers of status appear to be a promising approach to meaningful measures of status[110]. For instance, a composite score of vitamin B_{12} status including serum B_{12}, holo-transcobalamin, MMA, Hcy and which accounts for age and low folate status is likely a more superior marker of vitamin B_{12} status[109,111]. However, these integrated markers are not yet routinely used in clinical or research settings as further validation is required, and the challenge of differences in the sample type, processing, and specialist equipment required further limits their applicability[109,111].

Table 1.2: Summary of direct and functional markers of B vitamin status.

Vitamin	Type	Biomarker[1]	Advantage	Disadvantage
Folate	Direct	Circulating folate	• Reflects recent dietary folate intake.	• Impacted by alcohol ingestion. • Cut-off values are defined, but inconsistently used.
		Erythrocyte folate	• Reflects longer-term status. • Reflects tissue folate stores	• Cut-off values are defined, but inconsistently used.
	Functional	Circulating Homocysteine[2]	• Responsive to B vitamin depletion and repletion. • Stable at -80°C	• Poor specificity. • Impacted by genetics, renal impairment, age, medication.
Riboflavin	Direct	Circulating riboflavin, FAD, FMN	• Stable at -80°C.	• Impacted by age, sex, protein and alcohol intake. • High variability within and between-subjects of riboflavin
	Functional	EGRac	• Most widely used marker. • Reflects tissue saturation and long-term status. • Stable at -80°C.	• Does not reflect 'optimal' riboflavin status. • Assay is not standardised across labs.
Vitamin B_6	Direct	Plasma PLP	• Good specificity. • Most widely used marker. • Reflects tissue concentrations. • Responsive to depletion and repletion. • Stable at -80°C.	• Impacted by age, sex, protein and alcohol intake. • Sensitive to degradation at warmer temperatures and following light exposure.
		Erythrocyte PLP	• Responsive to depletion and repletion • Possibly more reliable than plasma PLP under inflammatory conditions/diseases.	• Assay is technically difficult, with variable recovery and low precision.
Vitamin B_{12}	Direct	Circulating vitamin B_{12}	• Good sensitivity. • Easily accessible and cheap.	• Poor specificity. • Does not reflect intracellular concentrations. • Functional deficiency can occur in normal ranges. • Not appreciably influenced by recent dietary intake. • Impacted by liver disease and renal impairment. • Cut-off values are defined, but inconsistently used.
		Circulating holoTC	• Good sensitivity • Measures 'functional' component of serum vitamin B_{12}. • Earliest indicator of vitamin B_{12} depletion. • Likely the best marker in older adults.	• Specificity not yet known. • Cut-off values are not standardised. • Limited availability of assay. • Impacted by renal impairment.
	Functional	Circulating or urinary MMA	• Excellent sensitivity and good specificity. • Reflects intracellular concentrations. • Early indicator of functional vitamin B_{12} deficiency.	• Impacted by renal impairment and bacterial overgrowth. • Expensive and specialist equipment required.

[1]Circulating is considered as plasma or serum. [2]Also used as an indicator of vitamin B_{12} status. Abbreviations: EGRac, erythrocyte glutathione reductase activation; FAD, Flavine Adenine Dinucleotide; FMN, Flavin Mononucleotide; GC-MS, Gas Chromatography coupled with Mass Spectrometry; holoTC, holo-transcobalamin; HPLC, High-Performance Liquid Chromatography; LC-MS, liquid chromatography coupled with Mass Spectrometry; MMA, Methylmalonic Acid; PLP, Pyridoxal 5' Phosphate

1.3.3.2.1 Relationship between direct and functional markers in older adults

Older adults are undoubtedly at greater risk for inadequate B vitamin status, although it remains uncertain whether deficiencies are actually more prevalent with advancing age[52]. Regardless, it is clear that the prevalence of B vitamin deficiency is greater when functional markers are used compared to direct biomarkers of status[112–114]. During the rise of functional indices, Joosten and colleagues[112] investigated whether the measurement of metabolites could provide a better indication of intracellular and functional deficiency of micronutrients. Indeed, the authors found a greater prevalence of elevated serum metabolites which reflect inadequate B vitamin status (including Hcy and MMA) compared to direct markers of deficiency in both healthy and hospitalised older adults, with the greatest discrepancy found for vitamin B_{12}. Following this, Naurath and colleagues[115] showed that intramuscular vitamin supplementation reduced serum Hcy and MMA even in those with baseline adequacy according to direct serum measures, strongly supporting the notion that direct markers of status under-report the true prevalence of deficiency.

The relationship between direct and functional markers also appears to be impacted by the status of other B vitamins. Chen and colleagues[116] found that the association between riboflavin inadequacy and elevated Hcy was influenced by the presence of vitamin B_6 or folate deficiency. Similarly, the strength of association between vitamin B_{12} inadequacy and Hcy increased approximately three-fold when combined with folate inadequacy, but was not affected by riboflavin or vitamin B_6 inadequacy[116]. Even with functional markers like Hcy, emerging evidence shows the benefit of more integrated measures of a biological pathway to provide greater insight into the relationship between B vitamin status and health outcomes. For example, analysing ratios of metabolites in the same pathway can better reflect functional B vitamin status than a single functional marker like Hcy[117,118], and correlate stronger with health outcomes like metabolic stress[119] and colorectal cancer[120] in both young and older populations. Although promising, studies using this integrated biomarker approach are scarce and require further validation in older adults.

Evidently, there is a need to better understand the relationship between functional markers of status and health outcomes, and this will require moving beyond single functional markers to a more integrated analysis of biological pathways. While there is currently a trade-off in choosing markers between practicality (cost, availability) and understanding the dynamic relationship between B vitamin status and health, the paradigm of how B vitamin status is monitored has the potential to transform with emerging -omics technologies.

1.4 ONE-CARBON METABOLISM

One-carbon (1C) metabolism is comprised of a series of reactions involving the transfer of methyl groups between molecules. The central methionine cycle, folate cycle, and choline oxidation and transsulfuration pathways are inter-connected in 1C metabolism by their mutual dependence on B vitamins. Together, these pathways regulate major cellular processes through the movement of 1C groups, including DNA synthesis and repair, and diverse methylation reactions.

1.4.1 A BRIEF OVERVIEW OF ONE-CARBON METABOLISM

Due to its cyclical nature, there are many points at which one could 'begin' describing the reactions and metabolites involved in regulating 1C metabolism. The focus of this review is largely on Hcy and the central methionine cycle, and so begins with the adenosylation of the essential amino acid methionine by methionine adenosyltransferase to produce *S*-adeonsylmethionine (SAM)[121]. A collection of methyltransferases then coordinate transmethylation reactions whereby SAM donates a methyl group to various methyl acceptors, notably including the methylation of DNA and histone tails which are two major epigenetic modifications. Every transmethylation reaction using SAM as a methyl donor forms *S*-adenosylhomocysteine (SAH), which is immediately hydrolysed to Hcy in a reversible reaction[122]. Here, the two fates of Hcy are either removal through the transsulfuration pathway or remethylation to methionine, reactions which are both sensitive to B vitamin status. Hcy transsulfuration occurs primarily in the liver by two PLP-dependent reactions. First, cystathionine β-synthase (CBS) catalyses the condensation of Hcy with serine to form cystathionine, which is then hydrolysed by cystathionine γ-lyase (CGL) to form cysteine[123,124]. Alternatively, Hcy remethylation is catalysed by methionine synthase or betaine-homocysteine methyltransferase (BHMT) which are B vitamin-dependent and independent pathways, respectively. Methionine synthase requires 5-methyltetrahydrofolate (5-MTHF) as a methyl donor and vitamin B_{12} in the form of methylcobalamin as a cofactor, while BHMT only requires betaine as a methyl donor[125,126]. Hcy remethylation by methionine synthase is the major point at which the folate cycle is linked to the methionine cycle, yielding tetrahydrofolate. Tetrahydrofolate then accepts a 1C unit from serine in a PLP-dependent reaction to form 5,10-methylenetetrahydrofolate, which is used either for thymidine and purine synthesis or is converted to 5-MTHF. 5-MTHF is the primary circulating form of folate, and its synthesis requires riboflavin as flavin adenine dinucleotide for the activation of MTHFR[125]. An overview of the inter-connected pathways comprising 1C metabolism is summarised in **Figure 1.1**.

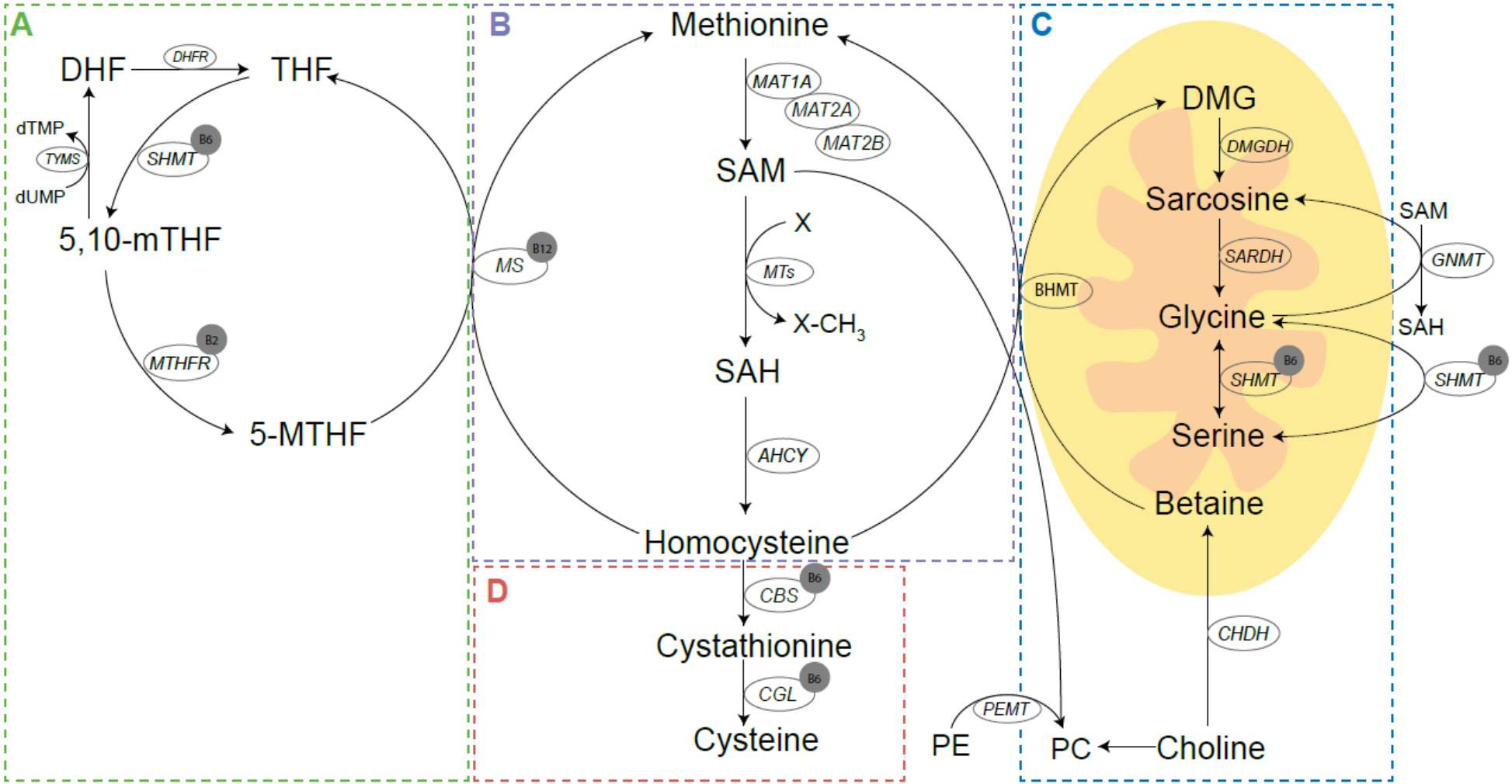


Figure 1.1: Overview of one-carbon metabolism.
Metabolites in the A) folate cycle, B) methionine cycle, C) choline oxidation pathway (predominantly occurring in the mitochondria) and D) transsulfuration pathway which comprise one-carbon metabolism. B vitamins acting as co-enzymes are in a grey circle, and enzymes are outlined in an oval. Abbreviations: AHCY, S-adenosylhomocysteine hydrolase; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; CGL, cystathionine λ-lyase; CHDH, choline dehydrogenase; DHF, dihydrofolate; DHFR, dihydrofolate reductase; DMG, dimethylglycine; GNMT, glycine N-methyltransferase; MAT, methionine adenosyltransferase; MS, methionine synthase; MTs, methyltransferases; PC, phosphatidylcholine; PE; phosphatidylethanolamine; PEMT, phosphatidylethanolamine methyltransferase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SARDH, sarcosine dehydrogenase; SHMT, serine hydroxymethyltransferase (SHMT1, cytosolic; SHMT2, mitochondrial); THF, tetrahydrofolate.

1.4.2 THE FINE BALANCE OF HOMOCYSTEINE REGULATION

1.4.2.1 Transmethylation reactions

Hcy resides at a critical branch-point in 1C metabolism and is accordingly considered an overarching marker of 1C and B vitamin status. Maintenance of Hcy concentrations in tissue and plasma is subject to competing demands of synthesis, remethylation, and catabolism[127,128], though the research focus has largely been on Hcy removal through reactions sensitive to B vitamin status. However, Brosnan and colleagues[129] have highlighted that the transmethylation reactions which produce Hcy should be equally addressed, as demand on methyltransferases has important consequences for Hcy regulation.

Transmethylation reactions, involving the transfer of methyl groups from donor to acceptor by methyltransferases, are the cornerstone of 1C metabolism. Mudd and Poole pioneered early attempts to estimate methyl balance in humans by investigating fluxes of labile methyl groups[130]. This research provided initial evidence that when methyl group intake (derived predominantly from choline, then followed by methionine and methylfolate) is less than output, *de novo* formation of methyl groups can balance the difference. It is now understood that requirements for methionine conservation govern the coordination of pathways comprising 1C metabolism. Methionine is required to synthesise SAM, which is considered the 'universal methyl donor' for critical transmethylation reactions involving DNA, RNA, and the central nervous system[125]. This requirement is largely achieved through a feedback mechanism, where transmethylation enzymes are stimulated when SAM accumulates, leading to excess SAM being scavenged and subsequent production of SAH. SAH is a potent inhibitor of methyltransferases, and the ratio of SAM/SAH therefore serves as an index of transmethylation potential[131]. Here, glycine *N*-methyltransferase (GNMT) is particularly important for ensuring that methyl group balance is 'optimised' for SAM-dependent transmethylation reactions and regulates the balance of SAM/SAH. GNMT is less sensitive to inhibition by SAM compared to other methyltransferases, and instead is allosterically inhibited by 5-MTHF[132]. This regulatory system ensures the conservation of methyl groups for SAM-dependent transmethylation reactions when methyl group availability is restricted, and promotes the utilisation of methyl groups when supply is abundant. The two SAM-dependent transmethylation reactions considered of most quantitative importance to Hcy synthesis are catalysed by guanidinoacetate methyltransferase and phosphatidylethanolamine methyltransferase (PEMT) which produce creatinine and phosphatidylcholine, respectively[129]. Historically, it was thought that guanidinoacetate methyltransferase consumed up to 70% of methyl groups derived from SAM. However, more recent evidence suggests that PEMT may actually be a greater consumer of methyl groups and therefore have the greatest impact on Hcy status[133]. Indeed, *Pemt*$^{-/-}$ mice have lower Hcy concentrations than wild-type mice, demonstrating the functional link between 1C metabolism and phosphatidylcholine biosynthesis[134].

Following Hcy synthesis through methionine and SAM, remethylation occurs through two pathways – either by methionine synthase (found ubiquitously throughout the body), or BHMT (primarily expressed in the liver and kidney). Despite focus on folate and vitamin B_{12} as key regulators of Hcy remethylation via methionine synthase, BHMT is thought to contribute equally to Hcy remethylation under normal conditions[135]. SAM also regulates enzymes involved in Hcy cycling and clearance. For instance, the autoinhibitory domain of CBS is relieved when SAM accumulates, promoting Hcy removal through transsulfuration[131]. In contrast, SAM inhibits both remethylation enzymes, although allosteric inhibition of BHMT only occurs at very high SAM concentrations[136].

1.4.2.2 The choline oxidation pathway

The choline oxidation pathway involves the mitochondrial conversion of choline to glycine through betaine, dimethylglycine (DMG) and sarcosine[137]. Choline is provided exogenously through dietary sources or by endogenous synthesis through the methylation of phosphatidylethanolamine to phosphatidylcholine by PEMT[138]. Choline's conversion to betaine in a two-step reaction by choline dehydrogenase and betaine aldehyde dehydrogenase begins the choline oxidation pathway. While betaine may also be provided exogenously through the diet, endogenous betaine synthesis from choline is considered essential for maintaining adequate concentrations[138]. Betaine has two primary functions in humans - first as an osmolyte, and second as a methyl donor for BHMT-mediated Hcy remethylation[139,140]. *Bhmt*$^{-/-}$mice have impaired methylation potential and elevated fasting Hcy that is not influenced by dietary folate intake, highlighting the critical involvement of this pathway[141]. In addition to methionine, BHMT-dependent Hcy remethylation yields DMG, which undergoes two demethylation reactions in the mitochondria to produce sarcosine and glycine. Glycine has several fates, but can be retained in 1C metabolism by GNMT or interconversion with serine by the PLP-dependent enzyme serine hydroxymethyltransferase (SHMT), expressed both in the cytosol (SHMT1) and mitochondria (SHMT2)[142]. The choline oxidation pathway influences both methylation demand and Hcy regulation through PEMT-dependent phosphatidylcholine synthesis and BHMT remethylation. Despite the integral role of choline metabolites in 1C metabolism, influencing both methylation demand (SAM-dependent phosphatidylcholine synthesis) and Hcy regulation (betaine-dependent remethylation), they have received little research attention compared to Hcy and B vitamins.

1.4.2.3 A brief summary

The regulation of 1C metabolism is influenced by endogenous and exogenous shifts in methyl groups and metabolites, which creates complexity when interpreting 1C metabolite status (**Table 1.3** and **Table 1.4**). Circulating Hcy reflects the interaction between competing demands for transmethylation reactions and its removal, which is governed by methyl group and B vitamin availability. While Hcy is at a critical intersection of 1C regulation, Hcy alone as a marker of 1C metabolism or B vitamin status tells a somewhat limited story of the fine balance of 1C metabolism.

Table 1.3: Overview of one-carbon metabolites in the circulation

Metabolite	Pathway	Endogenous synthesis		Exogenous dietary sources[1]		Interpretation[2]
			Precursor			
Betaine	Choline oxidation	✓	Choline	✓	Spinach, whole wheat, beetroot	↑ with decreased betaine-dependent remethylation
Choline	Choline oxidation	✓	Phosphatidylethanolamine	✓✓	Eggs, meat (particularly liver), legumes	Limited interpretation as a single marker, but ↑ secondary to choline sparing with decreased betaine-dependent remethylation
DMG	Choline oxidation	✓	Betaine	✗		↑ with decreased betaine-dependent remethylation
Betaine/choline	Choline oxidation					↑ with decreased betaine-dependent remethylation
DMG/betaine	Choline oxidation					↑ with upregulated betaine-dependent remethylation
Glycine	Choline oxidation Folate cycle	✓	Serine, Sarcosine	✓	Protein-rich foods, particularly animal-based sources	Limited interpretation as a single marker
Cystathionine	Transsulfuration	✓	Homocysteine	✗		↑ with vitamin B_6 deficiency or decreased transsulfuration flux
Cysteine	Transsulfuration	✓	Cystathionine	✓	Protein-rich foods, particularly animal-based sources	↑ with decreased transsulfuration flux
Serine	Transsulfuration Folate cycle	✓	Glycine	✓	Protein-rich foods, particularly animal-based sources	Limited interpretation as a single marker
5-MTHF	Folate cycle	✗		✓✓	Leafy green vegetables, citrus fruits, legumes, fortified breads/cereals	
Homocysteine	Methionine cycle	✓	Methionine	✗		↑ with inadequate B vitamin status, particularly folate
Methionine	Methionine cycle	✗		✓✓	Protein-rich foods, particularly animal-based sources	Limited interpretation as a single marker, but ↑ with greater homocysteine remethylation and transmethylation potential
SAH	Methionine cycle	✓	Methionine	✗		Limited interpretation as a single marker, but ↑ with greater transmethylation potential
SAM	Methionine cycle	✓	Methionine	✗		Limited interpretation as a single marker, ↑ with reduced transmethylation potential
SAM/SAH	Methionine cycle					↑ with greater transmethylation potential

[1]Dietary sources with ✓✓ are considered essential nutrients (endogenous synthesis of choline and methionine is inadequate to meet requirements). [2]Refers to interpretation of metabolites within 1C metabolism specifically. Abbreviations: DMG, dimethylglycine; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine.

Table 1.4: Overview of key enzymes involved in the regulation of one-carbon metabolism

Enzyme		Pathway	Function	Cofactor or coenzyme[1]	Regulation[2]
Cystathionine β-synthase	CBS	Transsulfuration	Condensation of homocysteine and serine to form cystathionine	Vitamin B_6 (PLP)	*S*-adenosylmethionine relieves autoinhibitory domain
Cystathionine λ-lyase	CGL	Transsulfuration	Hydrolysis of cystathionine to cysteine	Vitamin B_6 (PLP)	NA
Methionine adenosyltransferase	MAT1A MAT2A MAT2B	Methionine cycle	Transfers adenosyl group from adenosine triphosphate to methionine, synthesising *S*-adenosylmethionine	NA	Upregulated activity with higher methionine concentrations
Methionine synthase	MTR	Methionine cycle	Uses 5-methyltetrahydrofolate as a methyl-donor for the remethylation of homocysteine, producing tetrahydrofolate and methionine	Vitamin B_{12} (cobalamin)	NA
Methylenetetrahydrofolate reductase	MTHFR	Folate and methionine cycle	Synthesis of 5-methyltetrahdrofolate, the active folate form, from 5,10-methylenetetrahydrofolate	Riboflavin (FAD)	Inhibited by *S*-adenosylmethionine
Methyltransferases	$_x$MT	Methionine cycle	Donate methyl group from *S*-adenosylmethionine to various molecules (including DNA, RNA, proteins, phospholipids, histones, and neurotransmitters), producing *S*-adenosylhomocysteine	NA	Inhibited by *S*-adenosylhomocysteine, stimulated by higher *S*-adenosylmethionine concentrations.
Betaine-homocysteine methyltransferase	BHMT	Methionine cycle and choline oxidation	Uses betaine as a methyl-donor for the remethylation of homocysteine, producing dimethylglycine and methionine	NA	Inhibited at higher dimethylglycine and *S*-adenosylmethionine concentrations
Glycine *N*-methyltransferase	GNMT	Methionine cycle	Regulates the balance of *S*-adenosylmethionine and *S*-adenosylhomocysteine by scavenging excess *S*-adenosylmethionine	NA	Allosterically inhibited by 5-methyltetrahydrofolate, stimulated by *S*-adenosylmethionine
Phosphatidylethanolamine *N*-methyltransferase	PEMT	Choline oxidation	Synthesis of phosphatidylcholine from phosphatidylethanolamine	NA	Inhibited at low phosphatidylethanolamine and *S*-adenosylmethionine concentrations
Serine hydroxymethyltransferase	SHMT1, SHMT2	Folate cycle and choline oxidation	Reversible interconversion between glycine and serine, favouring either serine (SHMT1) or glycine (SHMT2) synthesis.	Vitamin B_6 (PLP)	NA

[1]Refers to B vitamin cofactors or coenzymes only, [2] Refers to regulation by other 1C metabolites only. Abbreviations: FAD, flavin adenine dinucleotide; NA, the enzyme does not have B vitamin cofactors/coenzymes or is not regulated by 1C metabolites.; PLP, pyridoxal 5'-phosphate

1.5 DIETARY REGULATION OF ONE-CARBON METABOLISM

Diet is intrinsically linked to the complex regulation of 1C metabolism, providing methyl donors (folate, choline, betaine, methionine), B vitamin coenzymes and cofactors (vitamins B_6 and B_{12}, riboflavin), and intermediary amino acids (glycine, serine, cysteine) (**Table 1.6**). Dietary intake influences diverse aspects of 1C regulation and metabolite concentrations, but the focus here will be predominantly on how diet impacts the central methionine cycle to maintain consistency across this literature review. The regulatory impact of B vitamins on 1C metabolism will be discussed alongside other methyl donors (betaine and its precursor choline, protein intake as a source of methionine) and macronutrients which although are not widely studied, may have additional impact in the broader dietary context. While this review elaborates on diet, it is important to note that other factors including age, sex, renal function, genetics, and lifestyle (smoking, alcohol intake, physical activity) also contribute to 1C status[143], but will not be expanded on in this Chapter (see Refsum *et al*[144] for a concise review of this topic).

1.5.1 B VITAMIN INTAKE

The influence of B vitamin intake on Hcy regulation gained momentum through the 1990s following evidence that implicated Hcy in cardiovascular diseases. In 1993, Selhub *et al*[145] provided the first report from a large cohort of older adults (*n*=1660, Framingham cohort) that dietary intakes of folate and vitamin B_6 were inversely associated with circulating Hcy. Riboflavin intake later emerged as a determinant of circulating Hcy concentrations in Framingham Offspring participants[146] (*n*=1960) and the Hordaland Homocysteine Study[147] (*n*=5812), though these studies were not cohorts of older adults specifically as Framingham participants were. The relationship between vitamin B_{12} intake and circulating Hcy concentrations is equivocal[145,146], explained in part by disparities in markers of vitamin B_{12} intake and status. It is generally considered that the individual effect of folate on Hcy is stronger and more consistent than other B vitamins[145–147]. A summary of epidemiologic evidence from these seminal studies can be found in **Table 1.5**

Table 1.5: Overview of landmark epidemiological studies describing the association between B vitamin intake and Homocysteine concentrations

		Framingham Cohort	Framingham Offspring	Hordaland Homocysteine
Study characteristics	Author, year	Selhub *et al*, 1993[145]	Jacques *et al*, 2001[146]	Konstantinova *et al*, 2007[147]
	Country	America	America	Norway
	Examination cycle	20th (1988 – 1989)	5th (1991 – 1994)	2nd (1997 – 1999)
Participant characteristics[1]	*n*	1160	1960	5812
	Age, range	67 - 96	28 - 82 years	47 – 74 years
	% males	39%	47%	46%
Association with homocysteine[2]	Folate	Inverse (p <0.01)	Inverse (p <0.01)[4]	Inverse (p <0.001)[4]
	Riboflavin	NR	Inverse (p <0.01)[4]	Inverse (p = 0.04)[4]
	Vitamin B_6	Inverse (p <0.01)	Inverse (p <0.01)[4]	NS
	Vitamin B_{12}	NS	NS	NS

[1]Refers to those in the analysis investigating the relationship between dietary variables and circulatory homocysteine concentrations (i.e. the specific cohort cycle), not the wider cohort. [2]Data presented for most completely adjusted models where the independent effect of each vitamin is examined (i.e adjusted for intake of other B vitamins). [3]Nutrient intake was according to combined food and supplemental intake. [4]Significant in non-supplement users only. Abbreviations: NR, Not reported; NS, not significant; PLP, Pyridoxal 5'-phosphate.

It is well established that oral supplementation with folic acid alone or in combination with vitamins B_6 and B_{12} normalises plasma Hcy[148–150]. While early studies investigating the potential of B vitamins to lower Hcy were with pharmacological folic acid doses of ≥5 mg/day[151], later research showed that ~400 µg/day was adequate to achieve a similar reduction of Hcy[152,153]. Physiologic doses of folic acid similar to RDIs have been shown to lower circulating Hcy in participants without elevated Hcy (<15 µmol/L) either alone[154] or in combination with vitamins B_6 and B_{12}[155]. Similarly, multivitamin and mineral (MVM) supplements containing B vitamins at doses similar to RDIs improve B vitamin and Hcy status in healthy older adults, even in those consuming a fortified diet[156].

Compared to the extensive evidence-base describing the relationship between B vitamin intake and Hcy regulation, similar research on other metabolites of the methionine cycle is scarce and inconsistent. While some suggest that folate and vitamin B_6 intakes are positively correlated with erythrocyte concentrations of SAM and SAM/SAH[157], others suggest that only vitamin B_{12} intake is associated with SAH concentrations[158], though neither of these studies were in older adults. High dose cobalamin supplementation (1000 µg/day) for three months is shown to reduce SAH in those with suboptimal vitamin B_{12} status[159], and riboflavin supplementation has also been shown to increase plasma SAM in individuals homozygous for the MTHFR C677T polymorphism[160]. In comparison, Green *et al*[161] reported that combined B vitamin supplementation lowered plasma Hcy in older adults with moderately elevated Hcy (>13 µmol/L), but with no change in SAM, SAH, or SAM/SAH.

While research on B vitamin intake and Hcy regulation has slowed through the 21st century, future research must extend beyond the narrow focus of folate intake and Hcy regulation which has previously commanded research attention. Inadequate intake of any of the B vitamins can perturb the complex regulation of 1C metabolism, leading to reduced methylation status, elevated circulating Hcy, and increased misincorporation of uracil into DNA[52,162,163]. These issues highlight the need for consistent inclusion of dietary riboflavin particularly and vitamins B_6 and B_{12} alongside folate in research pertaining to 1C metabolism.

1.5.2 CHOLINE AND BETAINE INTAKE

The available evidence tends to support an inverse association between choline and betaine intake with plasma Hcy[164–167]. This association appears to be stronger in men than women[164,165] due to women having higher folate status in these studies and greater *de novo* synthesis of choline[168]. However, others have found that higher betaine but not choline intake is associated with lower plasma Hcy concentrations[167], or that the sum of choline and betaine intakes are inversely associated with plasma Hcy but neither nutrient alone[166]. The impact of choline and betaine intake on plasma Hcy appears to be more pronounced with lower folate [164,166] or vitamin B_{12}[165] intake , supporting the hypothesis that Hcy remethylation is more dependent on betaine as a methyl donor when B vitamin intake is restricted.

Pharmacologic doses of betaine of around 6g/day are used to treat hyperhomocysteinemia[169,170], and near-physiologic supplemental doses and betaine-rich meals are also shown to reduce plasma Hcy[171,172]. Interestingly, ingestion of a betaine- and choline-rich meal was reported as more effective than supplementation in reducing postprandial plasma Hcy under standard conditions or attenuating post-methionine load Hcy concentrations[172]. Choline and betaine supplementation appear to be more effective at reducing post-methionine load plasma Hcy than folic acid supplementation does[173,174], leading to the hypothesis that folic acid combined with betaine or choline supplementation will further augment Hcy reduction[173]. However, the longer-term consequences have not been investigated and the potential of betaine and choline supplementation for improving 1C regulation has since been largely ignored. More recently, dietary intervention with egg protein has been shown to prevent the onset of hyperhomocysteinemia during folate restriction in rodents, though no effect on hepatic SAM or SAH was seen[175]. In comparison, 28 days of krill oil supplementation (a rich source of phosphatidylcholine), increased fasting plasma choline metabolites (choline, betaine, DMG), but had no effect on circulating Hcy or B vitamin status in healthy adults without elevated Hcy at baseline[176].

Although the epidemiologic and intervention evidence describing the impact of increasing choline and betaine on 1C regulation does seem promising, it is comparatively scarce to that for B vitamins and strong conclusions cannot be drawn. The limited evidence is not surprising though given that recommendations for choline intake were only established in 1998[177], and a database for quantifying choline and betaine intakes was developed soon after by the USDA and released in 2004[178] and later updated in 2008[179]. This American database is considerably restricted in its widespread implementation due to compatibility issues with local dietary analysis software and food supply. It is important to finally note here that the studies described above are predominantly conducted in young-to-middle aged adult populations. As such, it is unclear how modifying betaine or choline intake will impact 1C metabolism in older adults who are at greater risk of B vitamin deficiencies, and whose choline and betaine intakes have not been characterised.

1.5.3 MACRONUTRIENT INTAKE

There is evidence to suggest that the macronutrient composition of diets will impact 1C metabolite status. For example, Hcy was associated inversely with complex carbohydrate intake and positively with fat intake in the Hordaland Homocysteine Study, even after adjustment for dietary and plasma B vitamins[147]. In the same cohort, high saturated fat intake was associated with elevated circulating Hcy, whereas a higher intake of long-chain omega-3 fatty acids was inversely associated with plasma Hcy, which the authors attributed to differential effects on phosphatidylcholine synthesis[180]. Dietary fat has also been shown to increase genetic expression of *Chdh, Bhmt,* and *Dmgdh* genes*,* but downregulated *Cbs* and *Cgl* expression in rodents, indicating a PPARα-mediated upregulation of the choline oxidation pathway and restricted transsulfuration flux to conserve methionine synthesis[181]. Similarly, Obeid *et al*[182] have shown that flux through the betaine-dependent Hcy remethylation pathway increased following an oral fat tolerance test, while methyl flow through this pathway was restricted after an oral glucose tolerance test. These findings demonstrate the multifaceted influence of dietary intake on 1C metabolism beyond nutrients like B vitamins which are typically investigated.

The impact of protein intake on 1C metabolite status remains unresolved. Despite initial concerns that higher protein diets would elevate Hcy concentrations by increasing methionine intake, this evidence largely comes from methionine-loading studies which increase postprandial[183–185], but not fasting Hcy[186,187]. Rather, epidemiological evidence supports an inverse association between protein intake and circulating Hcy[146,188]. However, this association is typically lost following adjustment for B vitamin intake[146,189], which suggests that the complex supply of nutrients involved in Hcy co-regulation from protein-rich foods is driving the association between protein intake and Hcy status. As is the case for B vitamins, choline, and betaine – the association between macronutrient intake on concentrations of other 1C metabolites, particularly SAM and SAH remains to be characterised.

Table 1.6: Overview of dietary nutrients which are methyl donors or co-enzymes in one-carbon metabolism

	Dietary[1]			Supplemental[2]	
		Recommended daily intake			
Nutrient	Rich Source	Male	Female	Usual dose	Pharmacologic dose
Folate	Citrus, Leafy green vegetables, legumes, fortified cereals	400 µg/day	400 µg/day	0.4-0.8 mg/day[190]	5-40 mg/day[190]
Riboflavin	Milk and dairy products	1.6 mg/day	1.3 mg/day	1.6-5 mg/day[191]	25 mg/d[192]
Vitamin B_6	Meat, whole grains, vegetables, fruits	1.7 mg/day	1.5 mg/day	Doses vary from 20 – 120 mg/day[190].	
Vitamin B_{12}	Meat, poultry, fish, eggs, dairy	2.4 µg/day	2.4 µg/day	Doses vary from 0.2-2.0 mg/day[190]	
Choline	Eggs, meat, liver, milk, peanuts	425 mg/day	550 mg/day	Doses vary from 0.1 – 3g/day[172]	
Betaine	Spinach, whole-wheat, beetroot	NA, but usual intake of ~0.5 – 2.0 g/day[171]		0.5–1.5 g/day[172]	6 g/day[172]
Methionine	Animal-based protein sources	25 mg/kg body weight/day (methionine and cysteine combined[193])		-	1g/kg body weight (loading)

[1]Recommended daily intake according to the Australian and New Zealand Joint Nutrient Reference Values. Recommended daily intake is not available for choline, and an adequate intake is listed instead[87]. [2]Supplemental doses are based on studies considering the role of these nutrients within the regulation of 1C metabolism.

1.5.4 DIETARY INTERVENTIONS

The relationship between diet and 1C metabolism is evidently multifaceted. However, the impact of dietary interventions (compared to supplementation studies) which represent this complexity is under-represented in research aiming to improve 1C metabolite status through diet. Available studies have predominantly focused on increasing dietary folate, and support a decline in plasma Hcy with increased fruit and vegetable intake alone[194–198] or in combination with low-fat dairy products[199]. Dietary counselling to increase folate intake through regular consumption of folate-rich foods like fruits, vegetables, and legumes is also effective in reducing Hcy[200]. The authors suggested that dietary modification would be advantageous over fortification or supplementation strategies, as altering consumption patterns may lead to increased intake of other nutrients, such as vitamin B_6 intake in their participants following dietary counselling[200]. However, findings from another study comparing the effectiveness of increasing folate intake through folate-rich foods, fortified foods, or supplements found that only supplementation and fortified foods led to a reduction of plasma Hcy[201].

Beyond those focused on folate intake, other dietary interventions have considered the effect of modifying protein intake on Hcy concentrations. The objective of these studies was typically to address the hypothesis that increased protein or methionine intake would elevate Hcy concentrations. However, no changes in fasting Hcy concentration were found following increased animal-based protein intake in studies ranging from one-week to six-months in duration[187,202,203]. One study even demonstrated a 25% reduction in circulating Hcy after six-months of higher protein intake, although this did not reach statistical significance[203]. While an increase in post-methionine load Hcy with higher protein intake has been found, the physiological and clinical relevance of these findings is yet to be determined as the impact of increased post-methionine load Hcy on longer-term 1C regulation remains unclear[186,187]. As highlighted by these studies, dietary strategies to improve the regulation of 1C metabolism will undoubtedly be complex - even the seemingly simple concept of increasing methionine intake through protein is complicated by the supply of other co-regulatory nutrients in protein-rich foods, including B vitamins, choline, and intermediary amino acids. At this stage, there remains a paucity of data describing the role of wider dietary changes beyond focusing on single nutrients or foods to optimise 1C metabolite status, and again the impact on metabolites beyond Hcy remains unknown.

1.5.5 METHODOLOGICAL CONSIDERATIONS

1.5.5.1 Sample type

Most studies describing the diet-metabolite relationship use plasma or serum markers to measure Hcy and methylation status. Some studies have also reported erythrocyte SAM and SAH concentrations[204–207], but these cells are relatively inactive concerning 1C metabolism and peripheral blood mononuclear cells (PBMCs) are considered a more suitable measure for intracellular 1C metabolism[208]. Smith *et al*[208] found that while folic acid lowered plasma Hcy, no concurrent shifts to intracellular Hcy, SAM, or SAH concentrations in PBMCs were found. The authors suggested that folic acid supplementation may even disturb the physiological regulation of intracellular 1C metabolism, which is not apparent in circulating measures[208]. This evidently creates challenges for interpreting the evidence base. While declining plasma Hcy with various dietary interventions ranging from single nutrients to wider dietary modifications has been described in this review, there is currently very little insight into what implications this holds for intracellular 1C regulation, which is likely a more insightful marker of functional B vitamin status.

1.5.5.2 Nutrient status

Baseline nutrient status further impacts the interpretation of intervention studies and recommendations which stem from this. In saying this, the evidence base does appear to be distributed across participants with adequate and deficient baseline B vitamin status, and normal or elevated Hcy concentrations (be it through specific inclusion/exclusion criteria, chance, or during the implementation of fortification policies). As one would expect, B vitamin supplementation lowers Hcy in populations without folic acid fortification and lower folate status to a greater extent[149,209]. In more recent years, the focus has shifted towards excluding participants with adequate B vitamin status, particularly when looking to improve health outcomes[210]. While increasing B vitamin intake appears to provide additional benefit even for those with normal Hcy or B vitamin status[149,150,209], the public health relevance of improving status in the general population is unclear.

1.5.5.3 Time-frame of response

The duration of intervention varies considerably between studies, and large randomised controlled trials looking to improve health outcomes range from one month to several years in duration[150,211]. Metabolites appear to be responsive to short-term changes in nutrient intake, even after just five days of B vitamin supplementation in participants with adequate B vitamin status at baseline, though a peak effect tends to take around two weeks[115]. Similarly, a reduction of plasma Hcy has been found with betaine supplementation after just two weeks, to a magnitude of effect comparable to six weeks[171].

While 1C metabolism appears responsive to short-term dietary shifts, very little is known about the postprandial regulation of 1C metabolites outside of post-methionine load conditions, a dietary challenge developed to detect CBS deficiency and now used as a ‘stressor’ of Hcy metabolism[144]. Although valuable, the findings of such dietary loads do not apply to habitual dietary perturbations that older adults face several times a day (i.e. consuming mixed meals with or without supplements). Plasma Hcy does appear to immediately respond to mixed meals within an hour, with effects sustained during an eight-hour postprandial period, though the direction of response depends on meal type (particularly protein content)[185], or health status[212]. The postprandial period involves the concerted regulation of digestion and absorption for nutrient utilisation or storage, hormone fluxes, and the endogenous production or release of amino acids, glucose, and fatty acids. There is growing awareness that postprandial responses reveal aspects of metabolic health which would not be identified with fasting markers, particularly in healthy subjects where homeostasis masks early perturbations under fasting conditions[213–215]. This concept of metabolic flexibility is typically applied to macronutrients, though likely holds relevance to B vitamins and 1C metabolism given that insulin resistance[186,216], impaired amino acid and lipid metabolism[217], and reduced capacity to efficiently absorb and utilise B vitamins[75,177] frequently occur with advancing age – all of which contribute to 1C regulation. However, the postprandial 1C response has not been characterised, and the relevance of postprandial findings to long-term regulation is not yet adequately linked either.

1.6 ONE-CARBON METABOLISM AND HEALTHY AGEING

The realisation that Hcy is a risk factor for cardiovascular diseases was the impetus for early years of research on 1C metabolism, health, and ageing[218–221]. However, randomised controlled trials of Hcy-lowering with B vitamins on cardiovascular outcomes were underwhelming, particularly in secondary prevention settings[148,222–228]. Research attention has since shifted towards uncovering the role of B vitamins and 1C metabolism in the trajectory of cognitive ageing, which contributes significantly to morbidity and mortality in ageing societies. Cognitive decline is accelerated by both perturbed 1C and cardiometabolic regulation[229–232], which appear to be co-regulated[233–235]. This point of overlap likely holds relevance for how B vitamin status can be optimised to promote diverse aspects of healthy ageing, and will be the focus of this section.

1.6.1 COGNITIVE AGEING

By 2050, 131 million people are projected to have dementia[236]. Population ageing is driving the growing 'dementia epidemic'[237], and dementia in turn creates profound challenges for ageing societies due to the devastating nature of the condition for individuals and societies alike[236]. Developing strategies that prevent or delay the onset of cognitive impairment and progression of dementia has become a major public health priority for ageing societies, and here B vitamins have emerged as pivotal players.

Cognitive decline is considered a normal part of ageing, which is accompanied by a range of structural and metabolic modifications within the brain. These changes include brain atrophy[238], increased appearance of white matter lesions[239], the development of neurofibrillary tangles and Lewy bodies, progressive neuron loss, declining neurotransmitter production[239], and synaptic dystrophy[240,241]. While these changes are considered part of the physiology of ageing, accelerated cognitive decline may occur, which ranges from mild cognitive impairment (MCI) through to dementia at its most severe form. An individual is considered to have MCI when cognitive decline exceeds that expected for their age and education level but does not impact activities of daily living. In contrast, dementia is characterised by the inexorable deterioration of an individual's capacity for independent living attributed to cognitive impairment[242]. Notably, 50% of individuals with MCI are expected to develop dementia within five years of diagnosis[242].

1.6.1.1 The role of B vitamins and homocysteine

1.6.1.1.1 Epidemiological evidence makes a strong case

B vitamin deficiencies have long been linked with poor psychiatric health, reviewed by McCaddon[243] in an historical account. Even before it was recognised that vitamin B_{12} deficiency was associated with the pathology of pernicious anaemia, Addison noted in 1849 that "the mind occasionally wanders" in his patients (cited in McCaddon[243]). Barrett[244] then described histopathological changes in the cerebral cortex of patients with pernicious anaemia in the early 1900s, particularly neurodegeneration and damage to the white matter and blood vessels. By the mid-1900s, dementia was first associated with low vitamin B_{12} status independently of pernicious anaemia[245].

In 1983, Goodwin *et al*[246] reported an association between poor cognitive performance and low-to-normal serum concentrations of riboflavin, folic acid, and vitamin B_{12}. This data provided some of the earliest differentiation from the historical research in the sense that these associations were in seemingly healthy community-dwelling older adults, as opposed to institutionalised elderly. This was also some of the first evidence that subclinical malnutrition likely plays a role in age-related cognitive decline[246]. It was later proposed that for most people, it is more likely that these mild vitamin deficiencies contribute to the pathogenesis of cognitive ageing[247]. Soon after, hypotheses were developed where abnormal 1C metabolism was thought to play an important role in the aetiology of Alzheimer's disease[248,249]. Seminal reports emerged two decades ago now, where low folate and vitamin B_{12} status or elevated Hcy concentrations were associated with the diagnosis of dementia according to both clinical and histopathological criteria[250,251].

Following these landmark studies, a wealth of robust epidemiological data ensued. By 2008, A. David Smith, a pioneer in the field of B vitamins and cognitive ageing, noted that 77 cross-sectional studies and 33 prospective studies had shown associations between plasma Hcy and/or B vitamins and cognitive deficit or dementia, including associations across seemingly 'normal' concentrations of B vitamin status. This topic has since been extensively reviewed, all coming to conclusions similar to Smith[52,247,252–254]. Prospective evidence in particular makes a compelling case for elevated Hcy or low B vitamin status contributing to accelerated cognitive decline[252,255–258], including evidence from imaging studies of radiologic disease progression[251] and brain atrophy[259–263]. Elevated plasma Hcy is also associated with an increased likelihood of developing dementia[264], reported even to a similar magnitude as the genetic risk conferred by carrying the apolipoprotein E (ApoE) ε4 allele[265]. A recent consensus statement by Smith and colleagues[266] in 2018 posits that moderately raised plasma Hcy (11 μmol/L) increases the relative risk of dementia by 1.15 – 2.5 fold in older adults. It should be noted here that higher folate status, while generally associated with better cognitive performance, increases the risk of cognitive impairment in individuals with low vitamin B_{12} status compared to those with normal status of both vitamins in some[51,267–269], but not all studies[270,271]. This observation suggests that our understanding of what appears to be a long-standing relationship in the literature is by no means complete.

The evidence for vitamin B_6 and riboflavin is scarce compared to folate and vitamin B_{12}. Several epidemiological studies support an association between low vitamin B_6 status and poor cognitive performance or accelerated cognitive decline[258,272–276], Alzheimer's disease[277,278], while others report no association[277,279–283]. Although included in very few studies, riboflavin has been associated with better cognitive function and protection against cognitive decline in some[284,285], but not all[276] studies. As with folate and vitamin B_{12}, the matter of association is perhaps dependent on whether the vitamin is a limiting nutrient in terms of the extent of inadequate intake or status[276]. In the case of folate, studies conducted in countries with robust fortification policies or in populations with higher folate status do not show as strong support for the role of folate and Hcy in cognitive function[272,286], cognitive decline[287–289], or dementia[290].

1.6.1.1.2 B vitamin supplementation studies – A lost cause?

In contrast to a strong epidemiological evidence base, B vitamin supplementation studies were largely unsuccessful in improving cognitive function[289,291–293]. Several meta-analyses concluded that there was no beneficial effect of B vitamin supplementation[150,294] or Hcy-lowering[295] on cognition in older adults. However, these conclusions have been widely criticised, and generally not accepted by leading experts[264,296,297], largely due to methodological issues arising from the early intervention studies (eloquently reviewed by McCaddon & Miller[297]). For instance, studies tended to include participants with adequate B vitamin status or those who are not experiencing cognitive decline, as well as having a small sample size, short durations of supplementation, or using tests like the Mini-Mental State Exam which do not detect subtle changes in cognitive function. As highlighted by Morris[298], progress in this field has been hindered by poor quality randomised controlled trials, and ignoring a vast body of observational research in favour of these studies seems imprudent. Indeed, studies seeking to address these issues provide a compelling counter-argument, with evidence that B vitamin supplementation leads to improved measures of cognition[299–301], as well as a reduced rate of brain atrophy[300,302], effects which are more pronounced in participants with elevated plasma Hcy[301]. Further, omega-3 fatty acid status appears to affect the trajectory of cognitive decline in response to B vitamin supplementation, which is suggested to be connected to the role of B vitamins in forming phosphatidylcholine[303], though consensus regarding their impact has not been reached[303,304].

1.6.1.1.3 Mechanisms – homocysteine and methylation hypotheses

Research to date indicates two neurochemical mechanisms by which B vitamins could influence cognitive performance. First, the Hcy hypothesis posits that there is an indirect, and likely longer-term, effect of B vitamin inadequacies on the brain via the neurotoxic effects of Hcy[252]. Second, the hypomethylation hypothesis proposes that B vitamin inadequacy directly, and possibly acutely, affects cognitive function by inhibiting methylation reactions in the central nervous system involving proteins, membrane phospholipids, and neurotransmitters. SAM-dependent methylation reactions are particularly important in the brain, and a restriction tends to favour the accumulation of amyloid precursor protein and phosphorylated tau protein, hallmarks of Alzheimer's disease[305]. Furthermore, B vitamins are required to maintain DNA methylation patterns, and perturbations of this epigenetic regulation have been implicated in multiple disorders of ageing, including cognitive decline and dementia[306,307]. Other proposed mechanisms (extensively reviewed by Smith and Refsum[308]) include impaired endothelial function with reduced inducible nitric oxide synthase, augmented oxidative stress and decreased activity of key antioxidant enzymes, raised generation of the superoxide anion, altered lipid metabolism with increased cholesterol synthesis, and induction of thrombosis.

1.6.1.1.4 Apolipoprotein E genotype and its interaction with B vitamins and homocysteine

ApoE is a plasma glycoprotein involved in lipoprotein metabolism and neuronal repair, and is inherently linked to the ageing trajectory. The three genetic variants of ApoE are ε2, ε3, and ε4, with mean frequencies in the general population of around 8%, 78% and 14%, respectively[309,310]. Carrying at least one ApoE ε4 allele has been associated with elevated plasma cholesterol and risk for cardiovascular disease, decreased longevity, and is considered a risk factor for developing Alzheimer's disease[311]. Although the role of ApoE ε4 in modifying risk for cognitive decline is somewhat contentious, a meta-analysis indicated that the ApoE ε4 genotype does indeed impair cognitive performance in healthy older adults, although this is specific to certain domains of cognitive performance and with a relatively small impact[312].

Emerging evidence suggests an interactive effect of the ApoE genotype with B vitamin status or Hcy in modifying cognitive impairment. The association between low vitamin B_{12} status and poor cognitive performance in older age appears to be stronger in ApoE ε4 carriers[313,314]⁽³¹⁴⁾⁽³¹⁴⁾⁽³¹⁴⁾⁽³¹⁴⁾⁽³¹³⁾⁽³¹²⁾⁽³¹²⁾⁽³¹²⁾⁽³¹²⁾⁽³¹²⁾⁽³¹²⁾⁽³¹²⁾⁽³¹²⁾⁽³¹²⁾⁽³¹²⁾. Although some groups have not reported a similar interaction with Hcy[256,314], Elias *et al*[315] found that the association between plasma Hcy and cognitive performance was stronger with the ApoE ε4 genotype, and only persisted in ε4 carriers following adjustment for B vitamin status. These effects extend to brain volume, with reports that regional grey matter volume was only inversely associated with plasma Hcy and vitamin B_{12} concentrations in ε4 carriers with Alzheimer's disease[316]. While the evidence remains equivocal at this point due to the small number of studies, it should be highlighted that interactions with intake or status of vitamin B_6 or riboflavin has not been investigated, nor with metabolites of the choline oxidation or transsulfuration pathway in older adults.

1.6.1.2 Evidence for one-carbon metabolites beyond homocysteine

The strongest evidence for involvement of 1C metabolites beyond Hcy in cognitive function is that for choline and downstream metabolites (betaine, DMG), albeit currently limited in ageing human populations. Choline is connected to cognitive function through several mechanisms, including synthesis of the neurotransmitter acetylcholine and integral cell membrane components such as sphingomyelin and phosphatidylcholine. It is clear that a range of cholinergic abnormalities contribute to cognitive function in Alzheimer's disease, and perhaps in normal ageing, including alterations in choline transport, acetylcholine release, and nicotinic or muscarinic receptor expression[317]. Indeed, the available evidence supports an association between higher dietary choline intake[318–320], concentrations[321] or choline-containing phospholipid species[322] with improved cognitive function. To my knowledge, an association between transsulfuration metabolites (cysteine and cystathionine) and cognitive performance in older age has not been reported outside of psychiatric disorders[323], and as such the focus for the remainder of this section will be on choline metabolites.

1.6.1.2.1 Epidemiological evidence – choline metabolites and cognitive performance

The association between choline intake and cognition has been explored in two large cohorts, both providing robust evidence. In participants from the Framingham Offspring cohort (*n*=1391, mean age of 61 years), higher choline intake was associated with better memory performance in a cross-sectional analysis[319]. Further, choline intake 6-7 years prior was associated with lower white matter hyperintensity volume, which is considered pathologic in nature through their connection to brain atrophy, and the authors suggested that choline intake at midlife may be neuroprotective in later years[319]. This finding was recently supported by Ylilauri *et al*[318], who found that higher phosphatidylcholine intake was associated with a lower risk for dementia, and both total choline and phosphatidylcholine intakes were associated with better performance in verbal fluency and memory function tests in older adults part of a large prospective cohort in Finland (*n*=2497, mean follow-up of 21.9 years). Although other choline metabolites are infrequently reported, the association between betaine and cognitive performance does not appear to be as strong as that for choline[321]. DMG concentrations have been positively associated with memory performance[324], and lower DMG concentrations have also been reported amongst a panel of six metabolites which could discriminate patients with Alzheimer's disease from controls (alongside arachidonic acid, thymine, glutamine, glutamic acid, cytidine) or

five metabolites to discriminate those with amnestic MCI from controls (alongside arachidonic acid, thymine, 2-aminoadipic acid, and 5,8-tetradecadienoic acid)[325].

1.6.1.2.2 Supplementation studies – an inference from other populations

Intervention evidence for supplementation with choline or betaine is largely restricted to rodent studies or therapeutic treatments in Alzheimer's disease, though this does provide further evidence for the role of choline in maintaining cognitive performance across the life-course. Prenatal choline supplementation in rodents has led to improved memory function and later protection against age-related memory decline or cognitive performance in offspring[326–328]. This result has even been shown to have a transgenerational benefit, which was mechanistically linked to reduced brain Hcy levels[329]. Although most intervention studies in animal models have been during the maternal or perinatal period, rodent studies have also found that choline supplementation attenuates age-related cognitive dysfunction[330–333].

1.6.1.2.3 Interactions – folate, choline and cognitive performance

Rodent studies have explored the interaction of choline metabolites with B vitamins or Hcy, and suggest that the interconnected regulation of folate and choline metabolism moderates the trajectory of cognitive decline in ageing. Mice with a deficiency of methionine synthase reductase (characterised by elevated plasma Hcy and reduced methionine) show short-term memory impairment, global DNA hypomethylation, decreased choline, betaine and acetylcholine levels, and altered expression of enzymes involved in cholinergic neural networks[334]. These findings indicate that disturbances in folate- and vitamin B_{12}-dependent methionine synthesis, similar to that which occurs with dietary inadequacy, deplete choline availability in the brain as choline is prioritised to correct for perturbations to Hcy and 1C metabolism instead. Further, cognitive impairment in rats with folate deficiency has been associated with altered choline metabolism and depleted brain phosphatidylcholine[335], and folate deficiency has been shown to induce short-term memory impairment alongside impaired choline metabolism in the cortex and hippocampus[336]. These findings indicate that dysregulation of choline metabolism is likely an important, but under-appreciated, mechanism explaining cognitive dysfunction associated with B vitamin deficiency[335,336].

1.6.1.3 Summary of the evidence base

Experts have established considerable epidemiological evidence to support the role of suboptimal circulating B vitamin status, particularly folate, and/or elevated Hcy contributing to cognitive dysfunction and accelerated cognitive decline in ageing. However, the literature is rife with methodological heterogeneity. This ranges from measures of intake and status through to the various ways in which cognition is monitored[337], and several issues in the design of intervention studies which have complicated reviews of the evidence and hindered progress in the field[297]. At the same time, experts have cautioned that cognitive impairment is multifactorial, and there is no reason to expect B vitamin status to always be associated with cognitive function or decline in older adults[337]. The complexity of 1C metabolism also confounds the simple interpretation of associations between cognitive function with B vitamins and Hcy[338,339]. Animal studies have provided sound evidence for why the relationship between B vitamins or Hcy with cognition should be considered in the context of a more integrated 1C pathway, particularly including metabolites of the choline oxidation pathway based on available evidence, but this is yet to be explored in human participants.

1.6.2 CARDIOMETABOLIC HEALTH

Cardiometabolic dysregulation is common with advancing age and contributes to precocious ageing[340,341]. Particular concerns for older adults include insulin resistance, dyslipidaemia and sarcopenic obesity which drive the rising prevalence of metabolic disorders like cardiovascular disease, the leading cause of morbidity and mortality in ageing populations[342,343]. However, this review will not focus on cardiovascular diseases (extensively reviewed elsewhere[52,344,345]), but rather highlight the co-regulation of 1C metabolites and cardiometabolic parameters, given their overlap in the trajectory of cognitive ageing.

1.6.2.1 Homocysteine and impaired cardiometabolic regulation – cause or effect?

Elevated plasma Hcy is observed with increasing metabolic syndrome components (obesity, dyslipidaemia, insulin resistance, hypertension)[346–349], and in participants with the metabolic syndrome[235,350–352]. Methyl group availability likely plays an important role, particularly in associations between Hcy and lipid metabolism[233,234,353]. This centres around phosphatidylcholine availability, which is required for very-low density lipoprotein (VLDL) assembly and homeostasis. As a reminder, phosphatidylcholine can be provided through the diet, or is synthesised in the liver through two pathways connected to 1C metabolism. First, in a reaction catalysed by PEMT, which is a major methyl-group consuming pathway resulting in Hcy synthesis, or second via the cytidinediphosphocholine (or Kennedy) pathway which is linked to Hcy metabolism via the choline form required to maintain betaine concentrations. Therefore, the relationship between elevated Hcy and dyslipidaemia likely stems from a methyl group donor deficiency, leading to disturbed phosphatidylcholine metabolism and subsequent assembly or secretion of VLDLs[234]. In contrast, the question of cause or effect certainly remains unclear in the context dysglycaemia. Using insulin resistance as an example, hyperinsulinemia modifies genes involved in methylation and Hcy regulation[354], and hyperhomocysteinemia has been reported in animal models of hyperinsulinaemia[355]. Conversely, elevated plasma Hcy may lead to insulin resistance by modifying key pathways such as inhibition of insulin-receptor kinase activity[356]. Thus, while elevated Hcy is proposed as an additional component of the metabolic syndrome[235], interpretation of Hcy as a marker or driver of cardiometabolic perturbations is complicated and currently remains uncertain.

1.6.2.2 Complexity of diverse associations

Beyond Hcy, there is substantive evidence supporting the role of 1C metabolites in cardiometabolic health. For instance, a growing body of evidence indicates a protective role of glycine in cardiometabolic regulation[357], while cysteine is posited as an ‘obesogenic’ amino acid strongly associated with body mass index (BMI) and fat mass[358]. Perhaps the most compelling evidence again relates to choline metabolites. Divergent associations between plasma choline and betaine with markers of cardiometabolic health are consistently reported – with elevated choline typically associated with an unfavourable profile, while betaine with a more protective profile[359–362]. Higher plasma betaine has also been associated with lower incidence of diabetes in up to ten years of follow-up[363–365]. The longitudinal evidence for choline is less consistent across studies. Higher choline intake has been associated with a lower risk of incident diabetes after 19 years of follow-up[366]. In contrast, a large prospective study pooling data from the Nurses Health Study and Physicians Follow-Up Study reported that higher phosphatidylcholine intake was associated with an increased risk of diabetes, suggesting conversion to Trimethylamine N-oxide (TMAO) as a possible mechanism[367]. Evidence describing DMG is scarce again, although one study has suggested that lower plasma concentrations are associated with higher blood glucose, increased insulin resistance, and increased risk of incident diabetes[368].

1.6.2.3 Summary of the evidence base

It is challenging to summarise the relationship between 1C metabolism and cardiometabolic health given the disparate evidence base. Similar to that for cognitive performance, studies tend to focus on single metabolites, which creates further issues when considering cardiometabolic parameters. For instance, while lower plasma Hcy concentrations are favourable, what are the impacts of low Hcy combined with elevated choline, which is independently related to an adverse cardiometabolic profile? How do the relationships differ according to the numerous mechanisms acting to promote or perturb cardiometabolic health which may not even be related to the role of metabolites within the 1C pathway (for example, see Elshorbagy *et al* for a review on cysteine and body composition[358] or Alves *et al* for a review on glycine and cardiometabolic health[357])?

Regardless, the point of this review is not to labour the individual associations between individual metabolites and parameters of cardiometabolic health, but rather to emphasise that there is substantial evidence which supports diverse aspects of the co-regulation of 1C metabolites and cardiometabolic health. This observation should be an important consideration for interventions which aim to optimise B vitamin or 1C metabolite status, particularly for health outcomes also driven by cardiometabolic perturbations such as cognitive decline.

1.7 TOWARDS OPTIMISING B VITAMIN STATUS IN OLDER ADULTS – A PERSPECTIVE

Population ageing is rapidly occurring and must be countered with strategies which postpone the onset of age-related functional decline. Micronutrients are an essential consideration, providing the foundations of health through by diverse metabolic and physiological functions. Although classical micronutrient deficiency diseases are rare today, adequacy based on current thresholds does not sufficiently describe *functional* status - a critical component of the nutrient-health relationship. The impact of declining B vitamin adequacy on diseases which drive world-wide morbidity, mortality and rising health care costs is of particular interest for ageing populations. This is by no means a new problem, but characterisation of the relationship between B vitamins, function, and health in this context remains incomplete after decades of research, and the question remains, how must research progress towards optimising B vitamin status in older adults?

1.7.1 AGE-RELATED CHANGES ARE POORLY UNDERSTOOD

In paradox to the question at hand, it should first be stated that the need to optimise B vitamin status with advancing age is poorly understood. Older adults face increased risk for B vitamin inadequacies due to complex physiological, biological, and social changes, but this does not necessarily provide sufficient rationale for optimising B vitamin status *per se*. Indeed, age-related changes to intake, absorption, metabolism, and functional use of B vitamins are currently poorly characterised. Despite a wealth of literature describing cross-sectional comparisons of B vitamin intake or status in older compared to younger adults[369–372], longitudinal studies are required to describe the effects of age[373,374]. Further, the impact of ageing on absorptive and metabolic dynamics is incompletely understood, including B vitamins and their downstream use in functional pathways like 1C metabolism. While disentangling the effects of age on B vitamin and 1C metabolite status is complicated by the compounding effects of comorbidities, gastrointestinal dysfunction, and polypharmacy, this is necessary when considering how and when B vitamin status should be optimised for healthy ageing.

1.7.2 INTEGRATED PATHWAYS ARE REQUIRED

In order to characterise optimal B vitamin status, research attention must shift from the narrow focus on folate, vitamin B_{12} and Hcy, to studying inter-connected nutrients and metabolites within functional pathways. The need for integrated measures of B vitamin and 1C metabolites has been highlighted at several points throughout this review. To emphasise again, circulating Hcy levels within a 'normal' range do not necessarily mean that 1C metabolism is not disturbed[305]. For instance, DNA repair may be reduced due to low folate, riboflavin, and/or vitamin B_{12} concentrations, yet plasma Hcy concentrations can be maintained through catabolism in the transsulfuration pathway – known to be upregulated in the case of reduced methionine recycling[130]. This is important when considering the dynamic relationship between diet, 1C metabolism and health, which has been built on an evidence base considering lower plasma Hcy as a marker of optimal B vitamin status.

Dietary shifts, even targeted supplements, will not impact Hcy in an isolated manner. A clear example of this is the tightly connected co-regulation of folate and choline metabolism. Hepatic folate content in rodents decreases dramatically with consumption of a choline-deficient diet[375,376], and vice versa hepatic choline content declines in rats maintained on a folate-deficient diet[377]. Although not often the focal point of research, these metabolites outside of the central methionine cycle show independent relationships with health outcomes like cognitive decline and cardiometabolic dysregulation. These metabolites, and their interaction with nutrients and other 1C metabolites, must be taken into account when considering what an B vitamin and 1C metabolite profile or response to supplementation looks like.

Measuring integrated profiles has been an ongoing challenge due to the heterogeneity of molecular structures and concentration ranges even within a group of vitamins, creating labour-intensive and costly analyses. These difficulties have contributed to the vast array of methodologies available, and have hindered a more comprehensive analysis of interactions between nutrients and metabolites. However, the emergence of powerful analytical techniques like mass spectrometry within the wider field of -omics technologies make it possible to perform high throughput profiling of nutrients and metabolites in the research setting.

1.7.3 A DIFFERENT APPROACH TO INTERVENTIONS

A priority for research on healthy ageing is to develop feasible, practical, and safe interventions which confer multiple health benefits to older adults[378]. Ideally, strategies to optimise B vitamin status should be considered in the context of other interventions known to promote healthy ageing. For example, higher protein diets which are encouraged to prevent the onset of sarcopenia and frailty in older adults might also favourably impact B vitamin and 1C metabolite status, or be modified as such to provide wider-reaching health benefits for older adults. In the same vein, the inter-connected nature of many chronic diseases should be an important consideration moving forward. For instance, while choline sparing at higher folate intakes may be favourable for cognitive performance, higher choline concentrations are also related to cardiometabolic dysregulation. Further efforts are needed to understand the nuanced responses of an integrated pathway of metabolite and nutrients to dietary interventions, particularly in light of downstream health effects.

1.8 THESIS HYPOTHESIS

The research comprising this thesis centres around the primary hypothesis that ***a broader profile of 1C metabolites will allow for greater functional insight into the B vitamin-health relationship in older age, ultimately leading to improved monitoring and intervention strategies to optimise B vitamin status***. Specifically, it is hypothesised that:

1. B vitamin intake and metabolism will be perturbed with advancing age, which necessitates the need for optimisation in older adults.
2. Exploring the relationship between B vitamins, 1C metabolites and inter-related health outcomes like cognition and cardiometabolic health will shed light on an optimal plasma 1C metabolite profile.
3. Interventions which increase B vitamin intake through food or supplements will not only reduce circulating Hcy but also impact concentrations of metabolites in the choline oxidation and transsulfuration pathways.
4. Food-based interventions like a high protein diet may offer additional benefits to 1C regulation due to the complex supply of co-regulatory 1C nutrients (including B vitamins, methyl donors, and amino acids) compared to the limited spectrum of B vitamins in supplements.

1.9 THESIS AIM AND OBJECTIVES

The overarching aim of this thesis was to explore how B vitamin status can be optimised and monitored in older adults, using 1C metabolites as a measure of functional B vitamin status.

In line with the hypotheses above, the objectives of this work are broadly categorised into three 'themes'.

1. To describe **age-related changes to B vitamin intake and metabolism**. This will be achieved by conducting a systematic analysis of age-related changes to B vitamin intake and adequacy in Chapter 3, and by comparing the postprandial plasma 1C metabolite response to a MVM supplement and standard meal between young and older adults in Chapter 4.
2. To examine the relationship between **B vitamins, 1C metabolites, and health** in older adults. This will be achieved first by evaluating the relationship between B vitamins, plasma 1C metabolites and markers of cognitive and cardiometabolic health in Chapter 5. Second, the longitudinal relationship between 1C metabolites and cardiometabolic parameters in response to nutritional supplementation will be evaluated in Chapter 6.
3. To measure the **extent of changes to plasma 1C metabolites beyond Hcy alone in response to increased B vitamin intake through dietary intervention and nutritional supplementation.** In line with priorities for healthy ageing research, this will be analysed in the context of interventions already known to promote longevity, focusing on higher protein intake. Specifically, plasma 1C metabolites will be measured in response to (i) Six-months of intervention with a protein-based nutritional supplement containing B vitamins alongside exercise training in aged-care residents compared to exercise training alone or cognitive training (control) in Chapter 6; and (ii) A diet containing twice the current protein recommendations, focused on increasing animal-based protein sources, compared to current protein recommendations for ten-weeks in community-dwelling older males in Chapter 7.

METHODOLOGY

2

CHAPTER 2: GENERAL METHODS

2.1 GENERAL METHODS OVERVIEW

A detailed description of the study design, methodology, and statistical analysis used for the five studies included in this thesis is provided in their respective chapters, and will not be replicated here. Rather, this chapter is designed to provide the reader with a broader overview of how these chapters address the thesis objectives, and to summarise the techniques used and variables measured across clinical studies.

2.2 STUDY DESIGN OVERVIEW

This thesis is comprised of a systematic review (Chapter 3), one single-arm acute intervention (Chapter 4 – 'VIOME'), a cross-sectional cohort study (Chapter 5 – 'REACH'), and two randomised controlled trials (Chapters 6 – 'VAAS', and 7 – 'OptiMuM). Although diverse, these studies collectively address the primary thesis objective of exploring how B vitamin status can be optimised and monitored in older adult (**Figure 2.1**).

Except for the systematic review, the studies included in this thesis are all secondary analyses or outcomes from studies conceptualised prior to my candidature. As such, there are some variations in subject characteristics, exposures, and data availability between studies highlighted here and in **Table 2.1**. These studies have predominantly been conducted in community-dwelling 'early' agers (65-75 years), though Chapter 6 (VAAS) was conducted in aged-care residents with a mean age of 83 years. Sex is not evenly distributed throughout studies. The OptiMuM study recruited males only to reduce homogeneity that would need to be accounted for in a dietary intervention (e.g. differences in nutrient requirements and amino acid or skeletal muscle metabolism), whereas participants were predominantly female in VAAS (representative of the participant's age range) and REACH (resulting from difficulties in participant recruitment), and sex was balanced in VIOME. In the acute- and long-term supplementation studies, different supplements with varying B vitamin contents were used. A Centrum Advance General MVM supplement was used in the VIOME study, where the primary aim was to investigate differences in the postprandial micronutrient response between young and older adults. Whereas a Fortifit liquid supplement was used in the VAAS study alongside resistance training, where the primary aim was to investigate the effect of resistance training alone or with nutritional supplementation on functional parameters (**Table 2.2**).

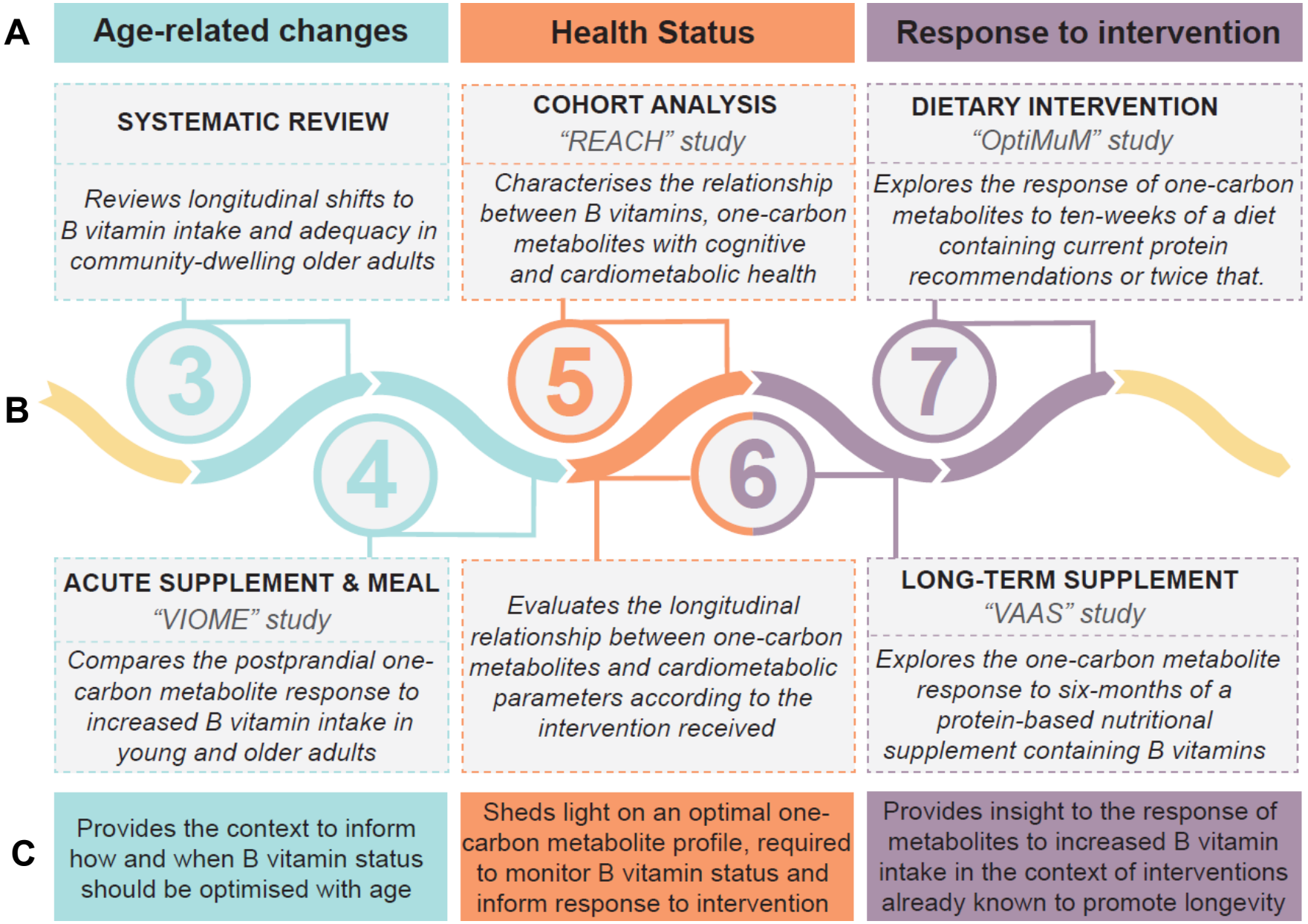


Figure 2.1: Overview of A) Thesis objectives, B) Studies and objectives of each Chapter, and C) Expected outcomes relating to the overarching thesis objectives.

Table 2.1: Overview of subject characteristics study design across results chapters

	Review – Chapter 3	VIOME – Chapter 4	REACH – Chapter 5	VAAS – Chapter 6	OptiMuM – Chapter 7
Subjects					
Sample size	8 studies, n=3119	n=40	n=313	n=95	n=29
Age range	≥65 years at follow-up	Younger: 19 – 30 years Older: 65 – 76 years	65 – 74 years	≥65 years	≥70 years
Setting	Community-dwelling	Community-dwelling	Community-dwelling	Aged-care	Community-dwelling
Sex (%male)	38%	50%	35%	12%	100%
Study					
Study design	Systematic Review	Open-label, single-arm acute intervention	Cross section	Randomised, controlled, parallel-group intervention	Randomised, parallel-group intervention
Duration	-	4 hours	-	6 months	10 weeks
Date, place of study	May 2018 – September 2020, Liggins Institute	July – Sep 2017, Liggins Institute Clinical Research Centre	April 2018 – Feb 2019, Massey University Human Nutrition Unit	July 2011 – Jan 2012, University of Vienna	April – Oct 2016, Liggins Institute Clinical Research Centre
Primary outcome[1]	NA	Differences in the postprandial micronutrient response to a MVM supplement between young and older adults.	The association between dietary patterns and cognitive performance and metabolic health in older adults.	The effect of resistance training with or without supplementation on functional parameters in the elderly (oxidative stress, DNA damage, chromosomal integrity)	Differences in skeletal muscle mass and function in healthy older males following ten weeks of a diet containing protein at current recommendations or twice that
Exposure	NA	MVM supplement (Centrum Advance General Multi) with standard meal	NA	1. Resistance training (2x/week) 2. Resistance training and nutritional supplementation (Fortifit supplement, 9x/week) 3. Cognitive training (2x/week)	1. High protein (1.6g protein/kg /day) 2. Current protein recommendations (0.8g protein/kg/day)
Statistical analysis	Prevalence of dietary inadequacy based on the estimated average requirement cut-point approach.	Repeated measures ANOVA including time*age interaction	Regression analysis - multivariate linear regression and modified poisson logistic regression	1. Repeated measures ANOVA including time*intervention interaction 2. Regression analysis – multivariate linear regression	1. Repeated measures ANOVA including time*intervention interaction 2. Regression analysis – multivariate linear regression
Thesis aims[1]					
Primary	Change in the prevalence of dietary inadequacy from baseline to follow-up.	Differences in the postprandial response of one-carbon metabolites between young and older adults	Association between B vitamin and one-carbon metabolite status with cognitive function and cardiometabolic health.	Differences in six-month changes in one-carbon metabolites according to intervention received.	Differences in ten-week changes in one-carbon metabolites according to intervention received.
Secondary	-	Impact of sex on the postprandial response of one-carbon metabolites in young and older adults.	Impact of apolipoprotein E genotype or interactions between B vitamins and metabolites on cognitive function and cardiometabolic health.	Cross-sectional and longitudinal associations between one-carbon metabolites with markers of cardiometabolic health, considering intervention impacts	Impact of intervention received on longitudinal association between changes in one-carbon metabolites and B vitamin intake.

[1]All analyses in this study from Chapters 4 – 7 were completed as secondary analyses, thus the primary outcomes of these studies are presented to provide background context for the study design. Abbreviations: MVM, multivitamin and mineral.

Table 2.2: Overview of B vitamin intake provided according to intervention exposures across intervention studies.

	Chapter 4 - VIOME		Chapter 6 - VAAS	Chapter 7 - OptiMuM	
Exposure					
Intervention	Centrum Advance Multivitamin and Mineral supplement and standard meal[1]		Fortifit supplement and resistance training	High protein diet (2RDA)	
Frequency	Single supplement		9x/week (supplement), 2x/week (training)	-	
Duration	Acute		6 months	10 weeks	
Comparison	-		1. Resistance training alone 2. Cognitive training (controls)	Current protein recommendations (RDA)	
Nutrients provided[2]	Supplement	Standard meal[1]	Fortifit supplement	2RDA	RDA
Energy, kJ	0.0	1,906	635	11,800 (640)	11,217 (2158)
Carbohydrate	0.0	83.3g	9.4g	47% TE	52% TE
Fat	0.0	8.3g	3.0g	28% TE	31% TE
Protein	0.0	8.7g	20.7g	20% TE	12% TE
Folate (DFE), µg[3]	-	79.0		878.3 (72.7)	684.8 (148.6)
Folic acid, µg	400	-	200	-	-
Riboflavin, mg	3.2	0.16	0.25	3.37 (0.38)	2.71 (0.64)
Vitamin B_6, mg	6.0	0.38	0.75	3.25 (0.27)	2.82 (3.25)
Vitamin B_{12}, µg	22.0	0.0	3.0	5.26 (0.56)	2.26 (0.71)

[1]Standard meal included white toast, margarine, honey, apple sauce, orange juice. [2]Nutrient intake is reported either according to a single dose (VIOME, VAAS) or average daily intake (OptiMuM). Nutrient intakes from supplements in VIOME and VAAS are based on details available from manufacturers. Nutrient intake from the standard meal in VIOME was evaluated using Foodworks Version 9 (Xyris, Australia), which uses data from the New Zealand Food Composition Database (NZ FOODFiles 2016). Nutrient intake in the OptiMuM study was calculated on Foodworks software from 7-day food records completed for each participant based on food consumption during their final week of intervention, and mean (standard deviation) for each group reported. [3]Folate intake from food is calculated as dietary folate equivalents, which considers differences in the bioavailability between natural food folates and folic acid found in fortified foods. Abbreviations; 2RDA, twice recommended daily allowance; RDA, recommended daily allowance; TE, total energy.

2.3 METHODOLOGY OVERVIEW

2.3.1 ONE-CARBON METABOLITE STATUS

The reverse-phase ultra-high-pressure liquid chromatography coupled with tandem mass spectrometry (UHPLC- MS/MS) method used to quantify fasting 1C metabolite in each clinical trial was developed in the Liggins Institute by Dr. Stephanie Andraos during my candidature. This method has been peer-reviewed and is available to read in full detail in *Analytical Biochemistry*[379]. In total, this method quantifies 37 metabolites and amino acids involved in several pathways including 1C metabolism, the citric acid cycle (ketogenic and glucogenic amino acids), the urea cycle, and branched-chain amino acid metabolism. A targeted panel of 11 metabolites involved in the methionine cycle (methionine, Hcy, SAM, SAH), the choline oxidation pathway (choline, betaine, DMG, glycine) and transsulfuration (cystathionine, cysteine, serine) were considered relevant to the objectives of this thesis, and included for further analysis

2.3.1.1 Standard preparation and quality controls

A labelled amino-acid premix solution (Cambridge isotope) was diluted to 33µM in 1% ascorbic acid and MilliQ® H_2O. Labelled 1C compounds were prepared in 0.1 M Hcl and diluted to 33µM in 1% ascorbic acid and MilliQ® H_2O from powders of; homocysteine d4 (Novachem (CIL)), cystathionine d4 (Novachem (CIL)), *S*-adehonsylhomocysteine d4 (Sapphire bioscience (Cayman)), betaine d3 (SciVac PTY. Ltd. (CDN)), dimethylglycine d3 (SciVac PTY. Ltd. (CDN)), and Taurine d4 (SciVac PTY. Ltd. (CDN)). Labelled amino acids and 1C compounds were mixed in a 1:1 solution, and used as the internal standard solution. A dilution series of unlabelled amnio acids (SIGMA® Chemical company, USA) and 1C metabolites were prepared at known concentrations, and mixed in a 1:1 ratio to create standards 1-8, which represents a wide range of concentrations that mirror physiological plasma concentrations of these compounds[379].

For each plate (maximum sample *n*=77), intra-assay recovery of standards and reproducibility of samples was checked using quality controls (QCs, 100µL in triplicate) which were dispensed into three different locations across the plate. This enabled quantification of fluctuations at different injection times across the 96-well plate. Stripped human plasma (SeraCare Life Sciences Inc.) was used to calculate compound recoveries. QC 1 was stripped plasma alone, QC 2 was spiked with 70µL of calibration standard 7, and QC 3 was pooled human plasma samples. Further details regarding the materials used throughout the method development and analysis can be found in the article by Andraos *et al*[379].

2.3.1.2 Sample preparation and robotic automation

In brief, whole blood was collected from participants into EDTA blood collection tubes in this thesis. Plasma was separated by centrifugation, and plasma samples were then separated into aliquots and stored at -80°C in Eppendorf tubes until required for analysis. Samples were thawed only immediately prior to analysis, and no sample in this thesis was subject to a freeze-thaw cycle. Samples were loaded onto an automated robotic liquid handling system (Eppendorf epMotion® 5075vt, Hamburg, Germany), which was configured to perform liquid handling procedures using the epBlue Client version 40.6.2.6 software. First, 300µl of 1% formic acid in methanol was pipetted into a 96-well IMPACT® protein precipitation plate (Phenomenex, California, USA). Next, 100µl of 1% ascorbic acid in MilliQ® H_2O was dispensed into the first two wells as blanks. Then, all standards (100µl), QCs (100µl), samples (100µl), and one blank well were spiked with 20µl of internal

standard solution, agitated for 5 minutes (800rpm), then filtered into a 96-well collection plate (Phenomenex, California, USA) by applying a vacuum (450mbar). Tris (2-carboxyethyl) phosphine (100µl) was then dispensed into each well, which reduces disulfide bonds in cystine and homocystine to allow for the separate quantification of cysteine and Hcy, respectively. Finally, 200µl of 1% ascorbic acid in MilliQ® H_2O was added to each well, the plate was then agitated for a further 5 minutes (800 rpm) and placed in the UHPLC auto-sampler unit (held at 10°C) for analysis.

2.3.1.3 Ultra-high-pressure liquid chromatography tandem mass spectrometry – sample run and data processing

The UHPLC-MS/MS technique was performed using a Vanquish UHPLC$^+$ system coupled with a TSQ Quantiva triple quadrupole mass spectrometer (Thermo Scientific) in positive electrospray ionization mode. A Kinetex® EVO C18 100 Å 150x2.1mm 1.7µm column (Phenomenex®), coupled with a Krudkatcher (Phenomenex®) pre-column filter was used, with a flow of 400µL/min starting at 2% Acetontrile and 98% mobile phase (5mM perfluorohexanoic acid in MilliQ® H_2O applied to the column, and gradually reaching 95% Acetonitrile for each sample. 7µL of samples were injected, and the run time for each sample was 15.5 minutes, with a total run time of 26-30h.

Xcalibur version 4.0.27.19 (Thermo) was used to complete data processing. Compounds were detected based on retention times and labelled standards were used for quantification. To account for missing values where a peak was not detected, half of the minimum value was input according to each plate[380]. Missing values were not calculated for compounds that were excluded from analysis based on QC failure. Metabolites were considered acceptable if standard recoveries were between 80-120% of expected values, and if coefficients of variance were below 20%. As outlined in **Table 2.3**, SAH did not satisfy requirements in any study, SAM did not meet these requirements in Chapters 4 or 6, and cystathionine did not for Chapters 5 or 6, and so were excluded from further analysis. The exclusion of cystathionine and SAH in particular is consistent with previous reports of these compounds being unstable across runs, encountering chromatographic complications (cystathionine), or being below the lower limit of detection (SAH) using the same UHPLC-MS/MS technique[379,381]. The average of coefficients of variance across studies is presented in **Table 2.3**, ranging from 3.56 – 12.4 for methionine and Hcy, respectively.

Table 2.3: Overview of measures of B vitamin and one-carbon metabolite status included across results chapters

Domain	Nutrient/Metabolite	Method	CV[1]	Chapter 4	5	6	7
Plasma 1C metabolites	Betaine	UHPLC-MS/MS	4.5	✓	✓	✓	✓
	Choline	UHPLC-MS/MS	6.4	✓	✓	✓	✓
	Cystathionine	UHPLC-MS/MS	8.9	✓	✗	✗	✓
	Cysteine	UHPLC-MS/MS	7.6	✓	✓	✓	✓
	Dimethylglycine	UHPLC-MS/MS	4.2	✓	✓	✓	✓
	Glycine	UHPLC-MS/MS	8.9	✓	✓	✓	✓
	Homocysteine	UHPLC-MS/MS	12.4	✗	✓	✓	✗
		Enzymatic assay	NA	✓	✗	✗	✓
	Methionine	UHPLC-MS/MS	3.6	✓	✓	✓	✓
	S-adenosylhomocysteine	UHPLC-MS/MS		✗	✗	✗	✗
	S-adenosylmethionine	UHPLC-MS/MS	11.3	✗	✗	✗	✓
	Serine	UHPLC-MS/MS	9.9	✓	✓	✓	✓
Direct B vitamin status	Serum folate	Electrochemiluminescence binding	NA	✓	✓	✗	✓
	Erythrocyte Folate	Electrochemiluminescence binding		✗	✗	✗	✓
		Radioimmunoassay		✗	✗	✓	✗
	Serum vitamin B_{12}	Electrochemiluminescence binding		✓	✓	✗	✓
		Radioimmunoassay		✗	✗	✓	✗
B vitamin intake	Folate	Food record	-	✓	✓	✗	✓
	Riboflavin	Food record		✓	✓	✗	✓
	Vitamin B_6	Food record		✓	✓	✗	✓
	Vitamin B_{12}	Food record		✓	✓	✗	✓

[1]Reported as the average coefficient of variance across included studies. Coefficient of variance for each plate is based on three quality control samples per plate to assess intra-assay variability and is calculated as follows: (standard deviation/mean)*100. Abbreviations: NA, data was not available to calculate the coefficient of variance. Chapter 4 – VIOME, 5 – REACH, 6 – VAAS, 7 – OptiMuM.

2.3.2 B VITAMIN INTAKE AND STATUS

2.3.2.1 Dietary intake

Habitual dietary intake was reported across all clinical studies except VAAS (Chapter 6), where data was not available. All studies reporting dietary intake use an estimated food record, though the length for which intake was recorded varied. In Chapters 4 (VIOME) and 7 (OptiMuM), a three-day food record was used, while a four-day food record was used in Chapter 5 (REACH), all of which included at least one weekend day. The food records used in REACH were used to validate a dietary questionnaire for older adults in a separate analysis, explaining why more detail was required than in VIOME and OptiMuM, where dietary intake was more used as a confounding variable in the analysis. Dietary intake during the ten-week intervention in OptiMuM was based on each participant's personalised seven-day meal plan, which captured participant's food choices and intake during the final week of intervention and was representative of foods consumed in earlier weeks of the intervention period. All food records were analysed by myself, including assistance of one other Registered Dietitian and one Nutritionist for REACH. Foodworks (Xyris, Australia; Version 9 – VIOME and OptiMuM, or Version 10 - REACH) was used to estimate nutrient intake from the food records, which aligns with the New Zealand Food Composition Database (New Zealand FoodFiles 2016, Version 01).

2.3.2.2 B vitamin status

Direct markers of vitamin B_{12} (serum) and folate (serum or erythrocyte) status were measured in all clinical trials included in this thesis. These were measured in Chapters 4, 6, and 7 prior to the 1C metabolite analysis, and for Chapter 5 in parallel with the 1C metabolite analysis. Except for Chapter 5, these markers of B vitamin status have been used as primary outcome measures in separate analyses to those included in this thesis, and so are treated as confounding variables in analyses presented here. Given that these markers have defined cut-off values for deficiency or suboptimal status, biochemical folate and vitamin B_{12} status also bolster B vitamin intake in contextualising Hcy status, as Hcy concentrations are not within expected reference ranges - described in further detail below. Markers of vitamin B_6 and riboflavin status were considered. However, issues were encountered across studies including inadequate sample preparation at the time of collection (e.g. a hemolysate is required for the erythrocyte glutathione reductase activity (EGRac) assay[382]) and limited sample availability (e.g. those that were not exposed to light or have gone through freeze-thaw cycles for PLP assays[383]). Thus, the decision was made to not proceed with riboflavin or vitamin B_6 analysis, and while this is not a major limitation of the work in this thesis, possible implications will be expanded on in later sections.

2.3.3 CROSS-PLATFORM HOMOCYSTEINE COMPARISON

Plasma Hcy concentrations between 5 -15 μM are considered normal, while concentrations ≥15μM are typically defined as elevated[384]. These ranges have been reported in adults using LC-MS techniques[385–387], however Hcy concentrations using the UHPLC-MS/MS technique for the research presented in this thesis are lower than expected ranges. This discrepancy can be explained by the lower concentration of Tris (2-carboxyethyl) phosphine used, an agent used in sample preparation to reduce disulfide bonds in cystine and homocystine to allow quantification of cysteine and Hcy, compared to other studies[388]. While the UHPLC-MS/MS technique used here has been peer-reviewed, and intra-assay QCs have been performed, it is worth ensuring that concentrations quantified using this technique correlate well with those within expected reference ranges. A cross-platform comparison between Hcy concentrations from the UHPLC-MS/MS analysis to those quantified using an enzyme-based test on a Cobas e411 autoanalyser (Roche, Mannheim, Germany) was therefore performed.

Pearson correlation analyses were performed on samples from Chapter 4 (VIOME, *n*=198) which had previously been analysed using the enzyme-based test, as well as a subsample from Chapter 5 (REACH, *n*=73, 23% of samples). The possible influence of sampling effects (batches), age (young v old), and sample type (fasting v postprandial) were separated out on the cross-platform comparison of VOME samples. REACH was a cross-sectional study and all samples were measured from a single UHPLC-MS/MS batch, therefore no further analyses were performed.

Table 2.4: Cross-platform comparison of homocysteine concentrations between liquid chromatography-mass spectrometry and autoanalyser techniques.

			UHPLC-MS/MS		Autoanalyser			
Study	Subsample analysis		Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	Correlation coefficient	*p*
REACH	-		2.2 (0.7)	2.1 (1.7 – 2.6)	11.4 (2.9)	11.1 (9.3 – 12.8)	0.72	<0.001
VIOME	-		2.4 (1.0)	2.3 (1.7 – 2.9)	11.8 (2.8)	11.2 (9.9 – 12.7).	0.71	<0.001
	Age	Older	2.6 (1.1)	2.5 (1.8 – 3.3)	12.4 (2.8)	11.7 (10.2 – 14.7)	0.65	<0.001
		Younger	2.1 (0.9)	2.1 (1.6 – 2.6)	11.2 (2.6)	10.8 (9.7 – 12.1)	0.74	<0.001
	Batch	1	2.2 (0.8)	2.1 (1.6 – 2.7)	11.9 (2.8)	11.3 (9.9 – 13.1)	0.82	<0.001
		2	2.7 (1.0)	2.5 (2.0 – 3.0)	11.6 (2.8)	10.9 (10.0 – 12.4)	0.73	<0.001
		3	2.1 (1.1)	1.8 (1.3 – 3.0)	11.8 (2.8)	11.5 (9.7 – 13.6)	0.72	<0.001
	Sample type	T0	2.9 (1.2)	2.7 (2.3 – 3.4)	13.1 (2.9)	12.3 (11.1 – 14.5)	0.58	<0.001
		T1	2.5 (0.9)	2.4 (1.9 – 2.9)	11.7 (2.6)	10.8 (10.2 – 12.6)	0.78	<0.001
		T2	2.2 (1.0)	2.1 (1.5 – 2.9)	11.4 (2.7)	10.9 (9.6 – 12.6)	0.75	<0.001
		T3	2.1 (0.7)	2.0 (1.6 – 2.6)	11.5 (2.8)	10.9 (10.0 – 12.4)	0.66	<0.001
		T4	2.2 (1.0)	2.1 (1.5 – 2.7)	11.1 (2.6)	10.5 (9.5 – 12.2)	0.77	<0.001

All concentrations are reported in μM. Abbreviations: IQR, interquartile range; SD, standard deviation.

The results from this cross-platform analysis confirmed that concentrations from UHPLC-MS/MS quantification were lower than those from the enzyme-based test, which reported concentrations in line with expected reference ranges. Although concentrations differed, this analysis showed that concentrations between the two techniques were strongly correlated for the total population analysed in VIOME (R=0.71, p <0.001) and REACH (R=0.72, p <0.001). These findings align with those from Andraos[381], where concentrations of 9 amino acids (valine, leucine, isoleucine, methionine, threonine, phenylalanine, tryptophan) were compared between the UHPLC-MS/MS technique used here and samples analysed by Nuclear Magnetic Resonance (0.5 < R <0.9, p<0.001). Concentrations of plasma Hcy between the two platforms were most variable at the lowest and highest concentrations (Figure 2.1), similar to those reported by Andraos[381]. According to the sub-group analyses reported here, Hcy concentrations remained strongly correlated regardless of age, UHPLC-MS/MS batch, fasting or postprandial status (0.55 < R <0.85, p<0.001). The weakest correlation was found in the fasting samples of VIOME (R=0.58, p<0.001,) compared to postprandial samples (0.65 < R <0.8, p<0.001), as presented in **Table 2.4**. As observed in **Figure 2.2**, the greatest variability is observed at the highest and lowest concentrations, and thus it logically follows that the correlation between methods is weaker at T0 (UHPLC-MS/MS = 2.9 ± 1.2 µM; Autoanalyser = 13.1 ± 2.9 µM) where concentrations are higher than during the postprandial period (UHPLC-MS/MS = 2.3 ± 0.9 µM; Autoanalyser = 11.4 ± 2.7 µM).

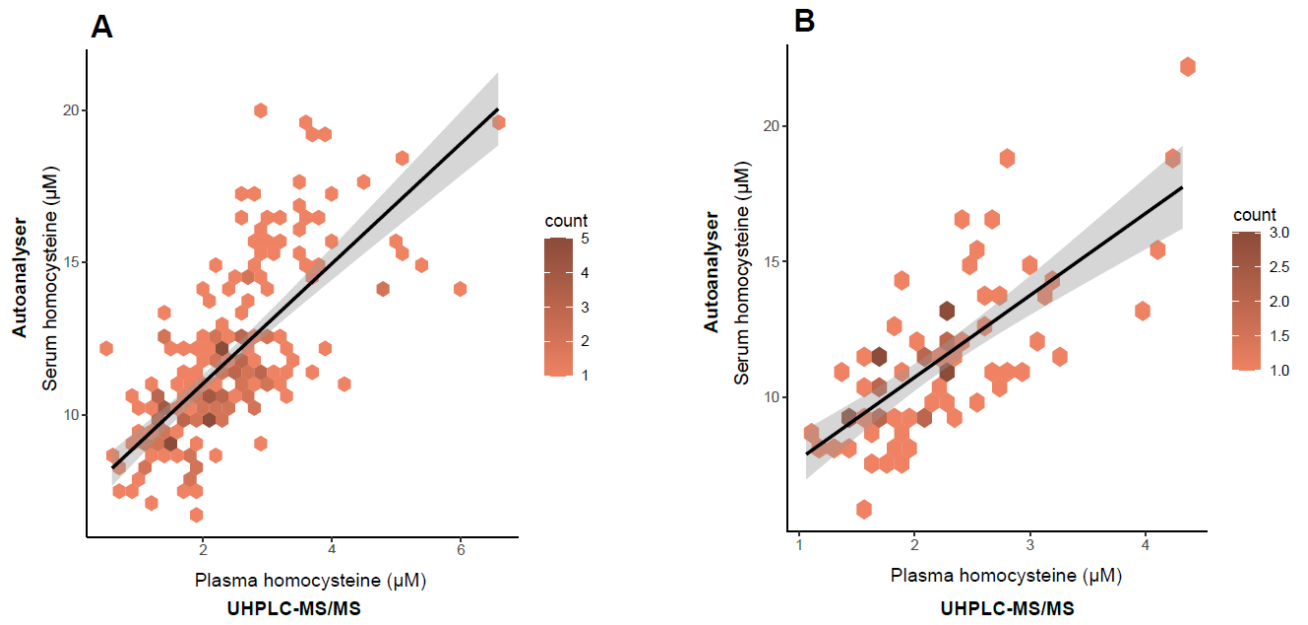


Figure 2.2: Comparison of homocysteine concentration quantified by UHPLC-MS/MS and autoanalyser techniques
Data from Pearson correlations are presented for A) VIOME participants in Chapter 4 (R = 0.71, p <0.001) and B) REACH participants in Chapter 5 (R = 0.72, p <0.001)

To summarise, plasma Hcy concentrations quantified from the UHPLC-MS/MS technique used in this thesis do not align with expected reference ranges according to differences in the disulfide-bond reducing agent used in sample preparation compared to other reported LC-MS/MS techniques. However, concentrations correlate sufficiently with those reported using an enzyme-based test, which quantifies samples within expected ranges, and plasma Hcy concentrations from the UHPLC-MS/MS technique can be reported with confidence in this thesis. However, Hcy concentrations in Chapter 4 (VIOME) are presented as those analysed by the enzymatic technique, as this was preferable for manuscript preparation and the complete sample set was available.

2.4 STATISTICS OVERVIEW

As outlined in **Table 2.1,** the primary statistical approaches were repeated measures analysis of variance (ANOVA) using mixed models for intervention studies, and regression analyses in both cohort and intervention studies. The specific models used in each Chapter are described in detail in their respective methods. This section comments on why these approaches were selected, and explains some minor inconsistencies between chapters.

2.4.1 MIXED MODELS FOR REPEATED MEASURES ANOVA

The primary objective of all intervention studies was to investigate differences in the 1C metabolite response to intervention according to either the intervention received (Chapter 6 – VAAS, Chapter 7 – OptiMuM) or age group (Chapter 4 – VIOME). Given that 1C metabolites were measured across more than one plate, the variation expected between plates owing to UHPLC-MS/MS batch effects also needed to be accounted for. Therefore, mixed models were the most appropriate approach to take rather than the classical repeated measures ANOVA, as the fixed effects of time and either intervention or age, and the interaction between these fixed effects could be taken into account alongside the random effects of participant and batch effects with more acceptable type I error rates[389]. Mixed models are also the preferred approach for handling missing data, and can properly account for correlation between repeated measures on the same subject[390].

2.4.2 MODEL BUILDING FOR REGRESSION ANALYSIS

Many approaches may be taken to building models for regression analysis, and arguments for and against each approach can be made[391,392]. These can broadly be considered as; 1) Step-wise approaches, where covariates are included if they fit within the bounds of a pre-determined statistical criteria; or 2) Fitting covariates in pre-defined models based on known causal relationships or variables which are likely confounding the exposure-outcome association[393]. This thesis contains both step-wise approaches (based on bivariate screening) and pre-defined model building (**Table 2.5**).

Table 2.5: Summary of model building approaches used in regression analyses

		Chapter 5 – REACH	Chapter 6 – VAAS	Chapter 7 – OptiMuM[1]
Approach		Stepwise with bivariate screening	Pre-defined models	Stepwise with bivariate screening
Dependent variable		Cognitive performance	Change in cardiometabolic markers	Change in metabolite concentration
Independent variable		One carbon metabolite concentrations	Change in metabolite concentration	Change in B vitamin intake
Covariates	Basic	Age, sex, education	-	Unadjusted
	Full	Age, sex, education, energy intake, physical activity, alcohol intake, polypharmacy, supplement use, batch effects	Age, sex, baseline metabolite concentration, BMI, glomerular filtration rate, batch effects	1. Baseline metabolite concentration and BMI 2. Baseline metabolite concentration and creatinine 3. Age
Interaction		1. Category of B vitamin intake, status, or other metabolites. 2. Apolipoprotein E genotype	Intervention group	Intervention group

[1]Not adjusted for batch effects or sex as only one UHPLC-MS/MS plate was run, and only male participants were included.

These differences stem from variations in sample size, data availability, and research questions across the studies in this thesis. For example, bivariate screening was performed to build multivariate models based on covariates associated with the dependent variable (p <0.150) from a list of possible confounders in both Chapters 5 and 7. The sample size in Chapter 7 (n=30) restricted the availability of including more covariates in the model and therefore required prioritisation based on the strength of association. Chapters 6 and 7 answered similar research questions by regression models, but different regression approaches were used relating to sample size differences. Pre-defined models were used in Chapter 6, as the larger sample size (n=95) allowed for all covariates to be fit that were considered for inclusion in Chapter 7. In Chapter 5, it would have been appropriate to use either approach given the sample size available to build robust models (n=313). While pre-defined models were considered, the analysis was more exploratory in nature rather than with the intent to build models with the greatest predictive power. Further, it was apparent on testing the extensive list of potential covariates that some showed very weak associations (0.150 < p ≤ 1.00) with the dependent variable, despite being considered relevant to 1C metabolite status and/or cognitive performance. Therefore, inclusion of all potential covariates in the fully-adjusted model would be unlikely to confound the association between independent and dependent variables and risk over-fitting the model.

SYSTEMATIC REVIEW

CHAPTER 3: TRAJECTORIES IN DIETARY ADEQUACY OF B VITAMINS WITH ADVANCING AGE

3.1 PREFACE

This first results chapter of this thesis presents a systematic review of studies which describe the longitudinal change of dietary B vitamin adequacy in older adults. This review aims to describe the nature of age-related change to B vitamin intake, which is poorly understood compared to the wealth of literature describing B vitamin intake in older compared to younger adults. This chapter aligns with the first objective of this thesis, describing age-related changes to B vitamin intake and metabolism.

Of course, intake represents only one aspect of the dynamic relationship between intake, status, function, and health. This chapter is however a starting point for understanding what happens to B vitamin intake with age, which can inform dietary recommendations for older adults and future research addressing the implications of progressive dietary shifts on functional status and health. A lack of evidence reporting longitudinal B vitamin intake has limited the capacity of this review to draw such conclusions, which will be discussed through this chapter.

This chapter contains the peer-reviewed manuscript “Exploring trajectories in dietary adequacy of the B vitamins folate, riboflavin, vitamins B_6 and B_{12} with advancing older age: a systematic review”, co-authored by Nicola A Gillies, David Cameron-Smith, Shikha Pundir, Clare R Wall, and Amber M Milan, and published in the *British Journal of Nutrition* (2020, pp:1-32). The *British Journal of Nutrition* has a 2019 impact factor of 3.51, and a three-year impact factor of 3.96 (Chapter 9, Appendix 1).

3.2 MANUSCRIPT:

Exploring trajectories in dietary adequacy of the B vitamins folate, riboflavin, vitamins B_6 and B_{12} with advancing older age: A systematic review

3.2.1 ABSTRACT

Maintaining nutritional adequacy contributes to successful ageing. B vitamins involved in 1C metabolism regulation (folate, riboflavin, vitamins B6 and B12) are critical nutrients contributing to Hcy and epigenetic regulation, and DNA synthesis. Although cross-sectional B vitamin intake has been characterised in various ageing populations, longitudinal changes are infrequently reported. This systematic review explores age-related changes in dietary adequacy of folate, riboflavin, vitamins B6 and B12 in community-dwelling older adults (≥65 years at follow-up). Following PRISMA guidelines, relevant databases (Medline, EMBASE, Biosis, CINAHL) were systematically screened, yielding 1579 records; 8 studies were included (n = 3119 participants, 2-25 years of follow-up). Quality assessment using a modified Newcastle-Ottawa quality scale rated all studies of moderate-high quality. The estimated average requirement cut-point method estimated the baseline and follow-up population prevalence of dietary inadequacy. Notably, riboflavin (7 studies, n=1953) inadequacy appears to progressively increase with age, with the prevalence of inadequacy increasing from baseline by up to 22.6% and 9.3% in males and females, respectively. Dietary folate adequacy (3 studies, n=2321) improved in two studies (by up to 22.4%), but the third showed increasing (8.1%) inadequacy. Evidence was similarly limited (2 studies respectively) and inconsistent for changes in dietary inadequacy of vitamins B6 (n=559; -9.9 – 47.9%) and B12 (n=1410; -4.6 – 7.2%). This review emphasises the scarcity of evidence regarding micronutrient intake changes with age, and highlights the demand for improved reporting of longitudinal changes in nutrient intake that can better direct micronutrient recommendations for older adults. This review was registered with Prospero (CRD42018104364).

3.2.2 INTRODUCTION

Population ageing is occurring rapidly, with estimates that the global proportion of adults aged over 65 years will increase to 16% by 2050[2]. This profound demographic shift has important individual and societal consequences due to the increased chronic disease risk associated with age[4,52]. Maintaining optimal nutrition status is a key modifiable factor that contributes to successful ageing, and hence is increasingly important for older adults[394–396]. Although older adults are at greater risk for nutritional deficiencies[54,57], changes in micronutrient intake specific to ageing have not yet been systematically reported.

Among essential nutrients, dietary adequacy of B vitamins involved in regulating 1C metabolism (folate, riboflavin, vitamins B_6 and B_{12}) is of particular interest in older adults given their co-regulation of Hcy, DNA synthesis, and methylation reactions[125]. Accordingly, B vitamins and Hcy as a marker of 1C metabolism,] are implicated in several diseases common in ageing populations, including vascular disease[220,221], cancers[397,398], cognitive impairment[273,399,400], and osteoporosis[401,402]. Research on the association between B vitamins, Hcy, and health outcomes has typically focused on understanding the involvement of folate, and to a lesser extent vitamin B_{12}[163,403–407]. However, folate metabolism is closely interlinked with these other vitamins involved in 1C metabolism, and inadequate status of any one of the four nutrients can perturb the complex pathways comprising one carbon metabolism[52].

Despite the importance of adequate nutritional intake, this becomes increasingly difficult to achieve for older adults owing to a complex interaction of physiological, psychological and biological factors. Older adults experience sensory changes, such as diminished taste and smell, neuroendocrine changes that affect appetite and satiety, and face challenges with swallowing, chewing, and limited mobility. Together with social-economic and psychological changes that are often concurrent with advancing age, older adults are particularly vulnerable to inadequate micronutrient intake and status[54,57]. Indeed, a systematic review by ter Borg *et al* [372] reported a high prevalence of inadequate dietary intake, upwards of 25 to 40%, of folate and related vitamins in older adults, including riboflavin, folate, and vitamin B_6. However, these findings, echoed by others, are generally limited to cross-sectional designs or cross-age comparisons[408–413]. Although these cross-sectional findings bolster the widespread notion that intake of B vitamins and other nutrients declines with age, they fail to describe how nutrient intake changes due to the ageing process.

While targeted efforts to better understand the intake of this group of B vitamins have been made[277,418], it remains unclear how dietary intake of these nutrients changes during ageing, and how this impacts health outcomes. Accordingly, to better understand how to optimise nutritional status of B vitamins, the nature of age-related changes in dietary intake must first be characterised. The current systematic review therefore aims to explore how the prevalence of dietary adequacy of folate, riboflavin, and vitamins B_6 and B_{12} alters with age in community-dwelling older adults.

3.2.3 METHODS

The present systematic review followed the reporting checklist as part of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement[415]. The protocol of the current review was registered at PROSPERO (CRD42018104364), an international database of prospectively registered systematic reviews in health and social care. The PICOS (Population, Intervention, Comparison, Outcomes, and Study design) criteria used to define our research question are summarised in **Table 3.1.**

Table 3.1: PICOS criteria employed to define the research question

Criteria	Description
Participants	Community-dwelling older adults (mean age 65 years or older at follow-up)
Intervention/Exposure	Dietary intake of folate, riboflavin, vitamin B_6 and vitamin B_{12}.
Comparisons	Prevalence of population adequacy of dietary folate, riboflavin, vitamin B_6 and vitamin B_{12} intake
Outcomes	Change in prevalence of population adequacy of dietary folate, riboflavin, vitamin B_6 and vitamin B_{12} intake from baseline to follow-up
Study design	Prospective cohort studies, randomised controlled trials, non-randomised controlled trials, case-control studies.

3.2.3.1 Search strategy

The electronic databases Biosis, CINAHL, EMBASE, and MEDLINE were searched for publications between 1990 - 8 March 2018. Medical Subject Headings (MeSH), MeSH major topics, and free text terms were developed under four group headings (B-group vitamins, dietary intake, older adults, and study design) for EMBASE, MEDLINE, and Biosis databases, which was modified for the CINAHL search preferences. MeSH terms were applied to subject headings where possible, and key-words were searched for in 'many places', or in a similar search field. All subject headings were joined with AND to develop the search string. An example of the full search strategy can be found as supplemental material (Appendix 1, Table 9.1 – *supplemental table 1 in the article*). All articles retrieved were exported to the reference manager Mendeley (v1.19, Elsevier Inc., Amsterdam, Netherlands), where duplicate articles were removed. Additional publications were identified by hand-checking reference lists of the relevant articles, searching for articles citing the relevant articles, and through relevant review articles, but not through searching the grey literature.

3.2.3.2 Study selection and data extraction

Titles and abstracts of all studies were screened in Mendeley to assess whether they met the inclusion criteria, presented in **Table 3.2**, for full-text review. If the decision on study inclusion or exclusion was unclear at this stage, the full text was obtained. Next, studies were further screened at the full-text level to ensure that they were eligible, were relevant to the research question, and presented data in an appropriate manner. The screening process was performed independently by two reviewers at both the title and abstract phase (N.G and A.M) and at the full-text review (N.G. and D.C-S.) in a systematic manner, with discrepancies resolved by a third reviewer if required (S.P). Details of studies excluded at the full text review with reason for exclusion are included in supplemental material (Appendix 1, Table 9.2 – *supplemental table 2 in the article*). Two review authors (N.G and A.M) then independently extracted key data into a prepared table, with discrepancies resolved by a third reviewer if required (S.P).

Authors were contacted if the full text was not available from our search. The following data was extracted from each study; country the study was performed in, study year, sample size, participant sex and mean age at follow-up (age range obtained if mean age unavailable), years of study follow-up, dietary assessment and statistical methods, whether dietary supplement use was reported or included in analysis, whether biochemical measures were included, whether nutrient intake was adjusted for energy or other measures, mean intake and standard deviation (SD) of nutrient intake at baseline and follow up for each nutrient assessed, whether a significant difference was observed between nutrient intake at baseline and follow-up according to author's statistical analysis. Details of the included studies' funding sources and potential conflicts of interest are included in supplemental material (Appendix 1, Table 9.3 – *supplemental table 3 in the article*).

Table 3.2: Inclusion and exclusion criteria applied in article screening

	Inclusion Criteria	Exclusion Criteria[1]
Participants	• Male and/or female participants • Community-dwelling (or described in such a way that this could be assumed) • Mean age of population at follow-up is ≥65 years[2] • Data are presented as mean and standard deviation, or with sufficient information to calculate these outcomes, at both baseline and follow-up • Data are presented for at least one of the nutrients of interest (folate, riboflavin, vitamin B_6, vitamin B_{12}) and energy.	• Participants included in the study that have malignancy or other clinically significant medical conditions. • Participants receiving palliative care • Homogenous population groups – i.e. populations where all participants share a common condition that could affect dietary intake (e.g. arthritis or cardiovascular disease).
Study design, data availability	• Longitudinal study with a follow-up period of at least two years[3] • Study is performed in developed countries, according to the United Nations list of developed economies(416)	• Studies that only report nutrient intake from supplements in total dietary intake estimates[4] • Dietary intake has not been determined through a validated method of dietary assessment (validated food frequency questionnaire, food records, 24-hour dietary recall, diet history) • Participants involved in dietary intervention studies or drug trials that would affect the dietary intake of the nutrients of interest • Data is adjusted for energy intake
Other criteria		• Full-text is not available in English

[1]Studies had to meet all inclusion criteria, and if any additional exclusion criteria were met the study was excluded from the final review. [2] 65 years is the chronological age used in commonly used indicators of ageing(417,418) .[3] A two-year follow-up period was chosen to align with the minimum follow-up period of previous cohort studies (e.g. the Framingham Cohort(419)), and to minimise the risk of detecting fluctuating changes in nutrient intake (e.g. with seasonality(420)) in shorter follow-up periods.[4]To strengthen conclusions that could be drawn around changes in dietary intake, given the inconsistent reporting of supplement use in a prior systematic review of micronutrient intake in older adults(372).

3.2.3.3 Quality assessment

A quality assessment scale applied by ter Borg et al[372], based on the Newcastle-Ottawa quality assessment scale for cohort studies[421], was adapted for use in review with additional criteria regarding the adequacy of follow-up of cohorts.. Summary quality scores of 0-2, 3-4, and 5-7 were rated as low, moderate, and high, respectively (**Table 3.3**). Studies were then categorised according to these ratings.

Table 3.3: Overview of the study quality assessment score

Component	Criteria	Points [1]
Predefined study population	Detailed information provided	1
	No information provided	0
Representativeness	Truly or somewhat representative of the average, elderly, community-dwelling resident	1
	Selected group of patients (e.g. certain socio-economic groups or areas) **or** No description of the derivation of cohort	0
Inclusion and exclusion criteria	Clearly stated	1
	Not stated	0
Validated dietary assessment method	Method clearly outlined	2
	Method outlined, little detail provided or no statement of validation	1
	Other method used, or no detail provided	0
Selective reporting bias	Reported data correspond with initial sample size	1
	Reported data do not correspond with initial sample size, rationale provided	1
	Reported data do not correspond with initial sample size, no information or incomplete rationale provided	0
Adequacy of follow up	Completed follow up – all subjects accounted for	1
	Subjects lost to follow up unlikely to introduce bias[2]	1
	Follow up rates <80% and no description of those lost	0
	No statement	0

[1]Summary score: 0-2 points = low quality, 3-4 points = moderate quality, 5-7 points = high quality.
[2]Number lost is ≤20%, or description of those lost suggested no different from those followed.

3.2.3.4 Statistical analysis

The population distribution of nutrient adequacy or inadequacy was based on the EAR cut-point method. The cut-point approach determines the population prevalence of inadequate intakes as the proportion with intakes below the EAR, a value based on the intake level sufficient to meet the needs of 50% of the population[422]. As it is not appropriate to compare group means to the EAR in this approach[422], z-scores were first calculated from the mean and SD reported in each study. From here, intake distributions were calculated and segmented into frequencies of intake at percentages of the EAR. The cut-point approach for risk of inadequacy can then be applied by combining the total proportion of the those estimated to have intakes below the EAR, as used in other reviews assessing micronutrient inadequacy on the mean and SD of published data[423,424]. This approach assumes that the requirement distribution is approximately symmetrical, and that intake distribution is more variable than the requirement distribution of the group[422]. As nutrient reference values vary between countries, this was standardised in our analysis by using the EAR from the Institute of Medicine on behalf of the United States of America and Canada[177]. These recommendations align with nutrient reference values from Australia and New Zealand[87], but not with recommendations from the United Kingdom[425], or for many European countries where discrepancies in recommendations are well documented[80,426].

Only one study[427] required transformation for analysis, as dietary data were presented as mean (standard error). SD was determined by applying the reported mean and 95% confidence intervals using the following calculation for SD[428]

$$\sigma = \sqrt{n} \times (\text{upper limit} - \text{lower limit})/3.92$$

3.2.4 RESULTS

3.2.4.1 Study selection and characteristics

A total of 1579 articles were identified as potentially relevant from the search strategy following removal of duplicates retrieved across different databases. After screening and eligibility assessment were completed, eight longitudinal studies were included in the final review (**Figure 3.1**). This resulted in the inclusion of n=3119 (38% male) community-dwelling older adults, with a range of 78-1166 participants included and 2-25 years of follow-up in each study. The studies were conducted in seven developed countries: three articles from European countries, three from Australasia, one from North America, and one from Asia (**Table 3.4**).

Dietary intake of riboflavin was assessed in seven studies (*n*=1953), folate in three (*n*=2321), and vitamins B_6 and B_{12} both in two studies each (*n*=559 and *n*=1410, respectively). None of the included studies assessed a particular group of micronutrients. Habitual dietary intake was assessed by diet history methods (three studies), validated food frequency questionnaires (three studies), combined 24-hour recall and food frequency questionnaire (one study), and a three-day diet record (one study). Overall, the risk of bias included in this review was relatively low – four studies were of high quality, four of moderate quality, while none of the included studies were found to be of low quality (**Table 3.4**).

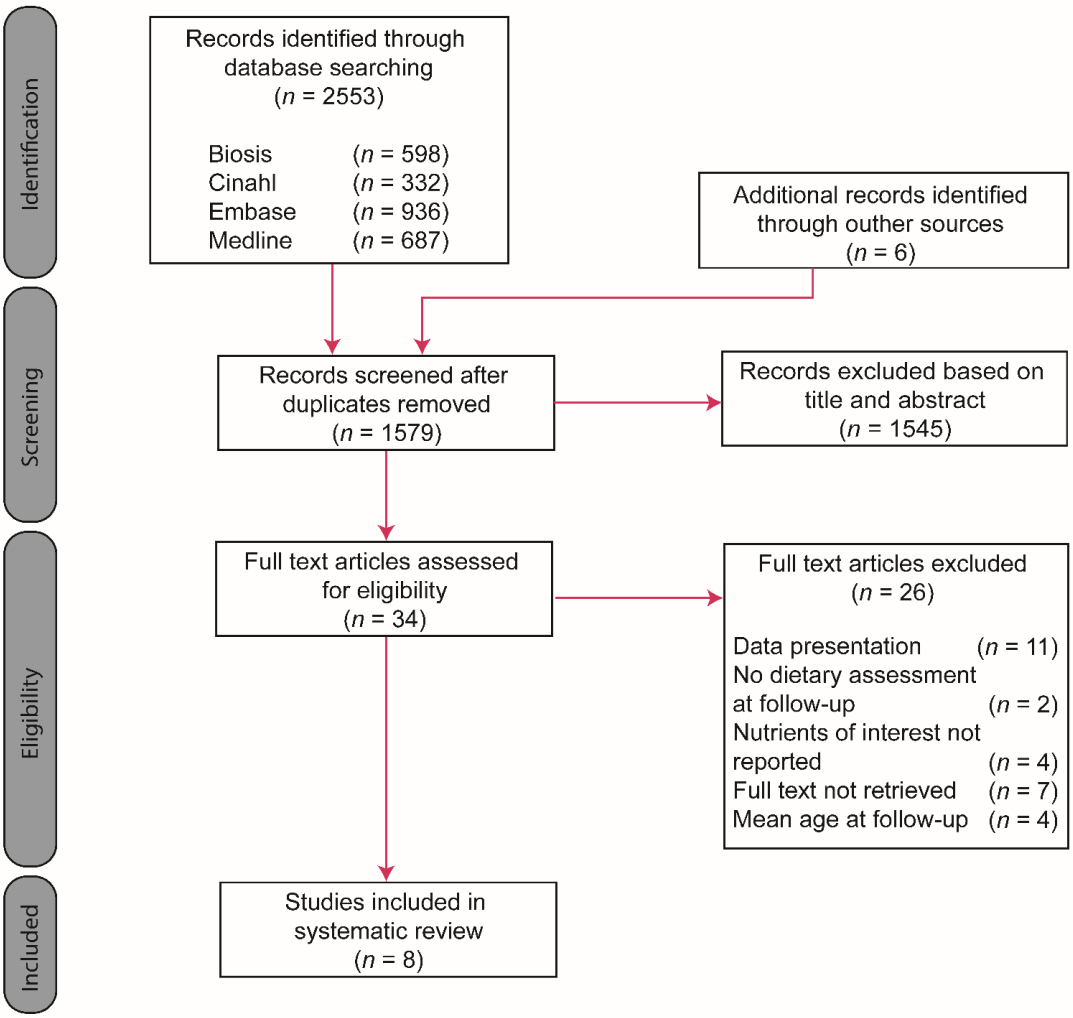


Figure 3.1: Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow chart of article selection and inclusion.

Table 3.4: Characteristics of included studies assessing longitudinal dietary intake of B vitamins in community-dwelling older adults.

Author, year, country of publication	FU years	Participants[1]				Dietary assessment method	Database used	Vitamins assessed	Supplements reported[2]	Quality Rating
		Sample size, *n* (sex)	Proportion lost to FU	Age						
				B	FU					
Chapman *et al.* 1996, USA[429]	2	209 (M)	30%	NA	71.8 (6.4)	Combined 24-hour recall and FFQ	USDA Food Composition Database 1963, 1975, 1976-86	Riboflavin	No	High
Fernyhough *et al*. 1999, New Zealand[430]	6	244 (M/F)	45%	>70y[3]	NA	120-item FFQ	New Zealand Food Composition Tables 1988	Folate Riboflavin Vitamin B_6 Vitamin B_{12}	Yes	High
Flood *et al.* 2010, Australia[427]	10	1166 (M/F)	60%	62.2 (3.2)	73.0 (3.6)	145-item FFQ.	NUTTAB90, NUTTAB95, AUSNUT	Folate Vitamin B_{12}	No	Moderate
Kromhout *et al.* 1990,The Netherlands[431]	25	315 (M)	64%	40-59y[3]	NA	Cross-check diet history	Netherlands Food Table 1985	Riboflavin Vitamin B_6	No	Moderate
Sjogren *et al*. 1994, Sweden[432]	6	98 (M/F)	Not reported	70 ± 0	76 ± 0	Diet history interview questionnaire (105-item questionnaire)	Not reported	Riboflavin	No	Moderate
Toffanello *et al*. 2011, Italy[433]	10	78 (M/F)	59%	M: 72.8 (1.7) F: 72.7 (1.6)	M: 81.9 (1.6) F: 82.0 (1.5)	Modified diet history	Italian Food Composition Table 1989	Riboflavin	Yes	Moderate
Yukawa *et al*. 2003, Japan[434]	8	98 (M/F)	61%	65-79y[3]	NA	3 day diet record coupled with direct interview.	Standard Tables of Food Composition in Japan 4th Edition 1990	Riboflavin	No	High
Zhu *et al.* 2010, Australia[435]	7	911 (F)	39%	74.9 (22.6)	81.9 (2.7)	74-item FFQ	Australian, exact database not specified.	Folate Riboflavin	No	High

[1]Age presented as mean (SD) unless stated otherwise if these values were not available. Sample size refers to the total number of participants included in both baseline and follow-up analyses for each study. The proportion of participants lost to follow-up includes the total of those no longer participating (e.g. due to death, illness, or change in location), and those with incomplete dietary intake data in the follow-up surveys. [2]Supplements were not included in the final analysis for any of the included studies that reported supplement use. It was assumed that if supplement use was not reported, then supplements were not included in the final dietary analysis. [3]Mean age at follow-up not available. Abbreviations: FU, follow-up; B, baseline; M, male; F, female; FFQ, food frequency questionnaire.

3.2.4.2 Change in prevalence of dietary inadequacy with time

3.2.4.2.1 Riboflavin

The prevalence of nutritional inadequacy for riboflavin (i.e., those with dietary intakes that do not meet the EAR) was estimated to be greater than 25% in three of the seven studies, and across both sexes at both baseline and follow-up[429,433,434]. At baseline, the greatest prevalence of inadequacy was 43.9% and 36.3%, while after follow-up was 50.0% and 45.6% in males and females, respectively. The magnitude of change in the prevalence of inadequacy from baseline to follow-up ranged from an increase of 0-22.6% and 0-9.3% in males and females, respectively (**Table 3.5**).

3.2.4.2.2 Folate

The prevalence of nutritional inadequacy for folate was estimated to be greater than 25% in all three studies, and across both sexes at baseline and follow-up[427,430,435]. At baseline, the greatest prevalence of inadequacy was 90.5% and 92.8%, while after follow-up was 85.5% and 83.5%, in males and females, respectively. The magnitude of change in prevalence of inadequacy from baseline to follow-up ranged from a decrease of 5.0-17.8% and 9.3-22.4% in males and females, respectively, while only one study showed an increase in the prevalence of inadequacy of 8.1% in females only (**Table 3.5**).

3.2.4.2.3 Vitamin B_6

The prevalence of nutritional inadequacy for vitamin B_6 was estimated to be greater than 25% in one of the two studies, and across both sexes at baseline[430], and in both studies across both sexes at follow-up[430,431]. At baseline, the greatest prevalence of inadequacy was 50% in both sexes, while after follow-up was 67.9% and 40.1% in males and females, respectively. The magnitude of change in the prevalence of inadequacy from baseline to follow-up ranged from an increase of 0-47.9% in males and a reduction of 9.9% in females (**Table 3.5**).

3.2.4.2.4 Vitamin B_{12}

The prevalence of nutritional inadequacy for vitamin B_{12} was not estimated to be greater than 25% for either study at either baseline or follow-up[427,430]. At baseline, the greatest prevalence of inadequacy was 14.0% and 19.8%, while after follow-up was 16.5% and 19.1%, respectively. The magnitude of change in prevalence of inadequacy from baseline to follow-up ranged from a 2.9% reduction to a 7.2% increase in males, and from a 4.6% reduction to a 2.2% increase in females[427,430] (**Table 3.5**).

Table 3.5: Calculated change in risk of micronutrient inadequacy between baseline and follow up of included studies.

Nutrient, EAR	Author	FU years	Sex	Time	Dietary intake[1]	% of individuals with dietary intake above and below EAR[2]						Change in % <EAR
						>100%	75-99%	50-74%	25-49%	<25%	% < EAR	
Folate, 320 µg/day	Fernyhough *et al*. [430]	6	M	B	236 (64)	9.5	38.0	40.7	11.0	0.74	90.5	-5.0
				FU	247 (69)	14.5	39.5	35.6	9.6	0.78	85.5	
			F	B	228 (63)	7.2	35.2	43.5	13.1	0.94	92.8	-9.3
				FU	247 (75)	16.5	37.2	34.0	11.0	1.3	83.5	
	Zhu *et al*. [435]	7	F	B	287 (92)	36.0	33.5	22.1	1.1	0.91	64.0	8.1
				FU	266 (92)	27.9	33.3	26.4	10.3	2.2	72.1	
	Flood *et al.* [427]	10	M	B	346 (106)	59.7	24.4	11.9	3.4	0.60	40.3	-17.8
				FU	425 (139)	77.5	13.3	6.3	2.2	0.65	22.5	
			F	B	325 (101)	52.0	28.0	14.9	4.4	0.76	48.0	-22.4
				FU	403 (127)	74.3	15.7	7.2	2.2	0.55	25.6	
Riboflavin, 1.3 mg/day (M) 1.1 mg/day (F)	Chapman *et al*.[429]	2	M	B	1.8 (0.9)	71.1	11.0	7.9	5.0	5.1	28.9	3.9
				FU	1.8 (1.1)	67.5	9.8	7.9	5.8	9.0	32.5	
	Fernyhough *et al*.[430]	6	M	B	1.7 (0.5)	78.8	13.8	5.6	1.5	0.30	21.2	0
				FU	1.7 (0.5)	78.8	13.8	5.6	1.5	0.30	21.2	
			F	B	1.6 (0.6)	79.8	10.4	5.8	2.6	1.4	20.2	0
				FU	1.6 (0.6)	79.8	10.4	5.8	2.6	1.4	20.2	
	Sjogren *et al*.[432]	6	M	B	2.0 (0.2)	99.96	<0.01	<0.01	<0.01	<0.01	0.04	0.58
				FU	1.7 (0.1)	99.4	0.62	<0.01	<0.01	<0.01	0.62	
			F	B	1.7 (0.1)	99.99	<0.01	<0.01	<0.01	<0.01	<0.01	0.10
				FU	1.5 (0.1)	99.9	0.10	<0.01	<0.01	<0.01	0.10	
	Zhu *et al*.[435]	7	F	B	2.4 (0.9)	92.6	3.4	2.0	1.1	0.91	7.4	-5.1
				FU	2.9 (0.6)	97.7	1.2	0.61	0.27	0.18	2.3	
	Yukawa *et al*.[434]	8	M	B	1.4 (0.4)	56.1	27.7	12.7	3.0	0.40	43.9	-3.0
				FU	1.3 (0.4)	53.1	28.8	14.1	3.6	0.50	46.9	
			F	B	1.4 (0.4)	63.7	24.1	9.8	2.1	0.27	36.3	9.3
				FU	1.4 (0.5)	54.4	25.3	14.2	4.9	1.1	45.6	
	Toffanello *et al.* [433]	10	M	B	1.6 (0.5)	72.6	16.9	7.7	2.3	0.5	27.4	22.6
				FU	1.3 (0.4)	50.0	20.6	15.5	8.7	5.2	50.0	
			F	B	1.5 (0.8)	69.1	10.9	8.2	5.5	6.3	30.9	3.6
				FU	1.3 (0.5)	65.5	17.3	10.4	4.7	2.0	34.5	
	Kromhout *et al*. [431]	25	M	B	2.2 (0.6)	93.4	4.7	1.5	0.34	0.06	6.6	11.8
				FU	1.8 (0.5)	81.6	12.2	4.7	1.2	0.24	18.4	
			F	B	1.5 (0.8)	69.1	10.9	8.2	5.5	6.3	30.9	3.6
				FU	1.3 (0.5)	65.5	17.3	10.4	4.7	2.0	34.5	

Nutrient, EAR	Author	FU years	Sex	Time	Dietary intake[1]	% of individuals with dietary intake above and below EAR[2]						Change in % <EAR
						>100%	75-99%	50-74%	25-49%	<25%	% < EAR	
Vitamin B_6, 1.4 mg/day (M) 1.3 mg/day (F)	Fernyhough *et al*. [430]	6	M	B	1.4 (0.4)	50.0	30.9	15.1	3.6	0.43	50.0	0
				FU	1.4 (0.5)	50.0	25.8	16.1	6.3	1.8	50.0	
			F	B	1.3 (0.4)	50.0	29.2	15.6	4.7	0.74	50.0	-9.9
				FU	1.4 (0.4)	59.9	25.7	11.4	2.6	0.36	40.1	
	Kromhout *et al.* [431]	25	M	B	1.8 (0.4)	80.0	14.9	4.3	0.69	0.06	20.0	47.9
				FU	1.3 (0.3)	32.1	46.3	19.5	2.0	0.05	67.9	
Vitamin B_{12}, 2.0 µg/day	Fernyhough *et al* [430]	6	M	B	4.7 (2.5)	86.0	4.0	3.1	2.3	4.6	14.0	-2.9
				FU	4.2 (1.8)	88.9	4.4	2.9	1.8	2.0	11.1	
			F	B	4.3 (2.4)	83.1	4.7	3.7	2.8	5.7	16.9	2.2
				FU	4.1 (2.4)	80.9	5.1	4.1	3.1	6.7	19.1	
	Flood *et al.* [427]	10	M	B	5.7 (2.8)	90.7	2.6	2.0	1.5	3.2	9.3	7.2
				FU	5.8 (3.9)	83.5	3.0	2.6	2.2	8.7	16.5	
			F	B	5.4 (4.0)	80.2	3.3	2.9	2.5	11.0	19.8	-4.6
				FU	5.5 (3.4)	84.8	3.2	2.7	2.2	7.1	15.2	

[1]Presented as mean (SD). [2]The population distribution of nutrient adequacy or inadequacy was based on the EAR cut-point method, using the EAR from the Institute of Medicine on behalf of the United States of America and Canada[177]. Abbreviations: EAR, estimated average requirement; FU, follow up; M, male; B, baseline; F, female.

3.2.5 DISCUSSION

Older adults are a vulnerable group regarding micronutrient intake and status owing to a complex interplay of physiological and psycho-sociological factors intrinsic to ageing, leading to the notion that micronutrient intake declines with advancing age. However, reports on nutrient intake in community-dwelling older adults vary widely, and rarely describe what changes occur due to ageing as opposed to the frequently reported cross-sectional differences in dietary intake compared to other age groups. The current systematic review presents the first pooled analysis of changes in micronutrient intake that occur with ageing. From eight longitudinal studies, evidence of decreased dietary adequacy of riboflavin with age was found, as well as a high prevalence of dietary inadequacy of folate, riboflavin, and vitamin B_6. More importantly, this study highlights the extreme scarcity of literature available to substantiate the often-reported claim that micronutrient intake declines due to ageing.

From the eight studies included, evidence for changes in dietary adequacy was limited for folate (three studies), and vitamins B_6 (two studies) and B_{12} (two studies). Indeed, a trend for changes in dietary adequacy of these nutrients could not be identified. Dietary riboflavin was most frequently reported, and dietary adequacy was found to decline into older age in five of the seven studies reporting on riboflavin intake. This decline supports the cross-sectional meta-analysis of dietary intakes in older adults by ter Borg *et al*[372], which identified riboflavin as a key nutrient of concern in older adults with an estimated prevalence of inadequacy of 31-41% across 30 studies. One study in the current review reported an increase in both occasional and regular multivitamin supplement use from baseline to follow-up, particularly in females[436], which although not considered in the analysis could of course mitigate the decline in riboflavin adequacy. Although one other study reported that participants were not taking supplements at baseline, follow-up data on whether habits around supplement intake changed with time was not reported[433], and the other five studies did not report on supplement use. Thus, it cannot be presumed that the decline in dietary riboflavin adequacy reported in this review reflects the total nutrient adequacy from food and supplements. It was not possible to disentangle changes in dietary adequacy from supplement use in the current review due to inconsistencies in reporting of supplement use, which as previously criticised by ter Borg *et al*[372], limits insight into the true prevalence of micronutrient inadequacies in older adults. This study supports demand for improved reporting of dietary intake data, including supplement sources of nutrients, to better understand changing dietary habits and status with advancing age. In the current review, original authors attributed the change in riboflavin intake to a reduction in energy intake, with smaller portions consumed[435], a reduced intake of milk products[435], meat or fish[433)], and an increased preference for sweet items with age[433]. Thus, ways to promote these and other widely available dietary sources of riboflavin (e.g. fortified cereals) throughout ageing should be considered in dietary recommendations and clinical practice to ensure nutritional adequacy with improved health outcomes for older adults.

Evidence for longitudinal changes was limited to eight studies including just 3119 participants. Irrespective of limited evidence, this review highlighted folate, riboflavin, and vitamin B_6 dietary inadequacy as a potential concern with advancing age. The prevalence of dietary inadequacy at follow-up was estimated to be greater than 25% for studies reporting on riboflavin (three studies), folate (three studies) and vitamin B_6 (two studies), though the prevalence of inadequate vitamin B_{12} intake was comparatively lower at less than 20%. Estimates of the prevalence of dietary inadequacy varied widely in the current analysis, particularly in the case of

riboflavin, which ranged from 0-43.9% at baseline and from 0-50% at follow-up, and results must accordingly be interpreted with some caution. However, this longitudinal analysis supports similar cross-sectional findings of micronutrient adequacy in older adults[372], as riboflavin, folate, and vitamin B_6 were reported to have an estimated prevalence of inadequacy of greater than 25% across 30 studies, while the prevalence of B_{12} inadequacy was less than 20%. Understanding longitudinal changes provides context to such cross-sectional analyses, helping to characterise their relevance to appropriate recommendations. Indeed, the analysis here showed that the decline in dietary riboflavin adequacy was progressive (up to 22.6% and 9.3% increase in dietary inadequacy in males and females, respectively), highlighting the need to consider dietary intervention prior to older age.

Factors contributing to changes in dietary intake with age are well established, ranging from physiological, such as sensory changes or increasing disability, to socioeconomic pressures and psychological perturbations[54,57]. While this review highlights that a progressive decline in dietary adequacy of riboflavin may occur with ageing, intake is only a single aspect of micronutrient status and function in pathways like 1C metabolism. Physiological functions that influence nutrient status are also known to be altered in older age including impaired absorption[57], low bioavailability from foods[438], and the impact of genetic polymorphisms on micronutrient status and function[439,440]. Such factors have led to controversy over the appropriateness of dietary reference values for older age, as these values, which are largely extrapolated from younger reference populations[80,426], have been suggested to contribute to discrepancies reported between dietary intake and nutrient status[441]. Although a direct correlation between intake and status of all B vitamins is not robustly established in older adults[441], concerns regarding the correlation between vitamin B_{12} intake and status in older adults serves to highlight the confounding influence of complex digestives processes on inferring adequacy from intake alone. Compromised vitamin B_{12} status is observed in older adults despite dietary adequacy due to progressive changes to the gastrointestinal tract with ageing that impair its absorption[442,443]. Accordingly, while vitamin B_{12} intake was largely adequate in this analysis, this does not rule out the possibility of a low biochemical status that would contribute to functional decline. Although the primary aim was to characterise changes in dietary adequacy with age, it is worthwhile noting that only one study included in the current review reported on longitudinal changes in both B vitamin intake and biochemical measures of B vitamin status status[429]. Hence, there remains a poor understanding of how changes in dietary adequacy with advancing age reported in this review extend to determine biological micronutrient status and health risk.

Despite the wealth of robustly studied longitudinal cohorts following participants into advancing age[444–447], our search for longitudinal dietary changes in a group of B-vitamins resulted in only eight studies included in the final review, and a total sample size of only 3119 participants. Unfortunately, some well-characterised, large, prospective cohorts did not meet the inclusion criteria for this review despite identification through our search strategy. Reasons included longitudinal data not being available for nutrients of interest (e.g. the Framingham Heart Study[145,419,448]), not meeting criteria for age at follow-up, inadequate presentation of nutrient intake (the Nurses' Health Study, the Health Professionals Follow-Up Study[449]), or the full-text not being available after request (e.g. the SENECA study[450]). While these studies could not be included in the final review, the longitudinal data published from the Nurse's Health Study and Health Professionals Follow-Up Study[449] would suggest that folate intake from both natural food sources and fortified foods increases with age, although participants did not reach older age by the time of follow-up. Data on B vitamin intake in the Framingham Cohort has only been published at the 20th follow-up[145,419,448], and although not able to be

combined to show longitudinal trends, cross-sectional data in this cohort (*n*=1160)[145] would also suggest that folate and vitamin B_{12} intake increases concurrently with categories of age. Both of these studies contrast the current understanding that micronutrient intake likely declines with advancing age, while the data synthesised from this review is equivocal given the paucity of longitudinal intake data reported on folate and vitamin B_{12} intake. Evidently, better or further reporting of longitudinal nutrient intake is needed, particularly in these large cohorts where the data is likely available. This data would help to better characterise the unique nutrient changes and requirements that occur with advancing age rather than relying on cross-sectional reports.

Although the included studies were all of moderate-high quality, participants were often not representative of the wider community-dwelling older population, tending to be of higher socioeconomic and health status[427,435], which is likely to impact dietary intake[451,452]. Indeed, dietary assessment methodology was robust for all included studies (diet history, diet records, validated food frequency questionnaires), but the variation of methods limits the comparison of dietary intake between studies included in this review. Adjusting nutrient intake for energy can help to address differences in dietary assessment methodology[453], however this was not possible in the current review without access to raw data. Further, it should be acknowledged here that the EAR cut-point approach is subject to over-inflation of prevalence estimates, and we were unable to adjust for within-person variation in nutrient intakes, and thus our results may over-estimate the prevalence of inadequacy[454]. It was not possible to adequately comment on sex-differences in age-related changes in intake, as several studies included only one sex, or provided conflicting findings. Similarly, we were unable to conduct sub-analyses of nutrient intake according to supplement use, as only two studies reported on supplement users, and this was not included in the final analysis. Additionally, although studies investigated changes in general micronutrient intake, the nutrients included were inconsistent, without strong rationale for inclusion or exclusion. This limited and inconsistent reporting restricts the strength of conclusions and recommendations from the current review, particularly around dietary intake of folate and vitamins B_6 and B_{12} which were reported in less than half of included studies. Further, as follow-up varied from 2-20 years, and there were not clearly defined age groups from the included studies, contrasts could not be drawn across either different lengths of follow-up or ageing groups.

Longitudinal studies, while useful for describing progressive changes in dietary adequacy, are subject to the confounding influence of changes in intake naturally occurring over time. Changes in the food supply or population dietary recommendations and behaviours may influence shifts in an individual's intake. In this review, three studies conducted time-sequential analyses by including cohort comparisons ranging from 6-25 years[430–432]. The reported changes in intake were inconsistent, some authors found increased dietary intake over time of riboflavin[432], vitamin B_6[430] and folate[430], while another author found decreased vitamin B_6[432] intake. Given the paucity of data, these analytical corrections are not sufficiently robust to influence the conclusions drawn from this review. Moreover, the effect of common population-wide influences limits the accuracy of estimating shifts in an individual's nutrient intake with time. This includes factors such as dietary assessment methods and differences in databases used between studies, and progressive database changes, which we were unable to correct for[455]. In particular, the limitations of comparing dietary folate intake between countries are widely acknowledged, stemming from differences in how different folate forms (naturally occurring folate and fortified folic acid) are estimated, and whether databases are up to date at the time of dietary analysis[394,456,457]. For example, studies reporting on folate intake in the current review[427,435,436] use food composition databases (Australian (AUSNUT, NUTTAB90, NUTTAB95)[427,435], New Zealand Food

Composition Tables[430]) that take folic acid voluntarily fortified by the industry (e.g. breakfast cereals) into account. Further, data in New Zealand[430] is largely based on the manufacturer's claims which may not reflect the true analytical value[89]. This issue is further compounded by changing folate fortification policies over time within countries. None of the studies reporting on changes in folate intake in this review were conducted in countries (Australia[427,435] and New Zealand[430]) at a time (between 1988 and 2006) where folic acid fortification was mandatory (only since 2009 in Australia[458], voluntary since 2009 in New Zealand[459]). However, changes in voluntary folate fortification practices in Australia were suggested by Flood *et al*[427] to be the reason why increasing folate intake was seen. Thus, it remains difficult to disentangle the effects of time and age on intake.

3.2.6 CONCLUSION

This systematic review provides evidence for alterations in dietary adequacy of B vitamins with the ageing process, including a progressive decline in riboflavin adequacy and a high prevalence of inadequacy for folate, riboflavin, and vitamin B_6, which complements and expands upon previous cross-sectional analyses. A limited number of studies and participants were included in this review, emphasising the lack of understanding around changes in nutrient intake with ageing, in contrast to the knowledge of differences in intake across age groups. Although supporting concerns around inadequate micronutrient intake in older adults, these concerns remain entangled with potential changes in overall dietary intake or micronutrient bioavailability and metabolism. To understand the true implications of these findings on related health outcomes and contribution to disease burden in ageing, a complementary understanding of concurrent biological changes to nutrient status is required. Hence, although informative on the risk of subclinical malnutrition in older adults, further evidence from longitudinal research including a comprehensive assessment of intake and markers of biochemical and functional status are required to direct micronutrient recommendations for older adults.

ACUTE MEAL AND SUPPLEMENT

VIOME study

4

CHAPTER 4: THE ACUTE ONE-CARBON METABOLITE RESPONSE IS MAINTAINED WITH AGE

4.1 PREFACE

Postprandial responses are increasingly recognised as insightful indicators of health status, revealing perturbations not captured in the fasting state where homeostasis is tightly controlled. While the longer-term response of Hcy to shifts in B vitamin intake is well described, the acute response has received comparatively little attention. Impaired amino acid, glucose, and lipid metabolism during the postprandial period are common with advancing age, likely impacting the acute regulation of Hcy in the day-to-day dietary perturbations that older adults face.

The second results chapter of this thesis presents data on the acute response of Hcy and other plasma 1C metabolites following increased B vitamin intake in older compared to younger adults. This was achieved by ingestion of a commercially available MVM supplement, providing a standardised quantity of B vitamins, alongside a simple meal designed to induce postprandial metabolic dynamics. This chapter builds on Chapter 3 in addressing the first objective of this thesis - to describe age-related changes to B vitamin intake and metabolism. Although Chapters 3 and 4 are not directly comparable in their study design and interpretation, Chapter 4 helps to infer what implications of progressive shifts to B vitamin intake on functional status might be with advancing age, which was not assessed in Chapter 3.

This Chapter reports on secondary outcomes of the VIOME (Multivitamin and Mineral Supplement Bioavailability and Metabolic Effects in Ageing) study. The primary outcome of the VIOME study was to evaluate the acute response of plasma and urinary B vitamins and vitamers. My role in the VIOME study was to complete the dietary analysis of three-day food records, and assist in study procedures (collecting anthropometric data, meal preparation, sample processing). For this Chapter, I completed the mass spectrometry analysis for 1C metabolites and wrote the manuscript as the first-author.

This chapter contains an altered version of the manuscript “The acute postprandial response of homocysteine to multivitamin and mineral supplementation with a meal is not impaired in older compared to younger adults”, co-authored by Nicola A. Gillies, Pankaja Sharma, Soo Min Han, Ruth The, Karl Fraser, Nicole C. Roy, David Cameron-Smith, and Amber M. Milan. This manuscript is awaiting submission to *The European Journal of Nutrition,* which has a 2019/2020 impact factor of 4.644, and a five-year impact factor of 4.348.

4.2 MANUSCRIPT:

The acute postprandial response of homocysteine and one-carbon metabolites to a multivitamin supplement and standard meal is maintained in older adults

4.2.1 ABSTRACT

Purpose: B vitamins are required for the complex regulation of Hcy and 1C metabolism, which are implicated in diseases predominant in ageing societies. Nutritional supplements are frequently used by older adults to counter nutritional inadequacies. However, the postprandial use of B vitamins from supplements in 1C metabolism is likely altered with age owing to impaired B vitamin absorption and metabolic dysregulation with comorbidities. Despite implications for health and nutritional status, this has not yet been characterised.

Methods: Healthy older (n=20, 65-76y) and younger (n=20, 19-30y) participants consumed a single commercial multivitamin and mineral supplement with a standard breakfast meal. Blood samples were collected at baseline and hourly for four hours following ingestion. Plasma 1C metabolites were quantified using liquid chromatography coupled with mass spectrometry, and serum homocysteine, folate, and vitamin B12 on a Cobas e411 autoanalyzer. Habitual dietary intake was evaluated from three-day food records.

Results: Older adults had higher fasting Hcy concentrations (older: 14.0 ± 2.9 μmol/L; younger: 12.2 ± 2.5 μmol/L; p=0.036) despite higher folate (older: 36.7 ± 17.4 nmol/L; younger: 21.6 ± 7.6 nmol/L; p = <0.001) and similar vitamin B12 concentrations (p=0.143) to younger adults; yet older and younger subjects had a similar decrease in postprandial homocysteine concentrations. Except for a faster decline of cystathionine in older adults (p=0.003), the postprandial response of other metabolites reflective of Hcy cycling (choline, betaine, methionine) and 1C flux (glycine, serine) was similar between young and older adults.

Conclusion: Healthy older adults appear to maintain postprandial responsiveness of 1C metabolism, supported by a similar postprandial decline in Hcy concentrations to younger adults.

4.2.2 INTRODUCTION

Population ageing is occurring rapidly, with the proportion of individuals aged 65 years or older expected to reach 16% by 2050, near double that of 2019[2]. Broadly, nutrition is a pivotal factor that can influence the ageing trajectory[5]. B vitamins have emerged as critical for maintaining health with age owing to their regulation of 1C metabolism. There is now a wealth of research describing associations between inadequate circulating B vitamin status and elevated concentrations of Hcy, a marker of dysregulated 1C metabolism, with diseases predominant in older adults, particularly cardiovascular disease and cognitive decline[52].

Despite the heightened importance of nutrition adequacy, ageing is concomitant with reduced appetite and changes in food intake, which increases the risk for inadequate B vitamin intake[54,75]. Supplements are a multi-billion dollar industry[460], promoted as a simple means to counter nutritional inadequacies in older adults. MVM supplements are the most frequently used supplement, particularly in ageing populations[461]. MVM supplements contain B vitamins essential for Hcy regulation, including those required for Hcy remethylation to methionine (folic acid, vitamin B_{12}, and riboflavin) and Hcy transsulfuration to cystathionine and cysteine (vitamin B_6), leading to improved biochemical B vitamin and Hcy status in older adults[156,462–464]. Although the primary motivation for older adults using MVM supplements is to improve or maintain overall health[462,465], concerns persist regarding their widespread use[466].

Compared to the long-term response of 1C metabolism to supplementation, little is known about the acute response to dietary perturbations outside of post-methionine load conditions[172–174], labelled isotope infusions[467,468] or fat and glucose tolerance tests[182]. Although these are valuable approaches, they do not reflect 'real-world' dietary perturbations older adults face[213,214], such as using MVM supplements alongside mixed-meals to maintain nutritional adequacy. Investigating the postprandial response to dietary perturbations reveals multiple aspects of metabolic health not captured by fasting markers. This also offers a more sensitive way to detect changes in healthy subjects given that homeostasis may mask early perturbations under fasting conditions[214,215]. Indeed, perturbations in Hcy regulation following a high protein diet have been apparent in the postprandial state in middle-aged adults, but not at fasting[186]. Candito *et al*[212] have also reported that while healthy older adults maintained Hcy concentrations within fasting ranges following a mixed meal, older adults with depression have elevated Hcy concentrations despite no differences in baseline concentrations.

Despite the value of integrating postprandial dynamics into measures of nutrient and health status, the postprandial response of 1C metabolism related to age *per se* is unknown. Hcy is the point at which the metabolism of amino acids, B vitamins, and choline converge. Plausibly, advancing age would impact the postprandial utilisation of Hcy given that insulin resistance, known to impact Hcy regulation[216,354], impaired amino acid and lipid metabolism[217,469], and increased risk for impaired B vitamin absorption[52,75,470] also frequently occur with ageing.

On the hypothesis that older adults would have a dysregulated postprandial 1C response, differences in the postprandial response of plasma 1C metabolite concentrations was investigated in healthy older compared to younger adults. This was achieved following ingestion of a commercially available MVM supplement, providing a standardised quantity of B vitamins, alongside a simple meal to induce postprandial metabolic dynamics.

4.2.3 METHODS

4.2.3.1 Study design

This study was an open-label, single-arm acute trial which has been described in full detail elsewhere[76]. Following an overnight fast, each participant consumed a single MVM supplement (Centrum Advance General Multi, Pfizer, New York City, NY, USA) with a standard breakfast meal within 20 minutes. Blood samples were collected at fasting and hourly for four hours following meal and supplement ingestion. Ethics approval was obtained from The University of Auckland Human Participants Ethics Committee (UAHPEC; Reference No. 019392) and the trial was prospectively registered with the Australian and New Zealand Clinical Trial Registry (ww.anzctr.org.au) as ACTRN12617000969369. Written informed consent was obtained from each participant before testing. The study was conducted at the Liggins Institute (University of Auckland) between July-September 2017. This article reports on secondary outcomes of the VIOME (Multivitamin and Mineral Supplement Bioavailability and Metabolic Effects in Ageing) study. The primary outcome of the VIOME study was to evaluate the postprandial response of plasma and urinary B vitamins and vitamers to MVM supplementation in older compared to younger adults[76]. The secondary outcomes reported in this article detailed assessment of plasma 1C metabolite concentrations.

4.2.3.2 Subjects

Twenty older (65-76 years) and twenty younger (19-30 years) adults, balanced for sex, were recruited to participate in the VIOME study through internal advertisements to the University of Auckland, and external advertisements in local newspapers and social media. Participants were healthy, with a BMI between 18-30 kg/m^2, free of current or prior major disease (including cardiovascular disease, diabetes, cancer) or gastrointestinal disease (Celiac Disease, Crohn's Disease, ulcerative colitis), and were non-smokers. Participants using vitamin or mineral supplements within the past three weeks, or using medications that might impact digestive or metabolic function (including proton pump inhibitors, calcium channel blockers, and thyroid medications) were excluded from the study, as were those with self-reported alcohol intake exceeding 28 units/week, or food allergies or intolerance to intervention foods.

4.2.3.3 Standardised meal

The test meal was designed to elicit a postprandial response, but without a complicated food matrix that would impact B vitamin absorption[471]. This meal was based on a test meal reported in a study investigating the bioavailability of micronutrients from an MVM supplement in the presence of a standardised meal[472].

The breakfast meal was prepared at the Liggins Institute before the trial day, with items purchased from a local supermarket (Countdown, Progressive Enterprises, Auckland, New Zealand). The breakfast contained two slices of white bread (74g), margarine (10g), honey (20g), unsweetened applesauce (100g), and unsweetened orange juice (250 ml). Water was freely available, but no other food or beverages were consumed until after the final sample collection. Combined nutrient content of the MVM supplement and standard meal are summarised in **Table 4.1.**

Table 4.1: Combined supply of B vitamins involved in one-carbon metabolism and macronutrients by test meal and multivitamin supplement.

Item	Folate, DFE (µg)	Folic acid (µg)	Riboflavin (mg)	Vitamin B_6 (mg)	Vitamin B_{12} (µg)	Energy (kJ)	Carbohydrate (g)	Fibre (g)	Protein (g)	Fat (g)
White toast (74g)	20.7	0.00	0.03	0.05	0.00	749	32.1	2.68	6.79	1.79
Margarine (10g)	0.00	0.00	0.00	0.00	0.00	223	0.00	0.00	0.03	6.01
Honey (20g)	0.40	0.00	0.01	0.00	0.00	272	15.9	0.00	0.08	0.00
Applesauce (100g)	1.80	0.00	0.02	0.02	0.00	317	17.4	1.08	0.2	0.23
Orange juice (250 ml)	56.1	0.00	0.10	0.31	0.00	346	17.9	0.71	1.61	0.26
Total (food)	79.0	0.00	0.16	0.38	0.00	1906	83.3	4.50	8.70	8.30
Supplement	0.00	400.0	3.20	6.00	22.0	0.00	0.00	0.00	0.00	0.00
Total	747µg Folate DFE		3.36	6.38	22.0	1906	83.3	4.50	8.70	8.30

Nutrient composition of the test meal was evaluated using Foodworks Version 8 (Xyris, Australia), which uses data from the New Zealand Food Composition Database (NZ FOODFiles 2016). Folate was consumed in two forms by our participants – naturally occurring folate in foods and folic acid. To account for differences in the bioavailability in folate between the two forms, dietary folate equivalents (DFEs) are used in the New Zealand Food Composition Database (DFE (µg) = 1.67 x folic acid (µg)).

4.2.3.4 One-carbon metabolites

Blood samples were collected into serum and EDTA-coated vacutainers (Becton Dickinson, NJ, USA), and then separated into aliquots and stored in Eppendorf tubes at -80°C until required for analysis. Samples were thawed immediately before analysis.

Serum concentrations of Hcy were measured using a Cobas e411 autoanalyser (Roche, Mannheim, Germany). Plasma concentrations of other 1C metabolites (betaine, choline, cystathionine, cysteine, DMG, glycine, methionine, SAH, SAM, serine) were measured using a UHPLC-MS/MS technique. Product/precursor ratios were calculated to provide insight into pathway regulation for betaine/choline, and DMG/betaine, which have been used as an index of endogenous betaine synthesis and its use, allowing inference of betaine-dependent remethylation of Hcy[119,120].

Samples were randomised across batches to ensure a balance of age group and sex, with all time points from the same participant included in a single batch. The methods have been reported in detail elsewhere[379]. Briefly, plasma samples were prepared using an automated robotic liquid handling system (Eppendorf epMotion® 5075vt, Hamburg, Germany). First, 300µl of 1% formic acid in methanol was pipetted into a 96-well IMPACT® protein precipitation plate (Phenomenex, Torrance, California, USA). Next, all standards (100µl), QCs (100µl), and samples (100µl) were spiked with 20µl of internal standard solution, agitated for 5 minutes (800rpm), then filtered into a 96-well square (2mL) collection plate (Phenomenex, Torrance, California, USA) by applying a vacuum (450mbar). Tris (2-carboxyethyl) phosphine (100µl) was then dispensed into each well to allow for the separate quantification of Hcy and cysteine.

Three sets of QC samples were included to assess recovery of standards and reproducibility of samples. Metabolites were considered acceptable if standard recoveries were between 80-120%, and coefficients of variance were below 20%. Coefficients of variance ranged from 0.75% (betaine) – 14.1% (glycine). SAH did not satisfy these requirements and was excluded from further analysis. SAM did not meet these requirements for one of three batches (23%, *n*=45), and was also excluded from further analysis given the likely issues that this reduction in sample size would cause for the statistical approach. For samples where a peak was not detected, a missing value was calculated as half of the minimum value of each batch.

4.2.3.5 Cardiometabolic health

Serum glucose and lipid profiles (total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, and triglycerides) were measured using a Cobas c311 autoanalyser by enzymatic colorimetric assay (Roche, Mannheim, Germany). Serum insulin was measured using a Cobas e411 autoanalyser by electrochemiluminescence immunoassay. Serum glucose, insulin, and triglycerides were measured both at fasting and postprandially, while cholesterol was measured at fasting only. Homeostatic model assessment of insulin (HOMA-IR) was calculated according to the method from Matthews *et al*[473], using fasting glucose and insulin concentrations. Body mass and height were measured following standardised anthropometric procedures, and BMI calculated as body mass relative to height (kg/m^2).

4.2.3.6 B vitamin status

A Cobas e411 autoanalyser was used to measure both serum vitamin B_{12}, by electrochemiluminescence immunoassay, and serum folate, by a binding assay.

4.2.3.7 Dietary intake

Habitual dietary intake was determined from three-day estimated food records, including at least one weekend day, completed before testing. Nutrient intake from food records and the test meal was analysed by a New Zealand Registered Dietitian using Foodworks (Version 9; Xyris Software, Australia), which utilises the New Zealand Food Composition Database (FOODfiles 2016, Version 01).

4.2.3.8 Statistical analysis

Before statistical analysis, the distribution of metabolites was graphically assessed, and logarithmically transformed as required to achieve a normal distribution. Incremental area under the curve was obtained with correction for baseline concentrations. All statistical analyses were performed using R 3.4.6 statistical software[474], and alpha set at p <0.05.

Differences in baseline participant characteristics or postprandial area under the curve according to age were assessed by unpaired Student's *t*-test. Differences in baseline 1C metabolite status according to age and sex were assessed by mixed models using the lme4 package, with age group and sex fitted as fixed factors, including an interaction term, and mass spectrometry batch effects as a random factor. The same mixed model was used to assess the postprandial response of 1C metabolites and cardiometabolic markers (not fitted with batch effects) to supplement and meal ingestion, adding time as a fixed factor and participant ID as a random factor. For postprandial analysis, two-way (time*age, time*sex, age*sex) and three-way interaction terms (time*age*sex) were fitted. Post-hoc analyses were performed using the Sidak correction to correct for multiple comparisons if a significant time effect (postprandial analysis) or interaction effect (postprandial and baseline analysis) was found.

4.2.4 RESULTS

4.2.4.1 Participant characteristics

Participant characteristics are presented in **Table 4.2**. In total, 20 older (70.1 ± 2.7 years) and 20 younger (24.2 ± 2.8 years) participants completed the study, and groups were balanced for sex. At baseline, waist circumference and fasting measures of blood glucose, total cholesterol, LDL-cholesterol, and triglycerides were higher in older participants (p < 0.05), while no differences in BMI, insulin HOMA-IR or HDL-cholesterol were found.

Higher folate intake (p=0.034) was reflected in higher serum folate status in older adults (p=0.001), but despite lower vitamin B_{12} intake for older adults (p=0.011), no difference in serum vitamin B_{12} was found between age groups. There were also no differences in habitual dietary intake of energy, riboflavin, and vitamin B_6 intake between age groups (**Table 4.2**).

Table 4.2: Baseline participant characteristics and dietary intake according to age group.

Characteristic	Variable	Older	Younger	*p*-value
	Age (years)	70.1 (2.7)	24.2 (2.8)	<0.001*
Cardiometabolic health	BMI (kg/m2)	25.6 (3.2)	24.3 (3.5)	0.239
	Waist circumference (cm)	88.4 (10.4)	79.2 (8.9)	0.005*
	Glucose (mmol/L)	5.00 (0.52)	4.64 (0.45)	0.022*
	Insulin (μU/mL)	7.38 (4.75)	6.81 (3.96)	0.686
	HOMA-IR	1.68 (1.12)	1.39 (0.81)	0.358
	Total cholesterol (mmol/L)	6.18 (1.32)	4.63 (0.93)	<0.001*
	HDL-cholesterol (mmol/L)	1.63 (0.41)	1.62 (0.47)	0.963
	LDL-cholesterol (mmol/L)	4.12 (1.23)	2.73 (0.84)	<0.001*
	Triglycerides (mmol/L)	1.41 (0.61)	0.93 (0.39)	0.005*
Habitual dietary intake	Energy (kJ/day)	7450 (1489)	7438 (2265)	0.985
	Folate, DFE (μg/day)	443 (270)	284 (170)	0.034*
	Riboflavin (mg/day)	1.93 (0.59)	1.67 (0.74)	0.240
	VitaminB6 (mg/day)	2.51 (0.88)	2.29 (1.16)	0.521
	Vitamin B12 (μg/day)	3.03 (0.94)	4.92 (2.72)	0.011*
B vitamin status	Folate (nmol/L)	36.7 (17.4)	21.6 (7.61)	0.001*
	Vitamin B12 (pmol/L)	327 (135)	385 (105)	0.143

Data presented as mean (SD), all biochemical markers of cardiometabolic health and B vitamin status measured as fasting serum concentrations. *Indicates a difference in baseline characteristics between age groups (p <0.05), determined by Students *t*-test.

4.2.4.2 Baseline one-carbon metabolite status according to age and sex

Table 4.3 outlines baseline circulating 1C metabolite concentrations. At baseline, older adults had higher fasting concentrations of Hcy and cystathionine, but lower concentrations of serine and methionine (age effect, p <0.05) regardless of sex. Regardless of age, males had higher baseline concentrations of DMG and methionine, but lower glycine concentrations (sex effect, p=0.037, p=0.040, p <0.001, respectively). Older males had higher baseline concentrations of choline and cysteine compared to older females (post-hoc, p=0.016, p=0.029), younger females (post-hoc, p=0.001, p=0.030), and younger males (post-hoc, p <0.001, p=0.006). No baseline differences according to either age or sex were found for betaine or DMG/betaine.

Table 4.3: Baseline one-carbon metabolite status according to age and sex.

Metabolites (plasma)	Older		Younger		Effect		
	Female (n=10)	Male (n= 10)	Female (n=10)	Male (n=10)	Age	Sex	Age*Sex
Betaine (μM)[1]	40.2 (8.79)	48.8 (11.6)	48.4 (16.8)	48.1 (12.5)	0.510	0.219	0.263
Choline (μM)	10.8 (1.31)[§]	13.4 (1.99)[†]	9.96 (1.39)[§]	9.34 (2.04)[§]	<0.001*	0.008*	0.004[#]
Cystathionine (nM)[1]	549 (434)	400 (187)	196 (75.5)	204 (73.3)	<0.001*	0.628	0.330
Cysteine (μM)[1]	90.3 (13.5)[§]	109 (14.9)[†]	90.3 (15.3)[§]	86.2 (10.0)[§]	0.006*	0.073	0.006[#]
DMG (μM)	2.89 (1.03)	3.36 (0.76)	2.78 (0.89)	3.53 (1.04)	0.922	0.037*	0.619
Glycine (μM)[1]	228 (32.5)	173 (21.7)	213 (43.3)	181 (24.7)	0.728	<0.001*	0.207
Homocysteine (μM)[1]	13.3 (3.05)	14.7 (2.78)	12.1 (3.34)	12.3 (1.47)	0.036*	0.238	0.637
Methionine (μM)	20.8 (3.08)	24.8 (3.46)	25.7 (3.08)	26.3 (4.97)	0.009*	0.040*	0.098
Serine (μM)	98.4 (13.7)	96.4 (14.1)	127 (16.1)	116 (20.3)	<0.001*	0.170	0.332
Betaine/choline	3.73 (0.77)	3.64 (0.74)	4.86 (1.57)	5.30 (1.58)	0.001*	0.604	0.564
DMG/betaine[1]	0.07 (0.02)	0.07 (0.02)	0.06 (0.02)	0.08 (0.03)	0.577	0.308	0.260

Data presented as mean (SD). p-values presented for main effects (age, sex), and an interaction effect according to linear mixed model analyses. *Indicates a significant main effect of age or sex, and [#] indicates a significant interaction effect (p = <0.05). For metabolites where a significant interaction was found, groups marked with [†] have a higher concentration than groups marked with [§] according to post hoc analyses. [1]Variables were log-transformed for analysis to achieve an approximately normal distribution. Abbreviations: DMG, dimethylglycine.

4.2.4.3 Postprandial response of one-carbon metabolites

Data for the area under the curve can be found in supplemental material as no differences were found between age groups for any metabolite (Appendix 2, Table 9.4). Following three-factor analysis, no interactions involving sex were found for differences in the postprandial response between age groups (Appendix 2, Tables 9.5 and 9.6). The findings below are presented for the interactions between time and age-group only. Metabolites are presented in groups based on their proximity to each other within pathways comprising 1C metabolism.

4.2.3.4.1 Homocysteine and methionine

Both age groups showed a sustained decline in circulating Hcy and methionine concentrations following supplement and meal ingestion, with the decline first seen at one hour after ingestion for Hcy and after 2 hours for methionine (post-hoc, p <0.001). Older adults had higher methionine (age effect, p=0.025) concentrations across all time points, and a trend towards maintaining higher Hcy concentrations (age effect, p=0.092) (**Figure 4.1, Table 9.6**).

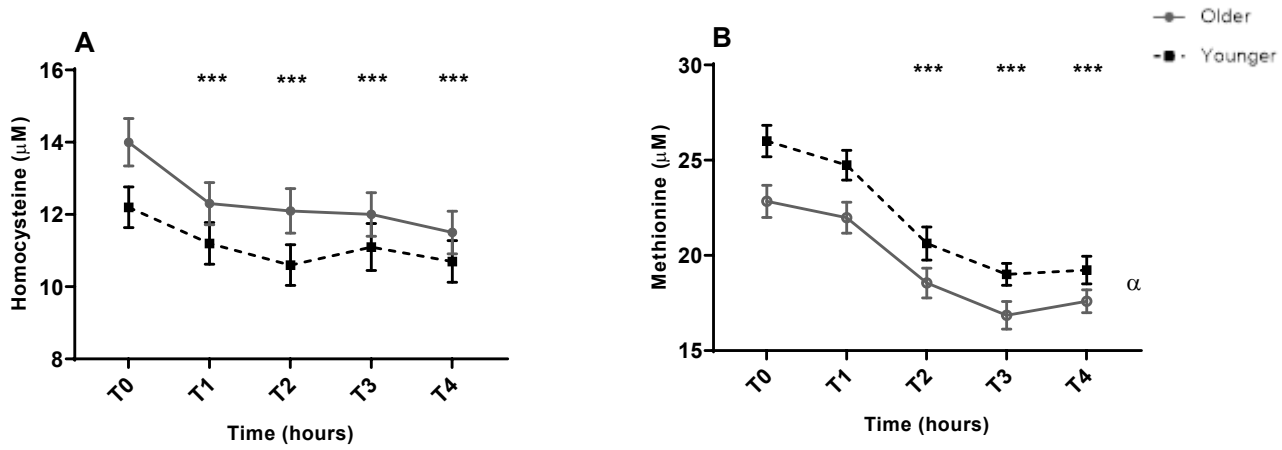


Figure 4.1: Postprandial response of homocysteine and methionine
*Mean ± SEM of (A) Homocysteine and (B) Methionine concentrations at fasting (T0), and hourly until 4 hours following supplement and meal ingestion (T1 – T4). Time difference from baseline for both age groups at the corresponding time point are indicated by * p <0.05, ** p <0.01, *** p <0.001. α indicates a difference between age groups across postprandial time points (p <0.05). No time x age interactions were found*

4.2.3.4.2 Cystathionine, cysteine, glycine, and serine

Plasma cystathionine declined from baseline in both age groups, and this decline was faster in older adults who showed a decline at 2 hours (post-hoc, p=0.004) compared to younger adults at 4 hours (post-hoc, p=0.048). Although no interaction between time and age was found, glycine declined from baseline after 4 hours, and serine after 2 hours (post-hoc, p <0.001). Older adults had lower serine and higher cysteine concentrations (age effect, p <0.001) than younger adults. Cysteine was not responsive to supplement and meal ingestion (**Figure 4.2, Table 9.6***).*

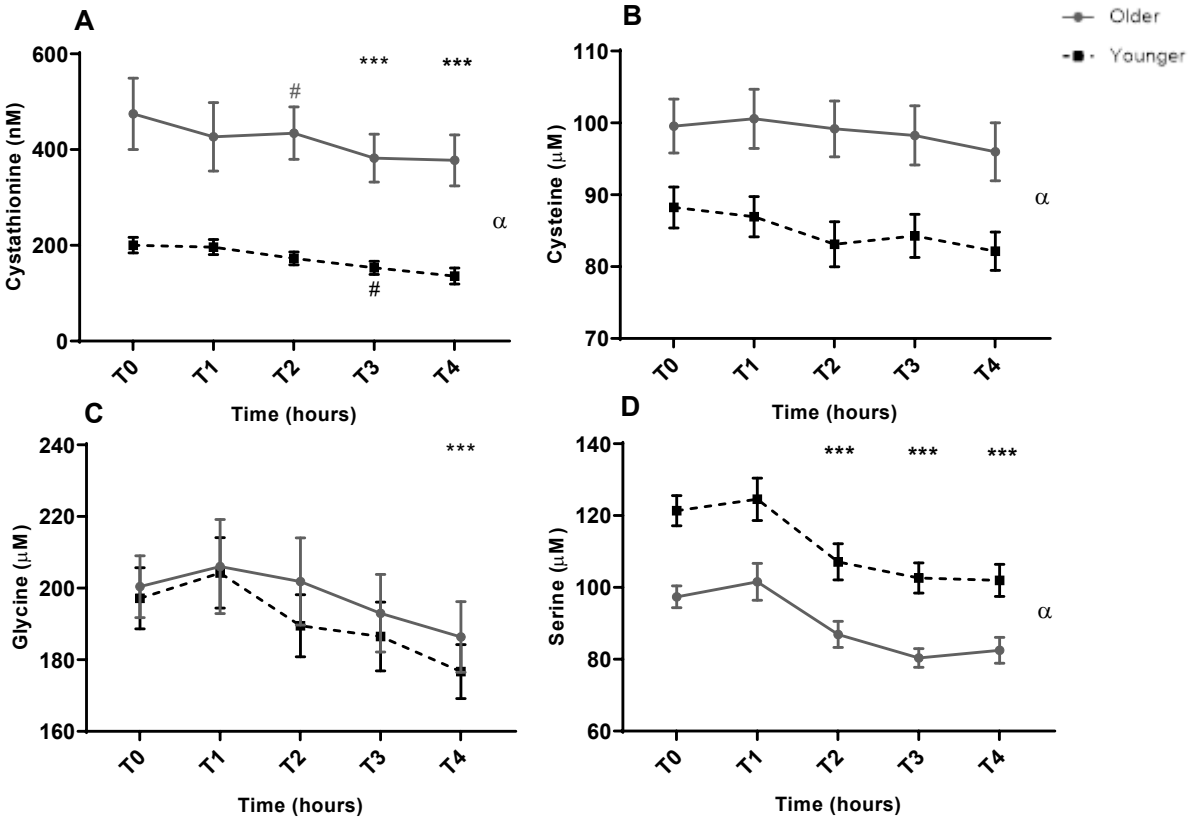


Figure 4.2: Postprandial response of cystathionine, cysteine, glycine and serine.
*Mean ± SEM of (A) Cystathionine, (B) Cysteine, (C) Glycine, and (D) Serine concentrations at fasting (T0), and hourly until four hours following supplement and meal ingestion (T1 – T4). Time difference from baseline for both age groups at the corresponding time point are indicated by * p <0.05, ** p <0.01, *** p <0.001. α indicates a difference between age groups across postprandial time points (p <0.05). # Indicates a time x age interaction for cystathionine, where older adults showed a faster decline of cystathionine concentrations at T2, compared to T3 for younger adults. No time x age interactions were found for cysteine, glycine, or serine.*

4.2.3.4.2 Betaine, choline, and dimethylglycine

No differences in the postprandial responsive of betaine, choline, DMG or betaine/choline were found according to age. One hour after supplement and meal ingestion, plasma concentrations of betaine and the ratio of betaine/choline were higher and the ratio of DMG/betaine was lower (post-hoc, p <0.001), which was sustained 4 hours after ingestion. The ratio DMG/betaine continued to decline from 1 hour to 2 hours after ingestion (post-hoc*, p*=0.008), but not in older adults, although no difference in status between age was found during the postprandial time points. Choline concentrations increased after one hour only (post-hoc, p=0.038), and DMG was not postprandially responsive. Across all time points, older adults had higher concentrations of choline (age effect, p=0.001) and lower betaine/choline status (age effect, p=0.004) (**Figure 4.3** Appendix 2, Table 9.6*).*

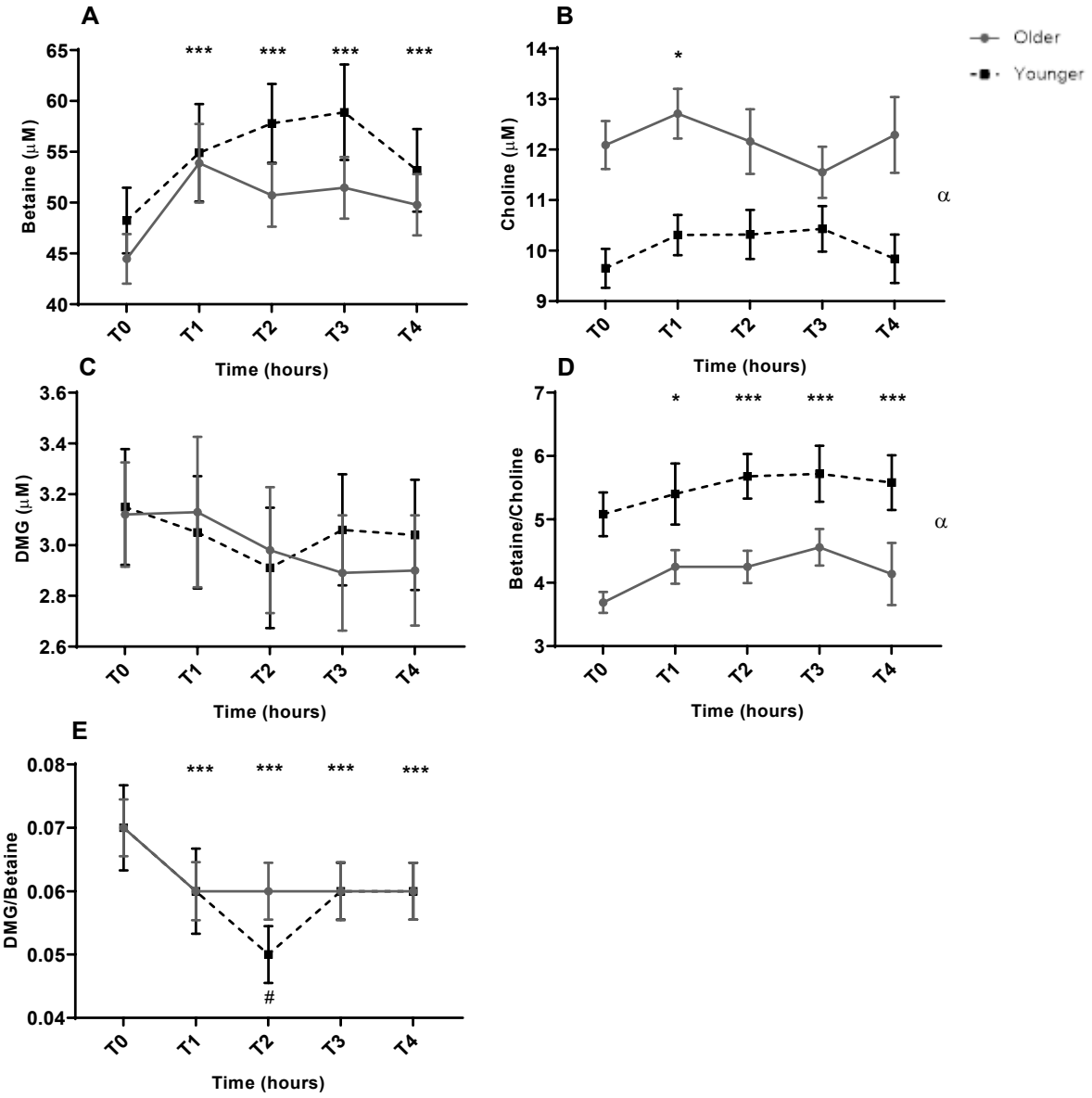


Figure 4.3: Postprandial response of betaine, choline, and dimethylglycine.
*Mean ± SEM of (A) Betaine, (B) Choline, (C) Dimethylglycine, (D) Betaine/Choline, and (E) Dimethylglycine/Betaine concentrations at fasting (T0), and hourly until four hours following supplement and meal ingestion (T1 – T4). Time difference from baseline for both age groups at the corresponding time point are indicated * p <0.05, ** p <0.01, *** p <0.001. α indicates a difference between age groups across postprandial time points (p <0.05). # indicates a time x age interaction for Dimethylglycine/betaine, where a decline was found between T1 and T2 for younger, but not older adults. No time x age interactions were found betaine, choline, dimethylglycine, or betaine/choline according to post-hoc analyses.*

4.2.5 DISCUSSION

Inadequate B vitamin status is implicated in diseases predominant in ageing populations, leading to elevated Hcy and dysregulated 1C metabolism. MVM supplements are frequently used amongst older adults, thought to help combat their increased risk of inadequate intake. Older adults have an increased risk for impaired B vitamin absorption[52,75,470], which alongside altered postprandial dynamics[217,469] led to the hypothesis that the acute response of 1C metabolites to an MVM supplement containing B vitamins with a standard meal might be impaired in older adults. However, the acute 1C metabolite response was not markedly different according to age in the current analysis. Beyond older adults showing a faster decline in plasma cystathionine concentrations, no differences in the response between young and older adults were found even though baseline differences in B vitamin intake, status, and 1C metabolite profiles were apparent. Thus, it appears that postprandial 1C cycling is maintained with age.

The Hcy responses following ingestion of an MVM supplement and standard meal were similar between younger and older adults, showing a sustained decline during the four-hour postprandial period. Older adults are considered to be at greater risk of impaired B vitamin absorption[52,75,470], yet this remains incompletely understood[57]. While the primary outcomes reported from the same study here would suggest impaired responsiveness of active B_6 vitamers, this did not extend to impaired 1C metabolite responses in the current study, but would perhaps be expected to be more apparent with different dietary challenges like methionine loading[144]. Further, the translation of proposed age-related differences in absorption to the inter-connected use of B vitamins in functional pathways like 1C metabolism remains unclear.

A rise in Hcy following post-methionine load challenges is well supported[172,173,186], though the response to food-based challenges is less clear[184,185], with a particular lack of data describing differences with advancing age. In younger adults, Guttormsen *et al*[185] reported a transient, non-significant rise in Hcy followed by a decline in free Hcy four hours after a mixed breakfast meal (slower than that observed after one hour in the current study), and a more rapid increase in Hcy following a protein-rich evening meal (51g). Hcy responses to a balanced meal have been investigated in older adults (*n*=24) by Candito *et al*[212], where healthy controls maintained Hcy concentrations within fasting ranges compared to depressed controls who showed elevated postprandial Hcy. However, details of meal components and nutrient composition were not provided in the study by Candito *et al*[212], and cannot be compared to the supplement and simple meal used for the analysis reported here. Similarly, the findings obtained here could not be compared to classical post-methionine load studies where a rise in Hcy is expected, and exacerbated in those with B vitamin deficiencies, given considerable differences in the dietary perturbation. The current study is perhaps most similar in design to a study investigating the acute response to a beverage fortified with B vitamins, where a decline in Hcy concentration was also found during a six-hour postprandial period, although that study population was only adolescents (13-19y)[475]. To my knowledge, this is the first study to investigate (i) the response of circulating Hcy to MVM supplements, and (ii) the potentially unique postprandial response in older compared to younger adults. While older adults appeared to maintain postprandial Hcy regulation, differences between age groups might be expected to emerge with a postprandial period longer than four hours, as previous studies have shown that Hcy returns to basal levels by around eight hours after feeding[184].

It was hypothesised that extending investigation to 1C metabolites reflecting Hcy remethylation and transsulfuration might provide further insight into the postprandial response of 1C metabolism. Baseline differences in 1C metabolites were evident in older adults, including higher cysteine, cystathionine, and choline but lower methionine and serine concentrations. All metabolites except cysteine and DMG were responsive to supplementation during the four-hour postprandial period, but for the most part no differences in response between young and old adults were apparent, and baseline differences in metabolite concentrations were maintained during the postprandial period.

There is limited available evidence to compare these age effects, and it is difficult to determine whether these findings are expected. Fasting amino acid profiles are better characterised in comparisons of healthy older adults or those who are frail or with comorbidities[476,477]. However, the results here are similar to those previously reported, particularly with respect to lower methionine[217,478–481] and serine[479–481], but higher cysteine[478,479,481] concentrations. Postprandial comparisons between healthy young and older adults are scarce for the metabolites and amino acids reported here. Notably, several amino acids showed decreased postprandial concentrations, which differs from those previously reported following some mixed meals[482,483], but not others[484]. This may reflect differential impacts on 1C enzymatic reactions dependent on increased acute availability of B vitamins relative to the macronutrient effects of the meal itself. Our laboratory has previously shown that age differences in postprandial choline concentrations were similarly sustained during the postprandial period, whereas other 1C metabolites demonstrating age effects were dependent on the postprandial time or meal type[483], highlighting that the age effects in the current study may not be generalisable to other postprandial challenges. In that same cohort, age differences in fasting amino acid concentrations were not maintained following the consumption of a mixed meal[217], though comparison to the current study is complicated by the use of a more complex meal (higher fat and protein) and incomplete data for individual amino acids. No effect of sex was observed on the postprandial response despite males having higher DMG and methionine, but lower glycine concentrations, and older males having higher choline and cysteine concentrations at baseline. However, noting that age was the primary comparison, the small sample size in the current study may have been insufficient to detect sex differences.

The postprandial decline in cystathionine was apparent sooner in older adults, indicating restricted flux of methyl groups through the transsulfuration pathway was restricted in the postprandial period. Evidence regarding the postprandial cystathionine response to meals or supplements is scarce. This observation contrasts the moderate postprandial rise in cystathionine concentrations after a simple breakfast meal and a greater rise after a methionine-rich meal in healthy younger adults[482]. The acute decline in cystathionine seen here more closely aligns to the reduction of cystathionine in healthy older adults reported after supplementation with B-complex vitamins for a median of 23 days[485]. In the same study, the authors indicated that lower cystathionine predicts lower unmetabolised folic acid in the circulation with enhanced 1C cycling following supplementation, which was attributed to improved vitamin B_6 status[485]. Therefore, the earlier response of cystathionine in the current population of older adults might be perceived as enhanced 1C cycling, perhaps to prioritise the conservation of methyl groups in response to perturbations in nutrient supply. However, the clinical or physiological relevance of this response remains to be determined in longer-term studies. The primary outcomes reported from the VIOME study showed that older adults had decreased plasma responsiveness of active B_6 vitamer forms to supplementation, yet the findings reported here would rather indicate no functional defect, at least with respect to 1C metabolism[76].

It is important to appreciate that numerous physiological changes characterise the postprandial period, and both exogenous and endogenous factors cooperate to facilitate the digestion, absorption, and utilisation of nutrients. Although the main interest here was the response of 1C metabolites to the increased availability of B vitamins in the MVM supplement, this is complicated by hormone fluxes, and endogenous production or release of amino acids, glucose, and fatty acids. Indeed, Obeid *et al*[182] found that the flow of methyl groups was restricted following an oral glucose tolerance test, with a rise in plasma choline and a decline of DMG concentrations, which have also been demonstrated *in vitro* after exposure to high glucose[486] – findings not dissimilar to the current study providing a similar carbohydrate load in the standard meal. In the same study, betaine and choline utilisation as methyl donors was increased with postprandial lipemia following an oral fat tolerance test, linked to phosphatidylcholine requirements to solubilise chylomicrons[182]. This observation may impact habitual postprandial 1C regulation in older adults given the frequent occurrence of postprandial lipemia with advancing age[487]. However, this common postprandial perturbation cannot be explored in the current study, as the fat content of the meal challenge was insufficient to demonstrate greater postprandial lipemia in older adults, despite their higher triglyceride concentrations at baseline (**Table 4.1,** Appendix 2, Table 9.6). Without employing stable isotope tracer protocols[467,468], the interdependent roles of most 1C compounds conveys difficulty in interpreting how acute shifts in circulating concentrations might reflect involvement in 1C metabolism and contribute to long-term status. While the full extent of postprandial impacts on acute regulation of 1C metabolism intermediaries remains unclear, healthy older adults appear to maintain 'metabolic flexibility' of this pathway, which is proposed as an essential descriptor of health status[214,215].

This study aimed to address the scarcity of literature describing changes in how nutrients are utilised in functional pathways like 1C metabolism which occur with age. A strength of the current study lies in the analytical approach used here, which allows for a more comprehensive understanding of the 1C pathway response compared to investigating Hcy alone. Further, the postprandial analysis reflects a more 'real-world' response to nutrient intake. This analysis is a particularly useful approach when characterising subtle age-related changes, as the ability to adapt to a nutritional challenge test offers a more sensitive way to detect changes in healthy subjects[213]. It should be noted, however, that fluxes in plasma concentrations observed in the current study do not necessarily reflect intracellular concentrations or activity. Plasma Hcy appears to correlate well with intracellular concentrations from PBMCs[208]. However, folic acid supplementation has been shown to reduce plasma but not intracellular Hcy, with a suggested loss of regulation between intracellular 1C metabolites[208]. Thus, extending the postprandial investigation to intracellular concentrations would likely reveal further insight into the 1C response to nutrient fluxes in older adults.

While it is important to characterise changes that accompany a healthy ageing process, findings from this small, healthy population might not be generalisable to those with inadequate B vitamin status, impaired metabolic health, gastrointestinal disorders, or other comorbidities[212]. These participants largely had adequate B vitamin status at baseline, which may not reflect the wider older population. Conceivably, the postprandial response of 1C metabolites would differ according to basal B vitamin status given the impact of either deficiency or excess on the upregulation or downregulation of B vitamin absorption, respectively[488]. However, the postprandial response was not affected by the higher folate status in older adults in the current study, and more apparent alterations in the postprandial response are perhaps expected in those with inadequate B vitamin status[467]. Similarly, subjects with higher Hcy concentrations show a more pronounced response to longer-term nutritional intervention[203], yet no difference was seen in the acute response in the

current study, despite older adults having higher baseline serum Hcy concentrations. The study population's health status will also inevitably influence findings. For example, insulin resistance impacts genes involved in 1C metabolism and Hcy regulation[354], yet no differences in baseline insulin concentrations or HOMA-IR were found between young and old adults in the current study despite evidence that insulin resistance increases with advancing age[489,490]. Similarly, postprandial Hcy regulation following a mixed meal was impaired in older adults with clinical depression but maintained in healthy older adults[212]. While the authors speculated that this in part may be due to differences in baseline B vitamin status, it is unclear how comorbidities such as depression in isolation would affect postprandial micronutrient metabolism and 1C regulation.

Although the exclusion criteria here were restrictive to define a 'healthy' older population, it should be noted that some participants were taking medication (e.g. beta-blockers, citalopram), non-vitamin or mineral supplements (glucosamine sulphate) or living with comorbidities (e.g. osteoporosis). Ageing is an inherently heterogeneous process, creating challenges in defining a generalisable study population of older adults[82], and evaluating effects of age *per se* without the confounding influences of functional, metabolic, and health status[63]. This study sought to first address changes to the postprandial 1C response which might be related to the ageing process. Future studies will need to address the same response in different ageing populations, such as community-dwelling compared to aged care residents, in those with a high prevalence of nutritional inadequacies, or those who regularly consume supplements, to better understand implications for ageing.

4.2.6 CONCLUSION

To conclude, the hypothesis that older adults would have an impaired postprandial response of 1C metabolites to an MVM supplement with a meal did not hold true. Rather, older adults showed a similar postprandial decline in serum Hcy concentrations to younger adults, which was supported by analysing a wider profile of 1C metabolites which provide insight into Hcy regulation. These findings suggest that healthy older people maintain metabolic flexibility of the 1C pathway, considered an essential marker of health status. Although not further explored here, other postprandial perturbations common in older adults, such as postprandial lipemia apparent with other types of meal challenges, likely hold important consequences for interpreting the findings reported here. The population of healthy older adults in this study provides an opportunity to examine changes related to the ageing process while minimising confounder influences such as gastrointestinal function and comorbidities. These findings should be investigated in more diverse populations to provide a broader understanding of the implications for health and nutritional status in ageing societies

COHORT ANALYSIS

REACH study

CHAPTER 5: ONE-CARBON METABOLITES, COGNITION AND METABOLIC HEALTH

5.1 PREFACE

This is the first chapter to align with the second objective of this thesis, and explores the relationship between B vitamins, 1C metabolites, and markers of cardiometabolic and cognitive health in older New Zealand adults. This analysis is central to the B vitamin-health relationship, which puts age-related shifts to B vitamin intake and status into perspective, and also informs the interpretation of 1C metabolite responses in the following chapters.

This Chapter was completed in collaboration with Associate Professor Kathryn Beck from Massey University, and reports a secondary analysis of the Researching Eating, Activity, and Cognitive Health (REACH) study. The primary outcome of the REACH study was to determine the association between dietary patterns and cognitive performance and metabolic health in older New Zealand adults. The secondary outcomes reported here include B vitamin intake, serum folate and vitamin B_{12}, plasma 1C metabolites, and cognitive and cardiometabolic markers. My role in the REACH study was the analysis of four-day food records, and assisting in study procedures and data collection (collecting anthropometric data, administering questionnaires and cognitive testing). For this Chapter, I completed the mass spectrometry analysis for 1C metabolites, autoanalyser analysis of B vitamins, and writing the manuscript as the first-author.

This chapter contains a modified version of the manuscript “B vitamins, one-carbon metabolites, and cognitive function: findings from a cross-sectional cohort in New Zealand”, co-authored by Nicola A. Gillies, Amber M. Milan, David Cameron-Smith, Cathryn A. Conlon, Pamela R. von Hurst, Crystal F. Haskell-Ramsay, Beatrix Jones, Karen D. Mumme, Nicole C. Roy, Jane Coad, Clare R. Wall, and Kathryn L. Beck. This manuscript is currently under preparation for submission to the Journal of Nutrition, which has a 2019/20 impact factor of 4.281, including additional data on genetic polymorphisms relevant to B vitamin and 1C metabolite status.

5.2 MANUSCRIPT:

B vitamins, one-carbon metabolites, metabolic health, and cognitive function: Findings from a cross-sectional cohort in New Zealand

5.2.1 ABSTRACT

Background: B vitamin inadequacies and dysregulated 1C metabolite status are associated with cognitive decline in older adults. 1C metabolites also appear to be connected to metabolic dysregulation, which also accelerates cognitive aging. Research has predominantly focused on folate, vitamin B12 and Hcy, which provides a narrow view of the complex association between 1C nutrients and inter-connected health outcomes in older adults.

Objective: To examine the interactive effects of B vitamins, 1C metabolites, metabolic health and cognitive function in healthy older adults.

Methods: 313 healthy older men and women (65-74 years, 65% female) were included in this cross-sectional analysis. Cognitive performance was assessed by the Computerised Mental Performance Assessment System and Montreal Cognitive Assessment. Fasting plasma 1C metabolites were quantified by ultra high performance liquid chromatography with tandem mass spectrometry, and four-day food records were analyzed for nutrient intake. Apolipoprotein E genotype was measured by polymerase chain reaction amplification. Interaction terms were fit in adjusted linear regression models between continuous (metabolites) and categorical (quartiles of B vitamins or metabolites, ε4 allele) variables.

Results: Higher glycine concentrations were associated with better global cognitive performance ($b = 1.340$, $p=0.017$), episodic memory ($b=1.396$, $p=0.016$) and location learning ($b=1.394$, $p=0.027$), although this relationship was not apparent in participants with higher choline concentrations or the apolipoprotein ε4 genotype (interaction, $p<0.05$). Conversely, the apolipoprotein ε4 genotype and higher vitamin B12 intake both attenuated the inverse association between Hcy and cognition across several domains of cognitive performance (interaction, $p<0.05$).

Conclusions: The relationship between cognitive performance and 1C metabolites, notably glycine and Hcy, is modified by vitamin B12 intake, apolipoprotein E genotype, and status of inter-connected metabolites. These findings point towards the need for a personalized approach to dietary interventions which protect against age-related cognitive decline.

5.2.2 INTRODUCTION

Dementia is projected to impact 131 million people world-wide by 2050 [236]. While global ageing drives the 'dementia epidemic'[237], this creates profound challenges for ageing populations due to the devastating nature of dementia for individuals and societies alike [236]. Gradual cognitive decline is considered part of the normal ageing process, which can progress to MCI when decline exceeds that expected for an individual's age and education level[242]. Notably, 50% of individuals with MCI are expected to develop dementia within five years of diagnosis[242]. As such, developing strategies to prevent or delay the onset of MCI has become a major public health priority.

Ranging from broader patterns to individual nutrients, one's diet contributes to the maintenance or deterioration of cognitive health[491]. B vitamins, both intake and status, have a long-standing relationship with cognitive health, attributed to their pivotal role in regulating 1C metabolism[253]. Here, B vitamins are required for several processes related to cognition, including DNA synthesis and repair, methylation reactions, and Hcy regulation. Over two decades ago now, seminal reports showed that elevated circulating Hcy and low folate or vitamin B_{12} status were associated with a diagnosis of dementia according to both clinical and histopathological criteria[250,251]. Following this, a wealth of epidemiological data ensued to support the association between cognitive function and B vitamin or homocysteine status in both cross-sectional and prospective settings[255,256,265,274,275,290,492–497]. Paradoxically, very high folate intake or status has more recently been associated with poor cognitive function or accelerated cognitive decline in older adults, particularly in those with suboptimal vitamin B_{12} intake[269,498,499]. Indeed, our understanding of what appears to be a long-standing relationship between B vitamins and cognition in the literature is by no means complete though, attributed in part to the predominant research focus of folate, vitamin B_{12}, and Hcy[276].

Hcy is at a critical branchpoint of 1C metabolism, where folate, methionine, choline oxidation and transsulfuration pathways intersect. Hcy is therefore considered an overarching marker of 1C metabolism, and has been the focus of research pertaining to B vitamins and 1C metabolism. While evidence relating to transsulfuration metabolites (cysteine, cystathionine, serine) is limited, the literature would suggest involvement of choline oxidation metabolites (choline, betaine, DMG) in cognitive function. In particular, higher dietary choline intake[500,501] and status[502] appear to be associated with improved cognitive performance and protection against cognitive decline in older adults. Betaine and DMG, involved in Hcy remethylation, have also been associated with memory performance in older adults[324], although evidence is too scarce to draw conclusions at this stage. Interestingly, rodent studies also suggest that the interconnected regulation of folate and choline metabolism moderates the trajectory of cognitive ageing[334].

While Hcy is an established clinical biomarker, relying on Hcy as a single marker is a simple interpretation of a complex pathway which has possibly impeded a more discerning understanding of the association between 1C nutrients (including B vitamins) and cognitive health outcomes. Adding further complexity is that 1C metabolites show divergent associations with cardiometabolic health[357,358,368,503,504]. Metabolic dysregulation accelerates cognitive ageing[229–232], and as such the intersection of 1C metabolism with cognitive and cardiometabolic health should be an important consideration in both disentangling the B vitamin-cognition relationship and informing future intervention strategies which promote healthy ageing.

The objective of this study was first to analyse the association of B vitamins and 1C metabolites with cognition and cardiometabolic health, and second to examine the interactive effects of B vitamins, the ApoE ε4 genotype and 1C metabolites on cognition in healthy older adults.

5.2.3 METHODS

5.2.3.1 Participants and study design

The Researching Eating, Activity, and Cognitive Health (REACH) study is a single-centre, cross-sectional study conducted between April 2018 - February 2019. Details of study design and data collection have been published elsewhere[505]. Briefly, a sample of 371 men and women aged 65-74 years living independently in the community were recruited to participate from the wider Auckland region. Participants were excluded if they reported a diagnosis of dementia or other conditions which may impair cognitive function (stroke, traumatic head or brain injury, or a neurological or psychiatric condition), if they were taking medication which may influence cognitive function, if they were colour blind (colour recognition was required for cognitive testing), or not proficient in English. Participants were further excluded if they experienced an event in the last two years which substantially impacted dietary intake or cognitive function (e.g. death or illness of a family member), or if another person in the household was enrolled in the study.

Participants attended one study visit at the Human Nutrition Research Unit, Massey University, Auckland. Prior to data collection, informed consent was completed with participants at the research facility. During their study visit, participants completed questionnaires, and anthropometric data was gathered before fasting blood samples were collected. Participants then received a standardised breakfast before completing two cognitive assessments to minimise any effects food may have on cognitive function. This study was approved by the Massy University Human Ethics Committee: Southern A, Application 17/69.

5.2.3.2 Cognitive testing

The Montreal Cognitive Assessment (MoCA) test was administered by trained examiners to assess global cognitive performance, with a score of <26 defined as MCI[506]. The Computerised Mental Performance Assessment System (COMPASS; Northumbria University, Newcastle upon Tyne, UK) was used to examine global cognitive performance and multiple cognitive domains. The COMPASS cognitive battery provides measures of participants' attention and vigilance, executive function, episodic memory, working memory, and location learning using the following tests; Simple and choice reaction times, digit vigilance task, Stroop test, immediate and delayed word recall, delayed word and picture recognition, Corsi blocks, and computerised location learning and recall (**Table 5.1**).

Cognitive testing with COMPASS took approximately 1 hour, with the first 15 minutes comprised of a training exercise, and a 5 minute break before actual assessment began. Testing was completed in a controlled environment, and participants were instructed to avoid undue stress, alcohol, recreational drugs, and non-routine physical activity prior to their study visit.

Table 5.1: Summary of tests used and cognitive domains measured from the Computerised Mental Performance Assessment System (COMPASS) battery of cognitive assessments.

Cognitive domain	Cognitive domain description	Tests
Attention and vigilance	**Attention**: Ability to concentrate on selected environmental aspects while ignoring others	Simple reaction time
		Choice reaction time
	Vigilance: Ability to maintain attention and alertness over time	Digit vigilance task
Episodic memory	Ability to retain memories that can be consciously recorded	Immediate and delayed word recall
		Delayed word recognition
		Delayed picture recognition
Executive function	Ability to co-ordinate cognitive responses	Stroop test
Location learning	Ability to co-ordinate visuo-spatial memory	Computerised location learning
		Computerised location recall
Working memory	Ability to retain information while carrying out more complex cognitive tasks	Corsi blocks
Global performance	Average performance across all cognitive domains	Average performance across all tests

5.2.3.3 Dietary intake and analysis

Dietary intake was determined from four-day estimated food records, which spanned four consecutive days with at least one weekend day included. During their study visit, participants were informed on the need to record all food and beverages consumed accurately, and the type of detail required (type of foods, brands, and cooking methods) by an instructional video and opportunity to confirm any questions with research staff.

Food records were completed within one-month of participants' study visit, and sent to the REACH study coordinator as electronic or hard copies. Food records were then checked for completion prior to analysis, and participants were contacted if further detail was required. Dietary analysis was completed by four trained Nutritionists and Dietitians using Foodworks (Version 10; Xyris Software, Australia), which utilises Food Composition Databases from New Zealand (FOODfiles 2016, Version 01) and Australia (AusFoods 2019, AusBrands 2019). Consistency of entries was achieved by using a register of common food items during data entry, which was checked once all food records were entered. Finally, one Dietitian checked every food record entered for accuracy and consistency. Following final data inspection, the plausibility of reported energy intake was compared against cut-off values specific for older adults[507]. For this analysis, variables of interest which were extracted from Foodworks included folate, riboflavin, vitamins B_6 and B_{12}, energy, and caffeine. Food records were excluded for further analysis if daily energy intake was <2100kJ (500kcal) or >14700 kJ (3500kcal) for women, and <3350 kJ (800kcal) or >16,800 kJ (4000kcal) for men.

5.2.3.4 Biochemical analysis

Following an overnight fast of at least nine hours, a qualified phlebotomist collected blood samples into serum and EDTA-coated vacutainers. Plasma samples were placed on ice, spun within two hours of collection, and centrifuged (Heraeus Labofuge 400R) for 15 minutes at 3500rpm at 4°C. For serum samples, whole blood was allowed to clot for 30 minutes, placed on ice and centrifuged as per above. Samples for analysis of blood glucose and the lipid profile were measured on the study day using point-of-care systems. All other samples were separated into aliquots and stored in Eppendorf tubes at -80°C until required for analysis, and thawed immediately before analysis.

5.2.3.4.1 One-carbon metabolites and B vitamins

Plasma concentrations of 1C metabolites (betaine, choline, cystathionine, cysteine, DMG, Hcy, methionine, SAH and SAM) was quantified by UHPLC-MS/MS. The methods for profiling 1C metabolites have been reported in detail elsewhere [379]. Briefly, an automated robotic liquid handling system (Eppendorf epMotion® 5075vt, Hamburg, Germany) was used to prepare plasma samples. First, 300µl of 1% formic acid in methanol was pipetted into a 96-well IMPACT® protein precipitation plate (Phenomenex, Torrance, California, USA). Next, all standards (100µl), quality controls (100µl) and samples (100µl) were spiked with 20µl of internal standard solution, agitated for 5 minutes (800rpm), then filtered into a 96-well collection plate (Phenomenex, Torrance, California, USA) by applying a vacuum (450mbar). Tris (2-carboxyethyl) phosphine (100µl) was then dispensed into each well, which reduces disulfide bonds in cystine and homocystine to allow for the separate quantification of cysteine and Hcy, respectively.

Three sets of quality control samples were included to assess recovery of standards and reproducibility of samples. Metabolites were considered acceptable if standard recoveries were between 80-120%, and coefficients of variance were below 20%. Cystathionine and SAH were excluded from further analysis because they did not satisfy CV requirements, as was Hcy from one of five batches (22%; n=77). To account for missing values where a peak was not detected, half of the minimum value was entered according to each plate [380]. A missing value was not calculated for the Hcy samples that were excluded from the analysis.

A Cobas e411 autoanalyser (Roche, Mannheim, Germany) was used to measure serum vitamin B_{12} by electrochemiluminescence immunoassay, and serum folate by a binding assay.

5.2.3.4.2 Cardiometabolic Health

Fasting blood glucose was measured by a HemoCue® Glucose 201 RT System (Radiometer Pacific Pty. Ltd., Victoria, Australia). A Cobas b 101 system (Roche, Mannheim, Germany) was used to measure the lipid profile (total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides). Diagnosis of metabolic syndrome was defined according to the criteria outlined by the American Heart Association/National Health, Lung and Blood Institute[508]

5.2.3.4.3 Apolipoprotein E genotype

Presence of the ApoE ε4 allele was measured at an accredited laboratory (Grafton Clinical Genomics, Auckland, New Zealand). Briefly, an automated system (QiaSymphony) extracted DNA using magnetic rods to shift nucleic acids through purification/wash steps and a final elute phase. Based on a sample input of 200uL of whole blood and an elution volume of 100uL, the DSP DNA Mini Kit (for DNA extraction) ensured the yield and concentration was sufficient for sample processing on the Genotyping platform. ApoE ε2, ε3, ε4 (SNP ID rs7412 and rs429358) alleles were detected using the Agena® MassARRAY® system[509].

5.2.3.5 Analysis of covariates

5.2.3.5.1 Sociodemographic, medical history, and physical activity

Sociodemographic and health information was collected through written questionnaires, which were completed in person and checked for completeness and sensibility. Variables included in this secondary analysis were age, sex, ethnicity, socioeconomic status, education (highest level achieved), physical activity, history of anxiety or depression, medication (polypharmacy defined as ≥5 medications used[510]), supplement use (B vitamin or MVM supplements), and frequency of alcohol consumption.

Socioeconomic status was defined by the New Zealand Index of Multiple Deprivation according to participants' residential address[511]. Physical activity was assessed by the International Physical Activity Questionnaire – short form[512], and categorised according to tertiles of physical activity. Alcohol intake was categorised according to self-reported frequency of intake (daily, weekly, monthly, never).

5.2.3.5.2 Anthropometry and blood pressure

Weight was measured to the nearest 0.01 kg (Tanita Electronic Scales, Amsterdam, Netherlands), and height was measured to the nearest 0.1 cm using a portable stadiometer. BMI was calculated as weight relative to height (kg/m^2). Waist and hip circumference were measured with a flexible steel tape (Lufkin W600 PM) following standard methods outlined by the International Society for the Advancement of Kinanthropometry (ISAK)[513].

Blood pressure was measured after participants were seated and resting quietly for five minutes. The average systolic and diastolic blood pressure was calculated from two measurements taken by a digital automatic blood pressure monitor (Omron HEM-907, Kyoto, Japan), with a one-minute rest period between measures.

5.2.3.6 Statistical Analysis

Sample size estimates for the REACH study were calculated for the primary outcome of a linear association between cognitive performance and dietary patterns, leading to a sample estimate of 350 participants required to see a medium size effect with 80% power[505]. This sample size is also likely sufficient to identify significant differences in secondary outcomes of associations between cognitive performance and B vitamins or 1C metabolites, with moderate (Pearson correlation 0.15) to small (Pearson correlation 0.3) size effects expected.

Prior to statistical analysis, the distribution of variables was graphically assessed and logarithmically transformed to achieve approximately normal distributions for DMG, glycine, Hcy, serum vitamin B_{12} concentrations and vitamin B_{12} intake. All statistical analyses were performed using R 3.6.3 statistical software[474]. Unless otherwise specified, Alpha was set at $P < 0.05$, and data presented as mean and SD.

Baseline participant characteristics were compared between males and females either by *t*-test or chi-square test for continuous and categorical variables, respectively. Simple associations were tested between potential covariates for multivariate regression modelling based on the current literature for those that influence 1C metabolite status (age, sex, alcohol intake, medication use, supplement use, caffeine intake, physical activity, and energy intake), or cognition (education, apolipoprotein E genotype, socioeconomic status, history of depression or anxiety, family history of cognitive impairment, and diagnosis of the metabolic syndrome). Covariates associated ($p < 0.150$) with either 1C metabolite status or cognitive performance were included in Model 2 in regression models described below.

Multivariate linear regression modelling was performed to investigate associations between B vitamin intake and status with one-carbon metabolite concentrations. Model 1 was adjusted for age, sex, and batch effects. Model 2 was further adjusted for energy intake, physical activity, frequency of alcohol intake, polypharmacy and supplement use. Model 3 was then further adjusted for intake or status of other B vitamins to test independent associations. The association between 1C metabolites and cardiometabolic parameters was also explored through multivariate linear regression models, using Models 1 and 2 as described above.

The relationship between B vitamin intake, status, and 1C metabolites with MCI according to MoCA scores and cognitive performance in COMPASS testing was assessed by multivariate logistic or linear regression, respectively. Model 1 was adjusted for age, sex, batch effects, and level of education. Model 2 was further adjusted for energy intake, physical activity, history of anxiety/depression and supplement use. The impact of 1) low (quartile 1) or high (quartile 4) B vitamin intake, B vitamin status, and other 1C metabolites, or 2) ApoE genotype on the relationship between 1C metabolites and cognitive performance in COMPASS testing was assessed in separate linear regression models which were fully adjusted for covariates described in Model 2 above. A sensitivity analysis was performed by excluding participants who used B vitamin or multivitamin supplements, and repeating the linear regression models to test the association between B vitamin intake, status, and 1C metabolites with cognitive performance.

Due to the number of tests performed across linear regression models, particularly where several interactions were explored, some significant results are expected by chance alone. Relationships which demonstrate consistency (e.g. vitamins or metabolites related to at least two cognitive domains, or cognitive domains which are impacted by at least two vitamins or metabolites) will be discussed.

5.2.4 RESULTS

5.2.4.1 Participant Characteristics

Of the 371 participants which completed the REACH study, 313 participants (69.8 ± 2.6 years, 65% female) of the REACH cohort were included in this secondary analysis (**Table 5.2**). Participants were excluded due to previous stroke or head injury which was not removed at screening (*n*=7), or missing data for cognitive performance (*n*=2), food records (*n*=38), or 1C metabolite and B vitamin status (*n*=11). Differences in participant characteristics between included and excluded participants are highlighted in Appendix 3, Table 9.7. Briefly, excluded participants were younger (69.0 ± 2.5 v 69.8 ± 2.6 years), and showed significant differences in education achieved.

The majority of REACH participants met EAR for intakes of riboflavin (males, 96%; females, 97%), vitamins B_6 (males, 98%; females, 96%) and B_{12} (males, 95%; females, 92%). However there was a relatively high prevalence of folate inadequacy with only 78% of males and 67% of females (p=0.006) meeting the EAR for folate of 320 μg/day. On average, males had higher intakes of folate (p=0.012), riboflavin (p <0.001), and vitamin B_6 (p <0.001), but similar vitamin B_{12} intake to females (p=0.089). Females had higher serum vitamin B_{12} concentrations than males (386 ± 159 v 352 ± 121 pmol/L, p=0.040). Markers of cardiometabolic health were largely similar between males and females, although females had higher concentrations of HDL- and LDL-cholesterol (p <0.001). Females had a greater prevalence of having a family history of cognitive impairment (p=0.028), although the personal history of anxiety or depression was similar between males and females. 26% of participants carried at least one apolipoprotein-ε4 allele, though this did not differ between males and females.

Table 5.2: REACH study population characteristics

Participant characteristics	Total population	Males	Females	*p*
n	313	111 (35)	202 (65)	
Demographics				
Age	69.8 (2.6)	70.3 (2.5)	69.5 (2.6)	0.019*
Highest education achieved, *n* (%)				0.011*
No qualification	5 (1.6)	1 (1.0)	4 (2.0)	
Secondary school	66 (21)	15 (14)	51 (25)	
Post-secondary school	127 (41)	42 (38)	85 (42)	
University	115 (37)	53 (48)	62 (31)	
Lifestyle				
Polypharmacy, *n* (%)	25 (8.0)	11 (9.9)	14 (6.9)	0.476
Supplement use, *n* (%)	53 (17)	18 (16)	35 (17)	0.926
Alcohol intake, *n* (%)				0.158
Daily	85 (31)	32 (32)	53 (30)	
Weekly	127 (46)	52 (52)	75 (43)	
Monthly	50 (18)	14 (14)	36 (21)	
Never	13 (4.7)	2 (2)	11 (6.3)	
B vitamin status				
Serum folate, nmol/L	27.9 (10.8)	26.4 (9.9)	28.8 (11.2)	0.059
Serum vitamin, B_{12} pmol/L	373 (147)	352 (121)	386 (159)	0.040*
Dietary intake				
Energy, kj/day	8095 (1918)	9378 (1956)	7463 (1510)	<0.001*
Folate, μg/day	429 (184)	468 (194)	412 (174)	0.012*
Riboflavin, mg/day	2.13 (0.79)	2.42 (0.87)	1.98 (0.70)	<0.001*
Vitamin B_6, mg/day	2.53 (0.89)	2.78 (1.00)	2.40 (0.79)	<0.001*
Vitamin B_{12}, μg/day	4.17 (3.60)	4.67 (3.54)	3.95 (3.73)	0.089
Dietary adequacy[1]				
Folate, *n* (%)	222 (71)	87 (78)	135 (67)	0.052
Riboflavin, *n* (%)	303 (97)	107 (96)	196 (97)	0.925
Vitamin B_6, *n* (%)	303 (97)	109 (98)	194 (96)	0.647
Vitamin B_{12}, *n* (%)	290 (93)	105 (95)	185 (92)	0.549
Cardiometabolic health				
BMI, kg/m^2	26.0 (4.29)	26.6 (4.5)	25.6 (4.5)	0.065
Glucose, mmol/L	4.40 (0.73)	4.50 (0.81)	4.35 (0.68)	0.093
Serum HDL-cholesterol, mmol/L	1.62 (0.40)	1.43 (0.35)	1.73 (0.40)	<0.001*
Serum LDL-cholesterol, mmol/L	2.97 (0.94)	2.63 (0.95)	3.16 (0.88)	<0.001*
Total/HDL-cholesterol	3.36 (1.11)	3.40 (1.18)	3.34 (1.07)	0.663
Serum triglycerides, mmol/L	1.25 (0.54)	1.26 (0.59)	1.23 (0.52)	0.605
Systolic blood pressure, mm/Hg	139 (17.9)	140 (14.5)	139 (19.6)	0.390
Diastolic blood pressure, mm/Hg	78.4 (10.4)	78.2 (11.1)	78.9 (9.10)	0.547
Metabolic syndrome, *n* (%)	34 (11)	10 (9.0)	24 (12)	0.554
Cognitive risk and status				
ApoE-ε4 genotype, *n* (%)	82 (26)	27(24)	55 (27)	0.649
History of anxiety/depression, *n* (%)	62 (20)	17 (15)	45 (22)	0.183
Family history, *n* (%)	94 (30)	24 (22)	70 (35)	0.019*
Prevalence of MCI, *n* (%)	93 (30)	34 (31)	59 (29)	0.842
Metabolites (plasma)				
Betaine	34.1 (9.59)	38.8 (10.0)	31.5 (8.3)	<0.001*
Choline	8.57 (2.19)	9.42 (2.21)	8.10 (2.04)	<0.001*
Cysteine	98.7 (14.9)	104 (14.2)	96.2 (14.6)	<0.001*
Dimethylglycine	2.14 (0.82)	2.39 (0.84)	2.01 (0.77)	<0.001*
Glycine	209 (57.3)	183 (41.5)	223 (60.0)	<0.001*
Homocysteine	2.26 (0.81)	2.57 (0.96)	2.10 (0.68)	<0.001*
Methionine	21.2 (3.33)	22.5 (3.17)	20.4 (3.18)	<0.001*
S-adenosylmethionine	138 (65.5)	149 (62.9)	132 (66.4)	<0.001*
Serine	79.6 (15.6)	75.2 (15.2)	82.0 (15.3)	<0.001*

Data presented as mean (SD) for continuous variables or count (%) for categorical data. Differences in participant characteristics according to sex are presented according to *t*-test and chi-squared tests for continuous and categorical data, respectively, with * indicating a significant difference (p <0.05). [1]Dietary adequacy is defined as the number (%) of participants meeting the estimated average requirement of folate, riboflavin, vitamins B_6 and B_{12} according to the Australia and New Zealand Nutrient Reference Values[87]. Dietary adequacy was not defined for the total population as recommendations are sex-specific. Abbreviations: MCI, mild cognitive impairment.

5.2.4.2 Association between B vitamins and one-carbon metabolites

The relationship between B vitamins and 1C metabolites is outlined in **Table 5.3.** Higher vitamin B_6 intake was associated with lower plasma Hcy concentrations (p=0.013), which remained significant after adjustment with covariates associated with 1C metabolite status (physical activity, supplementation, energy intake, polypharmacy, and frequency of alcohol intake) (p=0.008), and with further adjustment for folate, riboflavin, and vitamin B_{12} (p=0.008). Higher serum folate and vitamin B_{12} were also associated with lower Hcy concentrations, which remained significant in adjusted models (p <0.001). Plasma Hcy was not associated with intakes of folate, riboflavin, or vitamin B_{12} across regression models.

Serum folate and folate intake were inversely associated with serine concentrations (p <0.05), which remained significant with adjustment for covariates and was independent to other B vitamin intakes and serum vitamin B_{12} concentrations. Serum folate concentrations were also inversely associated with methionine concentrations (p <0.01), and positively with betaine concentrations (p <0.05), including after adjustment for covariates and vitamin B_{12} concentrations. Although betaine concentrations were positively associated with folate intake (p=0.044) and inversely with vitamin B_{12} intake (p=0.041) in simple age- and sex-adjusted models, this did not remain significant following further adjustment for covariates.

5.2.4.3 Association between B vitamins, one-carbon metabolites and cardiometabolic health

Overall, higher plasma betaine and glycine concentrations aligned with a favourable cardiometabolic profile, while higher choline, cysteine, and Hcy concentrations were related to greater cardiometabolic risk (**Table 5.4)**. Higher betaine and glycine, but lower choline, and Hcy concentrations were associated with a lower BMI and a favourable lipid profile (higher HDL-cholesterol, lower total/HDL-cholesterol and lower triglycerides). Diastolic blood pressure was positively associated with choline, but inversely with glycine concentrations. These associations in age- and sex-adjusted models largely remained significant with further adjustment physical activity, supplementation, energy intake, polypharmacy, and frequency of alcohol intake (p <0.05) (**Table 5.4)**. In comparison, few associations were found between B vitamin intake or status with cardiometabolic parameters. The most consistent association was found for serum folate and a favourable cardiometabolic profile. Serum folate was inversely associated with BMI, fasting blood glucose, total/HDL-cholesterol, and triglycerides, but positively with HDL-cholesterol (age- and sex-adjusted models, p <0.05), though this did not always remain significant when adjusted for confounding variables (**Table 5.4**).

Table 5.3: Associations between B vitamins and one-carbon metabolites in multivariate linear regression models.

	Model	Betaine		Choline		Cysteine		DMG[1]		Glycine[1]		Homocysteine[1]		Methionine		SAM		Serine	
		β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
Folate intake	1	0.006	0.044*	0.001	0.308	0.001	0.830	<0.001	0.234	<0.001	0.890	<0.001	0.883	<0.001	0.959	-0.008	0.486	-0.014	0.002*
	2	0.004	0.229	0.001	0.292	-0.001	0.864	<0.001	0.450	<0.001	0.651	<0.001	0.873	<0.001	0.798	-0.004	0.798	-0.016	0.001*
	3	0.008	0.053	0.001	0.374	0.007	0.249	<0.001	0.735	<0.001	0.261	<0.001	0.662	-0.001	0.497	0.012	0.527	-0.016	0.012*
Riboflavin intake	1	-0.480	0.465	-0.033	0.819	-0.306	0.745	-0.027	0.013*	-0.001	0.882	0.002	0.854	0.137	0.557	-5.088	0.080	-2.571	0.012*
	2	-0.721	0.360	-0.002	0.992	-1.558	0.177	-0.025	0.060	0.004	0.594	-0.001	0.969	0.209	0.473	-5.257	0.153	-2.167	0.086
	3	-0.143	0.175	-0.030	0.901	-2.215	0.151	-0.027	0.138	0.013	0.227	0.013	0.479	0.455	0.249	-7.513	0.132	-0.700	0.675
Vitamin B_6 intake	1	0.504	0.387	0.145	0.256	-1.239	0.136	-0.005	0.586	0.005	0.435	-0.027	0.013*	-0.183	0.374	-1.264	0.624	-1.199	0.187
	2	-0.003	0.996	0.202	0.200	-2.556	0.012*	-0.006	0.642	0.005	0.521	-0.034	0.008*	-0.221	0.395	0.067	0.984	-1.227	0.274
	3	0.074	0.920	0.230	0.167	-2.626	0.015*	0.001	0.968	0.006	0.445	-0.036	0.008*	-0.266	0.334	1.139	0.743	-0.535	0.646
Vitamin B_{12} intake[1]	1	-4.372	0.041*	-0.534	0.255	0.726	0.813	-0.045	0.206	-0.022	0.311	-0.021	0.565	0.117	0.877	-8.896	0.347	-2.558	0.444
	2	-3.751	0.101	-0.792	0.126	-1.439	0.669	-0.035	0.373	-0.009	0.708	-0.063	0.114	0.300	0.724	-6.248	0.560	3.051	0.407
	3	-2.732	0.290	-0.943	0.108	1.858	0.623	<0.001	0.996	-0.024	0.374	-0.072	0.108	-0.017	0.986	1.716	0.888	5.732	0.162
Serum folate	1	0.411	<0.001*	-0.009	0.710	-0.310	0.054	-0.001	0.467	<0.001	0.704	-0.010	<0.001*	-0.116	0.003*	-0.066	0.894	-0.393	0.023*
	2	0.344	0.004*	-0.021	0.450	-0.325	0.064	-0.001	0.616	-0.001	0.640	-0.011	<0.001*	-0.139	0.002*	-0.474	0.396	-0.480	0.012*
	3	0.292	0.019*	-0.032	0.264	-0.365	0.048*	-0.002	0.336	<0.001	0.808	-0.010	<0.001*	-0.143	0.002*	-0.465	0.427	-0.405	0.044*
Serum Vitamin B_{12}[1]	1	4.286	0.213	-0.227	0.764	-3.486	0.482	-0.015	0.803	0.022	0.530	-0.245	<0.001*	-0.674	0.582	-10.67	0.482	-3.899	0.466
	2	3.431	0.338	0.080	0.921	-3.413	0.521	0.024	0.706	0.016	0.664	-0.242	<0.001*	-0.067	0.960	-10.34	0.425	-3.155	0.583
	3	1.689	0.648	0.320	0.709	-1.562	0.776	0.052	0.424	0.001	0.982	-0.189	0.003*	0.540	0.695	-9.523	0.585	-1.223	0.838

β estimate and *p* values presented for each variable in domains of B vitamin intake or status fitted as an independent variables in models adjusted for 1) age, sex, batch effect; 2) Model 1 with further adjustment for physical activity, supplementation, energy intake, polypharmacy, frequency of alcohol intake; or 3) Model 2 with further adjustment for other measures of B vitamin intake and status.* indicates a significant association (p <0.05). Abbreviations: DMG, dimethylglycine; SAM, *S*-adenosylmethionine. [1]Fit as log-transformed variables in models

Table 5.4: Associations between B vitamins, one-carbon metabolites and cardiometabolic parameters in multivariate linear regression models.

	Model	BMI		Plasma glucose		HDL-cholesterol		LDL-cholesterol		Total/HDL-cholesterol		Triglycerides		Diastolic BP		Systolic BP	
		β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
Folate intake	1	-0.003	0.022*	<0.001	0.415	<0.001	0.663	<0.001	0.744	<0.001	0.290	<0.001	0.377	-0.004	0.252	0.001	0.876
	2	-0.001	0.534	<0.001	0.623	<0.001	0.830	<0.001	0.929	<0.001	0.862	<0.001	0.354	-0.004	0.287	-0.001	0.854
Riboflavin intake	1	-0.493	0.116	-0.083	0.125	0.024	0.391	0.092	0.177	-0.027	0.746	-0.010	0.809	-0.269	0.726	0.428	0.743
	2	-0.482	0.183	-0.106	0.110	0.038	0.266	0.147	0.070	0.022	0.829	-0.024	0.609	-0.445	0.633	0.300	0.852
Vitamin B_6 intake	1	-0.575	0.038	-0.045	0.341	0.047	0.059	<0.001	0.994	-0.119	0.106	-0.021	0.566	-0.583	0.389	-1.290	0.262
	2	-0.417	0.192	-0.074	0.208	0.067	0.025*	0.027	0.704	-0.050	0.577	-0.001	0.986	-0.576	0.492	-2.381	0.097
Vitamin B_{12} intake[1]	1	0.014	0.990	-0.084	0.632	0.179	0.055	0.537	0.018*	0.184	0.506	0.106	0.417	-0.396	0.874	1.365	0.747
	2	-0.063	0.953	-0.008	0.966	0.162	0.110	0.478	0.046*	0.266	0.374	0.118	0.398	-1.914	0.488	-1.906	0.688
Serum folate	1	-0.214	<0.001*	-0.020	0.025*	0.020	<0.001*	-0.006	0.624	-0.039	0.006*	-0.015	0.031*	-0.164	0.199	-0.285	0.187
	2	-0.158	0.004*	-0.009	0.377	0.016	0.002*	-0.019	0.123	-0.037	0.014*	-0.009	0.224	-0.243	0.090	-0.323	0.187
Serum Vitamin B_{12}[1]	1	-3.207	0.047*	-0.038	0.894	0.221	0.137	0.183	0.613	-0.436	0.324	-0.403	0.052	-6.909	0.085	-10.66	0.117
	2	-1.685	0.307	0.165	0.593	0.052	0.741	0.138	0.710	-0.093	0.841	-0.250	0.240	-3.843	0.372	-5.030	0.494
Plasma Betaine	1	-0.082	0.003	-0.009	0.048*	0.009	0.001*	-0.014	0.017	-0.029	<0.001*	-0.014	<0.001*	-0.083	0.219	-0.136	0.241
	2	-0.055	0.055	-0.008	0.112	0.008	0.002*	-0.012	0.063	-0.026	0.001*	-0.015	<0.001*	-0.042	0.575	-0.099	0.449
Plasma Choline	1	0.510	<0.001*	-0.026	0.211	-0.029	0.013*	-0.012	0.655	0.069	0.032*	0.041	0.007*	0.968	0.002*	1.232	0.019*
	2	0.529	<0.001*	-0.034	0.144	-0.029	0.015*	-0.014	0.631	0.074	0.037*	0.042	0.011*	0.942	0.005*	1.118	0.053
Plasma Cysteine	1	0.101	<0.001*	0.004	0.190	-0.004	0.037*	-0.004	0.366	0.003	0.491	0.003	0.247	0.035	0.451	0.034	0.675
	2	0.089	<0.001*	0.003	0.454	-0.002	0.220	-0.004	0.310	0.001	0.834	0.001	0.719	0.024	0.636	0.026	0.768
Plasma DMG[1]	1	2.436	0.144	-0.036	0.896	-0.241	0.105	-0.220	0.529	0.381	0.358	0.169	0.398	0.532	0.896	7.143	0.303
	2	2.195	0.185	-0.079	0.793	-0.140	0.370	0.028	0.940	0.466	0.308	0.181	0.407	-0.861	0.844	4.292	0.569
Plasma Glycine[1]	1	-8.682	0.001*	-0.523	0.238	0.802	<0.001*	-1.015	0.068	-2.487	<0.001*	-1.290	<0.001*	-19.47	0.003*	-20.43	0.066
	2	-7.032	0.010*	-0.382	0.444	0.720	0.004*	-0.841	0.163	-2.327	0.002*	-1.080	0.002*	-21.51	0.003*	-15.49	0.213
Plasma Homocysteine[1]	1	8.653	<0.001*	0.558	0.081	-0.521	0.003*	0.100	0.802	1.000	0.030*	0.553	0.027*	6.508	0.169	12.88	0.112
	2	8.268	<0.001*	0.615	0.077	-0.537	0.002*	0.043	0.918	0.963	0.051	0.663	0.013*	7.420	0.137	14.99	0.085
Plasma Methionine	1	0.150	0.057	0.017	0.190	-0.006	0.437	-0.038	0.025	-0.028	0.162	-0.006	0.507	0.310	0.104	0.555	0.089
	2	0.077	0.318	0.009	0.530	0.003	0.712	-0.032	0.069	-0.040	0.072	-0.012	0.232	0.134	0.514	0.172	0.626
Plasma SAM	1	0.017	0.008*	0.001	0.299	-0.001	0.246	-0.004	0.003	<0.001	0.816	0.001	0.348	0.019	0.209	0.026	0.321
	2	0.012	0.056	0.001	0.455	<0.001	0.502	-0.003	0.026	<0.001	0.930	0.001	0.279	0.014	0.385	0.012	0.672
Plasma Serine	1	-0.027	0.130	0.004	0.174	0.001	0.375	-0.002	0.629	-0.006	0.180	-0.007	0.001*	-0.092	0.034*	-0.073	0.323
	2	-0.018	0.312	0.004	0.184	0.002	0.242	0.001	0.857	-0.006	0.252	-0.006	0.010*	-0.068	0.153	-0.044	0.591

β estimate and *p* values presented for each metabolite fitted as independent variables in models adjusted for 1) age, sex, batch effect (metabolites only); or 2) Model 1 with further adjustment for physical activity, supplementation, energy intake, polypharmacy, frequency of alcohol intake.*Indicates a significant association (p <0.05). [1]Fit as log-transformed variables in models. Abbreviations: BMI, body mass index; BP, blood pressure; DMG, dimethylglycine; SAM, *S*-adeonsylmethionine.

5.2.4.4 B vitamins and one-carbon metabolites as predictors of cognitive function

According to scores from COMPASS testing, higher glycine concentrations were associated with better global cognitive performance (β=1.340, *p*=0.017), episodic memory (β=1.396, *p*=0.016) and location learning (β=1.394, *p*=0.027) z-scores in fully adjusted models, although this did not reach significance in models only adjusted for age, sex, and batch effects ($0.05 > p < 0.10$) (**Table 5.6**). Higher vitamin B_6 intake was associated with an increased risk for MCI based on MoCA test scores in the fully adjusted logistic regression model (risk ratio=1.295, 95% CI=1.04 – 1.60, *p*=0.020), though not in a model associated for age, sex, and education only (*p*=0.099) **(Table 5.5)**. This model contrasts the inverse association between vitamin B_6 intake and executive function from COMPASS testing which neared significance (β= -0.133, *p*=0.050) in a fully adjusted linear regression model. No other metabolites were associated with cognitive performance in COMPASS testing, nor was B vitamin intake or status (**Table 5.6**), or with altered risk of mild cognitive impairment according to MoCA scores (**Table 5.5**). These findings were similarly reported in sensitivity analyses where participants who regularly consumed B vitamin or MVM supplements were excluded (Appendix 3**,** Table 9.8).

Interestingly, choline status modified the relationship between glycine concentrations and cognitive performance. In those with the highest choline concentrations, glycine was inversely associated with global cognition (interaction, β= -2.968, *p*=0.020) and episodic memory (interaction, β= -3.278, *p*=0.013) z-scores compared to the positive association found with glycine concentrations as a main effect in both models (global cognition, β=1.897, *p*=0.005; episodic memory, β=2.052, *p*=0.004) (**Figure 5.1)**. The impact of interactions between all metabolites is presented in Appendix 3, Table 9.9.

Table 5.5: One-carbon metabolites as predictors of mild cognitive impairment according to multivariate logistic regression models

	Model 1			**Model 2**		
	Risk ratio	95% CI	*p*	Risk ratio	95% CI	*p*
Folate intake	0.999	0.99, 1.00	0.783	1.000	0.99. 1.00	0.776
Riboflavin intake	1.076	0.86, 1.33	0.520	1.204	0.93, 1.53	0.149
Vitamin B_6 intake	1.177	0.97, 1.42	0.099	1.295	1.04, 1.60	**0.020**
Vitamin B_{12} intake[1]	1.019	0.97, 1.06	0.378	1.021	0.97, 1.06	0.342
Serum Folate	1.003	0.97, 1.04	0.864	1.001	0.96, 1.04	0.970
Serum Vitamin B_{12}[1]	1.000	1.00, 1.00	0.759	1.00	1.00, 1.00	0.913
Plasma Betaine	0.990	0.97, 1.01	0.347	0.989	0.97, 1.01	0.327
Plasma Choline	0.952	0.88, 1.06	0.454	0.966	0.88, 1.06	0.470
Plasma Cysteine	0.996	0.98, 1.01	0.614	0.998	0.98, 1.01	0.814
Plasma DMG	0.484	0.15, 1.58	0.235	0.473	0.14, 1.58	0.229
Plasma Glycine[1]	0.665	0.09, 4.60	0.682	0.486	0.06, 3.69	0.490
Plasma Homocysteine[1]	0.684	0.16, 2.01	0.607	0.787	0.18, 3.48	0.751
Plasma Methionine	0.999	0.94, 1.05	0.959	0.998	0.94, 1.05	0.936
Plasma SAM	1.000	1.00, 1.00	0.758	1.00	0.99, 1.00	0.786
Serine	0.990	0.98, 1.00	0.136	0.988	0.97, 1.00	0.081

Risk ratios, 95% confidence intervals and *p* values presented for each metabolite fit in modified Poisson logistic regression models. Mild cognitive impairment was defined as a MoCA test score of <26. Model 1 is adjusted for age, sex, education, and batch effect. Model 2 was further adjusted for energy intake, exercise, history of anxiety/depression and supplement use. * Indicates a significant association ($p < 0.05$). [1]Fit as log-transformed variables in models. Abbreviations: DMG, dimethylglycine; SAM, *S*-adeonsylmethionine.

Table 5.6: Associations between B vitamins and one-carbon metabolites with domains of cognitive function according to multivariate linear regression models.

	Model	Global cognition		Attention/Vigalence		Episodic Memory		Executive function		Location Learning		Working Memory	
		β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
Folate intake	1	<0.001	0.461	<0.001	0.277	<0.001	0.625	<0.001	0.890	<0.001	0.344	<0.001	0.351
	2	<0.001	0.922	<0.001	0.727	<0.001	0.631	<0.001	0.740	<0.001	0.872	<0.001	0.180
Riboflavin intake	1	0.021	0.741	0.087	0.210	-0.011	0.869	0.024	0.729	-0.021	0.774	-0.036	0.607
	2	-0.039	0.594	0.033	0.682	-0.026	0.731	-0.019	0.807	-0.011	0.163	-0.073	0.364
Vitamin B_6 intake	1	-0.001	0.988	0.009	0.880	0.027	0.650	-0.083	0.176	0.019	0.768	-0.036	0.555
	2	-0.038	0.543	-0.043	0.527	0.032	0.616	-0.133	0.050	-0.028	0.690	-0.051	0.449
Vitamin B_{12} intake [1]	1	-0.003	0.990	-0.045	0.843	0.128	0.550	-0.291	0.195	-0.077	0.741	0.073	0.747
	2	-0.074	0.726	-0.137	0.555	0.128	0.564	-0.354	0.124	-0.177	0.461	0.029	0.900
Serum Folate	1	0.009	0.390	0.018	0.129	0.011	0.370	0.005	0.664	-0.012	0.293	-0.007	0.508
	2	0.012	0.299	0.018	0.133	0.015	0.218	0.002	0.843	-0.012	0.311	-0.005	0.646
Serum Vitamin B_{12}[1]	1	0.182	0.593	0.530	0.150	-0.083	0.814	0.228	0.533	0.055	0.883	-0.133	0.718
	2	0.302	0.378	0.594	0.109	0.039	0.914	0.225	0.543	0.183	0.630	-0.076	0.841
Plasma Betaine	1	0.004	0.519	0.002	0.726	0.001	0.946	0.002	0.739	0.009	0.180	0.004	0.507
	2	0.004	0.422	0.001	0.880	0.002	0.762	0.003	0.601	0.010	0.122	0.005	0.410
Plasma Choline	1	0.016	0.530	-0.008	0.776	0.027	0.316	0.016	0.573	0.008	0.770	0.006	0.830
	2	0.012	0.631	-0.014	0.630	0.026	0.334	0.009	0.743	0.007	0.823	0.007	0.808
Plasma Cysteine	1	<0.001	0.989	-0.004	0.362	0.002	0.551	0.001	0.794	-0.001	0.842	0.001	0.779
	2	-0.002	0.651	-0.006	0.161	0.001	0.731	-0.001	0.937	-0.002	0.620	0.001	0.815
Plasma DMG[1]	1	0.292	0.390	-0.053	0.885	0.207	0.552	0.460	0.211	0.051	0.894	0.610	0.096
	2	0.260	0.443	-0.098	0.790	0.180	0.608	0.500	0.174	0.013	0.972	0.603	0.102
Plasma Glycine[1]	1	0.912	0.095	0.618	0.299	1.075	0.055	0.353	0.552	1.028	0.093	-0.676	0.253
	2	1.340	0.017*	1.005	0.099	1.396	0.016*	0.376	0.539	1.394	0.027*	-0.428	0.486
Plasma Homocysteine[1]	1	-0.245	0.548	-0.618	0.157	0.307	0.479	-0.387	0.360	-0.327	0.476	-0.241	0.591
	2	-0.342	0.404	-0.618	0.160	0.195	0.658	-0.395	0.354	-0.472	0.307	-0.306	0.503
Plasma Methionine	1	-0.003	0.848	-0.009	0.619	0.003	0.865	0.013	0.439	0.003	0.862	-0.023	0.186
	2	-0.004	0.783	-0.011	0.539	0.002	0.905	0.012	0.495	0.002	0.891	-0.022	0.215
Plasma SAM	1	-0.001	0.490	-0.002	0.173	<0.001	0.912	0.001	0.409	-0.001	0.520	-0.001	0.588
	2	-0.001	0.384	-0.002	0.128	<0.001	0.815	0.001	0.583	-0.001	0.466	-0.001	0.675
Plasma Serine	1	-0.001	0.756	-0.001	0.756	0.001	0.850	-0.001	0.777	<0.001	0.952	-0.004	0.229
	2	<0.001	0.985	<0.001	0.917	0.001	0.734	-0.001	0.874	0.002	0.688	-0.004	0.300

β estimate and *p* values presented for each variable in the domains of dietary intake, serum status, or metabolite status fitted as an independent variable in models adjusted for 1) age, sex, education, and batch effect (metabolites only), or 2) Model 1 with further adjustment for energy intake, exercise, history of anxiety/depression and supplement use.*Indicates a significant association (p <0.05). [1]Fit as log-transformed variables in models. Abbreviations: DMG, dimethylglycine; SAM, *S*-adeonsylmethionine.

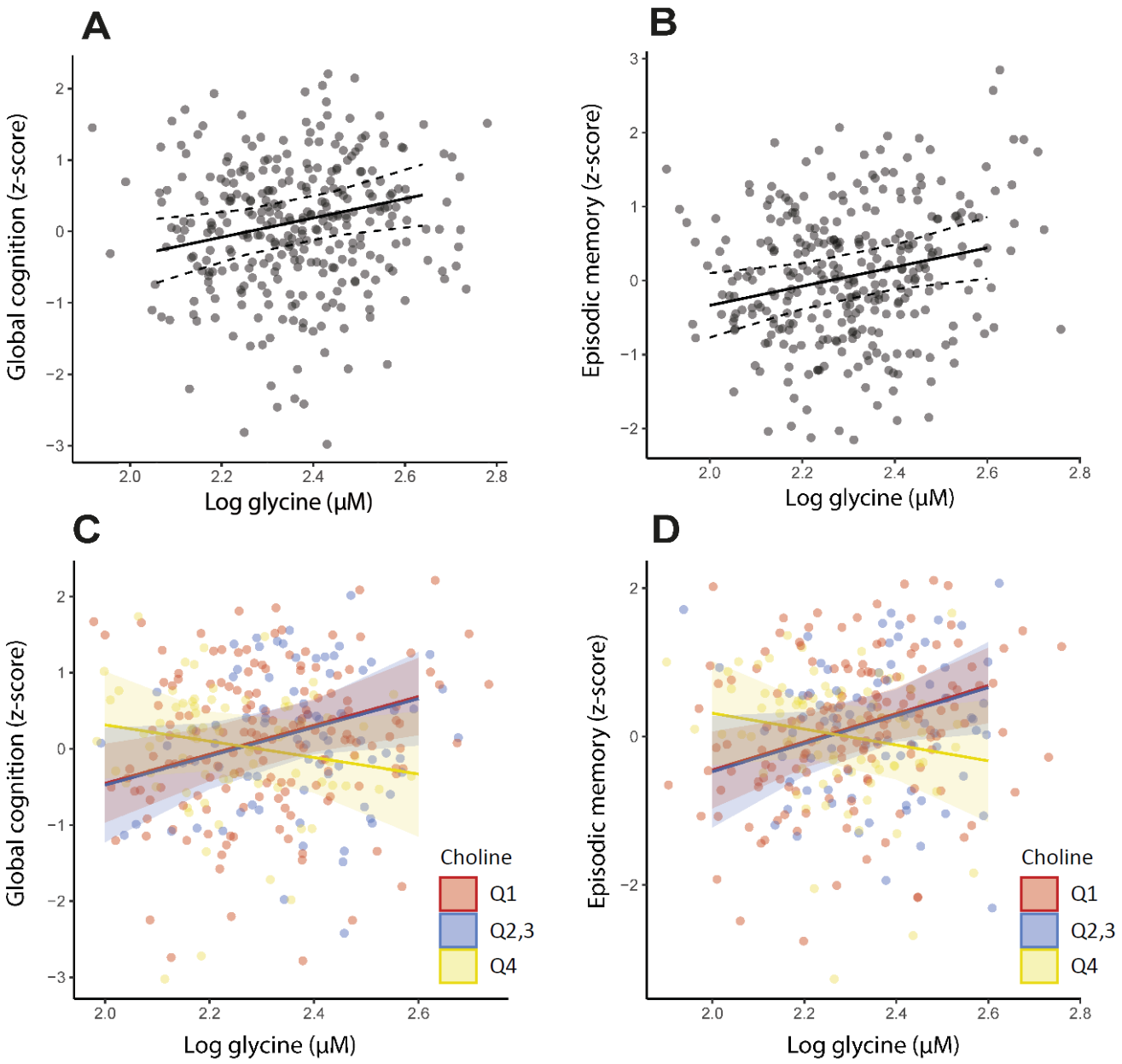


Figure 5.1: Association between plasma glycine concentration and cognitive performance
Data is presented for log-transformed glycine concentrations and A) global cognition (β=1.340, p=0.017), B) episodic memory (β=1.396, p=0.016), C) global cognition according to quartile of choline status (interaction with Q4 of choline concentrations, β= -2.968, p=0.020), and D) episodic memory according to quartile of choline status (interaction with Q4 of choline concentrations, β= -3.278, p=0.013). All regression models were adjusted for age, sex, batch effects, education, energy intake, exercise, supplementation, and history of anxiety or depression. Corresponding data is presented in Table 5.6 and Appendix 3, Table 9.9.

5.2.4.5 Impact of interactions between one-carbon metabolites and B vitamins on cognitive performance

Vitamin B_{12} intake and status appeared to have the greatest impact on the relationship between 1C metabolites and cognitive performance. Hcy concentrations in those with high vitamin B_{12} intake was positively associated with global cognition (interaction, β=2.134, *p*=0.018) and location learning (interaction, β=2.195, *p*=0.030) z-scores, whereas Hcy concentrations in those with the lowest vitamin B_{12} intake were inversely associated with attention z-scores (interaction, β= -1.891, *p*=0.049) compared to those with vitamin B_{12} intake in quartiles 2 and 3. Quartile of vitamin B_{12} intake was associated with global cognition, attention, and location learning as a main effect in each of these models ($p < 0.05$), though Hcy concentrations as a main effect were inversely associated only with location learning (β= -1.468, *p*=0.018) (**Figure 5.2)**. Glycine concentrations in those with the highest vitamin B_{12} intake (interaction, β = -3.341, *p*=0.006) or serum concentrations (interaction, β = -2.603, *p*=0.031) were inversely associated with working memory z-scores compared to those with intake or serum concentrations in quartiles 2 and 3, although glycine concentrations as a main effect were not associated with working memory in these models. SAM concentrations in those with the lowest riboflavin intake were inversely associated with global cognition (interaction, β= -0.004, *p*=0.018), episodic memory (interaction, β= -0.004, *p*=0.045) and working memory (interaction, β= -0.004, *p*=0.022) z-scores compared to those with riboflavin intake in quartiles 2 and 3, although SAM concentrations as a main effect were not associated with cognition in these models ($p > 0.05$). As outlined in the methods section, some significant findings are to be expected by chance alone due to the number of regression models tested, and this section therefore only describes interactions which showed the greatest consistency across metabolites or cognitive domains. The impact of interactions between all metabolites and B vitamins can be found in Appendix 3, Table 9.10.

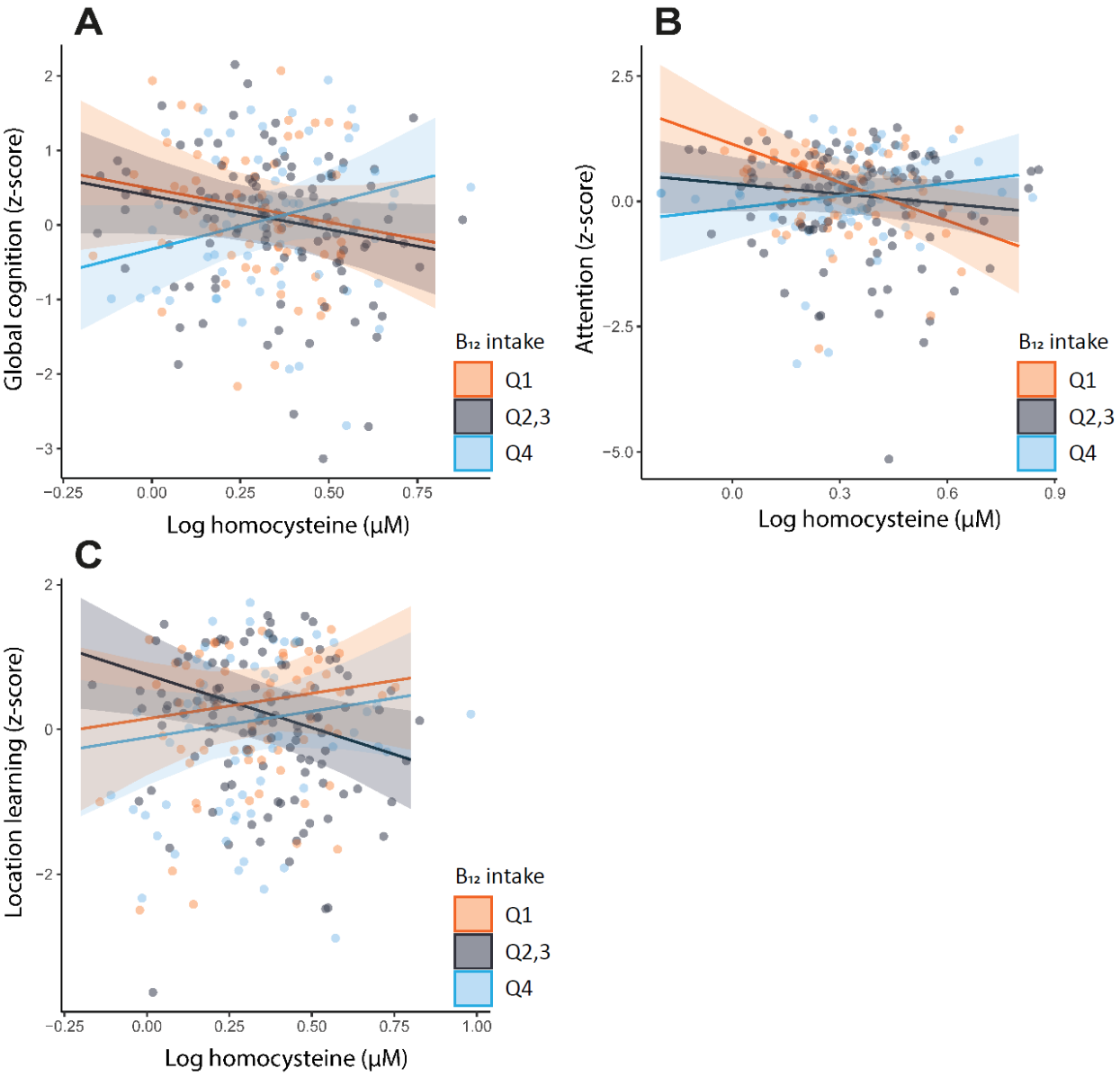


Figure 5.2: Association between one-carbon metabolites and cognitive performance according to vitamin B_{12} intake

Data is presented for A) log-transformed homocysteine and global cognition (interaction with quartile 4 of B_{12} intake, β=2.134, p=0.018), B) log-transformed homocysteine and attention & vigalence (interaction with quartile 1 of B_{12} intake, β = -1.891, p=0.049), C) log-transformed homocysteine and location learning (interaction with quartile 4 of B_{12} intake, β=2.195, p=0.030). All regression models were adjusted for age, sex, batch effects, education, energy intake, exercise, supplementation, and history of anxiety or depression. Corresponding data is presented in Appendix 3, Table 9.10

5.2.4.6 Impact of apolipoprotein ε4 genotype

These results show an interesting effect modification when an interaction term between 1C metabolites and the ApoE genotype was included in the regression model (**Table 5.7, Figure 5.3**). This result was most consistently seen in the cognitive domain of location learning. The ApoE genotype modified the relationship between cysteine, DMG, glycine, and Hcy with location learning such that higher cysteine (interaction, β=0.019, *p*=0.033), DMG (interaction, β=2.101, *p*=0.017), and Hcy (interaction, β=2.395, *p*=0.011) concentrations were associated with better location learning z-scores in those with the ApoE ε4 genotype compared to those who did not carry an ApoE ε4 allele. In these models, only Hcy was associated with location learning as a main effect (β= -1.226, *p*=0.024). Conversely, higher glycine concentrations were associated with lower location learning z-scores in those with the ApoE ε4 genotype (interaction, β= -2.280, *p*=0.043), compared to the positive association between glycine and location learning as a main effect in this model (β=2.071, *p*=0.004). Higher SAM concentrations were also associated with better global cognitive performance (interaction, β=0.004, *p*=0.016) and attention (interaction, β=0.004, *p*=0.019) z-scores in those with the ApoE ε4 genotype compared to the inverse relationship between attention and SAM as a main effect (β= -0.001, *p*=0.028), although this main effect did not reach statistical significance with global cognition as a dependent variable (β= -0.002, *p*=0.093).

Table 5.7: Effect of interaction between one-carbon metabolites and apolipoprotein ε4 genotype on cognitive performance in multivariate linear regression models

Metabolite (plasma)	Model Covariates	Global cognition		Attention		Episodic Memory		Executive function		Location Learning		Working Memory	
		β	*p*		*p*	β	*p*	β	*p*	β	*p*	β	*p*
Betaine	Betaine	0.002	0.800	-0.003	0.714	<0.001	0.983	0.002	0.794	0.008	0.292	0.006	0.455
	ApoE ε4	-0.509	0.211	-0.455	0.304	0.515	0.219	-0.049	0.913	-0.477	0.295	0.129	0.772
	Interaction	0.011	0.343	0.013	0.293	0.007	0.524	0.005	0.699	0.008	0.542	-0.003	0.918
Choline	Choline	0.001	0.976	-0.026	0.437	0.030	0.334	0.009	0.797	-0.021	0.554	-0.016	0.622
	ApoE ε4	-0.335	0.457	-0.333	0.498	0.006	0.989	0.125	0.799	-0.817	0.107	-0.485	0.326
	Interaction	0.024	0.646	0.038	0.499	-0.030	0.564	-0.001	0.991	0.072	0.213	0.067	0.231
Cysteine	Cysteine	-0.002	0.705	-0.007	0.144	0.004	0.362	0.001	0.896	-0.007	0.161	<0.001	0.946
	ApoE ε4	-0.367	0.636	-0.569	0.499	0.498	0.531	0.237	0.778	-2.038	0.019*	-0.488	0.565
	Interaction	0.002	0.764	0.006	0.501	-0.008	0.337	-0.001	0.886	0.019	0.033#	0.006	0.493
Dimethylglycine	Dimethylglycine	-0.138	0.717	-0.319	0.444	-0.146	0.722	0.029	0.489	-0.491	0.251	0.506	0.227
	ApoE ε4	-0.613	0.017*	-0.305	0.279	-0.587	0.027	-0.192	0.493	-0.823	0.004*	-0.064	0.820
	Interaction	1.640	0.037#	1.004	0.243	1.129	0.163	1.095	0.201	2.101	0.017#	0.563	0.513
Glycine[1]	Glycine	1.619	0.011*	0.988	0.155	1.689	0.010*	0.633	0.326	2.071	0.004*	-0.610	0.382
	ApoE ε4	1.783	0.441	-0.071	0.978	1.969	0.407	1.451	0.569	5.052	0.051	-0.203	0.426
	Interaction	-0.827	0.412	0.034	0.098	-0.961	0.353	-0.577	0.602	-2.280	0.043#	0.921	0.408
Homocysteine	Homocysteine	-0.266	0.584	-0.338	0.516	0.355	0.492	-0.317	0.531	-1.226	0.024*	-0.260	0.631
	ApoE ε4	-0.099	0.745	0.292	0.371	-0.236	0.466	0.151	0.633	-0.914	0.007*	0.274	0.418
	Interaction	0.104	0.902	-0.714	0.427	-0.035	0.969	0.022	0.980	2.395	0.011#	-0.227	0.807
Methionine	Methionine	<0.001	0.994	-0.017	0.382	0.009	0.638	0.024	0.234	0.004	0.847	-0.016	0.436
	ApoE ε4	-0.074	0.920	-0.717	0.374	-0.096	0.900	0.919	0.254	-0.152	0.855	0.582	0.472
	Interaction	-0.003	0.932	0.035	0.373	-0.008	0.828	-0.038	0.313	-0.003	0.948	-0.023	0.539
S-adenosyl methionine	*S*-adenosylmethionine	-0.002	0.093	-0.003	0.028*	-0.001	0.390	0.001	0.600	-0.002	0.176	-0.001	0.465
	ApoE ε4	-0.685	0.007*	-0.059	0.032*	-0.654	0.012*	0.048	0.862	-0.579	0.042*	-0.159	0.566
	Interaction	0.004	0.016#	0.004	0.019#	0.003	0.090	0.001	0.772	0.003	0.145	0.002	0.323
Serine	Serine	-0.001	0.739	-0.002	0.741	0<0.001	0.934	<0.001	0.948	0.001	0.857	-0.007	0.151
	ApoE ε4	-0.681	0.285	-0.300	0.665	-0.770	0.239	0.406	0.558	-0.581	0.416	-0.733	0.291
	Interaction	0.007	0.385	0.004	0.668	0.006	0.427	-0.004	0.673	0.005	0.595	0.010	0.229

β estimate and *p* values presented for main effects (one-carbon metabolite concentration and ε4 allele carrier) and the interaction between the main effects on cognitive outcomes. All models are adjusted for age, sex, education, batch effects, energy intake, exercise, history of anxiety/depression, and supplement use. * Indicates a significant main effect of metabolite concentrations or apolipoprotein E genotype, and # Indicates a significant interaction effect (p <0.05) [1]Fit as log-transformed variables in models.

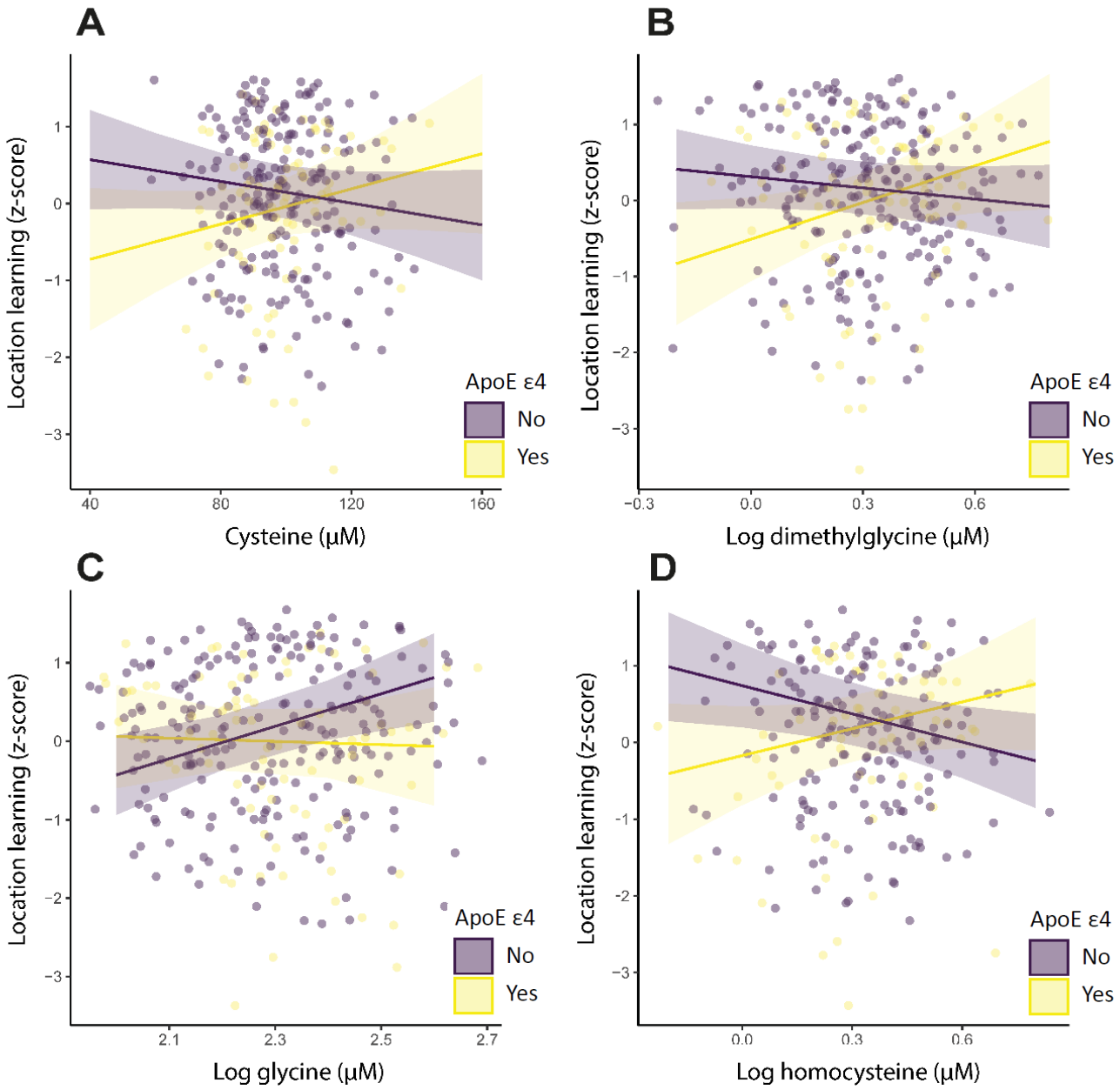


Figure 5.3: Association between one-carbon metabolites and location learning performance according to Apolipoprotein E genotype

Data is presented for A) Cysteine (interaction with ApoE *ε4 genotype, β=0.019, p=0.033), B) log-transformed dimethylglycine (interaction with* ApoE *ε4 genotype, β=2.101, p=0.017), C) log-transformed glycine (interaction with* ApoE *ε4 genotype, β = -2.280, p=0.043), D) log-transformed homocysteine (interaction with* ApoE *ε4 genotype, β = 2.395, p=0.011). All regression models were adjusted for age, sex, batch effects, education, energy intake, exercise, supplementation, and history of anxiety or depression. Corresponding data is presented in Table 5.7.*

5.2.5 DISCUSSION

The societal burden of cognitive impairment escalates in parallel with population ageing. Metabolic dysfunction accelerates cognitive decline and contributes to precocious ageing[341,514]. B vitamins and 1C metabolites are inherently linked to several processes underpinning both cognitive and metabolic health. However, research has predominantly focused on folate, vitamin B_{12}, and Hcy, providing a narrow view of the complex regulation of 1C metabolism. Indeed, neither B vitamins nor Hcy were consistently associated with cognitive function in 313 healthy older adults living in New Zealand, whereas glycine concentrations were linked to better performance across global function and cognitive domains according to COMPASS testing. Although the relationship between 1C metabolites and cardiometabolic health was more extensive than that found for cognition, our findings do highlight potentially important interactive effect of B vitamins, metabolites, and ApoE genotype on the relationship between 1C metabolites and cognition.

There is a wealth of evidence which supports the association between B vitamins, particularly folate and vitamin B_{12}, and Hcy with cognitive performance in older adults[255,256,265,274,275,290,492–497]. Although some studies have reported associations across seemingly normal ranges, it is generally agreed that associations between B vitamins or Hcy with cognitive function, or the effect of Hcy-lowering with B vitamins on cognitive performance, are stronger in the context of dietary or biochemical inadequacy[276,286,515,516]. Thus it is not surprising that associations between folate, vitamin B_{12}, and Hcy with cognition were not replicated in the current study. Although a relatively high prevalence of dietary folate inadequacy was reported (22% of males and 33% of females did not meet recommended daily intake of 400µg/day), intakes of other B vitamins and serum folate and vitamin B_{12} concentrations were generally within expected ranges in the REACH cohort[87,517,518].

Hcy was not associated with cognitive performance as a main effect in linear regression models. However, our findings demonstrate the interactive effects vitamin B_{12} intake and ApoE genotype in moderating the relationship between Hcy and cognition. In models fit with vitamin B_{12} intake, Hcy as a main effect was inversely associated with global cognition, location learning, and attention and vigilance. This was either exacerbated by lower vitamin B_{12} intake (attention and vigilance) or not apparent in those with the highest vitamin B_{12} intake (global cognition, location learning). Higher vitamin B_{12} intake therefore appears to attenuate the association between increasing Hcy and poor cognitive performance. Similarly, the inverse association between Hcy and location learning was not apparent in those carrying at least one ApoE ε4 allele. This finding does however contrast that from other authors, which have shown that the association between higher Hcy concentrations and cognitive impairment or reduced brain volume is stronger in ε4 allele carriers[519], or even only present in those with the ApoE ε4 genotype[520]. Others have found that ApoE genotype only influences the effects of vitamin B_{12} on cognitive performance but not Hcy[256,313,314]. However, to the best of our knowledge, this is the first study to show a more protective effect of the ApoE ε4 genotype. Mechanisms underlying the interaction between B vitamins or Hcy with ApoE genotype remain unclear, and the presence or direction of response is not yet consistent across enough studies to draw conclusions at this stage. However, these findings likely hold implications for stratifying dietary advice to maximise cognitive benefits according to ApoE genotype once such uncertainties have been resolved.

Interestingly, higher vitamin B_6 intake was associated with a greater risk for having MCI according to MoCA test scores. Although the evidence base is not as robust compared to that for folate or vitamin B_{12}, the literature generally supports an association between higher vitamin B_6 intake or status with better cognitive performance[521], reduced risk for cognitive decline[276], greater grey matter volume[286], and preservation of cortical structures in the brain[522], although others have found no associations[494]. The findings here were only significant in fully adjusted models, and biochemical measures of vitamin B_6 status were not obtained to evaluate functional status. Thus it is difficult to draw any firm conclusions from our findings, albeit an interesting consideration for future research. Riboflavin is scarcely reported in the literature. Similar to findings from Hughes *et al*[276], riboflavin intake was not associated with cognitive performance in the current study, although others have reported that dietary riboflavin intake promotes better global cognitive and verbal memory function and slower cognitive decline in middle-aged and older adults[523]. The lack of association reported here is likely explained by the adequate riboflavin intake found in the REACH population (96-97%). As Hughes and colleagues highlight, there is likely to be a stronger association between B vitamins and cognition with a higher prevalence of inadequacy, or with the "limiting nutrient" within the cohort[276]. Again, it is difficult to provide strong conclusions here without corresponding markers of functional riboflavin status.

In this population of healthy older adults, the most consistent association between 1C metabolites and cognition was found for glycine, which was positively associated with global cognitive performance, episodic memory and location learning z-scores according to COMPASS testing. The interpretation of this relationship is limited by a scarcity of literature reporting associations between glycine and cognition, and difficulties in disentangling the role of glycine within 1C metabolism. Glycine is the end-product following the degradation of metabolites involved in betaine-dependent Hcy remethylation, and is interconverted with serine via SHMT in the cytosol and mitochondria. Glycine is also a substrate for GNMT, and thus implicated in maintaining the balance of SAM and SAH, or methyl group availability[357]. Indeed, SAM-dependent methyl group transfers are critical for maintaining cognitive function, involving the methylation of proteins, membrane phospholipids, DNA and neurotransmitters, and impaired methylation capacity is thought to drive the relationship between B vitamin inadequacy or elevated Hcy with cognitive decline[524]. Whether this mechanism extends to the involvement of glycine remains unclear without a robust analysis of methylation capacity. Plausibly, the association between glycine and cognitive function in the current study could also stem from involvement in pathways outside of 1C metabolism. For example, glycine is a precursor of glutathione and contributes to anti-oxidative capacity[525], imbalances of which are considered a feature of Alzheimer's disease onset and progression[526,527].

While mechanisms between glycine and cognition are speculative due to the exploratory nature of this analysis, the interactive effects of glycine with choline status and ApoE genotype would indicate a connection to metabolic health. Contribution of the ApoE ε4 genotype to the risk of cognitive decline and dementia is also linked to the role of ApoE in lipid transport and metabolism[528,529]. Thus, disruption of the positive association between glycine and cognition in ApoE ε4 carriers in the current study is potentially linked to disturbances between glycine, cardiometabolic health, and cognitive performance. This proposed mechanism may also extend to the negative impact of choline status on the relationship between glycine and cognition, given that higher choline concentrations were associated with cardiometabolic disturbances, including higher BMI, fasting glucose, total/HDL-cholesterol, triglycerides, and blood pressure or lower HDL-cholesterol. The connection between glycine and health in older adults is likely complex and warrants future investigations

which aim to disentangle underlying mechanisms, which is of particular importance as glycine supplementation has recently been proposed as a potential strategy for the improvement of metabolic and related health outcomes[530].

It is interesting to note here that other metabolites of the choline oxidation pathway were not associated with cognitive parameters despite previous research demonstrating associations between higher dietary choline intake[318,319] and status[321] or DMG concentrations[324] with improved cognitive performance and protection against cognitive decline. Similarly, the inter-connected regulation of folate and choline metabolism is thought to be integral in the trajectory of cognitive ageing according to pre-clinical models[334–336], but was not supported by findings in this cohort. The lack of associations may indeed again be related to the B vitamin status of the REACH cohort, as the impact of choline or betaine on Hcy concentrations appears to be more pronounced with lower B vitamin status[164–166], suggesting that Hcy remethylation is more dependent on betaine as a methyl donor when B vitamin intake is restricted. This could theoretically be extended to associations with clinical outcomes like cognitive performance, whereby associations between choline metabolites and cognition is more pronounced in the context of B vitamin inadequacy.

Associations between 1C metabolites and cardiometabolic markers were much more extensive than those found with cognitive parameters. Of note, higher betaine and glycine concentrations were associated with a favourable cardiometabolic risk profile, while the inverse was true for cysteine, choline, and Hcy, and this was most consistent for glycine and choline across different parameters (i.e. BMI, fasting blood glucose, cholesterol, triglycerides and blood pressure). These findings are similar to those previously reported for glycine [531–533], betaine and choline[119,362,503,504,534], cysteine[535,536], and Hcy [235,350]. Thus, metabolites across the 1C pathways appear to be more closely related to cardiometabolic regulation than cognition in healthy older adults despite a higher prevalence of mild cognitive impairment (n=94, 29%) than metabolic syndrome (n=35, 11%) in the current population. The association between metabolites and cardiometabolic health might then provide an earlier indication of perturbations in the broader regulation of 1C metabolism, or a more sensitive indicator of an ‘optimal’ profile of 1C metabolites. This observation has potential relevance for the monitoring of health status and directing intervention strategies in older adults. However, this requires further understanding of how 1C metabolites contribute to the progression of metabolic disturbances to cognitive impairment in a cohort which allows for appropriate stratification of nutritional, metabolic, and cognitive status.

This study aimed to address the inter-connected role that 1C metabolites play in health outcomes which contribute significantly to the burden of ageing. The primary strength of this analysis lies in a broader profile of B vitamins and 1C metabolites than typically measured, including vitamin B_6 and riboflavin intake, and metabolites of the choline oxidation, transsulfuration, and methionine pathways. This is not a complete panel of B vitamins and metabolites, and the analysis could be strengthened with inclusion of SAH to provide an index of methylation capacity alongside SAM, or intracellular measures of 1C metabolites. The cognitive testing performed in this analysis was robust, including the MoCA test, a widely used benchmark for MCI[506], and the COMPASS battery which offers insight into domain-specific cognitive impairment and is sensitive to age effects[537]. While the dietary analysis was thorough with respect to the detailed direction provided, an experienced research team, and the rigorous internal checks performed there are still inherent limitations which all measures of dietary assessment are subject to. Of particular importance to the current analysis is that the Foodworks software relies on manufacturer claims of the added nutrient content in fortified foods, possibly leading to over-estimates of B vitamin intake[89]. While it is important to characterise the diet-health

relationship in healthy cohorts to direct strategies which promote and monitor healthy ageing, the findings from this study should also be considered in populations with lower socioeconomic or nutritional status, or a higher prevalence of metabolic dysregulation in order to understand their generalisability in ageing populations.

While several interesting interactions between nutrients, metabolites, and genotype were found, it should be highlighted that this analysis is exploratory in nature. The sample size is sufficiently powered to detect the relationship between B vitamins or 1C metabolites with cognition as main effects, but there are indeed limitations in examining the interactive effects which can inflate type I and II error rates. The results are accordingly discussed in a more stringent manner, with a focus on those interactions demonstrating consistency, and these interactive effects should be confirmed in larger, adequately powered cohorts.

5.2.6 CONCLUSION

This analysis provides insight into the nuanced relationships between B vitamins, 1C metabolites, and inter-connected health outcomes in older adults. Although B vitamin intake and status were not related to cardiometabolic or cognitive measures in the current study, higher vitamin B_{12} intake does appear to attenuate the association between increasing Hcy concentrations and poor cognitive performance. Higher glycine concentrations were associated with better cognitive performance although this was disrupted by higher choline status and the ApoE ε4 genotype, all of which are related to cardiometabolic regulation. These findings inform the development of an 'optimal' profile of nutrients and metabolites, which hold relevance to monitoring health in older adults and directing targeted intervention strategies which promote healthy ageing. Further investigation of the interactive effects of 1C metabolites with B vitamin status, cardiometabolic markers and genetic composition on the trajectory of cognitive ageing in prospective ageing cohorts should be considered.

LONG-TERM SUPPLEMENT

VAAS study

6

CHAPTER 6: ONE-CARBON METABOLITES, CARDIOMETABOLIC MARKERS, AND THEIR ASSOCIATION AFTER 6-MONTHS OF NUTRITIONAL SUPPLEMENTATION

6.1 PREFACE

Chapter 5 revealed the close correlation between cardiometabolic parameters and 1C metabolites. Data presented in the current chapter extends these findings by providing insight into the longitudinal relationship between 1C metabolites and cardiometabolic health, and further considering the impact of an intervention on this relationship. This chapter continues to address the second thesis objective regarding B vitamins, 1C metabolite and health status.

Chapter 6 is also the first to align with the final thesis objective of characterising the long-term response of 1C metabolites to intervention with B vitamins through dietary intervention and nutritional supplementation. The intervention evaluated in this Chapter, a broad-spectrum nutritional supplementation containing B vitamins alongside resistance training, is already recommended to improve health in older adults. The novelty in this analysis lies first in a wider profile of 1C metabolites than typically considered in response to interventions, and second by considering the response of a 'very old' population. Octogenarians and nonagenarians are the fastest growing segment of ageing population, but vastly under-represented in research to date despite heightened risk of nutritional inadequacies, gastrointestinal dysfunction, and comorbidities.

This chapter was completed in collaboration with Professor Karl-Heinz Wagner from the Platform Active Ageing, University of Vienna, and presents a secondary analysis of the Vienna Active Ageing Study (VAAS). The primary outcomes of this study were to evaluate the impact of resistance training alone or with nutritional supplementation on functional parameters in older adults, including chromosomal integrity, DNA damage, and oxidative stress. This study was completed in Vienna by Professor Karl-Heinz Wagner and the Platform Active Ageing group from 2011-2012 prior to my candidature. As such I was not involved in the completion of the trial. For this Chapter specifically, I was responsible for performing the mass spectrometry analysis of 1C metabolites and writing the manuscript as first-author.

This chapter contains an adapted version of the manuscript "Nutritional supplementation alters associations between one-carbo metabolites and cardiometabolic risk profiles in older adults: A secondary analysis of the Vienna Active Ageing Study", co-authored by Nicola A. Gillies, Bernhard Franzke, Barbara Wessner, Barbara-Schober-Halper, Marlene Hofmann, Stefan Ossen, Anela Tosevska, Eva-Maria Strasser, Nicole C.Roy, Amber M. Milan, David Cameron-Smith, and Karl-Heinz Wagner. This manuscript has been accepted for publication in the *European Journal of Nutrition*, which has a 2019/2020 impact factor of 4.644, and a five-year impact factor of 4.348.

6.2 MANUSCRIPT:

Nutritional supplementation alters associations between one-carbon metabolites and cardiometabolic risk profiles in older adults: A secondary analysis of the Vienna Active Ageing Study

6.2.1 ABSTRACT

Purpose: Cardiovascular diseases and cognitive decline, predominant in ageing populations, share common features of dysregulated 1C and cardiometabolic homeostasis. However, few studies have addressed the impact of multifaceted lifestyle interventions in older adults that combine both nutritional supplementation and resistance training on the co-regulation of 1C metabolites and cardiometabolic markers.

Methods: 95 institutionalised older adults (83 ± 6 years, 88.4% female) were randomised to receive resistance training with or without nutritional supplementation (Fortifit, containing folic acid, vitamins B12 and B6), or cognitive training (control for socialisation) for six months. Fasting plasma 1C metabolite concentrations, analysed by liquid chromatography coupled with mass spectrometry, and cardiometabolic parameters were measured at baseline and the three- and six-month follow-ups.

Results: Regardless of the intervention group, choline was elevated after three months, while cysteine and methionine remained elevated after six months (mixed model time effects, $p<0.05$). Elevated DMG and lower betaine concentrations were correlated with an unfavourable cardiometabolic profile at baseline (spearman correlations, $p<0.05$). However, increasing choline and DMG concentrations were associated with improvements in lipid metabolism in those receiving supplementation (regression model interaction, $p<0.05$)

Conclusion: Choline metabolites, including choline, betaine and DMG, were central to the coregulation of 1C metabolism and cardiometabolic health in older adults. Metabolites that indicate upregulated betaine-dependent Hcy remethylation were elevated in those with the greatest cardiometabolic risk at baseline, but . were associated with improvements in lipid parameters following resistance training with nutritional supplementation. The relevance of how 1C metabolite status might be optimised to protect against cardiometabolic dysregulation requires further attention.

6.2.2 INTRODUCTION

Metabolic dysregulation, leading to cardiovascular diseases, diabetes and cognitive decline are major causes of morbidity and mortality in the ageing population[341,538]. One central metabolic pathway impaired with ageing is that of 1C metabolism[52,145]. Traditionally identified on the basis of elevated Hcy, 1C metabolism is central to aspects of both cognitive[5,308] and cardiometabolic health[220,235].

Hcy is at a critical branch point in 1C metabolism, with circulating concentrations dependent upon competing actions of the 1C cycle. Hcy is synthesised from the conversion of methionine to SAM and SAH and is remethylated through either folate and vitamin B_{12} or betaine-dependent pathways to support the methionine cycle, or is removed through the vitamin B_6-dependent transsulfuration pathway to form cysteine[125].

There is substantive evidence supporting the association of 1C metabolites beyond Hcy with cardiometabolic health. Cysteine is proposed as an obesogenic amino acid[358] and elevated glycine with a favourable cardiometabolic profile[357]. Of particular interest is choline and its downstream metabolites, betaine and DMG. Choline plays a critical role in lipid metabolism, as phosphatidylcholine is required for VLDL synthesis and hepatic lipid transport. Evidence supports divergent associations between choline and betaine with markers of cardiometabolic health. Elevated choline concentrations are typically associated with an unfavourable metabolic profile, and conversely, elevated betaine with a more protective profile for indices of anthropometry, lipid metabolism, and insulin sensitivity[359–362].

Those in their oldest years of life represent a vulnerable group, with an increased risk of dysregulated 1C metabolism[52] and cardiometabolic disease markers[341], yet the relationship between these processes is not well understood. Although the pathways of 1C metabolism are tightly interconnected, there is little evidence from studies that consider metabolites beyond distinct, targeted 1C metabolites (e.g. Hcy or choline and betaine alone). Furthermore, nutritional supplementation becomes more frequent with advancing age to support dietary adequacy and counteract age-related functional decline[461,539]. Resistance training (RT) is another important lifestyle intervention particularly in elderly subjects, shown to improve cardiometabolic parameters[540] and 1C metabolism with evidence of circulating Hcy reduction [541,542], and favourably impact related health outcomes such as cognition[543,544]. Given the overlap of 1C metabolism and cardiometabolic markers in multiple related health outcomes, there is potential to provide various health benefits to older adults. However, the impact of RT and nutritional supplementation containing B vitamins on profiles of 1C metabolites and cardiometabolic parameters requires further investigation in an elderly population.

Thus, in this secondary analysis of the VAAS study, the objective was first to evaluate six-month shifts in 1C metabolites and cardiometabolic markers, and their correlation, in the context of lifestyle interventions including RT with or without nutritional supplementation (containing protein and micronutrients, including folic acid, vitamins B_6 and B_{12}). Second, by investigating the cross-sectional association between markers of cardiometabolic health and 1C metabolites and the influence of B vitamin status.

6.2.3 METHODS

6.2.3.1 Study design

The VAAS study uses a randomised, controlled, observer-blind design[545]. Participants were randomly assigned to three parallel intervention groups: RT, RT with nutritional supplementation (RTS), or cognitive training (CT) which acted as the control group. Blood samples and anthropometric measurements were taken at baseline, and after three- and six-months of intervention. Informed written consent was obtained from all participants before they were enrolled following the Declaration of Helsinki. The VAAS Study was approved by the ethics committee of the City of Vienna (EK-11-151-0811) and registered at ClinicalTrials.gov, NCT01775111. This article reports on secondary exploratory outcomes; the primary outcome of the VAAS Study was to evaluate the effect of RT or RTS on functional parameters, including markers of chromosomal damage[546], oxidative stress[547], and DNA damage[548]. The secondary outcomes reported in this analysis include the assessment of plasma 1C metabolite concentrations and markers of cardiometabolic health.

6.2.3.2 Subjects

Over six months, 117 institutionalised older men and women (aged 65-98 years) were recruited to participate in the VAAS Study from five senior residences in the area of Vienna (Curatorship of the Viennese Retirement Homes). At the six-month follow-up, 95 participants were included for analysis in the current analysis, and a detailed participant flow diagram describing the loss of participants during follow-up has previously been reported[545]. Participants were eligible if they were mentally (Mini-Mental State Examination ≥ 23)[549] and physically (Short Physical Performance Battery >4) able. Inclusion and exclusion criteria have previously been described in detail[545]. Briefly, participants were sedentary (<1 hour of physical activity or exercise per week), aged ≥65 years, and referring to the recommendations of the American Heart Association[550], were free of diseases that pose contraindication to medical training therapy, including serious cardiovascular diseases, diabetic retinopathy, and regular use of cortisone-containing drugs. Subjects were excluded if they performed regular resistance training (at least once/week in the six months before inclusion) or were using dietary supplements and not willing to abstain during the intervention.

6.2.3.3 Study intervention

6.2.3.3.1 Resistance training

Groups receiving the training-based intervention (RT and RTS) performed two sessions of RT per week. The training protocol was developed in line with the guidelines of the American College of Sports Medicine for RT with older adults[551]. Exercise sessions were conducted on non-consecutive days and supervised by a sport scientist who recorded attendance. The training program has been described in detail by Oesen *et* al[545]. Briefly, each session lasted approximately one hour, consisting of an initial ten-minute warm-up, a 30-40 minute strength training using exercise bands and a chair, and a ten-minute cool down, and the intensity and volume of the exercise were progressively increased.

6.2.3.3.2 Resistance training with nutritional supplementation

Participants in the RTS group followed the same training protocol as those in the RT group, additionally consuming a nutritional supplement at breakfast every morning and directly after the bi-weekly training session. The supplement was a 150 ml serving of FortiFit (Nutricia, Vienna, Austria), a supplement which is freely available to purchase and designed to prevent sarcopenia in older adults. Of note, each 150kcal serve of this supplement provided 200 µg of folic acid, 3.0 µg of vitamin B_{12}, 750 µg of vitamin B_6, 55 mg of choline, and 20.7 g of protein [56% of energy, 19.7 g of whey protein, 3 g leucine, >10 g essential amino acids], 9.3 g of carbohydrates (25% of energy), 3.0 g of fat (18% energy), and other vitamins (A, C, B_1, B_2, niacin, pantothenic acid, D3, biotin) and minerals.

6.2.3.3.3 Cognitive training

The CT group served as the control group to minimise the bias of being part of a social group activity, as those in the RT and RTS groups received twice a week during training sessions. Those in the CT group performed coordinative or cognitive tasks[552], which were mainly memory training and finger dexterity exercises in a sitting position.

Participants in all intervention groups were instructed to maintain regular food intake, which was checked with 24h diet recalls when blood samples were taken at three- and six-month follow-ups.

6.2.3.4 Cardiometabolic parameters

Waist and hip circumference, body mass and body height were measured following standardised anthropometric procedures. Body mass was measured to the nearest 0.1 kg (BWB 700, Tanita, Amsterdam, Netherlands) and height was measured to the nearest 0.5 cm using a portable stadiometer (SECA Model 217, Seca GmbH & Co. KG, Hamburg, Germany). BMI was calculated as body mass relative to height in meters squared (kg/m2).

Lipid profile (cholesterol (LDL, HDL, total/HDL) and triglycerides), high sensitive insulin, plasma glucose and glomerular filtration rate (GFR) were analysed immediately after blood sampling at a routine laboratory (study lab GmbH, Vienna, Austria). Renal function is a determinant of circulating Hcy concentrations[553,554], and GFR was therefore used as a confounding variable in regression models. To determine HOMA-IR as a marker of insulin resistance, fasting plasma glucose (mg/dl) and insulin (IU/ml) values were converted into mmol/L (glucose) and pmol/L (insulin), and then entered into the validated HOMA2 calculator (HOMA2 v2.2.3 © β, Diabetes Trials Unit, University of Oxford).

6.2.3.5 One-carbon metabolites

Following an overnight fast, blood samples were collected into EDTA-coated vacutainers. Samples were placed on ice prior to separation of plasma for analysis, and then separated into aliquots and stored in Eppendorf tubes at -80°C until required for analysis. Samples were thawed only immediately before analysis. Plasma concentrations of 11 metabolites (betaine, choline, DMG, SAH, and SAM) and amino acids (cysteine, cystathionine, glycine, Hcy, methionine, and serine) involved in the regulation of 1C metabolism was determined by UHPLC-MS/MS. Product/precursor ratios were calculated to provide insight into pathway regulation for betaine/choline, and DMG/betaine, allowing inference of betaine-dependent remethylation of Hcy[119,120].

Samples were randomised to ensure a balance of intervention group and sex across batches. The UHPLC-MS/MS methods are reported in detail elsewhere[379]. Briefly, plasma samples were prepared using an automated robotic liquid handling system (Eppendorf epMotion® 5075vt, Hamburg, Germany). First, 300µl of 1% formic acid in methanol was pipetted into a 96-well IMPACT® protein precipitation plate (Phenomenex, Torrance, California, USA). Next, all standards (100µl), quality controls (100µl) and standards (100µl) were spiked with 20µl of internal standard solution, agitated for 5 minutes (800rpm), then filtered into a (2mL) 96-well square collection plate (Phenomenex, Torrance, California, USA) by applying a vacuum (450mbar). 100µl of Tris (2-carboxyethyl) phosphine was then dispensed into each well to allow for the separate quantification of Hcy and cysteine.

Three sets of QC samples were included to assess recovery of standards and reproducibility of samples. Metabolites were considered acceptable if standard recoveries were between 80-120%, and coefficients of variance were below 20%. Cystathionine and SAH did not satisfy these requirements and were excluded from further analysis, as were SAM samples from one of three batches (34%, *n*=76). For samples where a peak was not detected, a missing value was calculated as half of the minimum value of each batch[380]. A missing value was not calculated for the SAM samples that were excluded from the analysis.

6.2.3.6 B vitamin status

Plasma vitamin B_{12} and erythrocyte folate status were analysed using a radioimmunoassay technique, according to Müllner et al[555]. Standard curves were drawn and sample values calculated according to the protocol provided by the kit producer (MP Biomedicals, Germany). Coefficient of variance was calculated as 5.5% for folate and 5.6% for vitamin B_{12}. Inadequate plasma vitamin B_{12} and erythrocyte folate status were defined as <150 pmol/L and <340 nmol/L, respectively according to recommendations by the World Health Organization[517,556].

6.2.3.7 Dietary intake

Dietary intake was assessed by interview-based 24 h recalls, which were performed at baseline and after 6 months. The evaluation of the records was performed using the nutritional software NUT.S (Dato Denkwerkzeuge, Vienna, Austria), which is based on the German Food Composition Database Version II.3 (Berlin, Germany) but was adapted for Austrian eating habits through the addition of typical Austrian recipes.

6.2.3.8 Statistical analysis

Before analysis, normality was assessed graphically, and HOMA-IR, DMG and the DMG/betaine ratio were log-transformed for analysis. Non-transformed values were used to construct tables and graphs of summary statistics. All statistical analyses were performed using R 3.6.3 statistical software[474]. Unless otherwise specified, Alpha was set at $P < 0.05$, and data presented as mean and SD.

Baseline differences in markers of metabolic health and B vitamin status between males and females were assessed by Students *t*-test, and 1C metabolites by linear mixed models to correct for batch effect as a random factor. Baseline correlations between markers of metabolic health and 1C metabolites were assessed by Spearman rank correlation. Changes in markers of metabolic health and 1C metabolites were analysed by linear mixed models, where subject and batch-effect (1C metabolites only) were fit as a random factor, and time and intervention were fit as fixed factors, including an interaction term between time and intervention. For significant interaction terms, the Sidak adjustment was applied to correct for multiple comparisons. Multiple regression modelling was performed to evaluate associations between shifts in 1C metabolites and markers of metabolic health, and the effect of the intervention received on this. Change (baseline – follow-up) in each metabolic marker was set as the dependent variable, and change in each metabolite was set as the independent variable in the following models. Model 1 – unadjusted; Model 2 – adjusted (age, sex, GFR, baseline metabolite status, and BMI for non-anthropometric dependent variables); Model 3 – adjusted model including an interaction term between the independent variable (change in metabolite concentration) and the intervention group.

6.2.4 RESULTS

6.2.4.1 Participant characteristics

This secondary analysis of the VAAS Study included 95 participants at baseline, of which 67 had data available for 1C metabolites and cardiometabolic parameters at the six-month follow-up. Participants had an average age of 83 (±6) years and were predominantly female (88.4%), which reflects the gender distribution of this age group near their statistical life expectancy in the houses of the Curatorship of Viennese Retirement Homes (**Table 6.1**).

Table 6.1: Baseline status of one-carbon metabolites, cardiometabolic parameters and B vitamins

Characteristic	Variable	Total (*n* = 95)	Males (*n*= 11)	Females (*n*= 84)	*p*-value
	Age (y)	83.1 (5.8)	83.4 (5.2)	82.4 (6.7)	0.110
Metabolic health	Body mass index (kg/m^2)	29.1 (4.8)	26.9 (3.4)	29.4 (4.9)	0.045*
	Waist/Hip ratio	0.86 (0.07)	0.93 (0.04)	0.85 (0.07)	<0.001*
	Serum LDL-cholesterol (mmol/L)	3.17 (0.97)	2.77 (0.98)	3.22 (0.96)	0.178
	Serum HDL-cholesterol (mmol/L)	1.65 (0.44)	1.51 (0.36)	1.67 (0.44)	0.200
	Serum Total/HDL-cholesterol	3.47 (1.01)	3.23 (0.66)	3.50 (1.04)	0.240
	Serum triglycerides (mmol/L)	1.31 (0.51)	1.09 (0.34)	1.33 (0.52)	0.051
	Plasma glucose (mmol/L)	5.71 (1.10)	5.43 (0.84)	5.74 (1.13)	0.280
	HOMA-IR	2.41 (2.30)	1.65 (1.18)	2.51 (2.39)	0.064
One-carbon metabolites (plasma)	Betaine (µM)	30.8 (9.36)	35.0 (10.5)	30.3 (9.13)	0.193
	Choline (µM)	14.3 (2.59)	14.4 (3.49)	14.3 (2.48)	0.477
	Cysteine (µM)	103 (21.9)	104 (13.6)	102 (22.8)	0.539
	DMG (µM)	2.84 (1.26)	2.74 (1.25)	2.85 (1.27)	0.749
	Glycine (µM)	195 (48.1)	157 (25.6)	200 (48.2)	0.003*
	Homocysteine (µM)	2.20 (1.07)	2.00 (1.12)	2.23 (1.07)	0.943
	Methionine (µM)	22.45 (3.19)	24.3 (3.21)	22.2 (3.12)	0.061
	S-adenosylmethionine (nM)	56.6 (16.8)	53.3 (22.0)	57.1 (16.1)	0.555
	Serine (µM)	95.8 (26.6)	79.9 (16.1)	97.9 (27.0)	0.050
	Betaine/choline	2.20 (0.69)	2.44 (0.46)	2.16 (0.71)	0.211
	DMG/betaine	0.10 (0.05)	0.09 (0.06)	0.10 (0.05)	0.940
B vitamin status	Plasma Vitamin B_{12} (pmol/L)	380 (333)	460 (410)	370 (324)	0.456
	Prevalence of vitamin B_{12} inadequacy	21%	20%	30%	0.479
	Erythrocyte folate (nmol/L)	181 (107)	185 (95.4)	181 (109)	0.962
	Prevalence of folate inadequacy	93%	94%	89%	0.589
Dietary intake	Energy (kcal)	1556 (354)	1705 (298)	1544 (357)	0.176
	Protein (% energy intake)	16.2	17.3	16.1	
	Fat (% energy intake)	35.0	34.4	35.1	
	Carbohydrate (% energy intake)	48.8	48.3	48.8	
	Fibre (g)	21.7 (30.6)	21.7 (7.0)	21.7 (31.8)	0.176
	Protein (g/kg/body weight)	0.83 (0.30)	0.85 (0.24)	0.83 (0.27)	0.701
	Folate (µg)	261 (124)	300 (121)	257 (125)	0.450
	Riboflavin (mg)	1.17 (0.41)	1.02 (0.35)	1.18 (0.42)	0.347
	Vitamin B_6 (mg)	1.56 (0.78)	1.73 (0.47)	1.55 (0.80)	0.205
	Vitamin B_{12} (µg)	5.05 (3.03)	5.59 (3.64)	5.01 (2.99)	0.618

Data presented as mean (SD). All biochemical measures are fasting samples. *Indicates a significant difference (p <0.05) between sex for variables at baseline. † Inadequate plasma vitamin B_{12} was defined as <150 pmol/L according to the World Health Organization[556]. ‡ Inadequate erythrocyte folate was defined as <340 nmol/L according to the World Health Organization[517]. Abbreviations: DMG, dimethylglycine

6.2.4.2 Differences in B vitamins, one-carbon metabolites, and cardiometabolic parameters according to sex

At baseline, females had a higher BMI (p=0.045), but lower waist/hip ratio (p <0.001) than their male counterparts (**Table 6.1**). Minor differences were seen between 1C metabolite status at baseline; females had higher plasma glycine concentrations at baseline (p=0.003). No difference in B vitamin status (plasma vitamin B_{12} and erythrocyte folate) or adequacy was found between males and females. Notably, there was a high prevalence of folate inadequacy in the total population (93% with concentrations <340 nmol/L) (**Table 6.1**).

6.2.4.3 Baseline correlations: one-carbon metabolites and cardiometabolic parameters

According to Spearman correlation analyses, plasma cysteine positively correlated with BMI (ρ=0.202) and the waist/hip ratio (ρ=0.223) (**Table 6.2**). The waist/hip ratio was also positively correlated with both methionine (ρ=0.219) and glycine (ρ=0.398). HDL-cholesterol was positively correlated with glycine (ρ=0.345), and the total/HDL-cholesterol ratio with DMG (ρ=0.208) and DMG/betaine (ρ=0.230). Choline metabolites were also correlated with triglycerides, with an inverse correlation between both betaine (ρ= -0.300) and betaine/choline (ρ= -0.287), while a positive correlation was found with both DMG (ρ=0.233) and DMG/betaine (ρ=0.335). An inverse correlation with betaine (ρ= -0.254) and positive correlation with DMG/betaine (ρ=0.207) was also found for HOMA-IR, but plasma glucose was not correlated with 1C metabolites (**Table 6.2**).

Table 6.2: Baseline correlation analysis between one-carbon metabolites and cardiometabolic parameters
Correlation coefficients presented according to Spearman correlation analysis. * p <0.100, ** p <0.05, *** p <0.01.

Metabolites (plasma)	BMI	Waist/hip ratio	LDL-Cholesterol	HDL-Cholesterol	Total/HDL-Cholesterol	Triglycerides	Glucose	HOMA-IR
Betaine	-0.191*	0.180*	0.076	0.196*	-0.116	-0.300**	-0.086	-0.254**
Choline	-0.135	0.050	0.031	0.032	-0.035	-0.053	-0.201*	-0.104
Cysteine	0.202**	0.223**	-0.094	-0.167	0.061	0.158	0.053	0.091
DMG	0.043	-0.022	0.122	-0.083	0.208**	0.233**	-0.182	0.077
Glycine	0.071	-0.398***	0.044	0.345***	-0.173*	-0.128	-0.125	-0.063
Homocysteine	0.170	0.065	-0.080	-0.145	0.045	0.097	0.101	0.080
Methionine	-0.046	0.219**	-0.099	-0.004	-0.127	-0.199*	0.039	-0.088
SAM	0.143	0.134	-0.032	-0.238	0.206	-0.150	0.083	0.142
Serine	0.109	-0.088	0.078	0.074	0.004	-0.049	-0.092	-0.046
Betaine/choline	-0.100	0.146	-0.019	0.180*	-0.147	-0.287**	0.043	-0.176
DMG/Betaine	0.144	-0.057	0.081	-0.160	0.230**	0.335***	-0.068	0.207**

Abbreviations: DMG, dimethylglycine; SAM, *S*-adenosylmethoinine

6.2.4.4 Changes in one-carbon metabolites and cardiometabolic parameters according to intervention group

Across all intervention groups, an increase of the waist/hip ratio (time effect, p=0.008) and LDL-cholesterol (time effect, p=0.028) was found at the six-month follow-up, while a decrease in HOMA-IR was found for the three-month follow-up, but was not sustained at the six-month follow-up (time effect, p <0.001) (**Table 6.3**, **Figure 6.1**). In the control group, a reduction in plasma glucose was found at the three-month follow-up, but again was not sustained at the six-month follow-up (interaction, p=0.006). Plasma choline concentrations increased at the three-month follow-up only (time effect, p=0.013), cysteine at both the three- and six-month follow-ups, and Hcy and methionine at the six-month follow-up (time effect, p <0.001). An interaction effect was found for serine (p=0.024), however, post-hoc comparisons were not significant upon applying the Sidak correction (**Table 6.3, Figure 6.1**). Changes in B vitamin concentrations from baseline to follow-up have been presented elsewhere[546]. Of relevance to the present findings, an increase in erythrocyte folate and plasma vitamin B_{12} concentrations were reported to increase in the RTS group only.

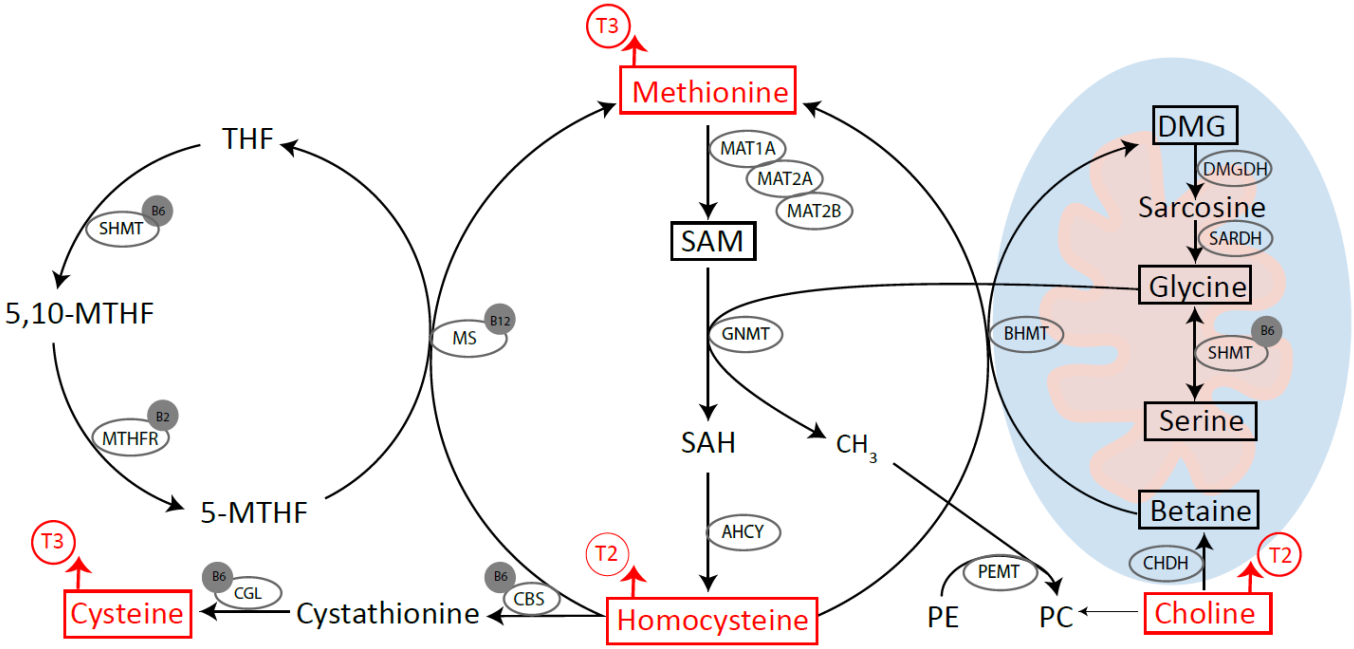


Figure 6.1: Overview of one-carbon metabolism and intervention effects on metabolite concentrations
This figure highlights the impact of intervention with resistance training (RT), resistance training with supplementation (RTS), or cognitive training (CT) for six months on one-carbon metabolites. Analysed metabolites are highlighted in bold and outlined in a box, B vitamins acting as coenzymes are in a grey circle, and enzymes are outlined in an oval. A red arrow indicates a change from baseline to follow-up (↑, increase; ↓, decrease) and time point (T2, three-month; T3, six-month) for metabolites where a significant time effect (p <0.05) was found in linear mixed models. No changes according to the intervention group were found for any metabolites (interaction, p >0.05). AHCY, S-adenosylhomocysteine hydrolase; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; CGL, cystathionine λ-lyase; CHDH, choline dehydrogenase; DMG, dimethylglycine; GNMT, glycine N-methyltransferase; MAT, methionine adenosyltransferase; MS, methionine synthase; PC, phosphatidylcholine; PE; phosphatidylethanolamine; PEMT, phosphatidylethanolamine methyltransferase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SARDH, sarcosine dehydrogenase; SHMT, serine hydroxymethyltransferase (SHMT1, cytosolic; SHMT2, mitochondrial); THF, tetrahydrofolate.

Table 6.3: Baseline, three- and six-month status of one-carbon metabolites and cardiometabolic parameters according to intervention group

		RT			RTS			CT			*p*-value	
Parameter	Marker	Baseline	FU-3mo	FU-6mo	Baseline	FU-3mo	FU-6mo	Baseline	FU-3mo	FU-6mo	Time	Interaction
Metabolic Health	BMI	29.0 (3.62)	28.9 (3.65)	29.0 (3.59)	29.3 (6.02)	27.7 (3.36)	29.1 (4.91)	28.9 (4.68)	27.4 (4.85)	28.3 (5.17)	0.154	0.982
	WHR	0.85 (0.06)	0.86 (0.06)	0.88 (0.06)†	0.85 (0.07)	0.84 (0.06)	0.86 (0.08)†	0.87 (0.07)	0.85 (0.07)	0.88 (0.05)†	0.008*	0.201
	LDL-C	123 (43.4)	121 (45.3)	128 (38.7)†	124 (33.5)	121 (36.8)	131 (41.9)†	124 (36.5)	120 (37.3)	126 (39.8)†	0.028*	0.974
	HDL-C	64.2 (16.5)	65.9 (16.9)	62.3 (13.9)	66.1 (15.6)	64.7 (18.2)	64.7 (17.2)	63.0 (19.0)	61.3 (14.5)	62.3 (16.8)	0.977	0.939
	Total/HDL-C	3.47 (1.18)	3.30 (0.82)	3.57 (0.88)	3.40 (0.91)	3.45 (0.95)	3.63 (1.12)	3.54 (0.92)	3.45 (0.81)	3.53 (0.83)	0.304	0.744
	Triglycerides	114 (49.0)	111 (47.6)	120 (46.0)	117 (37.8)	127 (48.3)	128 (42.7)	117 (48.6)	114 (50.3)	115 (41.5)	0.790	0.657
	Glucose	106 (24.1)	101 (20.2)	103 (17.8)	96.0 (10.5)	102 (15.2)	94.1 (12.3)	106 (20.7)	94.9 (14.6)†	97.8 (11.8)	0.001*	0.006#
	HOMA-IR	2.66 (2.99)	1.05 (1.56)†	2.60 (3.36)	2.24 (1.42)	1.06 (0.98)†	2.38 (1.93)	2.32 (2.20)	0.85 (0.92)†	2.06 (1.77)	<0.001*	0.989
1C metabolites (Plasma)	Betaine	29.3 (8.16)	32.8 (11.1)	30.9 (10.3)	31.1 (10.0)	32.2 (6.20)	32.9 (8.26)	32.2 (9.92)	33.0 (7.46)	30.0 (8.08)	0.623	0.316
	Choline	13.9 (2.66)	15.4 (2.50)†	13.9 (3.47)	14.3 (2.29)	15.4 (2.89)†	15.4 (3.08)	14.6 (2.81)	16.5 (3.01)†	15.5 (3.37)	0.013*	0.312
	Cysteine	102 (21.2)	109 (9.0)	115 (20.7)†	102 (23.3)	105 (18.2)	112 (23.0)†	103 (21.9)	112 (25.3)	114 (22.8)†	<0.001*	0.932
	DMG	2.53 (0.82)	2.50 (0.73)	2.42 (0.83)	3.14 (1.68)	2.67 (1.24)	3.34 (1.79)	2.88 (1.15)	3.20 (2.25)	2.80 (1.20)	0.624	0.231
	Glycine	200 (53.6)	204 (69.1)	207 (70.4)	203 (51.8)	215 (62.3)	200 (42.2)	184 (36.4)	183 (30.6)	185 (34.7)	0.323	0.812
	Hcy	2.23 (0.91)	2.39 (0.99)	2.52 (0.87)†	2.18 (0.89)	2.25 (0.91)	2.09 (0.68)†	2.19 (1.38)	2.58 (1.21)	2.64 (1.14)†	0.009*	0.264
	Methionine	22.7 (3.09)	24.6 (3.70)†	24.1 (3.95)†	22.6 (3.36)	25.7 (4.95)†	25.0 (3.99)†	23.1 (3.21)	23.1 (3.39)†	22.8 (3.47)†	<0.001*	0.274
	Serine	98.0 (26.7)	97.2 (19.6)	97.7 (26.5)	100 (30.9)	83.4 (13.7)	91.8 (25.1)	89.9 (21.3)	85.6 (13.3)	89.9 (16.6)	0.501	0.024#
	Bet/Cho	2.12 (0.44)	2.13 (0.54)	2.26 (0.61)	2.20 (0.73)	2.15 (0.57)	2.16 (0.53)	2.27 (0.84)	2.04 (0.49)	1.95 (0.39)	0.395	0.255
	DMG/Bet	0.10 (0.05)	0.08 (0.04)	0.09 (0.04)	0.10 (0.05)	0.09 (0.05)	0.11 (0.08)	0.10 (0.06)	0.10 (0.07)	0.10 (0.04)	0.609	0.434

Data presented as mean (SD). *p* values are presented for the main effect of time and interaction effect of time x intervention received. * Indicates a significant main effect of time according to analysis with mixed models (p <0.05), # Indicates a significant interaction effect according to analysis with mixed models (p <0.05), † Indicates which time points differ from baseline according to post-hoc comparisons adjusted with the Sidak correction (p <0.05). Abbreviations: 1C, one-carbon; Bet/Cho, Betaine/choline; BMI, body mass index; CT, cognitive training (control); DMG, dimethylglycine; DMG/Bet, DMG/betaine; FU-3mo, three-month follow-up; FU-6mo, six-month follow-up; Hcy, homocysteine; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; RT, resistance training; RTS, resistance training with supplementation; WHR, waist/hip ratio.

6.2.4.5 Longitudinal associations between changes in one-carbon metabolites and cardiometabolic parameters

Increasing Hcy concentrations were associated with a rise in the waist/hip ratio in both unadjusted (β=0.020, *p*=0.008) and adjusted models (β=0.020, *p*=0.042). A positive association between shifts in cysteine concentrations and the waist/hip ratio was significant in an unadjusted model only (β=8.2^{-4}, *p*=0.035). Increasing DMG concentrations were associated with a decline of BMI (β= -0.410, *p*=0.009) and LDL-cholesterol (β= -10.8, *p*=0.031), although only that with BMI remained significant when adjusted for confounding variables (β= -0.400, *p*=0.038). Increasing DMG was also associated with a rise in HOMA-IR in an adjusted model only (β=0.720, p=0.018). A positive association was also found between shifts in the ratio of total/HDL-cholesterol and DMG/betaine, a marker of upregulated betaine-dependent Hcy remethylation, in unadjusted (β=6.17, *p*=0.034) and adjusted (β=8.74, *p*=0.014) models (**Table 6.4**, **Table 6.5**).

The most consistent effect of the intervention was seen in markers of lipid metabolism. In the RTS group, increasing plasma concentrations of choline, cysteine, DMG, glycine, and serine were associated with a decline in the ratio of total cholesterol/HDL-cholesterol compared to the CT and RT groups (interaction, *p* <0.05) (**Figure 6.2)**. Similarly, increasing concentrations of choline, cysteine, DMG, and methionine were associated with reduced triglyceride concentrations in the RTS group compared to the CT and RT groups (interaction, *p* <0.05) (**Figure 6.2)**. Rising betaine and serine concentrations were also associated with a decline in HOMA-IR in the RTS group compared to the CT and RT groups. Increasing methionine and cysteine concentrations were associated with a decline in HOMA-IR in both the RTS and RT groups compared to those in the CT group. Full detail of these effects can be found in **Table 6.5**.

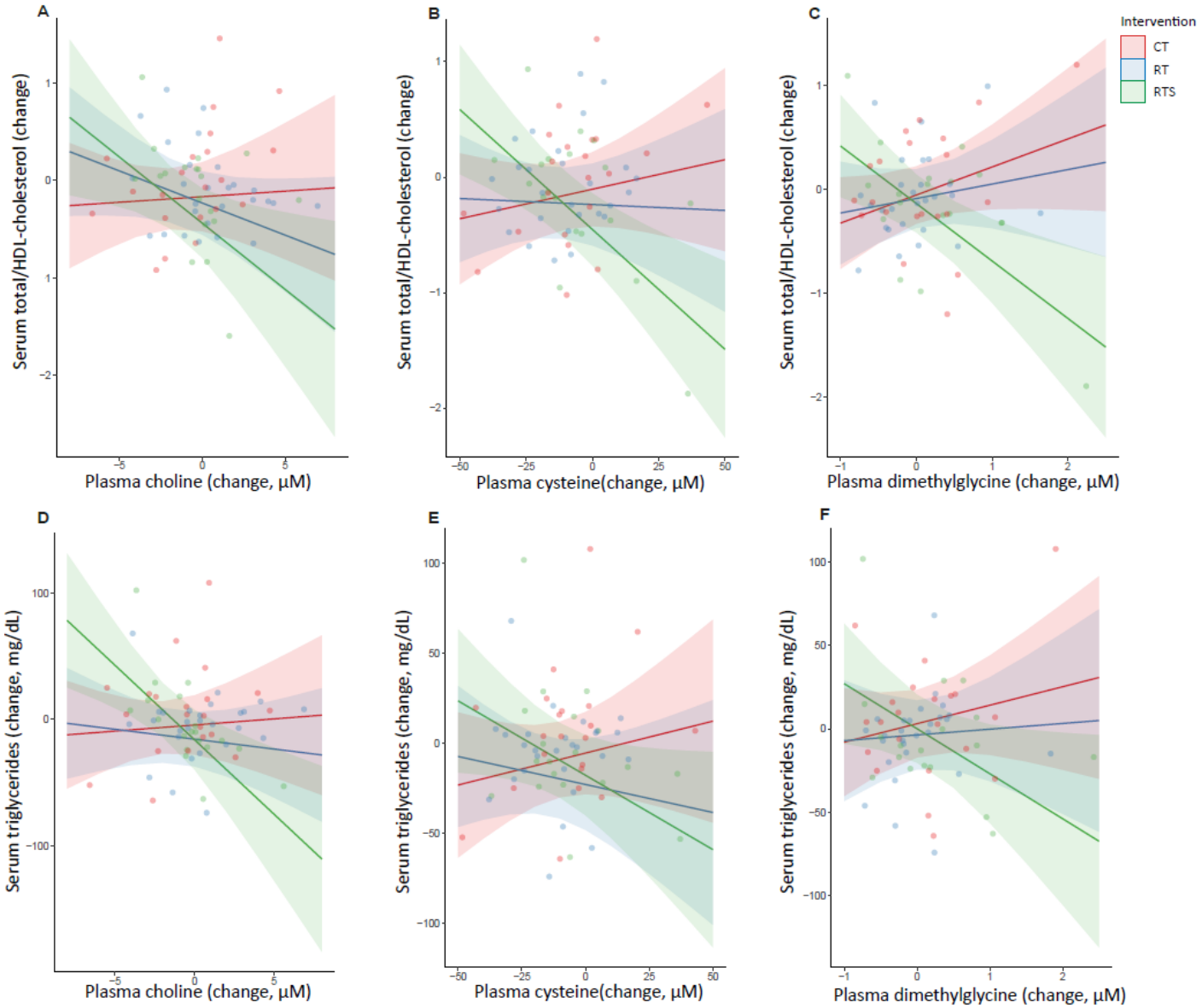


Figure 6.2: Effect of intervention on associations between six-month shifts in one-carbon metabolites and cardiometabolic parameters

Scatter plots are based on linear regression analyses with change (baseline – follow-up) in metabolites are set as dependent variables, and change in cardiometabolic markers are set as independent variables. Models are adjusted for confounding variables (age, sex, GFR, baseline metabolite concentrations and BMI), and include an interaction term between the intervention group (RT=resistance training, RTS=RT with supplementation, CT= cognitive training) and the dependent variable. Corresponding data is presented in Tables 6.4 and 6.5.

Table 6.4: Summary of the relationship between shifts in one-carbon metabolites and cardiometabolic parameters from baseline to six-month follow-up according to linear regression analysis

Metabolites (plasma)	Model	BMI		Waist/Hip ratio		LDL-C		HDL-C		Total/HDL-C		Triglycerides		Glucose		HOMA-IR	
		β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
Betaine	1	-0.020	0.273	0.001	0.297	-0.900	0.093	-0.030	0.853	-0.010	0.216	0.180	0.798	0.030	0.908	0.030	0.246
	2	-0.016	0.376	<0.001	0.547	-0.790	0.170	-0.033	0.832	-0.010	0.392	0.350	0.649	0.069	0.802	0.026	0.351#
Choline	1	0.020	0.763	-0.003	0.386	-0.560	0.716	0.340	0.516	-0.050	0.130	-2.990	0.134	-0.400	0.574	0.090	0.210
	2	0.018	0.742	-0.004	0.319	-1.090	0.529	0.340	0.571	-0.052	0.111#	-3.08	0.164#	0.520	0.492#	0.160	0.056#
Cysteine	1	0.001	0.891	0.01	0.035*	-0.190	0.313	-0.060	0.398	0.001	0.832	0.040	0.888	0.002	0.983	0.010	0.185
	2	0.002	0.755	<0.001	0.157	-0.320	0.184	-0.011	0.898	-0.006	0.166#	-0.220	0.473#	0.045	0.680	0.014	0.222#
DMG	1	-0.410	0.009*	0.010	0.534	-10.80	0.031*	-1.100	0.530	-0.060	0.566	-0.670	0.919	0.700	0.764	0.400	0.098
	2	-0.400	0.038*	0.009	0.510	-8.160	0.180	-2.410	0.257	-0.019	0.974#	-2.46	0.760#	4.02	0.175	0.72	0.018*
Glycine	1	<0.001	0.919	<0.001	0.782	0.090	0.357	-0.020	0.529	0.003	0.066	0.160	0.182	0.010	0.751	0.007	0.100
	2	-0.007	0.841	<0.001	0.588	0.094	0.368	-0.014	0.677	0.003	0.129#	0.170	0.200	0.021	0.657	0.006	0.222
Homocysteine	1	0.010	0.922	0.020	0.008*	-1.220	0.774	-1.620	0.264	0.050	0.505	1.610	0.770	0.800	0.682	0.180	0.373
	2	-0.002	0.990	0.020	0.042*	-2.740	0.587	-1.030	0.547	-0.019	0.838	-2.63	0.675#	1.65	0.473	-0.350	0.140
Methionine	1	0.020	0.602	0.002	0.343	-0.150	0.871	-0.250	0.436	0.020	0.387	0.600	0.617	0.650	0.126	0.160	<0.001*
	2	0.022	0.505	0.002	0.346	-0.820	0.412	-0.230	0.510	0.006	0.784	0.550	0.674	0.730	0.120#	0.140	0.002*, #
Serine	1	-0.010	0.266	<0.001	0.668	-0.001	0.774	-0.060	0.399	0.003	0.436	0.120	0.657	0.040	0.696	0.020	0.063
	2	-0.006	0.462	<0.001	0.576	0.062	0.848	-0.038	0.628	0.003	0.473#	0.100	0.757	0.092	0.416	0.019	0.103#
Betaine/choline	1	-0.320	0.201	0.030	0.104	-10.20	0.201	-2.830	0.305	0.030	0.837	13.80	0.186	2.430	0.512	0.190	0.614
	2	-0.280	0.337	0.022	0.240	-7.290	0.433	-3.05	0.338	0.130	0.456#	20.6	0.084	0.062	0.988	-0.096	0.826
DMG/betaine	1	-7.760	0.099	-0.120	0.700	20.70	0.891	-58.00	0.265	6.17	0.034*	101	0.608	-10.90	0.876	0.090	0.990
	2	-9.470	0.112	0.012	0.975#	161	0.395	-77.5	0.231#	8.74	0.014*	50.7	0.835	94.4	0.284	7.95	0.389

β estimates and *p* values presented for each metabolite fitted as an independent variable in unadjusted (Model 1) or models adjusted for age, sex, baseline metabolite status, GFR, and BMI for non-anthropometric dependent variables (Model 2). All independent and dependent variables refer to the change of each metabolite and metabolic marker (baseline - six-month follow-up). Further details for intervention effects are presented in **Table 6.5**. *Indicates an association between shifts in the dependent (change in metabolite concentration) and independent (change in cardiometabolic parameter) variables. # Indicates a significant interaction between the metabolite and intervention group. Abbreviations: DMG, dimethylglycine; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol.

Table 6.5: Effect of intervention with resistance training with or without nutritional supplementation compared to control on the association between changes in one-carbon metabolites and cardiometabolic parameters from baseline to six-month follow-up

Metabolites (plasma)		BMI		Waist/Hip ratio		LDL-C		HDL-C		Total/HDL-C		Triglycerides		Glucose		HOMA-IR	
		β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
Betaine	RT	-0.06	0.183	<0.01	0.157	-0.56	0.706	-0.75	0.136	0.03	0.360	0.77	0.691	-0.94	0.175	-0.07	0.326
	RTS	-0.02	0.650	<0.01	0.385	-1.29	0.370	0.20	0.684	-0.03	0.220	-0.88	0.639	-0.82	0.216	-0.17	0.013[#]
Choline	RT	-0.11	0.239	<0.01	0.971	-3.85	0.205	-0.06	0.956	-0.07	0.178	-2.51	0.485	-3.14	0.015[#]	-0.34	0.016[#]
	RTS	<0.01	0.944	-0.01	0.320	-2.24	0.530	0.85	0.484	-0.15	0.025[#]	-12.8	0.004[#]	-2.21	0.135[†]	-0.21	0.180[†]
Cysteine	RT	<0.01	0.467	<0.01	0.233	<0.01	0.976	0.11	0.488	<0.01	0.490	-0.65	0.259	-0.15	0.481	-0.05	0.013[#]
	RTS	<0.01	0.314	<0.01	0.705	-0.52	0.249	0.27	0.083	-0.03	0.002[#]	-1.16	0.039[#,§]	-0.29	0.162	-0.06	0.005[#]
DMG	RT	-0.05	0.910	-0.02	0.541	11.4	0.398	3.97	0.395	-0.13	0.594	-7.58	0.662	-6.50	0.299	-0.66	0.302
	RTS	-0.19	0.627	0.04	0.183	-10.2	0.419	7.70	0.081	-0.81	<0.001[#,‡]	-37.8	0.023[#]	0.56	0.924	-0.90	0.137
Glycine	RT	<0.01	0.621	<0.01	0.510	-0.21	0.355	<0.01	0.194	-0.01	0.016[#]	-0.55	0.052	-0.10	0.310	-0.02	0.067
	RTS	<0.01	0.174	<0.01	0.993[†]	-0.48	0.060	0.16	0.067	-0.02	<0.001[#]	-0.61	0.055	-0.14	0.227	-0.02	0.152
Homocysteine	RT	0.410	0.309	0.02	0.405	11.3	0.376	1.12	0.794	0.06	0.810	0.47	0.976	2.94	0.598	-0.57	0.331
	RTS	-0.02	0.959	<0.01	0.930	9.32	0.483	1.67	0.710	-0.09	0.709	-6.21	0.705[§]	-3.69	0.522	-0.87	0.150
Methionine	RT	-0.12	0.101	0.01	0.191	-2.03	0.386	-0.40	0.625	-0.04	0.377	-3.65	0.223	-2.29	0.030[#]	-0.34	0.001[#]
	RTS	-0.12	0.134	-0.01	0.214	-1.39	0.579	0.44	0.610	-0.08	0.084	-6.57	0.044[#]	-0.29	0.011[#]	-0.28	0.007[#]
Serine	RT	-0.02	0.289	<0.01	0.271	0.12	0.812	<0.01	0.560	-0.01	0.347	-0.94	0.152	-0.20	0.382	-0.04	0.071
	RTS	<0.01	0.507	<0.01	0.488	-0.51	0.425	0.21	0.318	-0.03	0.021[#]	-0.73	0.364	-0.16	0.567	-0.06	0.029[#]
Betaine/choline	RT	0.32	0.712	0.07	0.171	37.3	0.164	-8.27	0.367	1.25	0.012[#]	34.7	0.306	10.3	0.382	0.95	0.431
	RTS	0.27	0.753	0.07	0.160	12.0	0.649	3.74	0.678	0.65	0.175	50.4	0.135	-0.16	0.567	-1.31	0.273[†]
DMG/betaine	RT	-1.77	0.919	-3.13	0.002[#]	710	0.203	400	0.030[#]	-9.29	0.354	-860	0.217	10.3	0.382	1.77	0.947
	RTS	-18.7	0.295	0.01	0.928	270	0.639	170	0.363	-9.60	0.352[‡]	-1200	0.098	-2.56	0.826[†]	17.4	0.531

β estimates and *p* values are presented for the interaction term fitted between the independent variable (change in metabolite concentration from baseline to six-month follow-up) and intervention group (receiving the resistance training or resistance training with supplementation intervention compared to the control group). Data presented from each model is adjusted for age, sex, baseline metabolite status, GFR, and BMI (non-anthropometric variables). [#] Indicates a significant difference according to intervention, [†] Indicates that the dependent variable is inversely associated with baseline metabolite status in the corresponding model, [‡] Indicates that the dependent variable is lower in males in the corresponding model, [§] Indicates that the dependent variable is positively associated with baseline metabolite status in the corresponding model. Abbreviations: DMG, dimethylglycine; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol.

6.2.5 DISCUSSION

This study is a novel investigation into the impact of lifestyle interventions on a comprehensive profile of 1C metabolites and cardiometabolic parameters in a senior population (65-98 years). Metabolites reflecting upregulated betaine-dependent Hcy remethylation were associated with an unfavourable lipid profile at baseline. However, increasing DMG, cysteine, and choline concentrations were associated with improved lipid parameters in those receiving nutritional supplementation, which likely reflects underlying shifts in methylation status and choline availability, both of which play a critical role in lipid metabolism. This study highlights the complex relationship between 1C metabolism and cardiometabolic health, which choline metabolites appear central to. Notably, our findings highlight that the relationship between 1C metabolites and cardiometabolic health may be modified by resistance training combined with nutritional supplementation. These findings bolster the relevance of 1C metabolites in healthy ageing, and further research is required to understand whether optimising 1C metabolite status might confer cardiometabolic and related benefits in very old adults.

Hcy is an established risk factor for cardiovascular disease[220]. Despite growing interest in the association between Hcy and the metabolic syndrome and its components, this has yielded inconsistent findings and is poorly characteristed[235,347,348,350,351,557].

While associations between Hcy and cardiometabolic markers were limited, metabolites reflective of betaine-dependent Hcy remethylation were more closely associated with parameters of glucose and lipid metabolism. Our findings indicate that upregulation of the BHMT pathway to support Hcy remethylation is associated with an unfavourable cardiometabolic profile; betaine and the ratio of betaine/choline were inversely correlated with HOMA-IR and triglycerides, while DMG and the ratio of DMG/betaine were positively correlated with triglycerides, total/HDL-C and HOMA-IR. Shifts in DMG concentrations were associated with changes in cardiometabolic markers, yet the direction of response was inconsistent, as increasing DMG concentrations were associated with improvements in LDL-C and BMI, but worsening insulin sensitivity in adjusted models. These findings in part support previous reports of betaine being associated with a more favourable cardiometabolic profile, although based on the available evidence, we would have expected to find a more divergent association between betaine and choline with these metabolic markers rather than between betaine and DMG [359–362]. There is a relative paucity of literature regarding DMG, although lower concentrations have previously been associated with higher blood glucose, increased insulin resistance, and an increased risk of incident diabetes [368]. The only known pathway for DMG synthesis results from betaine donating a methyl group for the remethylation of Hcy, and thus is considered to reflect BHMT activity [558,559]. Upregulation of this pathway is evidently implicated in numerous aspects of cardiometabolic risk, which, for the most part, appears to be an unfavourable relationship.

Interestingly, supplementation modified the longitudinal relationship between shifts in these markers of Hcy remethylation and lipid metabolism. Increasing DMG, choline, and cysteine concentrations were associated with improvements in the lipid profile only in those receiving supplementation. Although we are unable to provide further insight into mechanisms, this is plausibly mediated by shifts in methylation status and choline availability for the synthesis of phosphatidylcholine. With increased flux through BHMT, phosphatidylcholine synthesis from choline is restricted, as there is greater demand for choline oxidation to support betaine availability. In turn, this places an increased demand on the synthesis of phosphatidylcholine from phosphatidylethanolamine, which requires labile methyl groups [560]. Indeed, betaine supplementation has

been shown to increase hepatic phosphatidylcholine concentrations and the ratio of phosphatidylcholine/phosphatidylethanolamine[561]. In another study, betaine supplementation improved whole-body glucose homeostasis and energy expenditure[562]. Supplementation with krill oil has been shown to reduce triglycerides, which was suggested to be through increased phosphatidylcholine intake (1750 mg/day) and choline availability leading to enhanced processes dependent on methyl donors, including flux over BHMT [176]. The Fortifit supplement used in the current study contains only a small amount of choline (55 mg). Although B vitamin supplementation may increase choline status [563], greater supplemental concentrations, or a longer follow-up period may have been required to see similar improvements in the lipid profile of our participants.

While increasing cysteine concentrations were associated with favourable changes in lipid parameters in those receiving supplementation, this should be considered alongside the relationship between cysteine and body composition. Cysteine concentrations were correlated with higher BMI and waist/hip ratio at baseline. Increasing cysteine concentrations were also associated with a rise in waist/hip ratio during the six-month follow-up in simple regression analyses regardless of the intervention received, but not when adjusted for confounding variables. These findings in part align with those from the Hordaland Homocysteine study [535,536], where cysteine was positively associated with BMI and fat mass in both cross-sectional and longitudinal analyses in a large cohort of middle-aged and older adults [358]. Beyond cysteine's link to methylation status, mechanisms have been extensively reviewed and proposed by Elshorbagy *et al* [358]. Our findings support the role of cysteine in body composition, but require careful interpretation in elderly populations, as there is a shift upwards in the BMI range that is considered healthy and protective against mortality in older adults [564]. Vitamin B_6 is required for cysteine synthesis (through cystathionine-β-synthase and cystathionine-λ-lyase), yet vitamin B_6 deficiency is prevalent amongst elderly populations [565,566]. The balance is complex, and further studies are required to better understand the mechanisms underlying the role of cysteine in the regulation of body composition and lipid metabolism, particularly in vulnerable populations that require special consideration, such as those in their oldest years.

It is interesting to note that Hcy did not decline in those receiving the RT or RTS intervention, an observation previously been reported with both B vitamin supplementation [155,156] and RT [541,542] in older adults. This might be explained by the nutritional supplement used here, which contained a moderate amount of 200 µg folic acid per serve compared to 400 µg shown to reduce Hcy in other elderly populations [155,156]. The Fortifit supplement also provided 20.7 g of protein per serve, thus increasing the supply of dietary methionine. Further, participants in the current study were older (mean, 83 ±6 years) than other elderly populations who have shown a decline in Hcy with RT interventions (range, 60-80 years) [541,542]. However, caution should be given to the interpretation of these findings given the analytical technique used. While the mass spectrometry technique used has been peer-reviewed with good internal reproducibility [379], the concentrations of Hcy are below expected reference ranges [567] which limits the clinical interpretation of our results. The discrepancy in Hcy concentrations can be explained by a lower concentration of Tris (2-carboxyethyl) phosphine used, an agent used in sample preparation to reduce disulfide bonds in cystine and homocystine to allow quantification of cysteine and Hcy, compared to other studies [388].

This study provides valuable insight into the response of 1C metabolism to intervention those in their oldest years of life. A key strength of our study lies in the elderly population included (83 ± 6 years). Octogenarians and nonagenarians are the fastest growing proportion of our ageing population [2], yet are under-represented

in research on 1C metabolism. Lifestyle interventions, such as supplementation and exercise, are promoted to optimise health in this vulnerable population, yet to our knowledge, this is the first study to investigate the effect of such interventions on cardiometabolic parameters in advanced age. These results, of course, should not be generalised to early agers where B vitamin status might be expected to be more adequate than in our cohort. Further, while the mass spectrometry technique used here gave a comprehensive profile of plasma 1C metabolites, but does not quantify sarcosine, trimethylamine N-oxide, or other choline species (e.g. phosphatidylcholine). The inclusion of these metabolites would help to interpret the findings in the current study, as would quantifying urinary 1C metabolites [365].

6.2.6 CONCLUSION

This study highlights the complex relationship between 1C metabolites and cardiometabolic health in an elderly institutionalised population, which choline metabolites were central to. While RT or RTS did not improve plasma 1C metabolites or cardiometabolic risk profiles after six months, more subtle changes in pathway regulation were indicated. 1C metabolites reflecting upregulated betaine-dependent Hcy remethylation were correlated with an unfavourable cardiometabolic profile. However, the direction of association between these 1C metabolites and the lipid profile was modified in those receiving RT with nutritional supplementation. These findings highlight the potential to promote successful ageing through improving health outcomes underpinned by these interconnected pathways, however further research is required to demonstrate the potential of optimising 1C metabolite status such that cardiometabolic benefit is conferred in older adults.

DIETARY INTERVENTION

OptiMuM study

7

CHAPTER 7: ONE-CARBON METABOLITES FOLLOWING 10-WEEKS OF A HIGH PROTEIN DIET

7.1 PREFACE

This chapter presents the final analysis in this thesis to understand the responsiveness of 1C metabolites to shifts in B vitamin intake. Chapters 6 and 7 both consider 1C metabolite responses in the context of interventions already recommended to promote health and longevity with advancing age. This research therefore aligns with the broader priorities for research on healthy ageing, which is to develop strategies that will confer multiple health benefits to older adults. It is hoped that these findings will advance the narrow approach to optimising B vitamin status previously employed.

Higher protein diets are increasingly recommended to promote skeletal muscle mass and physical function amongst older adults, but have traditionally been thought to unfavourably impact Hcy regulation due to the increased dietary methionine supply. It is recognised that increasing protein intake through food might have some benefit for 1C metabolism through the package of co-regulatory nutrients contained within animal-based proteins. However, the impacts on 1C metabolites, particularly beyond Hcy, have received very little attention.

Chapter 7 reports on secondary outcomes of the OptiMuM (Optimal nutrition in the elderly: High protein diets for muscular, metabolic, and microbiome health) study, where the primary outcome was to evaluate the acute differences in skeletal muscle mass and function in healthy ageing males. The current chapter presents data on the 1C metabolite response to increasing protein intake through a whole-food diet for ten weeks in older men compared to the current protein recommendations. This study was completed in April – October 2016 at the Liggins Institute prior to my candidature, and so I was not involved in the completion of the trial. For this chapter specifically, I was responsible for performing the mass spectrometry analysis for 1C metabolites, analysing participant's habitual and intervention dietary intake, and writing the manuscript as the first-author.

This chapter contains an altered version of the manuscript "Responsiveness of one-carbon metabolites to a high protein diet in older men: results from a 10-week randomised controlled trial", co-authored by Nicola A. Gillies, Amber M. Milan, Pamela P.H. Chia, Pankaja Sharma, Sarah M. Mitchell, Nina Zeng, Farha Ramzan, Randall F. D'Souza, Cameron J. Mitchell, Scott O. Knowles, Stephanie Andraos, Anders Sjödin, Karl-Heinz Wagner, Nicole C. Roy, and David Cameron-Smith. This manuscript has been accepted for publication in *Nutrition Journal*, which has a 2019/2020 impact factor of 3.359, and a five-year impact factor of 4.630.

7.2 MANUSCRIPT:

Responsiveness of one-carbon metabolites to a high protein diet in older men: Results from a ten-week randomised controlled trial

7.2.1 ABSTRACT

Dietary strategies to promote successful ageing are divergent. Higher protein diets are recommended to preserve skeletal muscle mass and physical function. Conversely, increased B vitamin intake, supporting 1C metabolism, reduces the risk of cognitive decline and cardiovascular disease. On the hypothesis that higher protein intake through animal-based protein sources will benefit 1C regulation due to the supply of B vitamins (folate, riboflavin, vitamins B6 and B12) and methyl donors (choline, methionine) despite higher methionine intake. Due to the complex supply of co-regulatory nutrients present in animal-based proteins, this study explored the impact of a higher protein diet on 1C metabolite status in elderly males compared to current protein recommendations. Elderly men (74 ± 3 years) were randomised to receive a diet for 10-weeks containing either the recommended dietary allowance of protein (RDA, 0.8g protein/kg body weight/day, n=14), or double protein recommendations (2RDA, n=15), with differences in protein accounted for by modifying carbohydrate intake with a higher ratio of animal:plant protein sources. Intervention diets were matched to individual's energy requirements based on the Harris-Benedict equation, and adjusted fortnightly as required depending on physical activity and satiety. Fasting plasma 1C metabolite concentrations were quantified by liquid chromatography coupled with mass spectrometry at baseline and after 10-weeks of intervention. Plasma Hcy concentrations were reduced from baseline to the 10-week follow-up with both diets (time effect, $p=0.002$). Changes in metabolite ratios, reflective of betaine-dependent Hcy remethylation were specific to the RDA diet, with an increase in betaine/choline and a decline in DMG/betaine (interactions, $p<0.05$). Comparatively, increasing folate intake was positively associated with a change in choline concentration and inversely with betaine/choline for those in the 2RDA group (interaction, $p<0.05$). Adding to the known benefits of higher protein intake in the elderly, these findings, this study which might suggest favourable regulation of 1C metabolism supports a reduction of Hcy with increased consumption of animal-based protein, thought the health impacts of differential response of choline metabolites to a higher protein diet remains uncertain. These findings add to the known benefits of higher protein intake in the elderly, and thus show potential to harmonize dietary advice that promotes successful ageing.

7.2.2 INTRODUCTION

The global population is rapidly ageing, with 16% of the population projected to be aged 65 years or older by 2050[2]. Ageing poses significant challenges to health care systems, due to the projected parallel increase in the prevalence of chronic diseases[6]. There is a need to identify and harmonise dietary advice to offer the most substantial health benefits to the aged.

Dietary strategies to counter age-related processes are divergent, reflective of major knowledge gaps that exist. For instance, higher protein intake is promoted to preserve skeletal muscle mass and function[40,568,569]. Thus, experts have proposed that protein recommendations need to be increased for older adults to almost twice that of current recommendations from global health agencies like the World Health Organization[40]. At the same time, evidence continues to emerge regarding the importance of achieving adequate B vitamin intake to optimise 1C metabolism in older age[52]. Although similar consensus statements are not yet available, experts have highlighted the potential of increasing B vitamin intake to reduce the risk of cognitive decline[5,308,491] and cardiovascular disease[220,221].

Hcy sits at a critical branch-point in 1C metabolism, where the folate and methionine cycles converge[570]. Hcy is accordingly considered a marker of overall 1C metabolism, which is frequently associated with age-related diseases, including increased risks for cardiovascular disease[220,221], dementia [253], and osteoporosis[571,572]. Given that elevated Hcy concentrations are common in older adults[52], the development of dietary advice to reduce circulatory Hcy and improve 1C regulation in older adults is important but unresolved – in part due to the complex regulation of Hcy. Hcy either enters the vitamin B_6-dependent transsulfuration pathway, or is remethylated to methionine which can then be converted to SAM. Remethylation can occur through two inter-connected pathways, either by methionine synthase which requires folate as a methyl donor and cobalamin (vitamin B_{12}) as a co-enzyme, or via BHMT which uses betaine as a methyl donor[125,573].

It is well established that B vitamin supplementation can improve 1C metabolite status[149,152]. Dietary intervention studies to improve 1C metabolite status have largely been focused on reducing circulating Hcy by increasing dietary folate intake through fruit and vegetable intake[194–198]. A comparable decline in Hcy is possible with betaine[171,172,574] and choline[173] supplementation, though evidence is scarce beyond an acute Hcy reduction[172]. Beyond micronutrients, evidence also suggests that the macronutrient composition of diets will impact 1C metabolite status. Hcy was inversely associated with complex carbohydrate intake and positively with fat intake in the Hordaland Homocysteine cohort, even after adjustment for dietary and plasma B vitamins[147], while the inverse relationship between protein and Hcy appears to be mediated by the B vitamin content of protein foods[189]. Despite the multifaceted nature of how diet regulates 1C metabolism, there is a paucity of data describing the role of wider dietary changes to optimise 1C metabolite status, and particularly in older adults who have greater risk for impaired B vitamin absorption and macronutrient metabolism[63]. Further, the impact of dietary modifications on closely interrelated 1C metabolites beyond Hcy, which holds valuable information on pathway regulation, remains unclear.

There is opportunity here to harmonise dietary advice for older adults, as dietary patterns higher in protein appear to be a promising strategy to reduce plasma Hcy concentration from both observational[188] and intervention data[203]. Despite prior concerns that increased methionine intake, the sole dietary precursor of Hcy, through animal-based proteins would lead to elevated Hcy, this evidence largely comes from methionine loading studies that increase postprandial[183–185], but not fasting Hcy[186,187]. Further, isolated changes in methionine intake does not reflect habitual protein intake, nor other nutrients in protein sources which

modulate the 1C metabolic response. Based on available evidence, increasing dietary protein intake through animal-based protein sources is hypothesised to reduce Hcy, owing to the supply of co-regulatory nutrients, including B vitamins (riboflavin, vitamins B_6 and B_{12}), choline, and amino acids which modulate the response to fluctuations in dietary methionine intake[187,575].

The objective of this study was to evaluate impact of increasing animal-based protein sources on 1C metabolite status, both to absolute changes in plasma 1C metabolite concentrations and to the relationship between B vitamin intake and 1C metabolite status.

7.2.3 METHODS

7.2.3.1 Study design

Participants were randomly assigned to treatment groups by sequences generated in www.random.org using a parallel-group design. Participants were allocated (1:1 ratio) using a locked spreadsheet to consume either the current recommended dietary allowance (RDA, 0.8g protein/kg body weight/day) or twice the RDA (2RDA, 1.6g protein/kg body weight/day) of protein for ten weeks, according to recommendations from global health agencies including the World Health Organization[576] and USDA[577]. Investigators were not blinded as they were responsible for diet preparation. Although participants were not advised of treatment allocation, they may have determined which group they were assigned to, based on their provided meals and snacks. Informed written consent was obtained from all participants before they were enrolled in the trial. The study was approved by the Southern Health and Disability Ethics Committee (New Zealand; 15/STH/236) and was conducted in accordance with the Declaration of Helsinki. The study was prospectively registered with the Australian and New Zealand Clinical Trial Registry (www.anzctr.org.au) as ACTRN12616000310460.

This analysis reports on secondary exploratory outcomes of the OptiMuM study. The primary outcome of the OptiMuM study was to evaluate differences in skeletal muscle mass and function in healthy ageing males following ten weeks of a diet containing protein at either current recommendations or twice that [578]. The secondary outcomes reported here included assessment of plasma 1C metabolite concentrations and B vitamin intake.

7.2.3.2 Participants

Thirty-one healthy older men (>70 years) were recruited to participate in the study by using advertisements in local newspapers. Participants were eligible if they were non-smokers, not taking regular dietary supplements for at least one month prior to the trial (three participants reported having taken multivitamin supplements on occasion), if their BMI was between 18-35 kg/m^2, and if their activities of daily living were performed independently without mobility aids. Participants were ineligible if they had a prior history of major diseases (including cancers, diabetes, and thyroid diseases). Participants with conditions affecting skeletal neuromuscular function, or those completing over 4h per week of structured physical activity (organised sport, resistance training, and vigorous-intensity aerobic exercise) were also excluded from the study, as were those with restrictive eating habits (e.g. vegetarians, and those with intolerances and allergies). Participants were recruited from Auckland, New Zealand, and all testing was conducted at the Liggins Institute (University of Auckland) between April and October 2016.

7.2.3.3 Intervention

The study procedure has been described in details previously[578]. Participants had all breakfasts, lunches (Muscle Chow NZ Ltd., New Zealand), dinners (Farmhouse Foods Ltd, New Zealand), and snacks delivered to their homes during the ten-week intervention period, and were instructed to maintain their normal lifestyle. All items were portioned, and only required minimal preparation or re-heating. Participants were able to drink water, tea, and coffee without restrictions. Participants were able to self-select options from a range of 7 breakfast, 18 lunch, and 9 dinner meals, with alterations to suit personal preferences made where feasible in order to maintain high compliance.

The energy content of each participant's intervention diet was individually calculated to match their estimated energy requirements. Individual energy requirements were based on the Harris-Benedict equation and adjusted for physical activity level [579,580]. Physical activity was assessed by wrist-worn accelerometers (Fitbit Charge HR) worn for 5-day periods prior to the start of intervention and at weeks 5 and 10. Energy intake was adjusted fortnightly based on physical activity level and self-reported satiety.

Participants were randomly assigned to consume either 0.8g protein/kg body weight/day (RDA group) or 1.6g protein/kg body weight/day (2RDA), with differences in protein intake accounted for by modifying carbohydrate intake. For both the RDA and 2RDA diets, dietary protein was provided from a diverse range of sources, including vegetarian proteins (whole grains, legumes, nuts, dairy products), white meat (chicken, fish), and red meat (beef). For the RDA diet, the portions of animal-based proteins were provided in smaller quantities, and as previously reported [578], the ratio of animal protein sources to plant protein sources was higher in the 2RDA diet. Both treatment groups adhered to the dietary guidance provided by the New Zealand Eating and Activity Guidelines [581], meeting minimum recommendations for major food groups (fruits and vegetables, grains, dairy products and alternatives, meat and alternatives).

Compliance records were checked at weeks 5, 6, 9, 10 of the study, where participants indicated the portion of each meal they consumed, and reported any non-provided study food that they might have consumed.

7.2.3.4 Dietary analysis

Participants were provided with detailed instructions on how to complete a three-day food record prior to the intervention commencing. Participants were instructed to weigh their food when possible, but could describe quantities consumed by either standard household measures or by visual guides provided with the food records if they were unable to do so. All records were checked for completeness by investigators. As all food was provided to participants, follow-up dietary intake was estimated using participants' seven-day personalised meal plan from week 10 of the intervention, which was representative of foods consumed in earlier weeks of the intervention period. Food and beverage intakes at both baseline and follow-up were analysed by a Registered Dietitian using FoodWorks (Version 9; Xyris, Australia), which aligns with the New Zealand Food Composition Database (New Zealand FOODfiles 2016, Version 01). As coffee intake has previously been reported as a determinant of Hcy[188], coffee consumption was evaluated at baseline according to cups of coffee consumed per day in participants' three day food records (RDA, 1.3 ± 1.3 cups; 2RDA, 1.2 ± 1.4 cups). Coffee consumption was not measured at follow-up, but it can be assumed that this would remain consistent as participants were able to freely consume coffee during the intervention.

Dietary intake was categorised into 17 food groups based on those previously published by Bohlscheid-Thomas *et al*[582], and Playdon *et al*[583] to evaluate differences in the profile of foods consumed between intervention groups. Briefly, average daily intake of foods in each of these food groups was calculated based on each participant's seven-day intervention diet record. An energy-adjusted variable was used for the average intake (g) of each food group based on nutrient residuals as described by Willett *et al*[584] to reduce variation from total energy intake. Participants' intake of B vitamins were compared to the RDI according to the Nutrient Reference Values for Australia and New Zealand[87]. The number and proportion of individuals from each intervention group considered inadequate were calculated at baseline and follow-up.

7.2.3.5 Biochemical analysis

On the morning of participant's study visit at baseline and the 10-week follow-up, blood samples were drawn from an antecubital vein by cannula at approximately 7am following an overnight fast. Blood samples were collected into EDTA-coated vacutainers, which were centrifuged at 1900*g* for 15 minutes at 4°C. Samples were then separated into aliquots and stored in Eppendorf tubes at -80°C until required for analysis, and thawed only immediately prior to analysis.

UHPLC-MS/MS was performed to determine plasma concentrations of 11 metabolites (betaine, choline, DMG, SAH, and SAM) and amino acids (cysteine, cystathionine, glycine, Hcy, methionine, and serine) involved in the regulation of 1C metabolism. Metabolite ratios were calculated to investigate product/precursor relationships for betaine/choline and DMG/betaine. These ratios of choline metabolites have previously been used as an index of endogenous betaine synthesis and its use, allowing inference of betaine-dependent remethylation of Hcy [119,120,585].

The methods are reported in detail elsewhere[379]. Briefly, plasma samples were prepared using an automated robotic liquid handling system (Eppendorf epMotion® 5075vt, Hamburg, Germany). First, 300µl of 1% formic acid in methanol was pipetted into a 96-well IMPACT® protein precipitation plate (Phenomenex, Torrance, California, USA). Next, all standards (100µl), quality controls (100µl) and samples (100µl) were spiked with 20µl of internal standard solution (labelled amino-acid premix solution, *Cambridge Isotope*; and labelled 1C compounds, SciVac PTY. Ltd. (CDN)), agitated for 5 minutes (800rpm), then filtered into a square (2mL) 96-well square collection plate (Phenomenex, Torrance, California, USA) by applying a vacuum (450mbar). 100µl

of Tris (2-carboxyethyl) phosphine was then dispensed into each well to allow for the separate quantification of Hcy and cysteine. Three sets of QC samples were included to assess both the recovery of standards and reproducibility of samples. Metabolites were considered acceptable if standard recoveries were between 80-120% and coefficients of variance were below 20%. Cystathionine and SAH did not satisfy these requirements and were excluded from further analysis.

Serum folate, erythrocyte folate, and serum vitamin B_{12} concentrations were measured by a Cobas e411 autoanalyser (Roche Diagnostics, Mannheim, Germany).

7.2.3.5 Statistical analysis

Sample size estimates for the OptiMuM study were calculated for the primary outcome of whole body lean mass, leading to a sample size estimate of 15 participants per group with a power of 80% and an alpha of 5% [(578)]. Prior to analysis, histograms were plotted to assess normality, with all metabolites showing a normal distribution. All statistical analyses were performed using the R programming environment version 3.6.3[(474)]. Alpha was set at P <0.05, and data presented as mean and SD unless otherwise stated.

One-way analysis of variance was used to evaluate whether average intake (g, energy-adjusted) of food within each pre-defined food group, macronutrients and relevant vitamins (folate, riboflavin, vitamins B_6 and B_{12}) differed between intervention groups. Linear mixed models were used to analyse the effects of the intervention group (RDA compared with 2RDA) and time (baseline compared with follow-up) on markers of 1C metabolite status. Using the “lme4” package[(586)], intervention group and time were defined as fixed factors, and subject as a random (repeated) factor, with an interaction term between intervention group and time included. Post-hoc comparisons were made if a significant interaction was found using the “emmeans” package [(587)] with the Sidak correction applied for multiple comparisons.

Multiple linear regression modelling was used to examine the impact of changes in dietary intake of each B vitamin on plasma 1C metabolites, and to investigate whether this relationship was modified by the diet received or confounding variables known to have an association with 1C metabolites (age, alcohol intake, medication use known to influence vitamin B_{12} metabolism, plasma creatinine, BMI, baseline metabolite concentration). Due to the small sample size used, backwards stepwise regression was performed and confounding variables were included in adjusted models if they were associated with a change in the concentration of the metabolite of interest from bivariate analysis (p <0.15), details of which are reported in Table 7.5. In each model, change in nutrient intake was set as an independent variable (each nutrient was assessed independently across all models), and change in concentration of each metabolite was set as the dependent variable. Model 1a was unadjusted, and Model 1b was unadjusted and included an interaction term between nutrient intake and intervention group. Model 2a was adjusted for confounding variables, and Model 2b was adjusted for confounding variables and included an interaction term between nutrient intake and intervention group. All regression models checked for normality of residuals, linearity, and homogeneity of variance.

7.2.4 RESULTS

7.2.4.1 Participant characteristics

In total, 29 elderly male participants (74.2 ± 3.6 years) completed the ten-week dietary intervention and were included in the final analysis, as previously described [578]. One participant withdrew prior to the intervention (RDA), and one participant was excluded from follow-up due to non-compliance (2RDA). No differences in participant characteristics at baseline were observed between intervention groups (**Table 7.1**).

Table 7.1: Baseline characteristics of participants in the OptiMuM study

		Intervention group	
Characteristic	Total population	RDA *(n=14)*	2RDA *(n=15)*
Age (years)	74.3 (3.6)	74.7 (3.9)	73.7 (3.3)
BMI (kg/m^2)	28.3 (4.2)	28.4 (5.1)	28.2 (3.3)
Alcohol intake (drinks)[1]	4.6 (4.6)	5.0 (5.3)	4.1 (3.8)
Plasma creatinine (µmol/L)	91.9 (16.9)	87.8 (17.6)	96.2 (15.2)
Medication use (*n*)	2	0	2
Serum folate (ng/mL)	5.96 (3.17)	6.99 (4.08)	4.93 (1.40)
Erythrocyte folate (ng/mL)	960 (167)	1015 (199)	890 (159)
Serum cobalamin (pg/mL)	482 (138)	513 (144)	448 (129)

Data presented as mean (SD), except for medication use which is presented as count based on medications that affect plasma B_{12} (proton pump inhibitors, histamine 2 receptor antagonists, metformin)[102]. Abbreviations: 2RDA, twice the recommended dietary allowance; BMI, body mass index; RDA, recommended dietary allowance.[1]Measured as self-reported standard drinks per week.

7.2.4.2 Differences in dietary composition according to the intervention group

Differences in dietary intake of food groups (energy-adjusted), macronutrients, and relevant micronutrients (folate, riboflavin, vitamins B_6 and B_{12}) between the RDA and 2RDA diet are presented in **Table 7.2**. The 2RDA diet provided a greater intake of animal-based proteins, including eggs (p 0.002), fish and seafood (p <0.001), meat and meat products (p <0.001), chicken (p <0.001), and milk and dairy products (p <0.001), as well as having higher intake of foods classified as spreads (p=0.025). Conversely, intake of participants consuming the 2RDA diet was lower in dietary intake of starchy vegetables (p=0.002), and plant-based alternatives such as soy milk (p=0.033), and foods classified as fats, oils, sauces and dressings (p <0.001). No differences in the consumption of cereals and grains, bread, non-starchy vegetables, fruit, desserts, snacks, herbs/spices, or legumes, nuts, and seeds were found between intervention groups (p >0.05). With respect to the macronutrient composition of the diets, the proportion of total energy intake from protein was higher in the 2RDA diet (p <0.001), while that from carbohydrate and fat was higher in the RDA diet (p <0.001). Although energy intake and consumption of fibre did not differ between intervention groups (p >0.05), this would be expected given the wide range of actual daily energy (7885-15110 kJ) and fibre (35.1-63.0g) intakes in both intervention groups. Those receiving the 2RDA diet had a greater intake of folate (p <0.001), riboflavin (p=0.003), vitamins B_6 (p=0.010) and B_{12} (p <0.001) compared to the RDA group.

Table 7.2: Comparison of dietary intake of food groups, macronutrients, and vitamins involved in one-carbon metabolism between intervention diets

Dietary variables	Food group or nutrient	RDA	2RDA	*p* value
Food groups[1]	Cereals & grains (g)	145 (35.4)	130 (37.2)	0.010
	Starchy vegetables (g)	214 (67.4)	137 (64.8)	0.002*
	Bread (g)	97.7 (35.1)	86.0 (28.6)	0.201
	Non-starchy vegetables (g)	266 (33.8)	252 (80.8)	0.222
	Fruit (g)	606 (220)	636 (233)	0.841
	Eggs (g)	57.0 (17.6)	92.8 (26.5)	0.002*
	Fish, seafood (g)	21.5 (8.6)	134 (50.0)	<0.001*
	Meat, meat products (g)	33.2 (10.7)	120 (40.3)	<0.001*
	Chicken (g)	77.1 (15.7)	180 (52.5)	<0.001*
	Milk, dairy products (g)	129 (80.3)	367 (130)	<0.001*
	Plant based alternatives (g)	158 (80.1)	9.5 (35.8)	0.033*
	Legumes, nuts, seeds (g)	59.3 (26.4)	69.7 (45.2)	0.513
	Snacks (g)	19.0 (22.5)	16.2 (19.0)	0.699
	Desserts (g)	221 (102)	207 (73.2)	0.515
	Fats, oils, sauces, dressings (g)	50.8 (4.8)	37.2 (13.9)	<0.001*
	Herbs, spices (g)	1.8 (2.1)	1.5 (0.8)	0.214
	Spreads (g)	11.3 (4.1)	18.6 (9.5)	0.025*
Macronutrients	Energy (kJ)	11,217 (2158)	11,800 (640)	0.340
	Carbohydrate (% energy intake)	52.1 (1.85)	46.6 (2.15)	<0.001*
	Fat (% energy intake)	31.0 (2.18)	28.1 (1.46)	<0.001*
	Protein (% energy intake)	11.6 (1.44)	19.8 (2.43)	<0.001*
	Fibre (g)	47.5 (8.91)	49.7 (4.81)	0.413
Vitamins	Folate, DFE (µg)[2]	685 (149)	878 (72.7)	<0.001*
	Riboflavin (mg)	2.71 (0.64)	3.37 (0.38)	0.003*
	Vitamin B_6 (mg)	2.82 (3.25)	3.25 (0.27)	0.019*
	Vitamin B_{12} (µg)	2.26 (0.71)	5.26 (0.56)	<0.001*

Data is presented as mean (SD), and refers to average intake/day. Data is calculated from seven-day personalised meal plans during the dietary intervention. * Indicates a significant difference between intervention groups (p <0.05). [1]Energy-adjusted variables according to the method described by Willett *et al* [584] are presented. [2]Dietary folate equivalents consider both naturally occurring food folates and folic acid in fortified foods, and are used to accommodate the relative bioavailability of both forms[87]. Abbreviations: 2RDA, twice the recommended daily allowance; DFE, Dietary Folate Equivalents; RDA, recommended daily allowance

7.2.4.3 Changes in B vitamin adequacy according to the intervention group

The proportion of individuals not meeting the RDI at baseline was high in both intervention groups for folate (28% - RDA, 27% - 2RDA) and riboflavin (28% - RDA, 33% - 2RDA), and moderate for vitamins B_6 (7% - RDA, 20% - 2RDA), and vitamin B_{12} (21% - RDA, 13% - 2RDA). During the dietary intervention, all participants met the RDI for folate, riboflavin, and vitamin B_6 in both intervention groups. All participants in the 2RDA group met the RDI for vitamin B_{12} intake during the intervention, while 57% in the RDA group were considered inadequate (**Table 7.3**).

Table 7.3: Comparison of nutrient intakes to recommended daily intakes at baseline and during dietary intervention with higher protein intake (2RDA) or current protein recommendations (RDA)

Nutrient	Recommend daily intake	RDA		2RDA	
		Pre	Post	Pre	Post
Folate	400 µg/day	4 (28%)	0	4 (27%)	0
Riboflavin	1.6 mg/day	4 (28%)	0	5 (33%)	0
Vitamin B_6	1.7 mg/day	1 (7%)	0	3 (20%)	0
Vitamin B_{12}	2.4 µg/day	3 (21%)	8 (57%)	2 (13%)	0

Data presented as number (%) in each group considered inadequate in each group compared to recommended daily intakes [87]. Abbreviations: 2RDA, twice recommended dietary allowance; RDA, recommended dietary allowance; RDI, recommended daily intake.

7.2.4.4 Response of one-carbon metabolites to dietary intervention

Despite changes in dietary B vitamin intake in both intervention groups, few changes in 1C metabolite concentrations from baseline to 10-week follow-up were observed. A main time effect for plasma Hcy concentration (p=0.013) was found, which decreased during the intervention in both groups and no difference in concentrations between groups at follow-up (**Figure 7.1, Table 7.4**). Changes to the ratio of choline metabolites were specific to diet received, with an increase of betaine/choline in the RDA group (p=0.031) and no change in the 2RDA group (p=0.973), and a decline in DMG/betaine in the RDA group (p=0.006), with no change in the 2RDA group (p=0.997) (**Figure 7.2**). No difference in status of either betaine/choline or DMG/betaine were found between intervention groups at follow-up. No other changes in metabolites were found (**Table 7.4**).

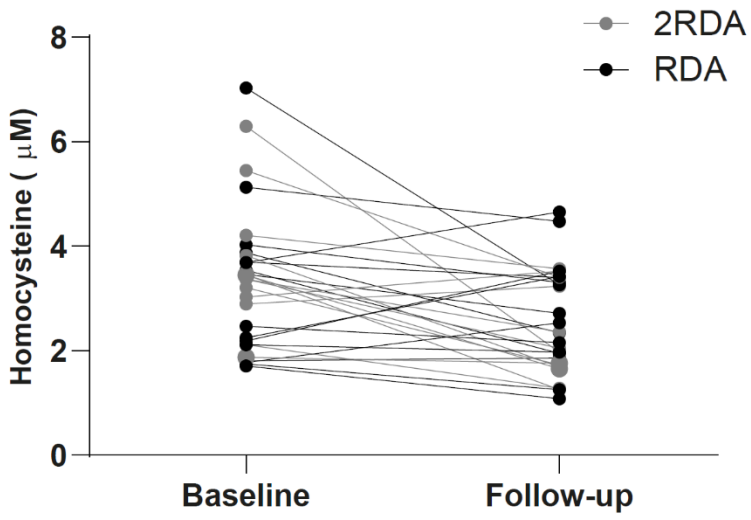


Figure 7.1: Paired differences of homocysteine concentrations at baseline and 10-week follow-up according to the intervention group
A decline in plasma homocysteine concentrations was found in both intervention groups from baseline to follow-up (time effect, p = 0.002), with no difference in concentration between groups at baseline or follow-up.

Table 7.4: One-carbon metabolite concentrations in plasma at baseline and following 10 weeks of a diet with higher protein intake (2RDA) or current protein recommendations (RDA)

Metabolites (plasma)	Baseline		Follow-up		*p* value	
	RDA	2RDA	RDA	2RDA	Time	Diet*Time
Betaine (μM)[1]	45.6 (9.3)	49.5 (14.0)	49.4 (8.6)	47.8 (13.3)	0.530	0.133
Choline (μM)[1]	17.0 (2.5)	17.9 (3.4)	16.4 (3.6)	18.2 (3.4)	0.710	0.443
Cysteine (μM)	91.1 (23.7)	87.6 (16.9)	91.5 (12.1)	77.6 (14.6)	0.062	0.157
DMG (μM)	2.9 (0.6)	3.1 (0.8)	2.7 (0.6)	3.0 (0.7)	0.502	0.483
Glycine (μM)[1]	231 (23.0)	208 (43.0)	225 (28.4)	208 (35.8)	0.946	0.805
Homocysteine (μM)	3.2 (1.5)	3.5 (1.2)	2.7 (1.1)	2.4 (0.9)	0.002*	0.215
Methionine (μM)[1]	22.9 (3.6)	23.1 (5.7)	22.5 (3.0)	22.1 (2.2)	0.419	0.325
SAM	153 (40.5)	156 (37.8)	157 (48.6)	185 (73.5)	0.065	0.238
Serine (μM)[1]	113 (30.7)	112 (20.0)	110 (15.6)	105 (11.2)	0.275	0.591
Betaine/Choline	2.74 (0.69)	2.81 (0.81)[†]	3.13 (0.82)	2.71 (0.93)[†]	0.438	0.011[#]
DMG/Betaine	0.07 (0.02)[§]	0.07 (0.02)	0.06 (0.01)[§]	0.07 (0.03)	0.603	0.006[#]

Data presented as mean (SD). *Indicates a significant time effect, and # indicates a significant interaction (p <0.05). [1]Variables previously reported elsewhere using different quantification methods[578,588]. According to post-hoc analyses, [†]Indicates a significant difference between baseline and follow-up in the RDA group (p=0.031), and [§] Indicates a significant difference between baseline and follow-up in the RDA group (p=0.006). Abbreviations: 2RDA, twice the recommended dietary allowance; DMG, dimethylglycine; RDA, recommended dietary allowance; SAM, *S*-adenosylmethionine.

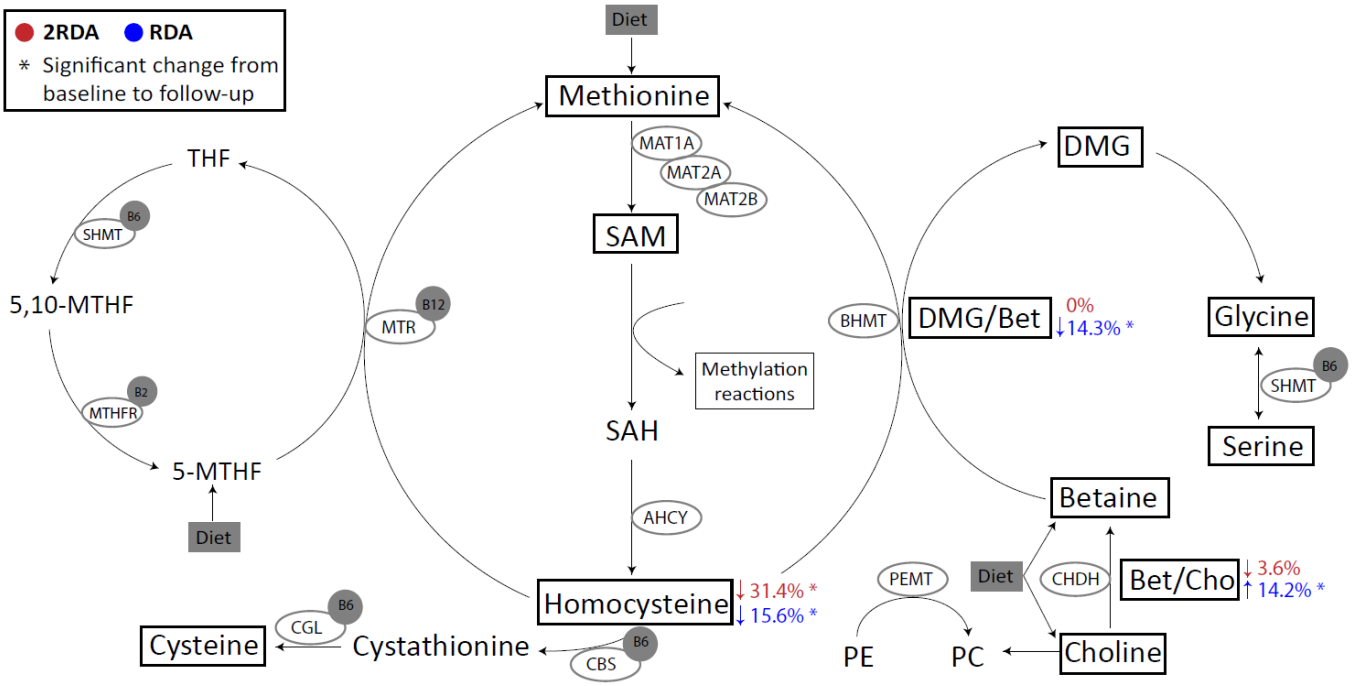


Figure 7.2: Pathway overview of one-carbon metabolism with changes according to intervention highlighted
*Significant % change from baseline to 10-week follow-up of metabolites are indicated by * according to each intervention group with a significant change (p <0.05). AHCY, S-adenosylhomocysteine hydrolase; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; CGL, cystathionine λ-lyase; CHDH, choline dehydrogenase; DMG, dimethylglycine; MAT, methionine adenosyltransferase; MTR, methionine synthase; PC, phosphatidylcholine; PE; phosphatidylethanolamine; PEMT, phosphatidylethanolamine methyltransferase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate.*

7.2.4.5 Associations between changes in B vitamin intake and plasma one-carbon metabolites

Linear regression modelling was performed to investigate associations between changes in B vitamin intake and plasma 1C metabolite concentrations, and whether the diet received impacted this relationship (**Table 7.5**).

7.2.4.5.1 Folate

In unadjusted models (Models 1a), a positive association was observed between changes in dietary folate intake and changes in DMG concentration (β=0.001, *p*=0.017), SAM concentration (β=0.126, *p*=0.014), and the ratio of DMG/betaine (β <0.001, *p*=0.003), while an inverse relationship was found with the ratio of betaine/choline (β= -0.001, *p*=0.015) (**Table 7.5**). These associations remained significant for DMG (β=0.001, *p*=0.005), DMG/betaine (β <0.001, *p*=0.004) and Betaine/choline (β= -0.001, *p*=0.046) in adjusted models (Model 2a). An adjusted model was not built for SAM, as no association with confounding variables in bivariate analyses were found (*p* >0.15). The relationship between changes in folate intake and choline concentration or the betaine/choline ratio was dependent on the intervention received (interaction, *p* <0.05) in unadjusted models (Models 1b) and adjusted models (Models 2b) (**Figure 7.3**).

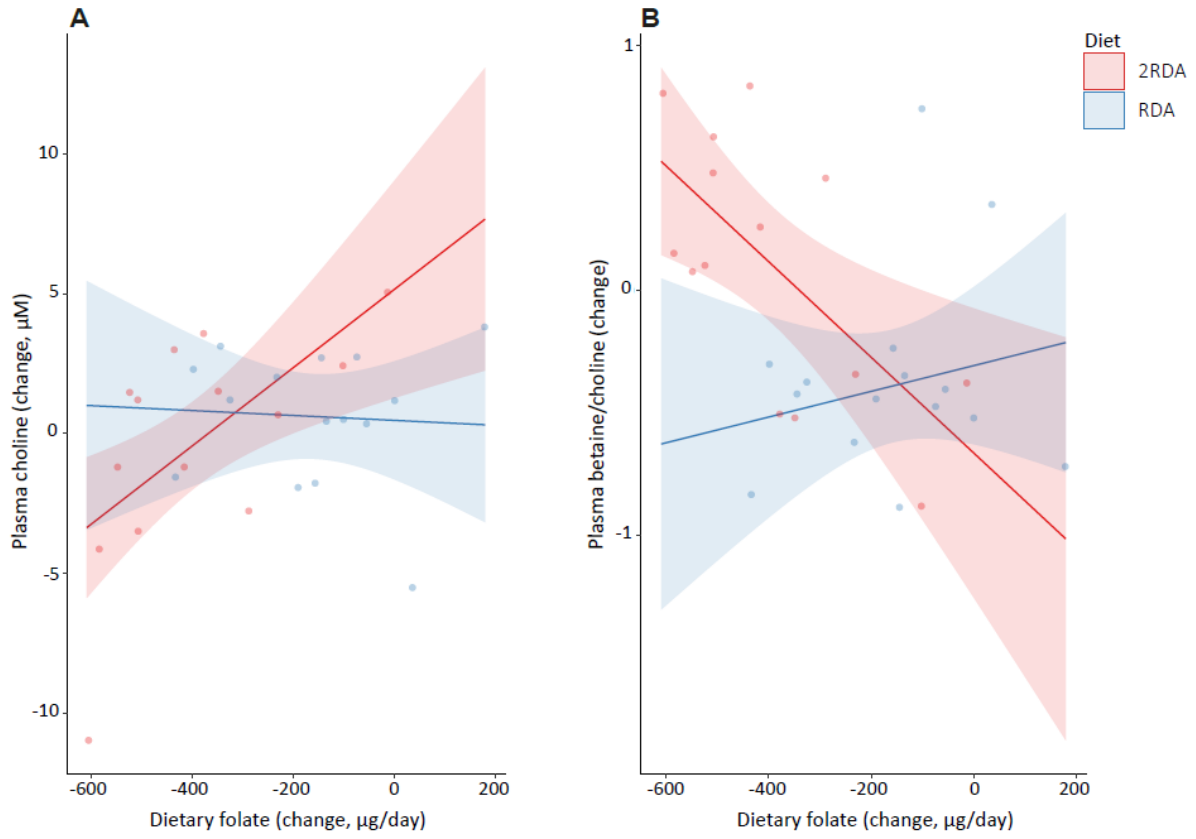


Figure 7.3: Scatter plot depicting associations between changes in folate intake with changes in choline and betaine/choline according to the intervention received.
Data is based on the linear regression analyses presented in Table 7.5, and refers to Model 2B (an adjusted model including an interaction term between the intervention group and change in folate intake) for both choline and betaine/choline as dependent variables.

7.2.4.5.2 Riboflavin, vitamins B_6 and B_{12}

In unadjusted models (Models 1a), a positive association was observed between changes in riboflavin intake and plasma glycine concentration (β=12.602, *p*=0.046) (**Table 7.5**). An inverse relationship was found between changes in vitamin B_6 intake and cysteine concentration (β= -8.014, *p*=0.034), as well as between vitamin B_{12} intake and Hcy concentration (β= -0.220, *p*=0.043). The association between shifts in vitamin B_{12} and Hcy did not remain significant when adjusted for baseline Hcy status, which was a determinant of changes to Hcy concentrations (β=0.664, *p* <0.001). An inverse relationship between vitamin B_6 intake and plasma Hcy neared significance (β= -0.483, *p*=0.050), but not when adjusted for baseline Hcy concentrations (β= -0.172, *p*=0.360).

Table 7.5: Association between changes in dietary intake of B vitamins and one-carbon metabolite concentrations from baseline to ten-week follow-up

		Folate		Riboflavin		Vitamin B_6		Vitamin B_{12}	
Metabolite	Model	β	*p*	β	*p*	β	*p*	β	*p*
Betaine	1a	0.009	0.919	-0.926	0.629	1.178	0.541	0.665	0.433
	2a[1]	-0.001	0.885	-2.413	0.160	0.076	0.964	-0.285	0.719
Choline	1a	0.006	0.052[#]	0.853	0.180	0.732	0.254	0.323	0.414
	2a[1]	0.007	0.013*,#	1.009	0.091	0.937	0.116	0.309	0.251
Cysteine	1a	-0.018	0.349	-2.502	0.521	-8.014	0.034*	-2.661	0.117
	2a[2]	0.013	0.304	-1.812	0.504	-3.221	0.264	-1.586	0.188
DMG	1a	0.001	0.017*	0.138	0.189	0.028	0.788	-0.003	0.955
	2a[2]	0.001	0.005*	0.135	0.145	0.110	0.261	0.035	0.419
Glycine	1a	0.053	0.087	12.602	0.046*	10.457	0.104	4.711	0.097
	2a[3]	-0.019	0.490	4.296	0.392	1.449	0.773	-0.684	0.772
Homocysteine	1a	-0.001	0.351	-0.205	0.417	-0.483	0.050	-0.220	0.043*
	2a[2]	<0.001	0.701	-0.124	0.486	-0.172	0.360	-0.122	0.124
Methionine	1a	0.001	0.776	0.259	0.785	-0.762	0.422	-0.064	0.879
	2a[2]	<0.001	0.871	0.213	0.674	-0.307	0.548	-0.155	0.488
SAM	1a	0.126	0.014*	8.393	0.453	12.009	0.283	1.759	0.724
	2a	NA	NA	NA	NA	NA	NA	NA	NA
Serine	1a	0.015	0.386	3.095	0.401	-0.818	0.826	2.143	0.185
	2a[3]	-0.003	0.779	0.651	0.773	-3.559	0.114	-0.88	0.426
Betaine/Choline	1a	-0.001	0.015*,#	-0.101	0.344	-0.154	0.146	-0.063	0.181
	2a[3]	-0.001	0.046*,#	-0.075	0.455	-0.105	0.307	-0.028	0.565
DMG/Betaine	1a	<0.001	0.003*	0.003	0.286	0.002	0.433	0.001	0.353
	2a[4]	<0.001	0.004*	0.002	0.369	0.002	0.422	0.001	0.310

Dependent variables used are the change in metabolite concentration from baseline to follow-up, and independent variables are the change in dietary intake from baseline to follow-up. *Modelling:* Model 1a was unadjusted, Model 2a was adjusted with confounding factors associated with the dependant variable in bivariate analysis (*p* <0.150). *Indicates a significant association between nutrient intake and the dependent variable (*p* <0.05) for Models 1a and 2a. β estimates and *p* values are not presented for Models 1b (unadjusted) and 2b (adjusted) which include an interaction term between nutrient intake and intervention group, variables for which there is an interaction (*p* <0.05) are indicated by # in the corresponding row for unadjusted (Model 1a) or adjusted (Model 2a) models. [1]Adjusted for baseline metabolite concentration or ratio, and BMI. [2]Adjusted for baseline metabolite concentration or ratio. [3]Adjusted for baseline metabolite concentration or ratio, and baseline creatinine concentration. [4]Adjusted for age. Abbreviations: DMG, dimethylglycine; SAM, *S*-adenosylmethionine.

7.2.5 DISCUSSION

Increasing protein intake through animal-based sources provides a rich source of essential and branched-chain amino acids that aid in the possible preservation of skeletal muscle and function in older adults. This same diet also provides a rich supply of nutrients, including B vitamins, choline, and amino acids involved in the co-regulation of 1C metabolism. However, there is comparatively limited analyses of the impact of increasing animal-based protein sources on 1C metabolite status in older adults. This analysis included 11 1C metabolites, providing a comprehensive overview of 1C status response to the dietary intervention, and enabling characterisation of the altered regulation of interconnecting cycles that act to coordinate the central metabolite Hcy. Plasma Hcy declined following ten weeks in the two well-balanced diets, which was determined by baseline Hcy status. Contrary to the hypothesis, higher intake of animal-based protein sources did not lower Hcy beyond that of the RDA diet, which was characterised by a high prevalence of dietary vitamin B_{12} inadequacy compared to the 2RDA diet. Evidence for upregulated choline to betaine conversion, but without upregulated betaine-dependent Hcy remethylation in the RDA group was found. While this study provides the first evidence for a longer-term reduction in Hcy with increasing intake of animal-based protein sources, which negates concerns around the supply of dietary methionine in the context of a whole-food diet, the longer-term consequences of modified choline metabolite regulation remains uncertain.

Dietary inadequacy of folate and riboflavin was high, while that of vitamins B_6 and B_{12} was moderate in this study population prior to dietary intervention. During the carefully controlled ten-week dietary intervention, dietary inadequacy was resolved for folate (through naturally sources, as well as breads and spreads fortified with folic acid), riboflavin, and vitamin B_6, with 100% of participants in both intervention groups exceeding the RDIs. Dietary adequacy of vitamin B_{12} was also achieved in 100% of participants in the 2RDA group, but declined in those receiving the RDA diet, with only 57% of participants achieving the RDI. The intention of the dietary intervention was to achieve the desired macronutrient balance between the RDA and 2RDA diets, and to ensure dietary adequacy of core food groups according to local recommendations. Although participants receiving the RDA diet consumed a combination of animal- and plant-based proteins, the decline in vitamin B_{12} adequacy in the RDA diet reflects the lower ratio of animal:plant proteins in a quantity which aligns with current protein recommendations. Intake of animal-based protein sources was higher in the 2RDA diet, including red meat, chicken, fish and seafood, eggs, milk and dairy products, all of which are rich sources of vitamin B_{12}. These animal-based proteins are also good dietary sources of other methyl donors, including choline and methionine, which not only influence Hcy concentrations but are involved in the wider functioning of 1C metabolism and methylation status.

Plasma Hcy concentrations declined from baseline to the ten-week follow-up by 24% in the total population, which aligns with findings from other dietary interventions focusing on changing food groups like fruits and vegetables with[199] or without[194–198] dairy products, or following dietary counselling[200], ranging from four weeks to one year in duration. There are, however, comparatively few studies investigating the impact of increasing dietary protein intake on plasma Hcy levels. Previously, Ward *et al*[187] demonstrated no change to Hcy following one-week of increasing animal-based protein sources. Similarly, Noakes *et al*[202] found no change to plasma Hcy concentration despite improvements in vitamin B_6 and B_{12} status, following 12 weeks of a higher protein diet. While Haulrik *et al*[203] demonstrated a 25% reduction in plasma Hcy following six months of a higher protein intake (22% of total energy intake), this change did not reach statistical significance.

Inconsistencies in the success of modifying dietary protein in reducing Hcy in the literature may stem from the spectrum of dietary manipulations, ranging from a focus on methionine intake alone through to wider dietary changes that reflect a more 'real-world' scenario in this study. Another reason may be that the success of modifying nutrients contained in dietary protein sources appears to be determined by the population's baseline Hcy status, found both in the current study and by Haulrik and colleagues[203]. Regardless, this is the first study to my knowledge to demonstrate a significant reduction of plasma Hcy following intervention with higher intakes of animal-based protein sources in an elderly population.

In contrast to the stated hypothesis, higher protein intake did not reduce plasma Hcy further than that found with the RDA diet. However, the findings do indicate differences in the regulation of choline metabolites involved in Hcy remethylation, with an increase in the ratio of betaine/choline and a decline in DMG/betaine in the RDA group only. These product/precursor ratios, although not entirely reflective of metabolite flux and use, can provide some inference of betaine-dependent Hcy remethylation[119,120,585]. Here, these shifts in product/precursor ratios might suggest a divergence in betaine synthesis and/or utilisation between the two dietary interventions. An increased betaine/choline ratio could indicate upregulated choline oxidation to betaine, thus compensating for a limited dietary supply of dietary vitamin B_{12} by increasing the availability of betaine as a methyl donor in the RDA group[589] (shown diagrammatically in **Figure 7.1**). With increased flux through BHMT, a rise in the ratio of DMG/betaine is expected rather than the decline observed. These findings perhaps reflect earlier shifts to pathway regulation in response to dietary fluctuations, which might be expected given baseline adequacy of vitamin B_{12} intake and status in this study population.

Specifically for the 2RDA diet, a positive correlation between changes in dietary folate intake and choline concentration, and inverse correlation with betaine/choline was identified. Two possible hypotheses are available which support these findings. First, adequate B vitamin status supports the *de novo* synthesis of choline via PEMT, by increasing the ratio of SAM/SAH and thus the methylation capacity required to catalyse PEMT[563]. Second, there may be choline 'sparing' at higher folate intakes, whereby folate supports the remethylation of Hcy via methionine synthase with a reduced flux through BHMT[138,590]. It might be expected that a decline in both betaine and DMG would be present with the increasing folate intake with the 2RDA diet [589,591]. However, increased folate intake was associated with increased DMG concentrations and the ratio of DMG/betaine in this study. This data then suggests that increasing choline availability enhances processes dependent on methyl donors, including betaine-dependent remethylation of Hcy, as found in a recent intervention study exploring krill oil supplementation[176]. Together, these results may indicate improved long-term regulation of Hcy in the 2RDA group, not only through the well-established B vitamin-dependent remethylation pathway, but also through the interconnected choline and betaine-dependent pathway, which to date has been under-appreciated in dietary intervention studies. It should also be noted here that differences in the fatty acid composition between the two diets also likely impact Hcy concentrations and methylation status via PEMT[180]. Indeed, omega-3 supplementation has been shown to increase Hcy remethylation and transsulfuration in mice[592], and higher intake of long-chain omega-3 fatty acids in human subjects have been inversely associated with Hcy compared to the positive association with saturated fatty acids, attributed to differential effects on phosphatidylcholine synthesis[180]. Although further discussion on this point is limited by the dietary analysis software used which does not provide estimates of omega-3 fatty acid intake, this is an important point to consider in future research as these findings suggest that while Hcy declined in both intervention groups, there may be differences in the regulation of methylation status with a higher protein diet.

The inter-dependence between B vitamin adequacy, particularly folate intake, with plasma choline metabolites has been suggested by several animal studies[375–377,563]. However, to the best of our knowledge, this is the first study in humans to demonstrate changes to the regulation of choline metabolites following shifts in B vitamin intake in a long-term dietary intervention. In saying this, it is important to acknowledge that changes to dietary intake of choline and betaine, although not quantified due to limitations in applying New Zealand foods to the USDA database[179], would also influence changes in the status of choline metabolites. Although no shifts in betaine, choline, or DMG status from baseline to follow-up were observed, the findings here may indicate early changes to metabolic regulation that could lead to longer-term perturbations in 1C metabolite status in the RDA group. Notably, the greater requirement for SAM as a methyl donor to support endogenous choline synthesis and flux through the BHMT pathway, which other groups have found to not fully compensate for Hcy remethylation by methionine synthase[593]. Thus, higher intake of animal-based protein sources in the current study appears to be a promising dietary intervention to deliver nutrients that not only reduce Hcy, but also may have additional benefits of sparing choline at higher folate intakes for its use in diverse functions[594], and conserving methyl donors required for its endogenous synthesis. The longer-term consequences of these differences in the co-regulation of Hcy and choline metabolites by B vitamins remain speculative, and have not yet been investigated in the literature. Although in the context of 1C metabolism choline sparing appears favourable for methylation status, choline, alongside betaine and L-carnitine (rich in animal-based proteins), are precursors of TMAO, a putative biomarker for cardiovascular risk[595,596]. Our research group has previously reported an increase in TMAO from baseline to follow-up in participants in the 2RDA group of the current study despite no shift in plasma choline concentrations, with a moderate increase in LDL-cholesterol[578]. This whole-diet intervention contrasts other research groups which found that targeted vitamin B and D supplementation lowers TMAO despite increasing concentrations of choline, betaine, and DMG[597], which led to the hypothesis that a ‘metabolic switch’ might occur between the use of TMAO-precursors (i.e. choline) between methyl-group donation in 1C metabolism and TMAO synthesis[381]. The findings from this analysis highlight the inherent complexities in a whole-food dietary intervention compared to a targeted approach through supplements. Future studies are needed to better understand the long-term consequences of shifts in regulation of 1C metabolites beyond Hcy alone, particularly given their diverse functions and competing effects on health outcomes such as choline in both methyl status and TMAO production.

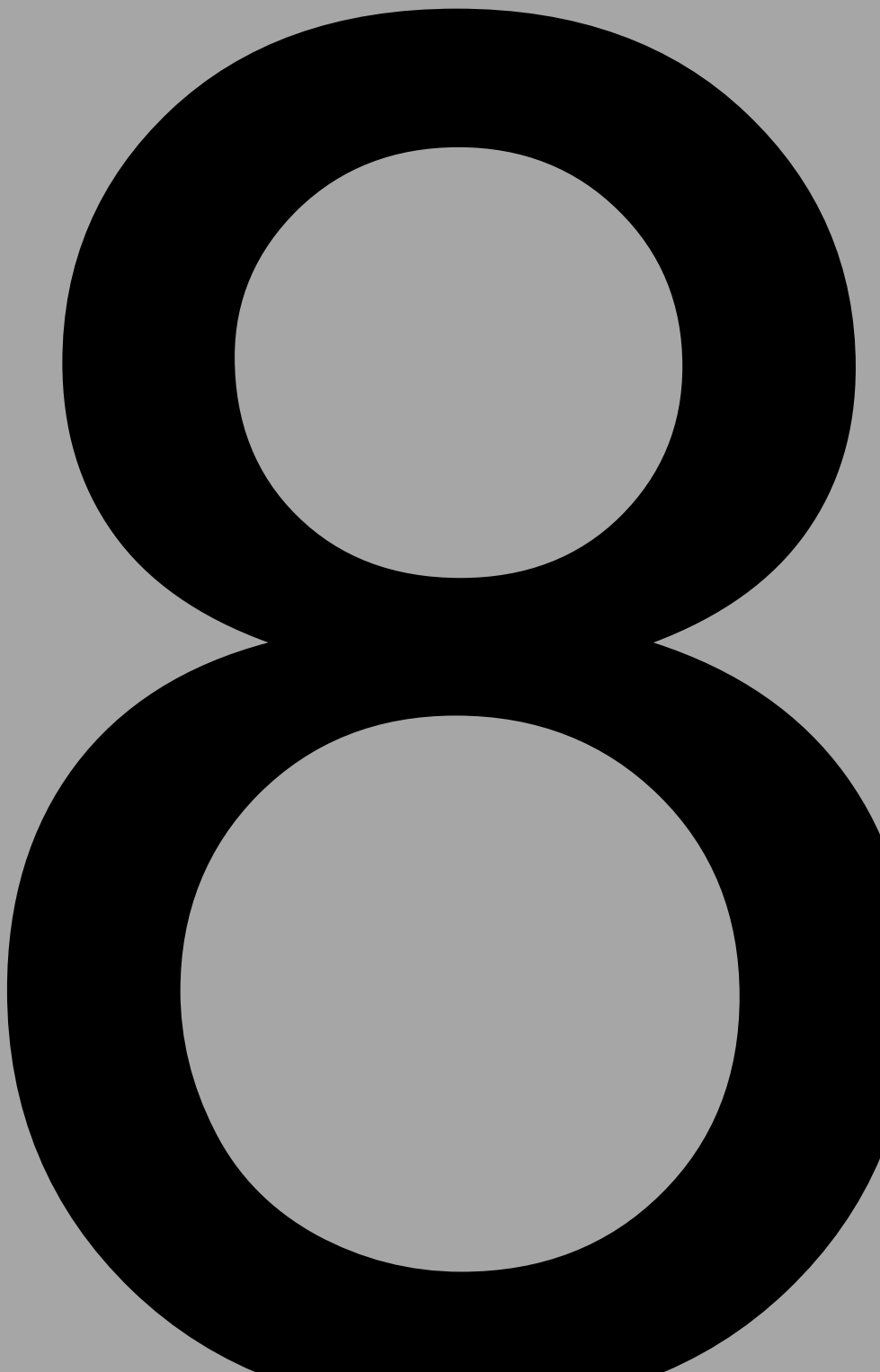
This well-controlled intervention study is the first to explore the impact of higher protein intakes on 1C metabolite status in older adults, analysing a more comprehensive array of metabolites than typically reported. In line with the primary outcomes of the OptiMuM study, only healthy older males were recruited to reduce the homogeneity that would need to be accounted for with differences in amino acid or skeletal muscle metabolism between sexes, and generalisations should not be extrapolated to females or frail elderly living with comorbidities. The sample size of this study (n=29) also limits power in statistical modelling, particularly regression analyses, where the number of confounding variables that could be included in each model was limited. It is important to note the concentration of Tris (2-carboxyethyl) phosphine, an agent used in sample preparation to reduce disulfide bonds present in cystine and homocystine to be quantified as cysteine and Hcy, respectively, is lower than in other published methods[388]. Accordingly, the plasma Hcy concentrations, although comparable at baseline and follow-up in the current study, cannot be directly compared to expected reference ranges[219,567,598] that are higher than the values reported here.

Further limiting the ability to remark on the clinical relevance of these findings, is the fact that although Hcy is indeed associated with an increased risk of age-related diseases, its causative action remains uncertain and a decline in plasma Hcy does not always lead to expected health outcomes, particularly in the case of cardiovascular health[52]. This observation is supported by the moderate increase in LDL-cholesterol in the 2RDA group, and no other changes to cardiometabolic markers in either intervention group in previous analyses[578], again perhaps reflecting the challenges of a whole-food dietary intervention, and action of 1C metabolites and co-enzymes in diverse metabolic pathways. Finally, one must consider how realistic the diet is for older adults to achieve in a community setting, compared to our motivated study group that had meals and snacks provided in a carefully planned intervention. Indeed both dietary arms were carefully formulated to provide adequate intake of fruits, vegetables, wholegrains, protein, and dairy products, which required minimal preparation. Future studies should consider different dietary patterns that achieve higher protein intakes, such as how this compares with increased plant-based proteins, alongside other factors such as how supplement intake might modify the response of 1C metabolites to these dietary changes.

7.2.6 CONCLUSION

Higher protein diets are known to favourably impact skeletal muscle mass and function with advancing age. This study provides evidence that a diet comprised of increased animal-based protein sources also benefits Hcy regulation in older adults, though the novelty lies in the comprehensive spectrum of 1C metabolites analysed. While higher protein intake did not lead to further reductions in plasma Hcy compared to the RDA diet, the approach used here provides insight into the divergent response of interconnecting cycles involved in Hcy regulation, which suggest favourable regulation of the wider 1C pathway in response to the 2RDA diet. However, these findings likely depend on dietary folate adequacy achieved in both diets through increasing intake of both natural and fortified sources, and the long-term consequences and relationship to functional health outcomes remains unclear at this stage. Although dietary strategies to support optimal 1C regulation are difficult to define, the decline of plasma Hcy seen at higher protein intakes highlights the potential for harmonising dietary advice that promote successful ageing.

GENERAL DISCUSSION



CHAPTER 8: GENERAL DISCUSSION

8.1 INTRODUCTION

This thesis sought to explore how B vitamin status can be optimised and monitored in older adults, using 1C metabolites as a measure of functional B vitamin status. To do so, three key themes were followed throughout this thesis;

1. What changes to B vitamin intake or metabolism occur with ageing?
2. What is the relationship between B vitamins, 1C metabolites and health status?
3. To what extent do 1C metabolites beyond Hcy respond to increased B vitamin intake through food or supplements?

The final discussion of this thesis will present a summary of key findings in the context of these themes, highlight some methodological considerations required for their interpretation, and the broader thesis implications and proposed future directions then return to the final question of ***"are we closer to understanding what optimal B vitamin status means for older adults, and how this can be achieved?***"

8.2 SUMMARY OF MAIN FINDINGS

The first results Chapter in this thesis systematically reviewed longitudinal shifts in dietary B vitamin adequacy in community-dwelling older adults, aiming to provide insight into the nature of how and when nutrient intake changes in older adults. While this revealed a progressive decline in riboflavin adequacy with age, this review primarily highlights the ***extreme paucity of data describing changes to intake that occur with age***, particularly compared to the wealth of data reporting on cross-sectional comparisons between older and younger adults.

Based on currently available evidence, it could be speculated that impaired absorptive and metabolic dynamics would compound progressive shifts in B vitamin intake in older adults. Rather, the VIOME study in Chapter 4 shows that ***the acute regulation of B vitamins within the 1C pathway appears to be maintained with age***. Here, the response of circulating Hcy and most 1C metabolites in older adults paralleled that of younger following ingestion of a MVM supplement alongside a standard meal, despite more divergent baseline metabolite profiles. The only difference found between age groups was a faster decline in cystathionine concentrations in older adults, perhaps suggesting enhanced 1C cycling in response to acutely increased B vitamin intake. Findings from Chapter 4 thus refute the hypothesis that ageing will be accompanied by a perturbed postprandial 1C metabolite to to B vitamin intake.

The relationship between B vitamins, 1C metabolites and health was considered in the context of cognitive (Chapter 5) and cardiometabolic (Chapters 5 & 6) health, disorders that contribute significantly to the societal and health burden in ageing populations. ***B vitamins and plasma Hcy were not consistently associated with either cognitive performance or cardiometabolic risk profiles, despite being the predominant focus of research to date***. Rather, choline metabolites (choline, betaine, and DMG) and cysteine were closely aligned with cardiometabolic regulation in Chapters 5 and 6, while higher glycine concentrations were protective against impaired cognitive performance and perturbed cardiometabolic regulation in both Chapters

5 and 6. However, the relevance of these findings to the question of how B vitamin status might be optimised to protect against cognitive decline and cardiometabolic disorders is difficult to address, given that glycine has several actions outside of the 1C pathway and is not typically assessed within the context of B vitamins and 1C metabolism. The connection between glycine and health in older age is likely complex, though the interactive effects of glycine and choline on cognitive function in Chapter 5 suggest a connection to the choline oxidation pathway, and thus to 1C metabolism.

The interactive effects of B vitamins, metabolites, and genotype on measures of cognition and cardiometabolic health was also highlighted in Chapters 5 and 6. For example, the ApoE ε4 genotype (conferring greater risk for dementia) and higher vitamin B_{12} intake attenuated the association between increasing plasma Hcy and poor cognitive performance in Chapter 5. A similar effect modification with higher B vitamin intake was found in the longitudinal analysis in Chapter 6, where increasing plasma choline and DMG concentrations were associated with improvements to the cardiometabolic lipid profile in those receiving nutritional supplementation only, which contrasts the inverse association between choline and DMG with cardiometabolic risk at baseline.

This thesis finally considered interventions which increase B vitamin intake and are already recommended to promote longevity in ageing populations, with a particular focus on protein, including a higher protein diet in Chapter 7 and a protein-based nutritional supplement in Chapter 6. A B-vitamin containing, protein-based supplement (Fortifit) did not lead to favourable 1C metabolite shifts in a senior study population (65 – 98y) compared to resistance training alone or controls. Regardless of the intervention received, Hcy, cysteine and methionine concentrations were increased at the six-month follow-up, and choline concentrations transiently increased after three-months only. ***These findings suggest that a more targeted approach is required to optimise B vitamin status to enhance 1C regulation in seniors where a high prevalence of B vitamin inadequacy is expected***. Comparatively, Hcy declined following ten weeks of a whole food diet containing either current protein recommendations or twice that in Chapter 7. This Chapter also provides novel insights into the interplay between choline, betaine, and folate in the remethylation of Hcy, ***perhaps indicative of enhanced 1C regulation with higher consumption of animal-based proteins***. On this note, it should be clarified that with a broader profile of 1C metabolites, it is difficult to interpret what fluxes in metabolites beyond a decline in Hcy would be considered 'favourable' – a point I will return to later in this discussion.

The overarching hypothesis of this thesis was that a broader profile of 1C metabolites will allow for greater functional insight into the B vitamin-health relationship in older age, ultimately leading to improved monitoring and intervention strategies to optimise B vitamin status. In support of this, ***the most common theme to emerge through the broad scope of research here has been the value, albeit with added complexity, in a more comprehensive analysis of B vitamins and 1C metabolites than typically considered***. Indeed, there were several instances where 1C metabolites revealed differential effects of age, intervention, or relationship with markers of cognitive and cardiometabolic health, where Hcy alone did not, and the importance of riboflavin intake with ageing was highlighted. This research holds relevance for informing future intervention strategies to promote healthy ageing, but the challenge is first disentangling how these findings contribute to understanding the B vitamin-health relationship, which will be the focus in subsequent sections of this discussion.

8.3 IMPLICATIONS

It is increasingly recognised that global population ageing must be met with strategies which prepare societies for healthy ageing over the 'usual' functional decline associated with age. Research attention on the role of B vitamins in maintaining health with advancing age piqued in the early 1990s, but has lost momentum in recent years following largely unsuccessful clinical B vitamin supplementation trials. It cannot be refuted that B vitamins are required for healthy ageing, supported by several decades of research, but is optimising B vitamin status in older adults indeed a lost cause as nutrition research advances?

In light of this question, the following section considers the broader implications of this research with respect to ageing, metabolic profiling, and dietary strategies, offering suggestions for how these findings might be extended in the context of future research trends, and perspectives on the direction research in this field might take in order to keep up with the rapid pace of nutrition research.

8.3.1 CHANGES TO B VITAMIN STATUS ATTRIBUTABLE TO AGEING ARE POORLY UNDERSTOOD AND DIFFICULT TO ADDRESS

Chapters 3 and 4 attempted to tease apart aspects of how B vitamin intake and metabolism is altered with advancing age, with the intent to inform how and when B vitamin status might be optimised in older adults, holding relevance for nutrition professionals and dietary guidelines.

With the novel finding that riboflavin intake progressively declines with advancing age, ***the data reported in Chapter 3 contributes to the emerging body of evidence suggesting that riboflavin inadequacies are a greater public health concern amongst ageing populations than previously acknowledged***[52,77,372,599]. Further implications for optimising B vitamin status during ageing from this Chapter were somewhat limited. A sub-group analysis looking at the impact of age, sex, follow-up period or supplement use would have provided insight into the nature of how riboflavin intake changes with the ageing process, but this was not possible with a paucity of studies and heterogeneity of their design. Although the systematic review in Chapter 3 was focused on dietary intake as one aspect of nutrient status, it should be acknowledged that the riboflavin-health relationship remains unclear in the wider literature as riboflavin has received scarce research attention, which is attributed in part to the laborious analytical approach. While riboflavin does appear to have a weaker direct relationship with health outcomes in the general population compared to other B vitamins[52], the adverse effects of riboflavin inadequacies are more pronounced in individuals homozygous for the MTHFR 677TT genotype, for example with elevated Hcy concentrations[600,601] or hypertension[602]. The active riboflavin vitamer forms are required as co-enzymes for folate-dependent Hcy remethylation and the activation of vitamin B_6 into the active form of PLP[599]. Shifts in riboflavin intake or status may hold greater relevance for the wider regulation of 1C metabolism, as opposed direct consequences with riboflavin inadequacy alone. Understanding trajectories of folate or vitamins B_6 and B_{12} intake in Chapter 3 would have provided further context to shifts in riboflavin adequacy, but was limited by a lack of data. Thus, it is difficult to provide clear recommendations from this systematic review, and ***questions persist around when nutrient intake starts to decline, what drives changes in nutrient intake, and how this might impact functional status and health for older adults***.

While these findings clearly demonstrate the need for more robust follow-up measures of nutrient intake, the true challenge is outlining how this might be addressed in future research. Capturing dietary intake is an ongoing challenge for nutrition researchers and, as outlined in Chapter 1, the currently available tools are inherently erroneous, impractical and expensive in large cohorts, and pose a considerable burden to researchers and participants alike. Metabolic profiling is undoubtedly going to replace dietary assessment in tracking nutrient status, which overcomes issues of subjectivity with self-reporting, reliance on up-to-date databases, and inter-individual factors which impact nutrient absorption, metabolism and status like genetic composition or the microbiome[603]. However, the scientific community has not reached a point where the extensive data generated from metabolic profiling is a useful reflection of dietary intake and we are not yet ready to completely harness the next era of monitoring nutrient intake and status[603,604]..

Chapter 4 shares the common realisation of how little is known about changes to B vitamin intake and metabolism that are attributable to ageing. While it was promising to find that healthy older adults had a comparable acute plasma 1C metabolite response to younger counterparts, implications regarding whether nutrient delivery (e.g. timing, delivery, amount) should be adapted with advancing age were not completely addressed with this simple meal challenge. The postprandial period offers a unique opportunity to understand age-related changes to metabolism given that homeostasis is tightly regulated at fasting and suggestions have already been made in this discussion for future study designs which might have greater sensitivity for revealing perturbations to postprandial B vitamin metabolism in older adults. What is relevant to the wider implications of this thesis is how few studies there are beyond our research group which include older adults in postprandial research, let alone provide comparisons to younger adults, which has been highlighted in the discussion of Chapter 4. Together with Chapter 3, ***these findings evoke concern around assumptions made with respect to older adults – how are we to direct interventions and recommendations to optimise B vitamin status amongst ageing populations if we do not understand the foundations of what changes to intake and metabolism occur with age.*** Through this research, it has become apparent that understanding age effects is a difficult question to address, and future studies must continue in attempts to do so. Age cannot simply be assumed as concurrent with comorbidities, or gastric and metabolic dysfunction. Targeted recruitment strategies are required in future research where populations can be stratified according to age, health, and nutrient status instead of convenience samples, an acknowledged limitation that is currently widespread in the nutrition research field[605].

8.3.2 DOES AN OPTIMAL ONE-CARBON METABOLITE PROFILE EXIST IN HEALTHY AGERS?

Hcy remains a valuable and widely-used marker of B vitamin and 1C metabolite status, but there are inherent limitations in using single markers to represent a complex network, where several pathways regulating metabolism and health intersect. Chromatographic techniques provide opportunity to measure integrated metabolite profiles in research and clinical settings, yet as technology continues to advance there are relatively few studies which report findings from a more comprehensive profile of 1C metabolites. This leads to the question of whether an optimal 1C metabolite profile can be characterised in older adults from the research presented here, which better reflects the B vitamin-health relationship than Hcy alone?

Drawing on findings from Chapters 5 and 6, higher plasma glycine concentrations were consistently related to better cognitive performance and a protective cardiometabolic profile. These findings also indicate that higher circulating betaine, but lower choline, cysteine, DMG, and Hcy concentrations might also be considered part of an optimal 1C metabolite profile - these metabolites were associated with favourable cognitive or cardiometabolic outcomes in at least one of the two chapters, or showed a relationship that was dependent on B vitamin intake (e.g. Hcy and cognition in Chapter 5) or the concentration of other metabolites (e.g. higher choline concentrations attenuating the favourable glycine-cognition relationship in Chapter 5). These findings broadly indicate that metabolites reflecting folate-dependent Hcy remethylation, with downregulated flux via BHMT or transsulfuration pathways, comprise an optimal metabolite profile (**Figure 8.1**). As previously discussed, the interpretation of glycine within this profile is currently unclear given its role in several reactions central to 1C metabolism. Further, lower choline concentrations do not necessarily align with this overarching characterisation, given that choline sparing and subsequently higher concentrations would be expected with restricted BHMT flux. It is pertinent to emphasise again that these metabolites participate in diverse action outside of the 1C pathway, and the relationship between metabolites like choline or glycine with health is possibly uncoupled from their direct role within 1C metabolism and the regulation of Hcy, methylation reactions and DNA synthesis, as illustrated in **Figure 8.1**

The interpretation of these metabolites as a marker of B vitamin and 1C metabolite status requires further disentangling, which must also extend these findings to address the use of metabolite profiles in monitoring intervention responses. Beyond Hcy, which is grounded in decades of research, our understanding of what fluxes in metabolites reflect enhanced 1C regulation in response to intervention remains incomplete. Chapter 6 indicates that while higher choline concentrations are perhaps unfavourable in the cross-sectional setting, an increase in response to nutritional supplementation is associated with improvements to the lipid profile. With uncertainty remaining as to whether higher circulating choline concentrations are good, bad, or benign from the current findings, this evokes questions of whether choline concentrations provide value within a 1C metabolite profile or create undue complexity. This discussion has used choline as one example, but the same argument might be extended to other metabolites which show a differential response according to intervention (e.g. DMG in Chapter 6), or which were not closely related to B vitamins (e.g. glycine in Chapter 5). While this thesis cannot completely address these questions, ***perhaps the most valuable implication then is that characterising a metabolite profile which reflects B vitamin and health status is exceedingly nuanced, and the associations reported here do not point towards a single interpretation of what comprises an optimal 1C metabolite profile.***

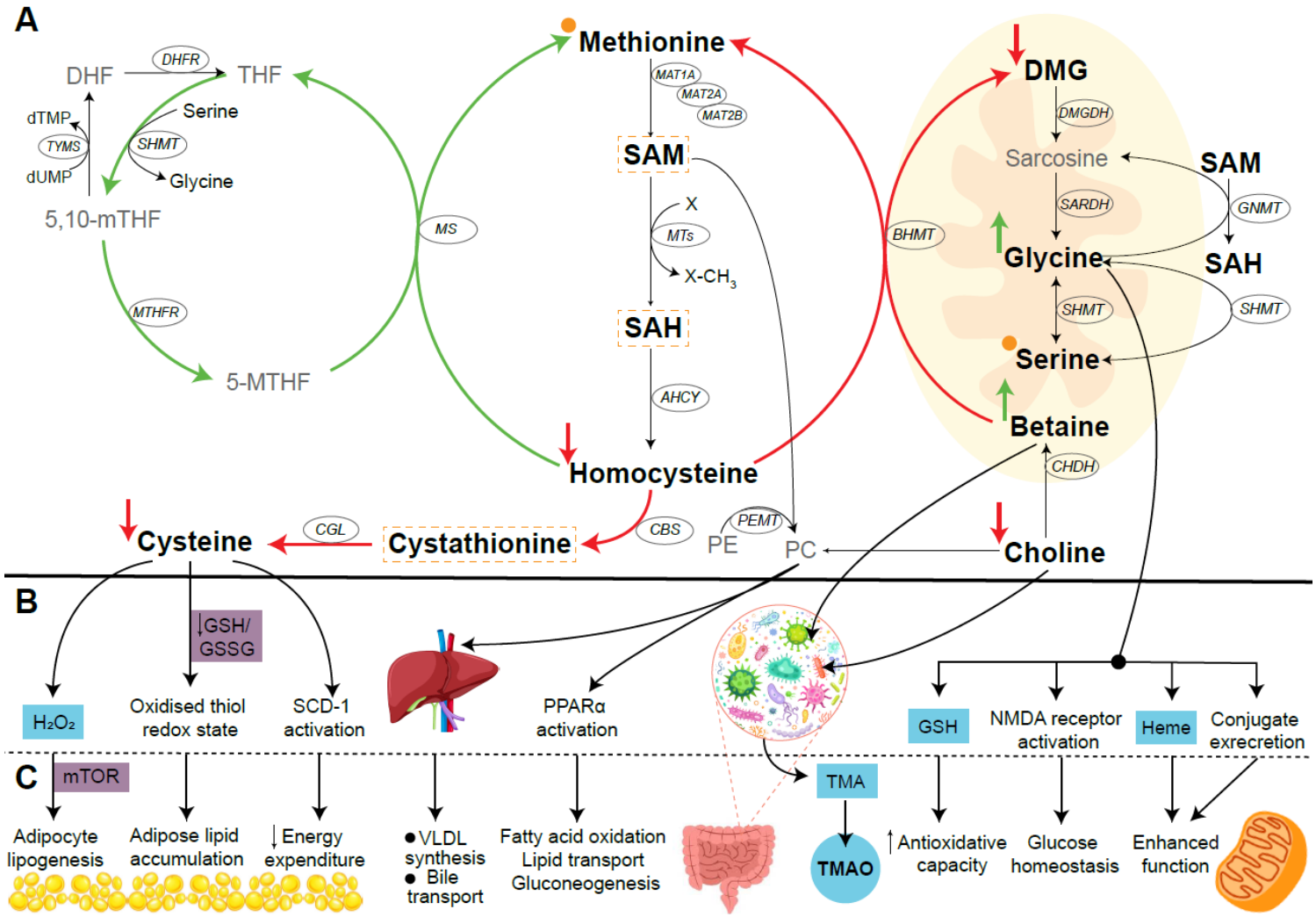


Figure 8.1: Towards an optimal one-carbon metabolite profile – indications and complications

Panel A highlights metabolites which were either favourable (betaine, glycine) or unfavourable (homocysteine, cysteine, choline, dimethylglycine) at higher concentrations in Chapters 5 and 6. Serine and methionine were neutral (orange circles), while cystathionine, S-adenosylhomocysteine, and S-adenosylmethonine were not consistently reported across chapters (dashed orange box). Based on these findings, it appears as though upregulated folate-dependent homocysteine remethylation and downregulated flux via BHMT or the transsulfuration pathways reflects optimal regulation (red and green arrows for pathways which are downregulated or upregulated, respectively). Using cardiometabolic health as an example of how the metabolite-health relationship may be uncoupled from the role within one-carbon metabolism, panels B and C highlight how metabolites are involved in the activation or synthesis of molecules outside of the one-carbon pathway and their respective consequences. Abbreviations: GSH, glutathione; GSSG, glutathione disulphide; mTOR, mechanistic target of rapamycin; NMDA, N-methyl-d-aspartate; PPAR, peroxisome proliferator-activated receptor; SCD-1, stearoyl-CoA desaturase-1; TMA; trimethylamine; TMAO, Trimethylamine N-oxide.

8.3.2.1 B vitamins, one-carbon metabolites and the gut microbiome – friend or foe?

Research on the human microbiome burgeoned during the 21st century, and will command significant attention in years to come[606]. The gut microbiota is comprised of trillions of organisms that play essential roles in nutrient digestion, absorption, and metabolism. Indeed, the gut may support B vitamin adequacy through the endogenous synthesis of B vitamins. Several studies have demonstrated improved folate status with the administration of probiotic strains containing *Bifidobacterium*[99,607,608], which can even lead to a clinically relevant reduction of Hcy[100]. Alternatively, the gut may contribute to disease progression through the microbial metabolism of choline and betaine to produce trimethylamine, which is further oxidised by the hepatic enzyme flavin monooxygenase 3 to produce TMAO, considered a potent cardiotoxic molecule[595,609]. However, vitamin B and D supplementation has been shown to lower circulating TMAO despite increasing concentrations of choline, betaine, and DMG[597]. These findings draw some parallels to that reported in Chapter 6 of this thesis, where increasing choline and DMG concentrations appear to favourably impact the lipid profile in the context of nutritional supplementation. Such findings have recently been hypothesised to reflect a 'metabolic switch' between the use of precursors (i.e. choline and betaine) between methyl-group donation in 1C metabolism and TMAO synthesis[381]. Although beyond the scope of this thesis, untangling the relationship between B vitamins, choline metabolites, and the gut microbiota in future research will undoubtedly reveal valuable insight into the role of choline metabolites within a 1C metabolite profile. Microbiome research will continue to make rapid advances. With a parallel rise in the availability of robust metabolic profiling techniques, it will be possible to delve into this dynamic relationship in established ageing cohorts such that both resources and time are maximised.

8.3.2.2 Are more sensitive benchmarks needed?

While I have suggested that the search for a single optimal profile may be futile, concern must be raised around what an optimal profile is compared to, both in this research and the wider literature. Using outcomes of cardiovascular disease and cognitive impairment has led to the understanding that lowering circulating Hcy is a marker of improved B vitamin function. However, biomarkers prior to disease progression might offer a more sensitive benchmark for quantifying optimal B vitamin and 1C metabolite status. Indeed, 1C metabolites were more closely related to parameters of cardiometabolic regulation rather than cognitive performance in the healthy ageing population in Chapter 5, which invites further investigation into whether other processes which drive disease rather than the resulting functional impairment might offer a more sensitive benchmark for an optimal 1C metabolite profile. This hypothesis could be tested with cardiometabolic parameters alongside hallmarks of ageing like genomic instability, telomere attrition, epigenetic alteration, DNA damage and oxidative stress[610], all of which are interconnected with 1C metabolism and diseases posing significant challenges to ageing societies including cardiovascular diseases, cognitive decline, and sarcopenia amongst others[611–615].

8.3.3 FOOD, SUPPLEMENTS, AND THE COMPETING INFLUENCE OF NUTRIENTS

Dietary advice for older adults is divergent, in part relating to the prior reductionist lens of nutrition research. To overcome this, strategies which promote healthy ageing must be practical, seek to maximise health benefits, and take into account the interconnected nature of the many pathways or processes that are dysregulated with advancing age[378]. B vitamin adequacy undoubtedly plays a crucial part in maintaining good health across the lifespan, though it is unrealistic to suggest that B vitamins should be modified in isolation as nutrition research moves beyond the era of focusing on single nutrients. However, the interactive actions of nutrients within a dietary pattern in contributing to functional B vitamin status has largely been neglected.

The findings reported in Chapters 6 and 7 highlight why B vitamins should not be separated from the competing or synergistic effects of other nutrients, with a particular focus on protein intake. Higher protein intake through food or supplements is increasingly recommended for older adults, yet is thought to perturb 1C metabolism with an increased dietary supply of methionine. In chapter 7, Hcy concentrations declined with higher protein intake in the context of a wider dietary pattern, characterised by higher intake of animal-based protein sources. While these findings extend the well-known benefits of higher protein intake with advancing age and offer the potential to harmonise dietary advice for older adults, an important caveat of the intervention in the OptiMuM study was that protein intake was increased in the context of a well-balanced diet which exceeded B vitamin recommendations. These findings cannot simply be interpreted as evidence that higher protein intake can optimise B vitamin intake such that 1C regulation is enhanced, and caution must be taken with how recommendations of a higher protein intake might be translated and applied. Indeed, Hcy did not decline in Chapter 6 where protein intake was increased with a liquid supplement alongside resistance training, despite a moderate supply of B vitamins in the Fortifit supplement. While I have suggested earlier in this discussion that a more targeted approach might be required to optimise B vitamin status in seniors where a high prevalence of B vitamin inadequacy is expected, an equally important recommendation is to understand whether B vitamin requirements should increase in parallel with higher protein recommendations. ***The competing influence of nutrients like protein and B vitamins is not sufficiently understood, which is central to harmonising dietary advice for older adults given their inter-related contribution to health***.

The issue of food versus supplements is of relevance to health professionals and policy-makers alike. Comparisons between food and supplements on B vitamin or 1C metabolite status are scarce beyond studies focusing on the folate response to folic acid supplements, fortified foods, or natural food folates[201]. While future research might compare the effectiveness of food and supplements in a single study, we must question what the most appropriate recommendations should be that will benefit the entirety of the ageing spectrum. There is ample epidemiological evidence demonstrating that micronutrient deficits exist amongst ageing populations[372], which might increase with age according to the findings in Chapter 3. However, wide-spread supplementation is likely not the answer, particularly with concerns regarding the potential harm caused by excessive supplemental folic acid intake[51,616]. Although wider dietary shifts were shown to have good effect in this research, relying on a balanced diet such as that provided in Chapter 7 becomes increasingly difficult with advancing age. Perhaps future research efforts should then be focused on functional foods.

8.4 STRENGTHS AND LIMITATIONS

A strength of this thesis is the extensive scope of research presented, including a systematic review, a cohort analysis, and acute- and long-term intervention studies. This approach has allowed for several aspects of the dynamic B vitamin-health relationship to be investigated in older adults, which would not have been possible with a singular study. This thesis has been a largely exploratory piece of research, and has been carefully interpreted as such. Within each chapter, more specific strengths and limitations in sample size, nutrient delivery, and population characteristics have been acknowledged and will not be repeated in detail here. Rather, this section will highlight more general methodological considerations which influence the broader interpretation of findings from this thesis in its entirety, with reflection on how the conclusions drawn from these studies might have been strengthened.

8.4.1 STUDY POPULATIONS – HETEROGENEITY AND GENERALISABILITY

Given the exploratory nature of the chapters comprising this thesis, there is a degree of heterogeneity across the broad scope of research presented. While each study in this thesis utilises a suitable population and study design to address their respective objectives, there is some difficulty comparing findings across chapters with differences in study design and populations used. For instance, the two studies investigating metabolite responses to longer-term interventions were conducted in different population groups – the most obvious of which was the older age of VAAS participants in Chapter 6 (83 ± 6y, 65 – 98y) compared to OptiMuM in Chapter 7 (74 ± 4y, 70 – 81y) (**Table 8.1)**.

Table 8.1: Comparison of study population characteristics across experimental chapters

	Review – Chapter 3	VIOME – Chapter 4	REACH – Chapter 5	VAAS – Chapter 6	OptiMuM – Chapter 7
Sample size	8 studies, n=3119	n=40	n=313	n=95	n=29
Age range	≥65 years at follow-up	Younger: 19 – 30y Older: 65 – 76y	65 – 74 years	≥65 years	≥70y
Setting	Community-dwelling	Community-dwelling	Community-dwelling	Aged-care	Community-dwelling
Sex (%male)	38%	50%	35%	12%	100%

While it has been suggested in this discussion that the divergent response of circulating Hcy to higher protein intake in Chapter 7 and broad-spectrum supplementation in Chapter 6 might indicate that a more targeted approach is needed in more senior adults, participants' health and nutrient status is also likely to impact the response to intervention[605]. The VAAS participants had an extremely high prevalence of biochemical folate inadequacy compared to other populations in this thesis, but were the least responsive to intervention regarding Hcy concentrations. This contrasts the general agreement that Hcy declines the most in those with inadequate B vitamin status at baseline. While this may point towards more of an age- or health-effect, it is difficult to comment at this stage due to heterogeneity in other features of study design such as differences in the quantity and delivery of nutrients.

The impacts of sex on 1C metabolite responses to intervention are difficult to tease apart from the current analyses given the unbalanced distribution of sex across study populations. Predominantly female populations were recruited in Chapter 6 (88.4%, reflecting the participants' age range) Chapter 5 (65%, stemming from difficulty in recruiting males), and even the systematic review in Chapter 3 (62%), whereas Chapter 7 included males only. The only study balanced for sex was VIOME in Chapter 4, which although did not show effects of sex on the acute response to supplementation, was perhaps not sufficiently powered to do so and should not be taken as evidence against an impact of sex. Plausibly, differences in sex hormones and cardiometabolic regulation (e.g. hyperinsulinemia, dyslipidaemia) might drive differences in baseline 1C metabolite profiles and response to dietary interventions according to sex. However, the effects remain uncertain in older age groups. For example, younger females might be expected to show more pronounced differences due to the effects of estrogen on choline metabolites in particular[168,617,618], which may hold relevance for the co-regulation of Hcy by folate and choline.

It is important to consider potential concerns around the 'healthy ager' phenotype here given its relevance to the interpretation and generalisability of findings from this thesis, although this will not be discussed at length given that this has been acknowledged in the discussion of each Chapter. This thesis presents findings from healthy ageing populations, generally excluding against history of major disease and obesity. There is some nuance across Chapters according to the primary outcome of each study (e.g. specifically excluding a history of diseases which would contraindicate exercise training in Chapter 6). Under no pretence do these populations represent the vast divergency of healthy status across the broader aged population. Though it is important to clarify here that the definition of a 'general' ageing population is contentious in itself, given the vast heterogeneity which characterises the ageing process[82]. While there are of course some limitations to the generalisability of these findings, the intent of this thesis was to characterise the relationship between B vitamins, 1C metabolites and health in healthy agers, which can then be used to inform future research in more complex ageing populations. Indeed, investigating potential changes in healthy agers compared to younger adults without the compounding influence of comorbidities, polypharmacy and gastrointestinal dysfunction (although common with advancing age but are not characteristic of age *per se*) has been largely overlooked, but is an important step towards understanding any special considerations which need to be taken into account with achieving optimal nutrient status to promote healthy ageing. The healthy ageing populations included in this thesis should therefore not be considered a limitation as such, but rather a suitable population to begin teasing apart the effects of age on nutrient intake, metabolism, response to intervention, and relationship with health.

8.4.2 INTERVENTIONS – CONFOUNDING AND CONTROLS

The most simple vehicle used for B vitamins was the MVM supplement in Chapter 4, which was ingested alongside a simple meal. The intent of this study was not to tease apart the effects of food and supplements on the 1C metabolite response, but rather to acknowledge that even supplemental micronutrients are not typically consumed in isolation. Indeed, the postprandial response is triggered several times a day following food and beverage intake. These dynamic hormone (e.g. insulin) and nutrient (e.g. glucose and amino acids) fluxes were hypothesised to impact the 1C metabolite response to acute shifts in B vitamin intake. Therefore, a strength of this intervention was the delivery of nutrients that reflect typical consumption patterns, providing an appropriate, relevant setting to investigate age-related differences in B vitamin metabolism. However, including a control arm (e.g. supplement or meal only), and/or another intervention arm with a complex mixed meal known to evoke perturbed postprandial responses in older adults (e.g. high fat), would have conceivably strengthened the conclusions drawn from this chapter, which could be considered in future research.

A MVM supplement was also used in Chapter 6, however it is difficult to compare findings between Chapters 4 and 6 given that Fortifit is a protein-based supplement and also contains a lower quantity of B vitamins than the Centrum Advance used in Chapter 4. It is possible that the higher methionine supply 'overwhelmed' the moderate increase in B vitamins through the Fortifit supplement as Hcy concentrations were not lowered. An additional intervention arm in Chapter 4 where the protein supply was matched to Chapter 6 might have provided further insight into questions surrounding age- and protein-effects on Hcy regulation, yet this was not possible with reporting on secondary outcomes and analyses in Chapters 4 and 6, respectively. Regardless, the primary objective of both Chapters 6 and 7 was to consider the impact of interventions already recommended to older adults to maintain health and function with advancing age. The competing influence of protein and B vitamins , and addressing the currently divergent dietary advice for older adults, is an important consideration in future research.

The two diets provided in Chapter 7 were not matched for B vitamin intake, which might be perceived as an issue of confounding. However, the aim of this Chapter was centred around 1C metabolite responses following changes to the wider dietary pattern, hypothesised to alter B vitamin intake through focusing on animal-based protein foods, not on isolated changes to protein itself. A strength of this analysis is the opportunity to move beyond the prior reductive focus on manipulating single nutrients, and rather consider the competing (or synergistic) actions of foods and nutrients in contributing to functional B vitamin status. However, this analysis could be strengthened with a more appropriate control arm than the current control group who still benefited from other dietary changes, evident in the decline in Hcy seen in both intervention arms. Indeed, selecting appropriate control groups is an ongoing source of contention which has considerable influence over the conclusions drawn from dietary intervention trials[619]. While the control used was suitable for the primary outcome of the OptiMuM study relating to skeletal muscle mass, the current analysis would benefit from understanding what impacts increasing animal-based proteins without the wider dietary changes also made in the OptiMuM study (e.g. adequate fruit, vegetable, whole grain, and dairy intake). This is perhaps also a more realistic application of a higher protein diet for many seniors.

8.4.3 ANALYTICAL APPROACH – EXTENSIVE BUT NOT COMPLETE

The primary strength of the research presented in this thesis is the targeted mass spectrometry technique used, allowing for circulating 1C metabolites of the inter-connected methionine, choline oxidation, and transsulfuration pathways to be quantified. There were several instances where these metabolites revealed differential effects of age or intervention, while Hcy did not. For instance, cystathionine concentrations declined faster in older compared to younger adults in response to acutely increased B vitamin intake in Chapter 4. Choline and DMG concentrations revealed differential impacts of nutritional supplementation on cardiometabolic health in Chapter 6, and the interplay between choline, betaine and folate indicated a differential response of 1C metabolites to higher protein intake in Chapter 7. The use of Hcy alone as a single marker would have markedly limited conclusions in each of these analyses. While this reinforces the value in using a more integrated 1C pathway approach, there is difficulty translating these findings to the central question of this discussion: what implications does this hold for understanding what optimal B vitamin status means for older adults? In complete transparency, there is not enough comparable data to completely interpret these findings in light of this question. As emphasised in Chapter 1, Hcy has been the overwhelming focus of 1C research to date. Even those studies aiming to provide a more comprehensive analysis tend to report on distinct aspects of the 1C pathway (e.g. the response of Hcy, SAM and SAH to B vitamins, or the relationship between choline and betaine with cognition), and very few studies have taken a more integrated approach. Robust evidence supports that a decline in circulating Hcy is a favourable outcome concerning 1C regulation – but the interpretation of other isolated or concurrent metabolite shifts is uncertain.

While a more integrated analysis has been discussed as a strength of this research, it is acknowledged that some gaps remain. The B vitamin analysis was somewhat superficial, largely relying on dietary intake, serum folate and B_{12}. Including robust markers like PLP, EGRac, MMA, and folate vitamers (e.g. 5-MTHF) would have strengthened this research, but as previously outlined in Chapter 2 was limited by sample availability and access to specialist equipment and assays. EGRac measures in Chapters 5 – 7 would have been particularly valuable in providing insight into the impacts of declining riboflavin intake with advancing age highlighted in Chapter 3. However, the EGRac assay requires laborious sample preparation and is not readily integrated alongside other assays for B vitamin status. Some metabolites were reported inconsistently across chapters due to technical issues. For instance, having measures of cystathionine in chapters investigating health-metabolite relationships would have provided further insight into the impacts of the age-related differences in cystathionine regulation found in Chapter 4. Similarly, quantifying SAM, SAH, and SAM/SAH would have strengthened the suggestions made around methylation status being connected to health-metabolite relationships and response to intervention, particularly in Chapters 6 and 7. Even with an extensive laboratory panel, trade-offs must be made as compounds within a pathway have variable concentration ranges, molecular mass and structures, which makes quantification with a single analytical platform challenging. Companies like BeVital (Bergen, Norway) overcome this by combining several platforms, but this approach becomes costly with respect to both expense and time.

These final points discussed are not only limitations for my research, but also provide some context as to why integrated analyses are an ongoing challenge. Measuring a ‘complete’ profile of B vitamins and 1C metabolites requires extensive sample preparation and several analytical procedures. The advent of -omics approaches and powerful chromatographic techniques offers hope for robust metabolite-health profiling, but we are not quite there yet with respect to comprehensive pathway analyses.

8.5 FUTURE DIRECTIONS - RESEARCH OPPORTUNITIES AND TRENDS

8.5.1 RESEARCH OPPORTUNITIES

The implications and limitations of this thesis create several opportunities for research. Priority areas for future research identified through this thesis include;

8.5.1.1 Addressing foundational knowledge through robust longitudinal ageing cohorts.

Chapter 3 revealed how little is known about age-related changes to B vitamin intake, and this of course is only one aspect of nutritional status. A critical gap in this understanding is not only the scarcity of reports on how and when nutrient intake starts to decline, but how this then impacts biochemical and functional B vitamin status*. Robust longitudinal cohorts are needed which concurrently measure dietary intake, biochemical and functional status, and health outcomes to understand the true impact of changes to nutrient intake with age on function and health.* This type of transdisciplinary research is essential to the question of how we best optimise B vitamin status for ageing populations, and will also help to disentangle the role of metabolites like glycine or choline as markers of B vitamin and 1C metabolite status.

8.5.1.2 Extending insights into the postprandial response

Following the results from Chapter 4*, there is scope for future research to consider the effects of a more complex mixed meal known to evoke a dysregulated postprandial response in older adults, such as a high fat meal, on the acute 1C metabolite response*. Further, *nesting acute postprandial challenges within longitudinal research* will help to understand whether this is indeed a sensitive way to understand early changes to the regulation of 1C metabolism.

8.5.1.3 Translational strategies to optimise B vitamin status

Practical approaches to optimising B vitamin status are needed for ageing populations. Wider dietary shifts appear promising from the research presented in Chapter 7, but are perhaps impractical in their translation. We can however use these principles to look at functional foods or beverages as a promising meeting point between food and supplements. Fortified foods and beverages are acceptable, feasible interventions applicable to early agers through to the very old. Fortified beverages have also shown benefits in improving B vitamin status[620,621], though insights into their impact on 1C metabolites is limited*. A protein and vitamin-enriched milk beverage is a convenient opportunity to analyse the competing influence of nutrients in optimising 1C metabolite status.*

8.5.2 FUTURE RESEARCH TRENDS

8.5.2.1 Preparing for future dietary trends

The future relevance of B vitamin research in ageing must be considered in the context of emerging dietary trends and public health messages, notably the promotion of plant-based diets. The EAT-Lancet Commission has stated that “A diet rich in plant-based foods with fewer animal source foods confers both improved health and environmental benefits”[622]. While there extensive health benefits conferred by a plant-based diet across the life-span, this recommendation evokes concern around the potential generational differences we might expect with respect to B vitamin status and 1C regulation as Millennials and Gen X reach older age with an increasing preference for vegetarian and vegan diets[623–625].

It cannot be refuted that foods of animal origin are a ‘convenient’ package of nutrients required for 1C regulation, and plant-based diets must be carefully planned to ensure that nutritional requirements are met. Routine vitamin B_{12} supplementation is recommended as animal-based foods are the only natural dietary source of vitamin B_{12}[626], yet deficiencies remain common amongst vegetarian and vegan populations with reports of absent or irregular supplement use[627–630]. Although vitamin B_{12} has been the focus, the broader supply and balance of methyl groups (folate, choline, methionine) must be considered. When B vitamin-dependent Hcy remethylation is restricted, choline requirements are increased to compensate for the increased Hcy flux via BHMT. Robust estimates of choline intake have been limited by incompatible databases, though the evidence available does raise concern. A representative Australian national nutrition survey found that less than 10% of the population in each age and gender group achieved adequate choline intake[631], similar to a sample of women of reproductive age in New Zealand where only 16% met recommendations[632], with animal-based foods being the major contributor to intake in both studies.

Healthy ageing is a continuum from the beginning to the end of life, and dietary choices made in early- and mid-life will undoubtedly impact the extent to which the nutrient-health relationship can be optimised in older age. With a societal shift towards plant-based diets an increasing presence of meat analogues in the market (e.g. the Beyond Burger) can be expected, which although provide consumers with convenient alternatives are not nutritionally matched to animal-based foods[633]. While the long-term impact of this global dietary trend is currently limited to speculation, the prospects for 1C regulation at a population level are not promising. Considerable efforts will be required from nutrition and public health professionals, researchers, and food innovators to deliver recommendations and products in the presence of public health and media messages which seemingly conflict those seeking to maximise health in older age.

8.5.2.2 Epigenetic clocks: the future of monitoring b vitamin status

Looking to future research avenues, it is imminent that the role of B vitamins in maintaining health with advancing age will be more sensitively revealed with markers of epigenetic age. Epigenetics refers to heritable changes of chromatin structure and gene function which does not involve the DNA sequence and is modified by environmental factors[634,635]. DNA methylation is the best understood epigenetic mechanism, involving the addition of a methyl group to the 5' position of the cytosine in cytosine-guanine dinucleotides (CpG), though other modifications involve histone tails (e.g. methylation, acetylation, phosphorylation, ubiquitination), and RNA (e.g. microRNAs or long non-coding RNAs)[636]. While DNA methylation is extensively programmed in early development[637], methylation patterns can change with time, which has been implicated in several age-related diseases, including cardiovascular disease, cognitive decline, and metabolic disorders[638–640].

Human studies have long pointed to the transgenerational impact of diet on genetic programming based on populations exposed to famine[641], and numerous studies have since shown that DNA methylation is labile in response to shifts in nutritional status[306]. B vitamins are central to the dietary regulation of DNA methylation, given that the interaction between B vitamins and other 1C nutrients (e.g. choline, betaine, methionine) determines SAM concentrations, which is the unique methyl donor for all DNA methyltransferase reactions in humans. Yet research describing the relationship between B vitamins and DNA methylation is remarkably scarce and equivocal. Some evidence suggesting a role of riboflavin intake[642], or an association with folate and vitamin B_{12} intake[643], while others report that betaine intake is associated with DNA methylation but intakes of B vitamins, choline, or methionine are not[644]. Further, these studies which measure global methylation have been limited in the extent to which they can determine the impacts of diet-related DNA methylation shifts on health and ageing.

A crucial breakthrough in this field over the last decade has been the establishment of three major epigenetic clock algorithms, which are based on specific CpG sites undergoing reproducible methylation changes with age as opposed to the stochastic changes which also occur[645]. Unlike global methylation, these clocks demonstrate high accuracy in predicting an individual's chronological age (Horvath clock, or DNA methylation age (DNAmAge))[645], age-dependent disease risk (PhenoAge)[639], and mortality risk (GrimAge)[646]. Applying epigenetic clocks to nutrition research is proposed to offer immense potential in unravelling the complex interplay between diet and disease to improve health outcomes with advancing age[306], and much more so than currently available functional markers of nutritional status or even biological markers of ageing like telomere attrition[647]. These novel clocks have already revealed an association between epigenetic age (and its acceleration) with a range of dietary factors. For instance, research from the Women's Health Initiative demonstrates a relationship between food groups (e.g. red meat, fruits, vegetables, and grains) or plasma nutrients (e.g. carotenes, tocopherols) with DNAmAge, PhenoAge, and GrimAge[639,646,648]. Omega-3 supplements lower GrimAge acceleration[649], and following a Mediterranean-style diet for one year was also shown to attenuate the acceleration of epigenetic age according to the Horvath clock[650].

Although currently scarce due to the novelty of these algorithms, research describing the association between B vitamins and markers of epigenetic age support this to be a promising future direction. A retrospective secondary analysis of the VA Normative Aging Study revealed that plasma measures of vitamin B_6 was inversely related to PhenoAge, while folate was associated with higher GrimAge and PhenoAge which depended on concentrations of other B vitamins and Hcy. Although this relationship for folate was somewhat surprising, there are similar paradoxical relationships between folate with other biological markers of age like

mitochondrial DNA copy number and telomere length[651,652], and with health outcomes like cognition[51]. Publicly available datasets of DNA methylation patterns from the B-PROOF study, a randomised controlled trial of folic acid and vitamin B_{12} supplementation or placebo in older adults, have also been applied to epigenetic clock algorithms. This analysis demonstrate that in addition to the differential methylation patterns that were previously reported by the B-PROOF study group[653], supplementation in women with the MTHFR 677CC genotype led to a reduction in epigenetic age measured by the Horvath clock[654].

This is a nascent field of research, and one with tremendous potential to further unravel the utility of B vitamins alongside other dietary components or patterns in maintaining normal epigenetic patterns for healthy ageing in the near future. Prior methodological barriers have been overcome with the advent of sophisticated methods to examine DNA methylation, such as the Illumina Infinium Human Methylation 450K BeadChip Array which provide measures of DNA methylation at a genome-wide scale[655,656], and which can then be input into epigenetic clock algorithms with streamlined analytical pipelines[657]. However, extensive research gaps remain. We simply need more data to inform how these epigenetic clocks can be used in future nutrition research from both observational data and secondary analyses of appropriate intervention studies, ultimately with the goal of targeted dietary recommendations for older adults which attenuate epigenetic ageing.

Regarding dietary interventions, questions persist regarding participants, doses, time-frames, and what nutrients, foods, or dietary patterns should be targeted as the evidence thus far suggests that B vitamins, omega-3 fatty acids and the Mediterranean diet are all promising candidates. This creates difficulty in proposing a more defined research opportunity in this field. I believe that the most constructive next step would be to continue developing our understanding of the diet-epigenetic age relationship with epidemiologic data from a robust follow-up study. As emphasised throughout this discussion, while it is important that B vitamins are considered in future research based on their integral role in methylation, it will be important to move beyond targeted B vitamin analyses alone. Rather, the synergistic and competing action of nutrients must be taken into account in order to inform a targeted nutritional intervention, be it supplements, functional foods, or diets, which can promote healthy ageing through attenuating epigenetic age.

8.5.2.3 Is a personalised approach needed?

Sequencing of the human genome in 2001 launched personalised health to the foreground of research attention in the 21st century. Personalised nutrition (PN) is grounded in the notion that we need to move beyond the 'one size fits all' approach, and rather leverage inter-individual differences in the interaction between endogenous (biochemistry, metabolism, genetics, and microbiota) and exogenous (dietary habits, physical activity) factors to optimise health and combat the chronic disease burden[658]. The evidence presented in this discussion undoubtedly points towards the need to adopt a personalised approach in improving B vitamin and 1C metabolite status, with baseline nutrient or health status, genetics, and microbial composition all critical considerations in how the 1C metabolite profile should be interpreted, targeted, and monitored. Indeed, a personalised approach has already demonstrated the success of riboflavin supplementation as an alternative nutrition-based therapy to lower blood pressure in hypertensive individuals with the MTHFR 677TT genotype[602,659]. Similarly, efforts to counter cognitive ageing appear to be moving towards a more targeted approach. The seemingly obvious conclusion that supplementation in those with inadequate B vitamin status or elevated Hcy will have a greater effect than seeking broad-spectrum benefits for all has been supported in robust randomised controlled trials[301], aligning with the foundations of PN.

Metabotyping, the clustering of individuals based on metabolic or phenotypic profiles, offers some hope in realising the promise of PN at a population level[660]. The pan-European Food4Me trial grouped individuals according to their baseline diet, phenotype, and genotype and found that individuals receiving advice tailored to their cluster transitioned towards a healthier diet more so than those receiving generic population-level recommendations, including an increase in folate intake[661]. Recommendations which are seemingly too complex at the population level can be resolved and even harnessed with a personalised approach, such as the issue presented in this thesis of how choline concentrations are interpreted depending on an individual's nutrient status and microbiota composition, or even the suggestions that interventions targeting epigenetic age likely need a personalised approach with findings that are dependent on sex, genotype, location, and baseline epigenetic age[650,654]. It is unrealistic to suggest that future algorithms are developed which tailor recommendations to the 1C metabolite profile alone, although these would of course prove useful in optimising B vitamin status. Rather, the true challenge for researchers is to prove the relevance of B vitamins and 1C metabolites (likely in a wider micronutrient network) within currently emerging PN approaches, as micronutrients have been largely absent from the PN conversation to date despite their fundamental role in maintaining diverse aspects of health[603,662].

As the scientific community continues to grapple with translating 'big data' generated from -omics technologies into relevant recommendations[663], personalised interfaces are already reaching the public. For instance, the health science company ZOE® has been launched by a team of scientists, tech developers and investors with the belief that new technologies 'could enable scientific research at an unprecedented scale'. The company is set to launch at-home test kits in 2021, aspiring to 'unlock the power of science for every body'. Based on research published by the team in *Nature Medicine*[664], the platform includes blood and stool tests, blood sugar sensors, a meal challenge, and a food diary to provide consumers with recommendations tailored to their unique biology. With daily insights a built-in feature, a future where real-time feedback is not too distant as these platforms emerge –might users be prompted to take a supplement if their diet has not satisfied requirements? Although rudimentary, it is plausible to see micronutrient optimisation taken in this direction if serious efforts are not taken to acknowledge the complexities of micronutrient metabolism within PN.

Indeed, micronutrients play an essential role in processes which regulate phenotypic flexibility, a cornerstone of PN, notably including oxidative stress and inflammation[662,665,666]. However, current understanding of postprandial micronutrient metabolism is nascent, and micronutrient profiling is typically excluded from personalised health interfaces like ZOE®. In the first study to investigate phenotypic flexibility in the context of micronutrient status, van den Broek and colleagues revealed differential associations between fat-soluble vitamins and carotenoids with inflammatory processes following a standard meal challenge, and called for the need to consider multi-micronutrient interaction networks in future postprandial research[662]. These findings were published in 2017, and with scarce research efforts since it remains unclear how micronutrient data should be incorporated to broaden our understanding of phenotypic flexibility and inform PN recommendations. 1C metabolites are well-positioned to be integrated within personalised health interfaces, providing a unique bridge between micronutrient status and metabolic health, and showing acute responsiveness to dietary challenges (Chapter 4). Further elucidating the competing influence of micro- and macronutrients on the postprandial 1C metabolite response and what impacts postprandial 1C perturbations have on longer-term nutrient and health status will be critical in ensuring this is realised.

PN is undoubtedly the future of nutrition research, and offers ground-breaking potential to overcome diet-health challenges which pose a significant burden to ageing populations. I must however conclude with the ethical question of whether interfaces which are being rapidly delivered to consumers will offer substantive benefits at a large scale as promised, or drive health inequalities by optimising health for the select few?

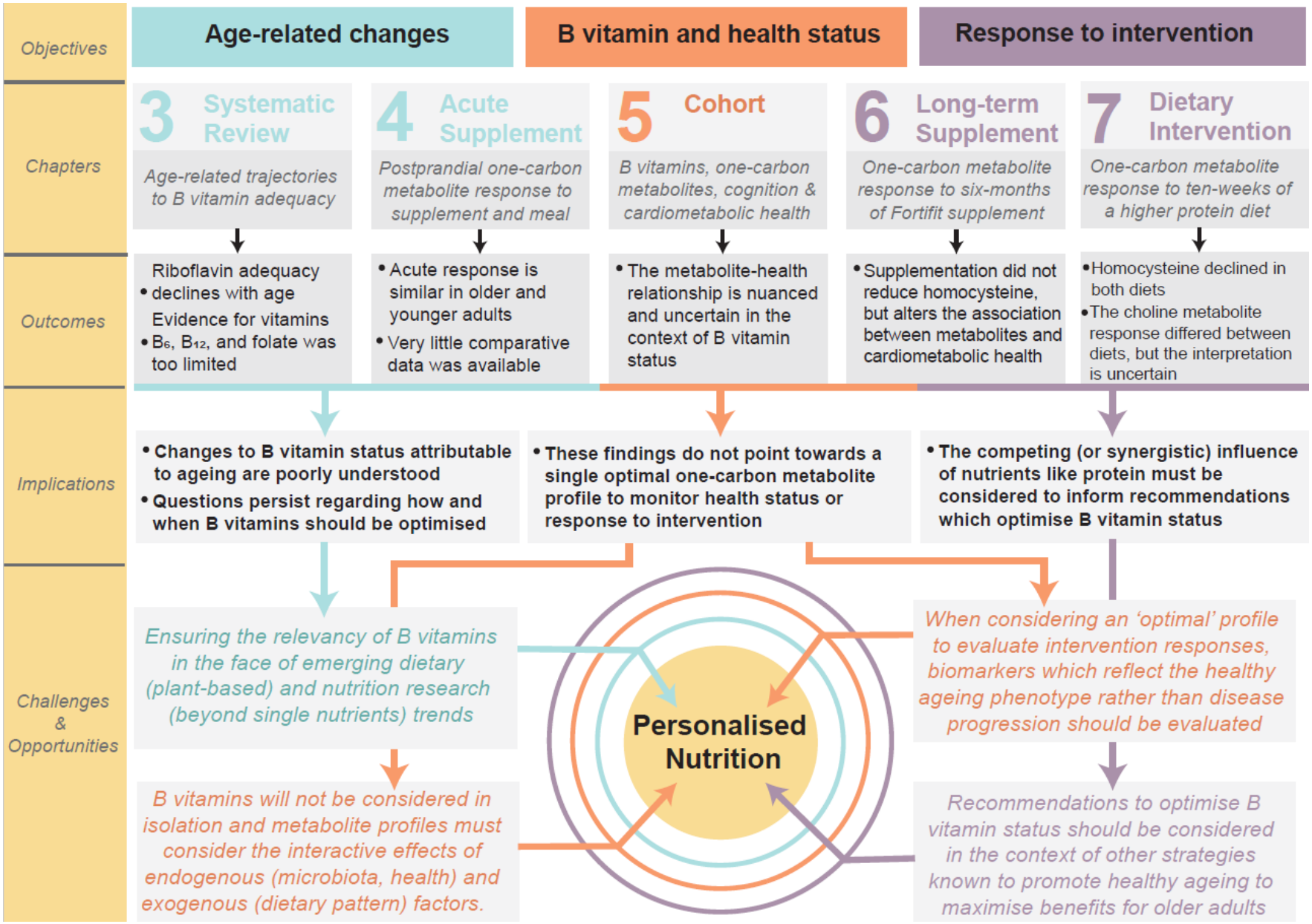


Figure 8.2: Overview of the outcomes, implications, and future directions generated from this thesis

8.6 CONCLUSIONS

The projected burden of population ageing will be devastating for individuals and societies alike if not countered with strategies which prevent or delay the expected functional decline of ageing. Optimising nutritional status will be instrumental in achieving this, but first requires practical recommendations which provide extensive benefits to older adults and robust monitoring tools to be developed. In line with these priorities, the research presented in this thesis explored how B vitamin status might be optimised to promote healthy ageing. Despite a wealth of epidemiological evidence which supports the need to optimise B vitamins in ageing, it was apparent through this research that changes to B vitamin intake and metabolism attributable to ageing have remarkably been neglected. These are the necessary foundations to inform the development of appropriate interventions for ageing populations, though ensuring that this is addressed will be a challenge as nutrition research transitions away from the single-nutrient focus. Advances in metabolite profiling offers potential to continue disentangling the effects of age on B vitamin status and function. However, this research also revealed the added complexities of a more integrated 1C metabolite profile. The findings presented in this thesis also emphasise that B vitamins should not be separated from competing exogenous (e.g. protein intake) or endogenous (e.g. metabolic health or microbiota composition) factors when interpreting 1C metabolite status. These challenges evidently restrict the characterisation of a single optimal 1C metabolite which reflects B vitamin status, health, or response to intervention in older adults. Opportunities for future research have however arisen in light of these challenges, and the research presented in this thesis ultimately points toward the need for a personalised approach to optimising B vitamin status, which will ensure the relevance of B vitamins within future research and dietary trends.

APPENDICES

APPENDICES

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CHAPTER 9: APPENDICES

9.1 APPENDIX 1:

Published article and Supplementary Material for the article presented in Chapter 3 (Systematic Review)

British Journal of Nutrition

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Exploring trajectories in dietary adequacy of the B vitamins folate, riboflavin, vitamins B_6 and B_{12}, with advancing older age: a systematic review

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Abstract

Maintaining nutritional adequacy contributes to successful ageing. B vitamins involved in one-carbon metabolism regulation (folate, riboflavin, vitamins B_6 and B_{12}) are critical nutrients contributing to homocysteine and epigenetic regulation. Although cross-sectional B vitamin intake in ageing populations is characterised, longitudinal changes are infrequently reported. This systematic review explores age-related changes in dietary adequacy of folate, riboflavin, vitamins B_6 and B_{12} in community-dwelling older adults (≥65 years at follow-up). Following Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines, databases (MEDLINE, Embase, BIOSIS, CINAHL) were systematically screened, yielding 1579 records; eight studies were included (*n* 3119 participants, 2–25 years of follow-up). Quality assessment (modified Newcastle–Ottawa quality scale) rated all of moderate–high quality. The estimated average requirement cut-point method estimated the baseline and follow-up population prevalence of dietary inadequacy. Riboflavin (seven studies, *n* 1953) inadequacy progressively increased with age; the prevalence of inadequacy increased from baseline by up to 22·6 and 9·3 % in males and females, respectively. Dietary folate adequacy (three studies, *n* 2321) improved in two studies (by up to 22·4 %), but the third showed increasing (8·1 %) inadequacy. Evidence was similarly limited (two studies, respectively) and inconsistent for vitamins B_6 (*n* 559; −9·9 to 47·9 %) and B_{12} (*n* 1410; −4·6 to 7·2 %). This review emphasises the scarcity of evidence regarding micronutrient intake changes with age, highlighting the demand for improved reporting of longitudinal changes in nutrient intake that can better direct micronutrient recommendations for older adults. This review was registered with PROSPERO (CRD42018104364).

Key words: **B vitamins: Elderly: Micronutrient intake: Nutrition**

Population ageing is occurring rapidly, with estimates that the global proportion of adults aged over 65 years will increase to 16 % by 2050[1]. This profound demographic shift has important individual and societal consequences due to the increased chronic disease risk associated with age[2,3]. Maintaining optimal nutrition status is a key modifiable factor that contributes to successful ageing and hence is increasingly important for older adults[4–6]. Although older adults are at greater risk of nutritional deficiencies[7,8], changes in micronutrient intake specific to ageing have not yet been systematically reported.

Among essential nutrients, dietary adequacy of B vitamins involved in the regulation of one-carbon metabolism (folate, riboflavin, vitamins B_6 and B_{12}) is of particular interest in older adults given their co-regulation of homocysteine, DNA synthesis and methylation reactions[9]. Accordingly, B vitamins and homocysteine, as a marker of one-carbon metabolism, are implicated in a number of common diseases of ageing, including vascular disease[10,11], cancers[12,13], cognitive impairment[14–16] and osteoporosis[17,18]. Research on the association between B vitamins, homocysteine and health outcomes has typically focused on understanding the involvement of folate, and to a lesser extent vitamin B_{12}[19–24]. However, folate metabolism is closely interlinked with these other vitamins involved in one-carbon metabolism, and inadequate status of any one of the four nutrients can perturb the complex pathways comprising one-carbon metabolism[2].

Despite the importance of adequate nutritional intake, this becomes increasingly difficult to achieve for older adults owing to a complex interaction of physiological, psychological and biological factors. Older adults experience sensory changes, such as

Table 9.1: Supplemental Material (Chapter 3): Search strategy as applied to Medline

Ovid MEDLINE(R) Epub Ahead of Print, In Process & Other Non-Indexed Citations, Ovid MEDLINE (R) Daily, and Ovid MEDLINE (R) 1946-Present		
#	**Search Statement**	**Results**
1	vitamin b complex/ or folic acid/ or riboflavin/ or vitamin b 12/ or vitamin b 6/	53160
2	vitamin B complex.mp.	8560
3	(folic acid or folate* or vitamin b9 or vitamin b 9 or folvite*).mp.	47327
4	(riboflavin or vitamin b2 or vitamin b 2 or vitamin g).mp.	12721
5	(vitamin b6 or vitamin b 6 or pyridoxine).mp.	14510
6	(vitamin b12 or vitamin b 12 or cobalamin* or cyanocobalamin* or eritron*).mp.	30325
7	micronutrient*.mp.	14353
8	or/1-7	104313
9	Energy Intake/	37738
10	((energy or vitamin) adj intake*).mp.	48407
11	(diet* adj (intake* or status)).mp.	25199
12	Nutritional Status/	38720
13	((nutrient or nutrition*) adj (intake* or status or assess*)).mp.	73621
14	Nutrition Assessment/	13274
15	(diet* adj (adequacy or adequate or inadequacy or inadequate or deficien* or sufficien*)).mp.	2789
16	(nutrition* adj (adequacy or adequate or inadequacy or inadequate or deficien* or sufficien*)).mp.	5994
17	(nutrient* adj (adequacy or adequate or inadequacy or inadequate or deficien* or sufficien*)).mp.	2176
18	or/9-17	137750
19	AGED/ or "AGED, 80 AND OVER"/	2826085
20	(elderly or geriatric*).mp.	294260
21	AGING/	215327
22	(aging or ageing).mp.	337560
23	((aged or ages or over) adj2 ("65" or sixty five or "80" or eighty)).mp.	839043
24	older.ti.	76512
25	advanced age*.ti,ab.	14769
26	or/19-25	3156164
27	cohort studies/ or follow-up studies/ or longitudinal studies/ or prospective studies/	1237147
28	observational study/	49518
29	(cohort or longitudinal or observation*).mp.	1474309
30	(followup or follow-up or prospective).tw.	1248042
31	or/27-30	2820523
32	8 and 18 and 26 and 31	730
33	limit 32 to yr="1990 -Current"	701

Table 9.2: Supplemental material (Chapter 3): Reference list of studies excluded from full-text review

Author, date	Reason for exclusion					
	Age of participants at follow-up	Full text not available	Full text not available in English	Nutrient intake not reported at follow-up	Nutrients of interest not reported	Presentation of nutrient intake data
Amorim Cruz *et al*, 1996[667]		✓				
Bailey *et al*, 1997[668]					✓	
Beydoun *et al*, 2018[669]					✓	
Decarli *et al*, 1998[670]		✓				
del Pozo *et al*, 2003[671]			✓			
Fidanza *et al*, 1991[672]		✓				
Flynn *et al*, 1992[673]					✓	
Forman *et al,* 2005[674]	✓					✓
Fung *et al*, 2003[675]						✓
Gose *et al*, 2016[676]	✓					
Hughes *et al,* 2017[276]				✓		
Jacques *et al*, 2005[677]						✓
Kang *et al*, 2014[678]						✓
La Rue *et al*, 1997[679]						✓
Larsson *et al*, 2005[680]						✓
Lee *et al*, 2011[449]						✓
Michaud *et al*, 2000[681]						✓
Mori *et al*, 2008[682]		✓				
Nicolas *et al*, 2000[683]		✓				
Skarupski *et al*, 2010[684]						✓
Taylor *et al*, 2002[685]						✓
Voorripes *et al*, 2000[686]				✓		
Winkvist *et al*, 2017[687]					✓	
Yoo *et al*, 2009[688]		✓				
Yoon *et al*, 2016[689]						✓
Zhang *et al*, 2002[452]	✓					

Table 9.3: Supplemental Material (Chapter 3): Details of funding sources and potential conflict of interest of included studies

Author, year, Country of publication	**Funding Source**	**Conflict of interest**
Chapman *et al.* (1996) USA[429]	Not available	Not available
Fernyhough *et al*. (1999) New Zealand[430]	1. Health Research Council of New Zealand. 2. University of Otago Medical School	Not available
Flood *et al.* (2010) Australia[427]	1. Australian National Health & Medical Research Council 2. Meat and Livestock Australia	No conflict of interest to declare
Kromhout *et al.* (1990) The Netherlands[431]	1. Prevention Foundation 2. Netherlands Nutrition Council	Not available
Sjogren *et al*. (1994) Sweden[432]	Not available	Not available
Toffanello *et al*. (2011) Italy[433]	Not supported by any grants	No conflict of interest to declare
Yukawa *et al*. (2003) Japan[434]	Not available	Not available
Zhu *et al.* (2010) Australia[435]	1. Healthway Health Promotion Foundation of Western Australia 2. Australian Menopause Society 3. Australian National Health And Medical Research Council	Not available

9.2 APPENDIX 2:

Supplementary Material for the article presented in Chapter 4 (VIOME)

Table 9.4: Supplemental Material (Chapter 4): Differences in one-carbon metabolite incremental area under the curve in response to a multivitamin and mineral supplement and standard meal according to age

Metabolite	Older	Younger	*p*-value
Betaine (μM)[1]	1893 (3027)	1867 (2506)	0.894
Choline (μM)	143.5 (777.2)	151.2 (284.1)	0.224
Cystathionine (nM)[1]	-7594 (54709)	-3742 (14999)	0.741
Cysteine (μM)[1]	-208.5 (2639)	-825.8 (2940)	0.702
DMG (μM)[1]	-36.0 (110.9)	-24.2 (124.7)	0.915
Glycine (μM)[1]	-189.4 (1730)	-350.0 (1566)	0.418
Homocysteine(μM)[1]	-434.1 (614.1)	-232.0 (253.9)	0.186
Methionine (μM)	-395.3 (238.1)	-459.2 (296.6)	0.200
Serine (μM)	-689.1 (1302)	-921.7 (1252)	0.085

Data is presented as mean (SD) of the incremental area under the curve values, which was calculated after subtracting fasting values. *Indicates a difference in baseline characteristics between age groups (p <0.05), determined by students t-test. [1]Variables were log-transformed for analysis. Abbreviations: DMG, dimethylglycine.

Table 9.5: Supplemental Material (Chapter 4): Effects of age, sex, and time on the postprandial response of one-carbon metabolites, glucose, insulin and triglycerides to multivitamin and mineral supplement and standard meal.

	Age	Sex	Time	Age*Time	Age*Sex*Time
Betaine	0.657	0.309	<0.001*	0.541	0.469
Choline	0.001*	<0.001*	0.018*	0.099	0.261
Cystathionine	<0.001*	0.581	<0.001*	0.003†	0.016#
Cysteine	<0.001*	0.010*	0.025*	0.394	0.568
DMG	0.780	0.047*	0.402	0.083	0.220
Glycine	0.741	<0.001*	<0.001*	0.732	0.620
Homocysteine	0.081	0.006*	<0.001*	0.545	0.026#
Methionine	0.025*	0.079	<0.001*	0.068	0.140
Serine	<0.001*	0.006*	<0.001*	0.817	0.214
Betaine/Choline	0.004*	0.742	<0.001*	0.471	0.215
DMG/Betaine	0.533	0.155	<0.001*	0.037#	0.173
Glucose	<0.001*	0.023	<0.001*	<0.001#	0.581
Insulin	0.536	0.488	<0.001*	0.891	0.999
Triglycerides	0.005*	0.274	<0.001*	0.158	0.091

p-values presented for main effects (age, time, sex), and an interaction effect (age*time or age*time*sex) according to linear mixed model analyses. *Indicates a significant main effect, # Indicates a significant interaction that was not maintained after post-hoc analysis, †Cystathionine declined in older adults at time point 2 and in younger adults at time point 3. Abbreviations: DMG, dimethylglycine.

Table 9.6: Supplemental Material (Chapter 4): Response of one-carbon metabolites, glucose, insulin, and triglycerides to multivitamin and mineral supplement and standard test meal according to age, sex and time

		Older					Younger				
Variable	Sex	T0	T1	T2	T3	T4	T0	T1	T2	T3	T4
Betaine (μM)[1]	M	48.8 (11.6)	58.9 (18.0)	54.2 (10.0)	53.1 (14.1)	52.3 (13.3)	48.1 (12.5)	54.5 (18.6)	55.6 (11.0)	53.1 (12.2)	50.4 (9.7)
	F	40.2 (8.8)	49.4 (15.2)	47.2 (16.6)	50.0 (12.9)	47.2 (13.8)	48.4 (16.8)	55.3 (25.0)	60.0 (22.5)	64.7 (26.7)	56.0 (21.6)
Choline (μM)	M	13.4 (2.0)	13.0 (1.4)	13.3 (2.1)	12.2 (2.0)	13.0 (2.7)	9.34 (2.0)	9.82 (1.6)	9.74 (2.4)	10.2 (1.9)	9.53 (2.2)
	F	10.8 (1.3)	12.5 (2.7)	11.0 (3.1)	11.0 (2.3)	11.6 (3.9)	10.0 (1.4)	10.8 (1.9)	10.9 (1.9)	10.6 (2.2)	10.2 (2.2)
Cystathionine (nM)[1]	M	400 (187)	343 (141)	392 (178)	364 (180)	332 (141)	204 (73.3)	203 (73.3)	168 (59.8)	150 (54.9)	132 (44.7)
	F	549 (434)	502 (404)	478 (301)	398 (257)	423 (308)	196 (75.5)	190 (70.9)	178 (63.4)	156 (69.4)	140 (51.8)
Cysteine (μM)[1]	M	109 (14.9)	109 (14.6)	108 (12.1)	109 (16.8)	104 (19.1)	86.2 (10.0)	88.7 (13.8)	83.4 (17.4)	82.7 (15.8)	84.1 (14.2)
	F	90.3 (13.5)	93.1 (18.0)	90.4 (18.0)	88.9 (13.7)	88.0 (13.5)	90.3 (15.3)	85.2 (11.6)	82.8 (10.7)	85.9 (11.2)	80.2 (9.6)
DMG (μM)[1]	M	3.36 (0.8)	3.42 (1.0)	3.28 (0.8)	3.11 (0.6)	3.16 (0.8)	3.53 (1.0)	3.43 (1.0)	3.19 (1.2)	3.24 (1.3)	3.28 (0.9)
	F	2.89 (1.0)	2.89 (1.5)	2.68 (1.3)	2.70 (1.2)	2.64 (1.1)	2.78 (0.9)	2.67 (0.9)	2.63 (0.9)	2.89 (0.6)	2.81 (1.1)
Glycine (μM)[1]	M	173 (21.7)	165 (34.4)	166 (27.0)	164 (22.3)	158 (30.0)	181 (24.7)	188 (36.4	168 (21.1)	173 (22.1)	160 (19.0)
	F	228 (32.5)	243 (48.1)	238 (51.1)	219 (48.8)	215 (37.8)	213 (43.3)	220 (46.8)	211 (40.7)	200 (54.9)	193 (37.4)
Homocysteine (μM)[1]	M	14.7 (2.8)	13.6 (2.2)	13.4 (2.3)	13.0 (2.3)	13.5 (2.0)	12.3 (1.5)	11.0 (1.4)	11.0 (1.0)	11.2 (1.2)	10.8 (1.0)
	F	13.3 (3.1)	10.4 (0.9)	9.97 (1.0)	10.2 (1.2)	9.5 (1.0)	12.1 (3.3)	11.4 (3.5)	10.2 (3.5)	11.0 (4.1)	10.7 (3.6)
Methionine (μM)	M	24.8 (3.5)	23.2 (3.8)	20.0 (3.1)	17.8 (3.5)	18.7 (3.0)	26.3 (5.0)	25.3 (4.5)	20.0 (5.0)	18.7 (2.6)	19.7 (3.8)
	F	10.8 (3.1)	20.9 (3.1)	17.1 (3.4)	16.0 (2.7)	16.5 (1.9)	25.7 (3.1)	24.2 (2.1)	21.2 (2.6)	19.3 (2.7)	18.8 (2.6)
Serine (μM)	M	96.4 (14.1)	89.2 (16.1)	81.0 (13.2)	77.6 (12.3)	75.7 (14.7)	116 (20.3)	115 (27.2)	94.4 (20.4)	99.3 (19.0)	95.1 18.6)
	F	98.4 (13.7)	113 (21.2)	92.9 (17.3)	82.8 (10.3)	89.3 (14.9)	127 (16.1)	134 (23.2)	120 (17.3)	106 (18.9)	109 (20.0)
Betaine/Choline	M	3.64 (0.7)	4.52 (1.3)	4.14 (1.0)	4.48 (1.3)	4.04 (0.8)	5.30 (1.6)	5.65 (2.1)	5.96 (1.7)	5.37 (1.8)	5.67 (2.3)
	F	3.73 (0.8)	4.01 (1.0)	4.37 (1.3)	4.64 (1.3)	4.24 (1.2)	4.86 (1.6)	5.16 (2.3)	5.41 (1.5)	6.07 (2.2)	5.49 (1.7)
DMG/Betaine[1]	M	0.07 (0.02)	0.06 (0.02)	0.06 (0.02)	0.06 (0.02)	0.06 (0.02)	0.08 (0.03)	0.07 (0.03)	0.06 (0.02)	0.06 (0.03)	0.07 (0.02)
	F	0.07 (0.02)	0.06 (0.02)	0.06 (0.01)	0.05 (0.02)	0.06 (0.02)	0.06 (0.02)	0.05 (0.02)	0.05 (0.02)	0.05 (0.02)	0.05 (0.02)
Glucose (mmol/L)	M	5.27 (0.6)	7.10 (1.4)	5.22 (1.0)	4.60 (0.7)	4.58 (0.6)	4.78 (0.5)	5.66 (1.5)	4.05 (0.7)	4.37 (0.5)	4.66 (0.4)
	F	4.74 (0.3)	6.10 (1.32)	4.84 (1.2)	4.38 (0.8)	4.69 (0.5)	4.49 (0.4)	4.59 (1.5)	4.20 (0.9)	4.04 (0.9)	4.27 (0.5)
Insulin (μU/mL)[1]	M	8.24 (5.2)	62.8 (34.4)	26.9 (14.0)	12.4 (8.4)	6.53 (2.8)	6.24 (4.6)	57.2 (52.9)	15.7 (20.8)	6.40 (6.6)	5.17 (4.8)
	F	6.51 (4.4)	43.2 (43.3)	27.5 (48.6)	8.86 (11.5)	4.54 (4.0)	7.39 (3.3)	41.9 (33.5)	22.0 (12.5)	8.61 (6.2)	5.67 (2.3)
Triglycerides (mmol/L)	M	1.51 (0.6)	1.47 (0.4)	1.54 (0.7)	1.73 (0.7)	1.70 (0.7)	1.06 (0.47)	1.13 (0.47)	0.99 (0.52)	1.01 (0.53)	1.04 (0.51)
	F	1.31 (0.7)	1.39 (0.7)	1.30 (0.7)	1.38 (0.8)	1.48 (0.8)	0.79 (0.24)	0.99 (0.37)	0.82 (0.34)	0.91 (0.42)	0.98 (0.41)

Data presented as mean (SD). [1]Variables were log-transformed for analysis to achieve an approximately normal distribution. Abbreviations: DMG, dimethylglycine; F, female; M, male.

9.3 APPENDIX 3:

Supplementary Material for the article presented in Chapter 5 (REACH)

Table 9.7: Supplemental Material (Chapter 5): A comparison of characteristics between included participants and those excluded for missing dietary or biochemical data[1]

	Included (*n* = 313)	Excluded (*n* = 58)	*p-value*
Age, years	69.8 ± 2.6	69.0 ± 2.5	0.028*
Male, *n* (%)	111 (35)	24 (41)	0.591
Highest education achieved, *n* (%)			0.011*
No qualification	5 (1.6)	5 (8.6)	
Secondary school	66 (21)	10 (17)	
Post-secondary school	127 (41)	20 (34)	
University	115 (37)	24 (41)	
Polypharmacy[2], *n* (%)	25 (8.0)	8 (14)	0.252
Supplement use[3], *n* (%)	53 (17)	10 (17)	1.000
Alcohol intake, *n* (%)			0.073
Daily	85 (31)	13 (22)	
Weekly	127 (46)	20 (34)	
Monthly	50 (18)	8 (14)	
Never	13 (4.7)	5 (8.6)	
Prevalence of MCI, *n* (%)	93 (30)	15 (25)	0.398

[1]Refers to those participants just for the current secondary analysis, not for primary outcomes. [2] Polypharmacy is defined as prescribed use of ≥ 5 medications. [3] Supplement use is defined according to regular use of B vitamin or multivitamin and mineral supplement

Table 9.8: Supplemental Material (Chapter 5): Sensitivity analysis of associations between B vitamin intake, status, and one-carbon metabolites with domains of cognitive function in multivariate linear regression models in participants not regularly consuming B vitamin or multivitamin supplements.

	Model	Global cognition		Attention		Episodic Memory		Executive function		Location Learning		Working Memory	
		β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
B vitamin intake													
Folate	1	<0.001	0.535	<0.001	0.362	<0.001	0.840	<0.001	0.750	<0.001	0.402	<0.001	0.638
	2	<0.001	0.883	<0.001	0.883	<0.001	0.886	<0.001	0.745	<0.001	0.774	<0.001	0.753
Riboflavin	1	0.066	0.331	0.106	0.151	0.028	0.680	0.062	0.423	-0.023	0.766	0.024	0.736
	2	0.003	0.969	0.050	0.547	-0.003	0.966	-0.006	0.947	-0.093	0.280	0.018	0.818
Vitamin B_6	1	0.015	0.806	0.008	0.898	0.051	0.382	-0.050	0.462	0.014	0.828	-0.049	0.429
	2	-0.034	0.589	-0.049	0.478	0.041	0.528	-0.105	0.164	-0.024	0.737	-0.072	0.280
Vitamin B_{12}[1]	1	-0.038	0.862	0.020	0.934	0.098	0.652	-0.204	0.416	-0.211	0.390	-0.120	0.598
	2	-0.090	0.684	-0.024	0.922	0.075	0.737	-0.256	0.323	-0.251	0.317	-0.146	0.529
B vitamin status													
Folate	1	0.001	0.913	0.017	0.226	-0.005	0.667	0.003	0.862	-0.012	0.413	-0.002	0.864
	2	-0.001	0.920	0.013	0.323	-0.007	0.604	0.002	0.918	-0.015	0.281	<0.001	0.977
Vitamin B_{12}[1]	1	0.433	0.228	0.335	0.391	0.207	0.565	0.195	0.636	0.495	0.211	0.410	0.270
	2	0.468	0.186	0.392	0.306	0.214	0.553	0.294	0.479	0.534	0.177	0.399	0.282
One-carbon metabolites													
Betaine	1	0.005	0.470	0.003	0.637	-0.001	0.921	0.007	0.333	0.010	0.147	0.003	0.623
	2	0.004	0.478	0.002	0.745	<0.001	0.971	0.008	0.252	0.010	0.155	0.003	0.697
Choline	1	0.037	0.191	0.019	0.528	0.042	0.135	0.028	0.389	0.018	0.561	-0.002	0.946
	2	0.028	0.324	0.009	0.758	0.037	0.185	0.015	0.629	0.011	0.726	<0.001	0.991
Cysteine	1	0.003	0.422	-0.001	0.752	0.006	0.146	0.002	0.677	0.004	0.426	-0.001	0.751
	2	0.002	0.703	-0.003	0.463	0.005	0.205	<0.001	0.988	0.003	0.550	-0.002	0.572
Dimethylglycine[1]	1	0.279	0.439	0.102	0.794	0.230	0.520	0.481	0.245	-0.105	0.795	0.293	0.431
	2	0.264	0.459	0.050	0.897	0.238	0.509	0.484	0.236	-0.141	0.729	0.325	0.379
Glycine[1]	1	0.993	0.097	0.157	0.808	1.299	0.028*	0.200	0.771	1.302	0.051	-0.050	0.935
	2	1.195	0.047*	0.369	0.573	1.371	0.024*	0.235	0.734	1.388	0.042*	0.290	0.643
Homocysteine[1]	1	-0.248	0.560	-0.392	0.392	0.116	0.792	-0.133	0.782	-0.379	0.435	-0.375	0.397
	2	-0.238	0.573	-0.333	0.461	0.096	0.828	-0.160	0.735	-0.398	0.415	-0.341	0.437
Methionine	1	0.002	0.910	-0.005	0.758	0.011	0.501	0.012	0.522	0.002	0.907	-0.023	0.169
	2	0.001	0.936	-0.007	0.705	0.011	0.505	0.012	0.530	0.001	0.945	-0.024	0.178
S-adenosyl-methionine	1	<0.001	0.903	<0.001	0.853	<0.001	0.893	0.002	0.256	<0.001	0.874	-0.001	0.344
	2	<0.001	0.899	-0.001	0.665	<0.001	0.935	0.001	0.416	<0.001	0.967	-0.001	0.430
Serine	1	-0.002	0.642	-0.003	0.473	0.002	0.547	-0.004	0.430	-0.001	0.884	-0.006	0.126
	2	-0.001	0.789	-0.002	0.562	0.003	0.463	-0.003	0.574	<0.001	0.973	-0.007	0.124

β estimate and *p* values presented for each variable in the domains of diet, serum status, or metabolite status fitted as an independent variable in models adjusted for 1) age, sex, education, and batch effect (metabolites only), or 2) Model 1 with further adjustment for energy intake, physical activity, and history of anxiety/depression. * Indicates a significant association ($p < 0.05$) [1]Fit as log transformed variables in models.

Table 9.9: Supplemental Material (Chapter 5): Effect of interaction between one-carbon metabolites on cognitive performance in multivariate linear regression models.

Dependent variable	Model terms		Global cognition		Attention		Episodic Memory		Executive function		Location Learning		Working Memory	
			β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
Betaine	Betaine	Low	0.147	0.759	-0.056	0.914	0.074	0.881	0.085	0.869	0.886	0.097	-0.238	0.648
		High	-0.243	0.641	-0.269	0.632	-0.348	0.518	-0.478	0.397	0.540	0.352	0.151	0.790
			0.005	0.521	0.002	0.811	<0.001	0.990	-0.001	0.942	0.021	0.024*	0.005	0.611
	Interaction	Low	-0.005	0.765	0.003	0.848	-0.004	0.813	-0.005	0.772	-0.024	0.157	0.007	0.662
		High	0.005	0.723	0.003	0.856	0.009	0.527	0.014	0.327	-0.015	0.324	-0.004	0.795
	Cysteine	Low	0.203	0.669	0.900	0.080	0.310	0.528	0.293	0.570	-1.031	0.052	-0.887	0.086
		High	0.297	0.528	0.365	0.473	0.215	0.659	0.445	0.384	-0.422	0.422	0.238	0.640
	Betaine		0.011	0.190	0.011	0.193	0.008	0.354	0.006	0.461	0.002	0.781	0.004	0.674
	Interaction	Low	-0.008	0.586	-0.025	0.112	0.013	0.394	-0.004	0.779	0.028	0.085	0.021	0.180
		High	-0.012	0.326	-0.015	0.277	-0.010	0.437	-0.008	0.553	0.009	0.544	-0.009	0.516
	Dimethylglycine	Low	-0.475	0.308	-0.593	0.239	-0.207	0.667	0.046	0.927	-0.078	.881	-0.749	0.140
		High	0.075	0.884	-0.035	0.951	0.352	0.511	-0.479	0.393	0.456	0.430	-0.488	0.386
	Betaine		-0.001	0.952	-0.005	0.576	<0.001	0.982	-0.003	0.763	0.014	0.140	-0.004	0.680
	Interaction	Low	0.016	0.262	0.025	0.122	0.006	0.675	-0.005	0.739	0.004	0.811	0.022	0.161
		High	0.001	0.918	0.004	0.766	-0.006	0.669	0.016	0.284	-0.013	0.376	0.013	0.368
	Glycine	Low	-0.380	0.391	-0.528	0.275	-0.278	0.543	0.007	0.988	-0.989	0.045*	0.747	0.125
		High	0.677	0.173	0.285	0.599	0.760	0.139	0.336	0.539	0.858	0.121	-0.209	0.701
	Betaine		0.005	0.494	-0.001	0.873	0.003	0.680	0.006	0.497	0.006	0.509	0.011	0.197
	Interaction	Low	0.006	0.628	0.010	0.446	0.004	0.769	-0.003	0.835	0.026	0.060	-0.021	0.117
		High	-0.013	0.374	-0.004	0.789	-0.014	0.357	-0.010	0.547	-0.019	0.246	0.005	0.753
	Homocysteine	Low	0.262	0.608	0.152	0.780	0.023	0.967	0.951	0.071	-0.100	0.862	0.220	0.700
		High	-0.456	0.383	-1.015	0.069	-0.082	0.884	-0.147	0.784	0.292	0.620	0.249	0.669
	Betaine		<0.001	0.976	-0.012	0.229	0.001	0.952	0.012	0.217	0.006	0.595	0.007	0.491
	Interaction	Low	-0.007	0.640	<0.001	0.980	-0.003	0.838	-0.026	0.074	0.003	0.840	-0.004	0.798
		High	0.007	0.619	0.025	0.115	<0.001	0.982	-0.002	0.890	0.001	0.940	-0.009	0.591
	Methionine	Low	-0.300	0.537	-0.630	0.228	-0.084	0.868	0.245	0.643	-0.522	0.335	0.161	0.761
		High	0.363	0.470	0.106	0.845	0.455	0.382	0.321	0.558	0.362	0.519	-0.197	0.733
	Betaine		0.006	0.427	<0.001	0.963	0.003	0.05	0.007	0.410	0.011	0.188	0.008	0.353
	Interaction	Low	0.009	0.532	0.011	0.496	0.008	0.584	-0.009	0.570	0.017	0.315	-0.003	0.868
		High	-0.012	0.381	-0.008	0.568	-0.008	0.537	-0.009	0.529	-0.014	0.351	<0.001	0.979

Dependent variable	Model terms		Global cognition		Attention		Episodic Memory		Executive function		Location Learning		Working Memory	
			β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
Choline	*S*-adenosylmethionine	Low	0.474	0.350	0.303	0.581	-0.025	0.962	0.602	0.277	0.986	0.080	0.480	0.386
		High	0.117	0.796	0.370	0.450	0.034	0.942	0.072	0.884	0.096	0.849	-0.375	0.449
	Betaine		0.011	0.166	0.009	0.290	0.002	0.784	0.008	0.358	0.021	0.021*	0.007	0.406
	Interaction	Low	-0.018	0.219	-0.015	0.363	-0.002	0.917	-0.016	0.320	-0.035	0.035#	-0.015	0.350
		High	-0.009	0.465	-0.017	0.200	-0.002	0.903	-0.004	0.779	-0.012	0.398	0.005	0.696
	Serine	Low	0.024	0.957	0.622	0.194	-0.492	0.283	0.115	0.810	0.293	0.553	-0.147	0.758
		High	-0.336	0.492	-0.085	0.872	-0.180	0.721	0.165	0.756	-0.516	0.344	-0.795	0.133
	Betaine		0.003	0.716	0.005	0.516	-0.003	0.719	0.004	0.619	0.010	0.255	-0.001	0.881
	Interaction	Low	0.001	0.964	-0.015	0.248	0.011	0.377	0.001	0.922	-0.009	0.496	0.010	0.435
		High	0.011	0.446	0.002	0.899	0.006	0.696	-0.003	0.860	0.016	0.316	0.025	0.097
	Betaine	Low	0.369	0.471	0.077	0.889	-0.008	0.988	1.245	0.025*	0.537	0.350	0.138	0.805
		High	0.085	0.881	-0.338	0.583	0.034	0.954	0.500	0.416	-0.122	0.849	0.682	0.271
	Choline		0.020	0.600	-0.021	0.605	0.022	0.573	0.055	0.184	0.014	0.743	0.022	0.597
	Interaction	Low	-0.074	0.245	-0.048	0.487	-0.005	0.944	-0.163	0.018#	-0.087	0.221	-0.047	0.496
		High	-0.012	0.837	0.019	0.774	0.001	0.991	-0.045	0.488	0.013	0.844	-0.076	0.247
	Cysteine	Low	-0.423	0.435	0.665	0.255	-0.660	0.238	0.418	0.477	-1.574	0.009*	-0.665	0.258
		High	-0.061	0.909	0.557	0.333	-0.336	0.541	0.194	0.737	-1.097	0.064	0.354	0.540
	Choline		0.012	0.731	0.033	0.388	0.012	0.743	0.016	0.688	-0.058	0.143	0.006	0.876
	Interaction	Low	0.049	0.468	-0.070	0.340	0.075	0.284	-0.035	0.636	0.179	0.017#	0.057	0.435
		High	-0.009	0.874	-0.077	0.204	0.020	0.736	-0.005	0.934	0.109	0.080	-0.047	0.443
	Dimethylglycine	Low	0.160	0.767	-0.533	0.359	0.743	0.180	-0.223	0.702	0.639	0.289	-0.544	0.354
		High	0.351	0.519	0.119	0.839	0.644	0.251	-0.314	0.596	0.077	0.900	0.076	0.899
	Choline		0.016	0.685	-0.038	0.381	0.060	0.144	-0.024	0.583	0.030	0.510	-0.011	0.807
	Interaction	Low	-0.018	0.783	0.086	0.234	-0.095	0.168	0.015	0.838	-0.086	0.252	0.062	0.399
		High	-0.025	0.663	0.005	0.941	-0.061	0.296	0.047	0.448	-0.012	0.853	-0.005	0.935
	Glycine	Low	-1.039	0.054	-1.016	0.085	-0.968	0.081	-0.756	0.203	-0.632	0.299	0.508	0.395
		High	0.65	0.123	-0.117	0.829	1.053	0.039*	0.105	0.848	0.984	0.080	0.360	0.513
	Choline		0.006	0.853	-0.041	0.264	0.028	0.425	-0.006	0.873	0.015	0.698	0.031	0.406
	Interaction	Low	0.092	0.107	0.092	0.141	0.087	0.141	0.072	0.255	0.056	0.389	-0.055	0.385
		High	-0.065	0.274	0.032	0.625	-0.094	0.125	-0.012	0.853	-0.092	0.175	-0.053	0.425
	Homocysteine	Low	0.447	0.437	0.720	0.245	0.114	0.854	0.249	0.678	0.371	0.569	0.038	0.954
		High	0.766	0.163	0.799	0.175	0.706	0.232	-0.291	0.610	0.332	0.592	0.493	0.423
	Choline		0.080	0.058	0.056	0.213	0.066	0.144	0.047	0.281	0.054	0.259	0.034	0.468
	Interaction	Low	-0.049	0.497	-0.075	0.332	-0.021	0.789	-0.023	0.756	-0.042	0.607	0.010	0.903
		High	-0.112	0.063	-0.110	0.090	-0.092	0.157	0.006	0.923	-0.069	0.310	-0.063	0.354
	Methionine	Low	-0.183	0.711	-0.690	0.191	-0.035	0.945	-0.093	0.861	0.528	0.338	0.090	0.866
		High	-0.298	0.575	0.061	0.914	-0.207	0.705	-0.682	0.236	0.058	0.923	-0.548	0.341

Dependent variable	Model terms		Global cognition		Attention		Episodic Memory		Executive function		Location Learning		Working Memory	
			β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
Cysteine	Choline		0.003	0.946	-0.022	0.584	0.010	0.786	-0.014	0.729	0.036	0.390	0.008	0.849
	Interaction	Low	0.019	0.747	0.048	0.440	0.026	0.668	0.003	0.959	-0.070	0.285	-0.004	0.948
		High	0.025	0.657	-0.026	0.667	0.037	0.530	0.074	0.231	-0.024	0.708	0.038	0.537
	S-adenosylmethionine	Low	-0.014	0.980	-0.171	0.774	0.047	0.935	0.363	0.547	0.096	0.877	-0.302	0.619
		High	0.509	0.305	0.691	0.197	0.522	0.311	0.592	0.274	-0.040	0.943	-0.649	0.232
	Choline		0.047	0.192	0.026	0.498	0.051	0.174	0.042	0.285	0.030	0.465	-0.011	0.775
	Interaction	Low	-0.014	0.824	-0.002	0.980	-0.014	0.835	-0.034	0.619	-0.034	0.629	0.032	0.641
		High	-0.080	0.145	-0.103	0.084	-0.066	0.251	-0.074	0.217	-0.026	0.673	0.056	0.352
	Serine	Low	-0.531	0.277	0.080	0.880	-1.094	0.030*	0.111	0.833	0.055	0.920	-0.120	0.820
		High	-0.558	0.290	-0.200	0.727	-0.541	0.318	-0.257	0.653	-0.311	0.600	-0.542	0.345
	Choline		-0.019	0.571	-0.018	0.635	-0.020	0.565	0.002	0.959	0.002	0.960	-0.016	0.672
	Interaction	Low	0.066	0.231	0.001	0.898	0.115	0.041#	0.004	0.943	-0.012	0.842	0.037	0.536
		High	0.069	0.255	0.021	0.752	0.065	0.293	0.040	0.545	0.039	0.568	0.070	0.282
	Betaine	Low	-0.103	0.911	-0.403	0.684	-0.610	0.523	1.142	0.254	-0.018	0.986	0.887	0.373
		High	0.297	0.719	-0.331	0.710	-0.109	0.899	0.810	0.367	0.110	0.906	1.689	0.060
	Cysteine		-0.002	0.741	-0.008	0.169	-0.001	0.920	0.004	0.532	-0.002	0.681	0.007	0.230
	Interaction	Low	-0.001	0.913	0.001	0.892	0.006	0.568	-0.012	0.242	-0.001	0.914	-0.012	0.265
		High	-0.003	0.703	0.002	0.847	0.002	0.848	-0.007	0.429	-0.001	0.921	-0.017	0.054
	Choline	Low	-0.236	0.785	0.134	0.886	-0.086	0.924	0.178	0.850	-0.469	0.629	-1.023	0.278
		High	-0.140	0.881	-0.185	0.854	-0.111	0.909	-0.741	0.464	0.282	0.787	0.373	0.713
	Cysteine		-0.003	0.637	-0.005	0.424	0.001	0.871	-0.002	0.717	-0.003	0.613	-0.002	0.755
	Interaction	Low	0.002	0.800	-0.002	0.873	0.001	0.941	-0.003	0.792	0.006	0.565	0.010	0.290
		High	0.001	0.902	<0.001	0.961	0.001	0.919	0.008	0.421	-0.002	0.835	-0.003	0.745
	Dimethylglycine	Low	0.327	0.723	-1.567	0.115	2.048	0.032*	1.343	0.179	-0.486	0.640	-1.628	0.106
		High	0.085	0.919	-0.384	0.669	0.840	0.328	-0.434	0.631	-0.094	0.920	-0.607	0.505
	Cysteine		-0.002	0.676	-0.011	0.055	0.007	0.227	-0.001	0.922	-0.004	0.547	-0.004	0.475
	Interaction	Low	-0.004	0.716	0.018	0.086	-0.022	0.028#	-0.015	0.141	0.005	0.662	0.016	0.120
		High	0.001	0.950	0.005	0.540	-0.007	0.382	0.005	0.545	0.001	0.903	0.006	0.484
	Glycine	Low	-0.650	0.443	-0.048	0.959	-0.985	0.259	-0.423	0.648	-1.030	0.280	0.756	0.417
		High	-0.245	0.782	-0.274	0.776	-0.418	0.648	0.938	0.335	0.179	0.858	-0.674	0.491
	Cysteine		-0.003	0.565	-0.006	0.283	-0.001	0.841	0.001	0.792	-0.004	0.543	0.001	0.866
	Interaction	Low	0.005	0.565	-0.001	0.904	0.008	0.332	0.003	0.727	0.009	0.324	-0.007	0.425
		High	0.005	0.571	0.004	0.662	0.008	0.420	-0.010	0.336	0.001	0.942	0.006	0.525
	Homocysteine	Low	0.703	0.473	1.475	0.159	-0.169	0.872	1.330	0.189	1.009	0.359	-0.975	0.366
		High	0.481	0.661	0.103	0.30	-0.699	0.553	1.353	0.233	1.102	0.371	1.978	0.102
	Cysteine		0.004	0.504	<0.001	0.975	0.001	0.926	0.011	0.104	0.005	0.534	0.006	0.385
	Interaction	Low	-0.007	0.497	-0.015	0.173	0.001	0.928	-0.013	0.222	-0.011	0.367	0.012	0.274
		High	-0.007	0.519	-0.003	0.816	0.005	0.613	-0.015	0.152	-0.013	0.262	-0.019	0.093
	Methionine	Low	0.006	0.994	-0.027	0.977	0.302	0.737	0.709	0.457	-2.210	0.024*	0.689	0.470

Dependent variable	Model terms		Global cognition		Attention		Episodic Memory		Executive function		Location Learning		Working Memory	
			β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
	Cysteine	High	-0.939	0.263	0.913	0.309	-1.463	0.090	-0.253	0.782	-2.324	0.013*	-0.330	0.717
			-0.005	0.428	-0.002	0.722	-0.004	0.538	<0.001	0.946	-0.014	0.024*	0.004	0.573
	Interaction	Low	-0.001	0.949	-0.003	0.769	-0.002	0.864	-0.008	0.410	0.022	0.027#	-0.007	0.502
		High	0.009	0.289	-0.011	0.222	0.016	0.062	0.002	0.792	0.022	0.016#	0.001	0.892
	S-adenosylmethionine	Low	-0.455	0.562	-0.399	0.637	-0.629	0.440	0.480	0.575	-0.605	0.492	0.237	0.782
		High	-0.291	0.763	-0.256	0.806	-0.390	0.697	0.179	0.865	-0.275	0.800	0.137	0.897
	Cysteine		-0.003	0.607	-0.007	0.273	-0.001	0.802	0.002	0.753	-0.003	0.603	0.003	0.594
	Interaction	Low	0.003	0.697	0.002	0.833	0.006	0.494	-0.004	0.619	0.004	0.643	-0.003	0.765
		High	0.001	0.882	0.001	0.936	0.004	0.702	-0.002	0.834	0.001	0.961	-0.003	0.769
	Serine	Low	-0.112	0.886	1.731	0.040*	-1.692	0.036*	0.970	0.254	0.614	0.495	-0.896	0.292
		High	-0.477	0.600	0.059	0.952	-0.613	0.512	0.104	0.916	-0.125	0.903	-0.956	0.333
	Cysteine		-0.003	0.553	<0.001	0.961	-0.005	0.328	0.003	0.613	-0.001	0.907	-0.004	0.533
	Interaction	Low	0.001	0.861	-0.017	0.044#	0.016	0.046#	-0.008	0.327	-0.007	0.435	0.011	0.188
		High	0.005	0.578	-0.001	0.943	0.006	0.507	<0.001	0.981	0.001	0.887	0.010	0.303
Dimethylglycine[1]	Betaine	Low	-0.316	0.195	-0.611	0.020*	-0.138	0.585	0.268	0.310	0.040	0.883	-0.377	0.155
		High	-0.115	0.753	-0.411	0.295	0.093	0.806	-0.163	0.680	0.195	0.634	-0.078	0.845
	Dimethylglycine		-0.157	0.758	-0.879	0.109	0.009	0.987	0.662	0.230	0.140	0.807	0.136	0.806
	Interaction	Low	0.522	0.541	1.335	0.147	0.364	0.681	-1.078	0.244	-0.757	0.431	0.862	0.354
		High	0.287	0.766	0.742	0.474	-0.112	0.911	0.711	0.495	-0.508	0.639	0.161	0.878
	Choline	Low	-0.210	0.393	-0.321	0.227	-0.107	0.675	-0.309	0.246	0.368	0.183	-0.241	0.369
		High	-0.169	0.609	-0.078	0.827	-0.244	0.475	-0.223	0.532	0.422	0.255	-0.260	0.469
	Dimethylglycine		-0.011	0.983	-0.307	0.579	-0.065	0.903	-0.044	0.937	0.619	0.283	0.192	0.732
	Interaction	Low	1.001	0.241	1.587	0.085	0.331	0.708	1.290	0.163	-1.022	0.286	1.001	0.281
		High	0.329	0.703	-0.195	0.834	0.640	0.473	0.770	0.409	-1.106	0.252	0.699	0.455
	Cysteine	Low	-0.130	0.617	0.294	0.292	-0.237	0.375	0.412	0.141	-0.537	0.064	-0.510	0.069
		High	-0.332	0.301	0.276	0.427	-0.597	0.072	0.031	0.929	-0.357	0.320	-0.361	0.299
	Dimethylglycine		0.096	0.861	0.572	0.332	-0.279	0.620	0.750	0.204	-0.653	0.285	0.045	0.940
	Interaction	Low	0.277	0.739	-0.686	0.446	0.497	0.562	-0.950	0.291	1.394	0.135	1.058	0.240
		High	0.519	0.539	-1.247	0.173	1.293	0.138	0.204	0.824	0.794	0.402	0.741	0.418
	Glycine	Low	-0.657	0.038*	-1.056	0.002*	-0.584	0.073	0.021	0.951	-0.202	0.572	0.410	0.239
		High	0.392	0.108	-0.041	0.875	0.500	0.048*	0.303	0.259	0.375	0.176	0.142	0.597
	Dimethylglycine		0.279	0.542	-0.667	0.179	0.296	0.561	0.884	0.080	0.182	0.727	0.996	0.049
	Interaction	Low	1.465	0.104	2.741	0.005#	1.320	0.156	-0.386	0.696	0.305	0.765	-1.272	0.201
		High	-0.527	0.473	0.566	0.478	-0.731	0.335	-0.953	0.239	-0.412	0.621	-6.190	0.445
	Homocysteine	Low	0.058	0.853	0.242	0.476	-0.054	0.873	0.199	0.544	0.122	0.731	-0.332	0.344
		High	-0.417	0.200	-0.108	0.756	-0.415	0.234	-0.415	0.218	-0.215	0.557	-0.258	0.473
	Dimethylglycine		0.002	0.997	0.159	0.770	-0.195	0.718	0.409	0.434	-0.141	0.804	-0.022	0.969
	Interaction	Low	-0.098	0.921	-0.440	0.678	-0.103	0.922	-0.511	0.617	-0.369	0.740	1.504	0.171
		High	0.614	0.479	-0.173	0.853	0.952	0.306	0.528	0.556	-0.149	0.879	0.629	0.513

Dependent variable	Model terms		Global cognition		Attention		Episodic Memory		Executive function		Location Learning		Working Memory	
			β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
Glycine[1]	Methionine	Low	-0.053	0.827	-0.653	0.012*	0.228	0.361	-0.050	0.848	0.137	0.612	0.315	0.227
		High	0.099	0.783	0.102	0.789	0.219	0.552	-0.052	0.894	0.058	0.886	-0.295	0.444
	Dimethylglycine		0.327	0.481	-0.424	0.393	0.302	0.529	0.468	0.353	0.352	0.500	0.986	0.050
	Interaction	Low	0.144	0.854	1.375	0.100	-0.194	0.809	0.055	0.948	-0.674	0.442	-0.866	0.304
		High	-0.474	0.616	-0.723	0.473	-0.256	0.793	0.071	0.945	-0.597	0.573	0.133	0.898
	S-adenosylmethionine	Low	-0.110	0.731	-0.667	0.053	0.018	0.956	0.561	0.105	0.128	0.719	0.045	0.897
		High	-0.065	0.817	-0.556	0.066	0.300	0.300	0.189	0.532	-0.069	0.825	-0.183	0.548
	Dimethylglycine		0.520	0.296	-0.675	0.209	0.564	0.274	1.226	0.023*	0.563	0.312	0.864	0.111
	Interaction	Low	-0.046	0.959	1.419	0.138	-0.242	0.792	-1.446	0.133	-0.986	0.321	-0.098	0.979
		High	-0.484	0.545	1.264	0.144	-1.136	0.171	-1.103	0.203	-0.716	0.425	-0.232	0.790
	Serine	Low	0.149	0.603	0.514	0.096	-0.368	0.214	0.307	0.322	0.404	0.207	0.110	0.722
		High	0.112	0.673	0.393	0.171	-0.071	0.798	-0.008	0.979	-0.058	0.845	0.071	0.804
	Dimethylglycine		0.431	0.367	0.614	0.233	-0.116	0.814	0.567	0.272	0.299	0.575	0.571	0.269
	Interaction	Low	-0.344	0.668	-1.320	0.126	0.829	0.316	-0.470	0.587	-1.425	0.112	0.323	0.709
		High	-0.315	0.691	-1.395	0.103	0.265	0.746	0.249	0.771	2.484	0.779	-0.095	0.912
	Betaine	Low	-3.366	0.184	-2.596	0.345	-2.287	0.384	-2.634	0.344	-3.827	0.178	-0.253	0.927
		High	0.046	0.986	1.474	0.606	-0.105	0.969	-0.872	0.763	2.273	0.442	-3.794	0.190
	Glycine		0.984	0.153	0.831	0.267	1.148	0.109	0.029	0.969	1.163	0.133	-0.739	0.328
	Interaction	Low	1.371	0.209	1.020	0.390	0.958	0.398	1.129	0.347	1.604	0.191	0.005	0.997
		High	-0.026	0.982	-0.718	0.566	0.072	0.952	0.429	0.734	-0.987	0.445	1.650	0.191
	Choline	Low	-0.005	0.998	0.006	0.998	0.235	0.931	0.236	0.935	-2.011	0.496	1.013	0.727
		High	6.702	0.020*	4.187	0.183	7.437	0.013*	4.036	0.202	3.349	0.303	-0.301	0.925
	Glycine		1.897	0.005*	1.309	0.078	2.052	0.004*	0.767	0.304	1.485	0.053	-0.342	0.649
	Interaction	Low	-0.009	0.994	0.003	0.998	-0.122	0.916	-0.123	0.921	0.893	0.479	-0.456	0.713
		High	-2.968	0.020#	-1.913	0.168	-3.278	0.013#	-1.748	0.211	-1.454	0.310	0.142	0.920
	Cysteine	Low	-1.398	0.585	-2.680	0.335	-0.604	0.820	-6.296	0.024*	0.200	0.945	5.453	0.051
		High	-1.973	0.454	-3.843	0.179	0.219	0.936	-3.902	0.172	0.779	0.792	-0.460	0.872
	Glycine		0.965	0.175	0.277	0.719	1.348	0.067	-0.619	0.422	1.496	0.062	0.018	0.982
	Interaction	Low	0.579	0.599	1.205	0.314	0.218	0.849	2.780	0.021#	-0.154	0.901	-2.455	0.041#
		High	0.813	0.479	1.620	0.193	-0.144	0.904	1.774	0.154	-0.376	0.770	0.163	0.896
	Dimethylglycine	Low	-2.147	0.418	-1.337	0.645	-3.168	0.248	-0.386	0.894	-2.309	0.442	2.605	0.376
		High	3.902	0.133	3.554	0.210	3.399	0.204	5.204	0.067	-0.902	0.758	0.168	0.953
	Glycine		1.497	0.036*	1.074	0.167	1.424	0.053	0.901	0.247	1.132	0.159	-0.053	0.947
	Interaction	Low	0.893	0.433	0.613	0.622	1.317	0.264	0.111	0.929	0.955	0.459	-1.145	0.365
		High	-1.652	0.145	-1.507	0.223	-1.436	0.220	-2.226	0.073	0.395	0.758	-0.070	0.957
	Homocysteine	Low	1.765	0.541	-0.162	0.957	1.541	0.604	0.919	0.751	2.044	0.512	2.664	0.391
		High	-1.160	0.703	-0.933	0.776	-1.469	0.652	-1.186	0.709	-1.173	0.732	2.032	0.551
	Glycine		1.156	0.168	0.538	0.551	1.331	0.139	-0.057	0.949	1.250	0.186	0.267	0.776
	Interaction	Low	-0.745	0.531	0.121	0.925	-0.697	0.585	-0.375	0.763	-0.868	0.517	-1.108	0.406

Dependent variable	Model terms		Global cognition		Attention		Episodic Memory		Executive function		Location Learning		Working Memory	
			β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
Homocysteine[1]		High	0.408	0.756	0.331	0.815	0.589	0.676	0.420	0.760	0.387	0.793	-0.901	0.541
	Methionine	Low	0.609	0.809	0.884	0.745	-0.602	0.817	2.272	0.410	1.670	0.554	-0.624	0.820
		High	-2.635	0.309	-0.503	0.858	-2.891	0.279	2.588	0.362	-2.366	0.415	-5.302	0.062
	Glycine		1.248	0.089	1.188	0.134	1.011	0.181	0.911	0.256	1.523	0.064	-0.853	0.286
	Interaction	Low	-0.277	0.800	-0.510	0.666	0.336	0.765	-1.013	0.398	-0.744	0.544	0.291	0.807
		High	1.095	0.328	0.116	0.924	1.296	0.262	-1.128	0.358	0.944	0.425	2.217	0.071
	S-adenosylmethionine	Low	-0.281	0.908	-2.563	0.331	0.717	0.776	2.329	0.384	-1.564	0.566	0.930	0.729
		High	1.909	0.500	1.714	0.577	2.069	0.481	0.321	0.918	-0.465	0.883	1.165	0.709
	Glycine		1.526	0.040*	0.894	0.266	1.712	0.026*	0.693	0.394	1.181	0.155	-0.183	0.822
	Interaction	Low	0.057	0.957	1.020	0.370	-0.346	0.750	-0.979	0.395	0.589	0.616	-0.412	0.721
		High	-0.906	0.465	0.845	0.529	-0.908	0.479	-0.151	0.912	0.100	0.943	-0.578	0.672
	Serine	Low	-0.536	0.833	-1.165	0.674	1.986	0.450	-1.704	0.540	-2.431	0.395	-2.112	0.450
		High	-1.430	0.586	-0.949	0.740	-1.006	0.711	-0.368	0.898	-1.803	0.541	-0.880	0.760
	Glycine		1.376	0.093	1.152	0.196	1.583	0.062	0.301	0.736	1.073	0.244	-0.676	0.452
	Interaction	Low	0.289	0.795	0.585	0.628	-0.882	0.443	0.826	0.497	1.082	0.387	1.005	0.411
		High	0.582	0.606	0.363	0.767	0.393	0.736	0.179	0.885	0.743	0.558	0.415	0.738
	Betaine	Low	0.170	0.607	-0.106	0.764	-0.044	0.901	0.575	0.094	0.676	0.070	0.050	0.891
		High	0.253	0.451	-0.101	0.779	0.138	0.703	0.479	0.169	0.416	0.271	0.320	0.391
	Homocysteine		0.075	0.893	-0.564	0.341	0.275	0.646	0.348	0.545	0.288	0.645	0.161	0.794
	Interaction	Low	-0.879	0.344	-0.195	0.844	0.048	0.962	-1.764	0.067	-1.928	0.065	-0.921	0.371
		High	-1.005	0.285	-0.627	0.530	-0.355	0.725	-1.038	0.285	-1.087	0.303	-1.108	0.287
	Choline	Low	0.061	0.841	0.361	0.262	0.072	0.824	-0.213	0.496	-0.319	0.348	-0.093	0.781
		High	0.356	0.403	0.478	0.292	0.251	0.583	0.099	0.822	-0.245	0.609	0.390	0.411
	Homocysteine		-0.136	0.813	0.061	0.921	0.039	0.530	-0.631	0.289	-1.072	0.098	-0.303	0.635
	Interaction	Low	-0.308	0.725	-1.248	0.181	-0.350	0.709	0.338	0.709	1.295	0.189	0.275	0.777
		High	-0.770	0.480	-1.537	0.190	-0.570	0.629	0.328	0.773	1.016	0.411	-0.736	0.547
	Cysteine	Low	-0.310	0.333	0.193	0.574	-0.245	0.474	-0.077	0.818	-0.628	0.082	-0.698	0.048*
		High	-0.320	0.480	-0.329	0.498	-0.569	0.239	0.505	0.283	-0.333	0.513	0.369	0.458
	Homocysteine		-0.612	0.293	-0.599	0.337	0.033	0.957	-0.518	0.391	-1.069	0.103	-0.651	0.309
	Interaction	Low	0.368	0.733	-0.551	0.634	-0.052	0.964	0.530	0.636	1.532	0.207	1.259	0.289
		High	0.587	0.587	0.766	0.509	0.901	0.436	-0.727	0.518	0.744	0.540	-0.928	0.435
	Dimethylglycine	Low	0.678	0.061	0.312	0.420	0.845	0.029*	0.676	0.070	0.246	0.548	-0.230	0.570
		High	0.197	0.545	0.370	0.289	0.117	0.737	0.139	0.679	-0.140	0.705	-0.015	0.967
	Homocysteine		-0.023	0.966	-0.459	0.423	0.597	0.295	0.010	0.986	-0.523	0.389	-0.371	0.536
	Interaction	Low	-1.876	0.073	-0.320	0.775	-2.606	0.020#	-2.511	0.020#	-0.360	0.761	0.603	0.606
		High	-0.136	0.874	-0.433	0.639	-0.018	0.984	-0.088	0.921	0.376	0.701	-0.084	0.831
	Glycine	Low	0.656	0.204	1.083	0.050	0.237	0.669	0.144	0.789	0.144	0.805	-0.230	0.570
		High	0.797	0.377	0.847	0.378	0.534	0.582	0.365	0.697	0.293	0.774	-0.015	0.967
	Homocysteine		1.886	0.173	2.010	0.173	1.397	0.347	0.771	0.592	0.686	0.660	-0.371	0.536

Dependent variable	Model terms		Global cognition		Attention		Episodic Memory		Executive function		Location Learning		Working Memory	
			β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
Methionine	Interaction	Low	-1.932	0.323	-4.201	0.045#	-0.400	0.849	0.354	0.862	0.056	0.980	0.603	0.606
		High	-2.594	0.197	-2.652	0.216	-1.695	0.433	-1.395	0.505	-1.317	0.562	-0.084	0.931
	Methionine	Low	0.349	0.356	0.431	0.281	0.123	0.759	0.433	0.267	-0.219	0.606	0.401	0.487
		High	-0.266	0.399	0.264	0.428	-0.350	0.297	-0.400	0.219	-0.688	0.053	0.449	0.656
	Homocysteine		-0.420	0.510	0.446	0.506	-0.562	0.406	-0.565	0.389	-1.232	0.085	0.777	0.615
	Interaction	Low	-1.064	0.331	-2.162	0.062	0.245	0.833	-1.644	0.146	0.798	0.515	-1.185	0.588
		High	0.649	0.439	-1.482	0.095	1.596	0.074	1.072	0.215	1.485	0.115	-1.255	0.577
	S-adenosylmethionine	Low	-0.370	0.274	0.202	0.576	-0.511	0.158	-0.147	0.677	-0.652	0.086	0.459	0.276
		High	0.043	0.910	0.379	0.346	-0.144	0.720	-0.014	0.971	-0.222	0.599	0.060	0.863
	Homocysteine		-0.591	0.327	-0.059	0.926	-0.479	0.459	-0.603	0.339	-0.971	0.152	0.142	0.842
	Interaction	Low	1.119	0.210	-0.422	0.657	1.738	0.069	0.316	0.735	1.518	0.130	-1.230	0.313
		High	-0.447	0.657	-1.606	0.136	0.424	0.694	0.313	0.766	0.067	0.953	-0.441	0.637
	Serine	Low	-0.105	0.741	0.170	0.615	-0.326	0.338	0.353	0.283	-0.280	0.432	-0.199	0.598
		High	-0.034	0.730	-0.089	0.830	0.054	0.895	0.236	0.555	-0.580	0.182	0.095	0.822
	Homocysteine		-0.361	0.528	-0.414	0.499	-0.013	0.983	0.103	0.863	-0.850	0.186	-0.209	0.756
	Interaction	Low	0.302	0.729	-0.383	0.681	0.764	0.413	-0.710	0.432	0.399	0.683	0.250	0.802
		High	-0.371	0.731	-0.231	0.841	-0.399	0.730	-1.066	0.340	1.031	0.394	-0.711	0.526
Methionine	Betaine	Low	-0.554	0.514	1.257	0.171	0.124	0.888	0.067	0.943	-1.263	0.188	0.361	0.697
		High	1.213	0.129	0.957	0.266	1.044	0.209	0.507	0.561	0.698	0.437	0.517	0.552
	Methionine		0.004	0.857	-0.008	0.733	0.014	0.534	0.015	0.517	-0.002	0.930	-0.015	0.527
	Interaction	Low	0.018	0.659	0.051	0.256	-0.009	0.835	-0.004	0.937	0.058	0.220	-0.031	0.493
		High	-0.055	0.123	-0.050	0.195	-0.045	0.227	-0.018	0.643	-0.030	0.451	-0.024	0.546
	Choline	Low	0.180	0.830	-1.130	0.210	0.918	0.287	-0.213	0.814	1.351	0.147	-0.414	0.648
		High	-0.031	0.969	-0.159	0.857	-0.316	0.709	-0.025	0.978	1.534	0.094	-0.418	0.640
	Methionine		-0.002	0.912	-0.018	0.423	0.007	0.766	0.007	0.752	0.033	0.164	-0.033	0.158
	Interaction	Low	-0.010	0.814	0.057	0.195	-0.047	0.260	0.009	0.833	-0.060	0.182	0.015	0.729
		High	-0.003	0.995	<0.001	0.994	0.014	0.715	0.004	0.918	-0.069	0.099	0.021	0.607
	Cysteine	Low	1.516	0.067	1.615	0.070	1.168	0.172	0.694	0.440	-0.140	0.881	1.097	0.221
		High	0.709	0.388	1.873	0.035*	-0.588	0.488	1.260	0.159	-0.347	0.708	0.801	0.369
	Methionine		0.021	0.322	0.034	0.152	0.008	0.718	0.032	0.172	-0.002	0.919	-0.003	0.886
	Interaction	Low	-0.078	0.054	-0.074	0.090	-0.063	0.129	-0.025	0.560	-0.001	0.978	-0.068	0.122
		High	-0.039	0.289	-0.093	0.021#	0.020	0.607	-0.051	0.208	0.011	0.798	-0.040	0.315
	Dimethylglycine	Low	-0.440	0.636	-1.969	0.049*	1.065	0.268	0.840	0.405	-1.020	0.328	-1.195	0.238
		High	0.935	0.224	0.652	0.429	0.874	0.271	0.676	0.418	0.778	0.367	-0.129	0.877
	Methionine		0.002	0.940	-0.017	0.459	0.018	0.426	0.023	0.333	0.006	0.805	-0.034	0.150
	Interaction	Low	0.023	0.618	0.105	0.032#	-0.054	0.256	-0.045	0.363	0.050	0.326	0.054	0.274
		High	-0.037	0.293	-0.024	0.523	-0.035	0.335	-0.026	0.490	-0.035	0.366	0.007	0.843
	Glycine	Low	0.356	0.649	0.281	0.741	-0.231	0.775	1.073	0.211	-0.254	0.773	1.208	0.159
		High	-0.357	0.641	-0.679	0.416	0.088	0.912	0.272	0.746	-0.538	0.534	-0.538	0.522

Dependent variable	Model terms		Global cognition		Attention		Episodic Memory		Executive function		Location Learning		Working Memory	
			β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
S-adenosyl-methionine	Methionine		-0.014	0.542	-0.023	0.361	-0.010	0.684	0.028	0.276	-0.017	0.521	-0.013	0.616
	Interaction	Low	-0.027	0.471	-0.024	0.560	0.004	0.927	-0.056	0.173	0.007	0.872	-0.060	0.146
		High	0.029	0.410	0.039	0.303	0.011	0.771	-0.012	0.747	0.037	0.345	0.022	0.561
	Homocysteine	Low	1.937	0.034*	1.305	0.182	1.581	0.106	1.781	0.060	0.83	0.402	0.822	0.420
		High	0.136	0.884	0.904	0.368	-0.361	0.719	-0.275	0.776	-0.170	0.872	0.340	0.746
	Methionine		0.018	0.478	0.010	0.697	0.016	0.541	0.033	0.211	0.002	0.941	-0.005	0.856
	Interaction	Low	-0.091	0.034#	-0.057	0.216	-0.079	0.085	-0.082	0.064	-0.040	0.406	-0.035	0.463
		High	-0.016	0.695	-0.048	0.285	0.011	0.808	0.001	0.987	-0.005	0.924	-0.018	0.706
	S-adenosylmethionine	Low	0.249	0.745	-0.999	0.223	0.284	0.721	1.608	0.053	0.403	0.638	0.630	0.450
		High	0.960	0.257	1.500	0.099	0.753	0.392	0.501	0.586	0.413	0.663	-0.850	0.357
	Methionine		0.016	0.488	0.001	0.981	0.015	0.518	0.040	0.104	0.021	0.394	-0.019	0.448
	Interaction	Low	-0.018	0.607	0.039	0.309	-0.017	0.645	-0.074	0.060	-0.028	0.481	-0.032	0.407
		High	-0.052	0.178	-0.078	0.062	-0.036	0.373	-0.027	0.516	-0.031	0.471	0.033	0.436
	Serine	Low	-0.568	0.487	0.217	0.806	-0.538	0.524	0.488	0.581	-0.364	0.690	-2.112	0.016*
		High	-0.729	0.366	-0.183	0.834	0.090	0.914	-0.281	0.748	-1.807	0.045*	-1.797	0.038*
	Methionine		-0.025	0.345	-0.009	0.757	-0.007	0.808	0.015	0.599	-0.034	0.248	-0.078	0.005*
	Interaction	Low	0.028	0.471	-0.007	0.868	0.021	0.604	-0.015	0.714	0.013	0.759	0.107	0.009#
		High	0.036	0.337	0.008	0.841	-0.003	0.933	0.015	0.706	0.085	0.041#	0.089	0.026#
	Betaine	Low	-0.596	0.037*	-0.751	0.015*	-0.618	0.037*	0.344	0.269	-0.035	0.915	-0.259	0.408
		High	0.037	0.902	0.043	0.893	-0.129	0.676	0.498	0.126	0.061	0.856	-0.067	0.838
	S-adenosylmethionine		-0.002	0.188	-0.003	0.104	-0.002	0.238	0.002	0.231	-0.001	0.536	-0.001	0.512
	Interaction	Low	0.003	0.130	0.004	0.069	0.004	0.042#	-0.003	0.204	-0.001	0.734	<0.001	0.988
		High	<0.001	0.940	-0.001	0.577	0.001	0.468	-0.003	0.177	<0.001	0.926	<0.001	0.836
	Choline	Low	-0.243	0.438	-0.226	0.503	-0.438	0.178	0.311	0.359	0.281	0.423	-0.199	0.561
		High	0.143	0.633	0.173	0.592	-0.027	0.931	0.490	0.133	0.449	0.183	-0.408	0.213
	S-adenosylmethionine		-0.001	0.541	-0.001	0.400	-0.001	0.590	0.002	0.210	<0.001	0.954	-0.002	0.245
	Interaction	Low	0.002	0.413	0.002	0.423	0.003	0.167	-0.003	0.260	-0.001	0.539	0.001	0.649
		High	-0.001	0.572	-0.002	0.323	<0.001	0.880	-0.003	0.136	-0.003	0.197	0.003	0.135
	Cysteine	Low	0.254	0.386	0.552	0.082	0.058	0.849	0.486	0.127	-0.518	0.117	0.123	0.702
		High	0.363	0.237	0.361	0.276	0.108	0.735	0.774	0.020*	0.119	0.731	0.065	0.845
	S-adenosylmethionine		0.001	0.729	<0.001	0.951	0.001	0.733	0.003	0.122	-0.001	0.501	<0.001	0.911
	Interaction	Low	-0.002	0.238	-0.003	0.115	-0.001	0.569	-0.003	0.259	0.003	0.224	-0.003	0.200
		High	-0.003	0.078	-0.003	0.100	-0.002	0.397	-0.004	0.038#	-0.001	0.496	-0.001	0.623
	Dimethylglycine	Low	-0.366	0.232	-0.595	0.071	-0.046	0.885	-0.037	0.912	-0.004	0.271	-0.235	0.483
		High	0.298	0.288	-0.023	0.939	0.415	0.154	0.286	0.348	0.040	0.899	0.174	0.571
	S-adenosylmethionine		-0.002	0.305	-0.003	0.056	<0.001	0.931	0.001	0.678	-0.002	0.352	-0.001	0.630
	Interaction	Low	0.002	0.226	0.005	0.022#	<0.001	0.986	<0.001	0.899	0.002	0.308	0.001	0.649
		High	-0.001	0.522	0.001	0.591	-0.002	0.269	-0.001	0.531	<0.001	0.916	-0.001	0.566
	Glycine	Low	-0.204	0.518	-0.102	0.766	-0.272	0.403	0.199	0.563	-0.428	0.227	0.092	0.790

Dependent variable	Model terms		Global cognition		Attention		Episodic Memory		Executive function		Location Learning		Working Memory	
			β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
	S-adenosylmethionine	High	0.097	0.735	0.079	0.802	0.155	0.602	0.102	0.747	0.006	0.986	-0.173	0.586
			-0.001	0.480	-0.002	0.270	<0.001	0.788	0.002	0.359	-0.002	0.290	-0.001	0.697
	Interaction	Low	<0.001	0.911	<0.001	0.811	0.001	0.667	-0.002	0.363	0.002	0.305	-0.001	0.807
		High	0.001	0.558	<0.001	0.856	0.001	0.552	-0.001	0.807	0.002	0.395	0.001	0.736
	Homocysteine	Low	-0.307	0.376	0.048	0.896	-0.587	0.115	-0.159	0.658	0.193	0.621	-0.138	0.721
		High	-0.177	0.595	0.236	0.505	-0.317	0.376	-0.573	0.099	-0.018	0.963	0.026	0.945
	S-adenosylmethionine		-0.002	0.197	-0.003	0.141	-0.003	0.193	0.001	0.604	-0.001	0.531	0.001	0.793
	Interaction	Low	0.002	0.299	<0.001	0.852	0.004	0.148	0.002	0.529	-0.001	0.618	0.002	0.503
		High	<0.001	0.980	-0.002	0.259	0.002	0.455	0.002	0.282	-0.002	0.499	-0.001	0.790
	Methionine	Low	0.005	0.896	-0.546	0.095	0.237	0.455	0.191	0.568	0.164	0.635	0.129	0.697
		High	0.214	0.450	0.288	0.339	0.415	0.157	-0.050	0.872	0.106	0.738	-0.646	0.036*
	S-adenosylmethionine		<0.001	0.883	-0.001	0.428	0.001	0.676	0.001	0.605	<0.001	0.965	-0.001	0.411
	Interaction	Low	<0.001	0.879	0.002	0.410	<0.001	0.828	-0.002	0.416	-0.002	0.492	-0.001	0.733
		High	-0.002	0.297	-0.003	0.087	-0.002	0.302	<0.001	0.895	-0.002	0.407	0.003	0.096
	Serine	Low	0.196	0.482	0.053	0.860	-0.183	0.726	0.497	0.100	0.410	0.189	0.590	0.052
		High	0.264	0.412	0.268	0.440	-0.004	0.992	0.288	0.408	0.258	0.473	0.364	0.296
	S-adenosylmethionine		<0.001	0.811	-0.002	0.352	<0.001	0.759	0.002	0.294	<0.001	0.925	0.001	0.687
	Interaction	Low	-0.001	0.523	<0.001	0.861	0.001	0.767	-0.003	0.196	-0.004	0.100	-0.003	0.150
		High	-0.002	0.428	-0.002	0.395	<0.001	0.951	-0.001	0.483	-0.002	0.457	-0.002	0.330
Serine	Betaine	Low	-0.385	0.555	0.281	0.689	-0.734	0.276	-0.065	0.927	-0.027	0.970	-0.498	0.480
		High	-0.143	0.825	-0.012	0.986	0.107	0.872	0.207	0.768	-0.340	0.640	-0.918	0.190
	Serine		-0.002	0.718	0.001	0.862	-0.001	0.878	-0.001	0.894	<0.001	0.965	-0.009	0.099
	Interaction	Low	0.002	0.774	-0.007	0.448	0.008	0.313	<0.001	0.955	-0.001	0.900	0.003	0.746
		High	0.002	0.842	-0.002	0.824	-0.001	0.927	-0.001	0.894	0.004	0.616	0.011	0.189
	Choline	Low	0.503	0.440	0.116	0.869	-0.050	0.941	0.975	0.166	1.075	0.140	0.639	0.367
		High	0.341	0.602	0.123	0.862	0.238	0.726	0.480	0.498	0.509	0.486	-0.030	0.966
	Serine		0.003	0.584	0.001	0.899	0.002	0.699	0.004	0.469	0.006	0.252	-0.002	0.684
	Interaction	Low	-0.006	0.426	-0.001	0.898	<0.001	0.969	-0.013	0.142	-0.012	0.168	-0.009	0.313
		High	-0.005	0.553	-0.004	0.684	-0.003	0.714	-0.005	0.554	-0.006	0.516	0.001	0.946
	Cysteine	Low	-0.401	0.543	0.481	0.499	-0.691	0.310	-0.416	0.559	-0.588	0.426	-0.230	0.747
		High	-0.592	0.363	-0.898	0.201	-0.020	0.976	-0.643	0.361	-0.725	0.320	0.002	0.997
	Serine		-0.003	0.598	-0.001	0.825	<0.001	0.961	-0.005	0.391	-0.002	0.719	-0.005	0.388
	Interaction	Low	0.004	0.603	-0.005	0.585	0.007	0.373	0.007	0.427	0.005	0.552	<0.001	0.963
		High	0.006	0.472	0.010	0.272	-0.002	0.855	0.010	0.244	0.008	0.392	-0.001	0.902
	Dimethylglycine	Low	0.954	0.168	0.897	0.232	0.652	0.361	0.360	0.633	0.623	0.424	0.553	0.464
		High	1.297	0.034*	1.076	0.105	1.472	0.020*	0.236	0.723	-0.273	0.692	0.727	0.277
	Serine		0.007	0.180	0.005	0.341	0.008	0.125	0.001	0.870	0.003	0.644	<0.001	0.993
	Interaction	Low	-0.012	0.160	-0.009	0.304	-0.008	0.328	-0.005	0.547	-0.008	0.397	-0.008	0.394
		High	-0.015	0.051	-0.012	0.138	-0.017	0.029#	-0.002	0.856	0.003	0.686	-0.009	0.276

Dependent variable	Model terms		Global cognition		Attention		Episodic Memory		Executive function		Location Learning		Working Memory	
			β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
	Glycine	Low	-0.988	0.135	-0.830	0.249	-0.767	0.262	1.030	0.156	-0.886	0.234	0.351	0.630
		High	-0.194	0.773	-0.111	0.879	0.009	0.989	-0.491	0.505	-0.096	0.899	-0.279	0.707
	Serine		-0.009	0.104	-0.007	0.237	-0.006	0.264	-0.007	0.235	-0.006	0.343	-0.003	0.570
	Interaction	Low	0.010	0.231	0.008	0.376	0.008	0.376	0.012	0.194	0.010	0.290	-0.005	0598
		High	0.006	0.430	0.004	0.651	0.004	0.606	0.007	0.440	0.005	0.587	0.003	0.752
	Homocysteine	Low	-0.467	0.497	-0.918	0.212	-0.488	0.510	1.273	0.073	0.149	0.848	-0.662	0.386
		High	-0.054	0.945	-0.997	0.231	-0.005	0.996	0.797	0.322	0.101	0.909	0.876	0.312
	Serine		-0.006	0.288	-0.012	0.063	-0.003	0.659	0.003	0.654	-0.001	0.909	-0.004	0.534
	Interaction	Low	0.006	0.465	0.013	0.155	0.005	0.578	-0.016	0.077	-0.002	0.866	0.010	0.310
		High	-0.002	0.856	0.011	0.298	-0.001	0.918	-0.013	0.207	-0.005	0.678	-0.012	0.275
	Methionine	Low	0.664	0.316	0.484	0.495	0.519	0.447	0.019	0.979	1.975	0.007*	-0.777	0.279
		High	0.266	0.675	0.127	0.852	0.468	0.475	-0.195	0.778	0.456	0.517	-0.386	0.575
	Serine		0.003	0.518	0.002	0.657	0.004	0.489	-0.001	0.784	0.010	0.067	-0.006	0.287
	Interaction	Low	-0.009	0.284	-0.010	0.262	-0.004	0.606	-0.001	0.903	-0.026	0.005#	0.010	0.246
		High	-0.004	0.594	-0.004	0.624	-0.004	0.601	0.002	0.771	-0.008	0.370	0.003	0.743
	S-adenosylmethionine	Low	1.869	0.003*	1.621	0.019*	1.540	0.021*	1.309	0.063	0.312	0.667	0.916	0.194
		High	1.090	0.109	1.620	0.028*	0.724	0.308	0.701	0.350	-0.249	0.747	0.132	0.861
	Serine		0.011	0.030*	0.012	0.028*	0.009	0.086	0.006	0.292	0.004	0.511	<0.001	0.993
	Interaction	Low	-0.027	0.001#	-0.024	0.007#	-0.021	0.012#	-0.016	0.068	-0.007	0.470	-0.012	0.172
		High	-0.015	0.057	-0.022	0.011#	-0.009	0.281	-0.009	0.315	<0.001	1.000	-0.003	0.703

β estimate and p values presented for models fit with one-carbon metabolite as continuous variables and other one-carbon metabolites as categorical variables (low – quartile 1, high – quartile 4, reference – quartiles 2 and 3), and an interaction term between these main effects for each cognitive domain fitted as the dependent variable. All models are adjusted for age, sex, education, batch effects, energy intake, physical activity, history of anxiety/depression, and supplement use. [1]Fit as log-transformed variables in models. A significant association ($p < 0.05$) between the main effects and dependent variable is indicated by *for main effects, and #for the interaction term.

Table 9.10: Supplemental Material (Chapter 5): Effect of interactions between one-carbon metabolites on cognitive performance in multivariate linear regression models.

			Global cognition		Attention		Episodic Memory		Executive function		Location Learning		Working Memory	
Dependent variable	Model terms		β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
Betaine	Folate intake	Low	0.230	0.645	-0.768	0.156	0.434	0.402	0.687	0.205	0.527	0.345	0.406	0.455
		High	0.338	0.434	0.447	0.339	0.005	0.991	0.951	0.043*	0.248	0.608	-0.250	0.596
	Betaine		0.009	0.259	0.003	0.772	0.005	0.480	0.016	0.085	0.014	0.134	0.004	0.695
	Interaction	Low	-0.002	0.890	0.025	0.116	-0.013	0.392	-0.014	0.382	-0.009	0.591	-0.003	0.841
		High	-0.008	0.491	-0.015	0.258	0.001	0.923	-0.024	0.060	-0.003	0.792	0.009	0.500
	Riboflavin intake	Low	-0.067	0.888	-0.690	0.177	-0.152	0.723	0.388	0.450	1.022	0.052	0.099	0.848
		High	-0.122	0.786	-0.152	0.757	-0.083	0.859	0.247	0.617	0.064	0.898	-0.420	0.397
	Betaine		0.007	0.494	-0.003	0.807	0.004	0.667	0.007	0.476	0.019	0.077	0.003	0.772
	Interaction	Low	<0.001	0.985	0.016	0.279	<0.001	0.976	-0.007	0.624	-0.025	0.094	-0.003	0.818
		High	-0.003	0.817	-0.001	0.922	-0.004	0.736	-0.005	0.699	-0.005	0.695	0.010	0.479
	Vitamin B_6 intake	Low	-0.633	0.181	-0.486	0.346	-0.374	0.448	-0.455	0.378	-0.714	0.179	-0.254	0.626
		High	0.269	0.555	0.676	0.174	0.241	0.612	-0.542	0.277	0.041	0.936	-0.112	0.824
	Betaine		0.003	0.706	0.005	0.611	0.001	0.916	-0.003	0.728	0.005	0.582	0.003	0.737
	Interaction	Low	0.022	0.098	0.015	0.297	0.014	0.311	0.017	0.257	0.024	0.123	0.011	0.466
		High	-0.009	0.478	-0.021	0.125	-0.005	0.718	0.009	0.531	<0.001	0.991	<0.001	0.974
	Vitamin B_{12} intake	Low	-0.370	0.423	-0.338	0.502	-0.395	0.408	0.326	0.518	0.251	0.628	-0.758	0.135
		High	0.452	0.319	-0.052	0.917	0.762	0.105	0.187	0.706	0.356	0.485	-0.115	0.818
	Betaine		0.004	0.625	-0.005	0.560	0.006	0.481	0.004	0.673	0.014	0.149	-0.001	0.902
	Interaction	Low	0.015	0.245	0.016	0.249	0.011	0.407	-0.002	0.913	-0.003	0.835	0.021	0.133
		High	-0.010	0.439	0.005	0.698	0.020	0.140	-0.005	0.737	-0.011	0.435	0.006	0.675
	Serum folate	Low	-0.597	0.272	-0.716	0.224	-0.272	0.631	-0.357	0.549	-0.580	0.322	-0.106	0.851
		High	0.045	0.922	0.359	0.475	-0.294	0.543	0.180	0.724	0.188	0.708	-0.014	0.976
	Betaine		0.003	0.771	-0.001	0.887	-0.001	0.927	0.006	0.533	0.018	0.214	0.002	0.855
	Interaction	Low	0.017	0.291	0.024	0.184	0.002	0.890	0.007	0.679	0.024	0.173	0.009	0.618
		High	<0.001	0.996	-0.003	0.838	0.006	0.624	-0.007	0.597	-0.008	0.562	0.002	0.882
	Serum vitamin B_{12}	Low	-0.731	0.141	-1.216	0.022*	0.101	0.845	-0.852	0.113	0.008	0.988	-0.948	0.082
		High	0.128	0.778	-0.487	0.315	0.310	0.513	0.422	0.390	0.974	0.051	-0.537	0.821
	Betaine		0.003	0.708	-0.011	0.241	0.008	0.384	0.003	0.776	0.022	0.017*	-0.007	0.461
	Interaction	Low	0.016	0.254	0.027	0.075	-0.006	0.699	0.023	0.135	-0.002	0.892	0.028	0.069
		High	-0.007	0.584	0.016	0.249	-0.015	0.244	-0.011	0.401	-0.032	0.020#	0.016	0.238
Choline	Folate intake	Low	0.243	0.628	-0.452	0.404	0.169	0.745	0.263	0.630	0.727	0.195	0.796	0.145
		High	0.502	0.322	0.867	0.115	-0.126	0.811	0.404	0.466	0.524	0.357	0.412	0.457
	Choline		0.028	0.404	0.001	0.987	0.027	0.445	0.018	0.617	0.029	0.441	0.030	0.416
	Interaction	Low	-0.010	0.859	0.059	0.340	-0.020	0.743	-0.006	0.918	-0.059	0.362	-0.058	0.357
		High	0.052	0.356	-0.105	0.086	0.018	0.753	-0.035	0.573	-0.046	0.462	-0.042	0.497
	Riboflavin intake	Low	0.378	0.431	-0.275	0.598	0.330	0.505	0.680	0.195	0.983	0.067	0.067	0.899

Dependent variable	Model terms		Global cognition		Attention		Episodic Memory		Executive function		Location Learning		Working Memory	
			β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
	Riboflavin intake	High	0.034	0.948	0.234	0.675	-0.186	0.725	0.582	0.300	-0.386	0.500	-0.321	0.570
	Choline		0.033	0.356	-0.004	0.921	0.044	0.237	0.042	0.287	0.022	0.587	0.003	0.949
	Interaction	Low	-0.052	0.336	0.015	0.801	-0.055	0.326	-0.062	0.298	-0.093	0.127	-0.008	0.889
		High	-0.023	0.696	-0.050	0.421	-0.005	0.928	-0.061	0.337	0.028	0.658	0.026	0.676
	Vitamin B_6 intake	Low	-0.745	0.133	-1.251	0.020*	0.135	0.794	-0.219	0.685	-0.729	0.192	-0.947	0.081
		High	-0.087	0.864	0.195	0.724	0.385	0.469	-0.690	0.216	-0.603	0.296	-0.699	0.212
	Choline		-0.015	0.686	-0.046	0.249	0.037	0.329	-0.011	0.787	-0.040	0.332	-0.043	0.286
	Interaction	Low	0.104	0.069	0.154	0.013#	-0.003	0.957	0.039	0.633	0.097	0.133	0.127	0.043#
		High	0.005	0.927	-0.026	0.671	-0.037	0.527	0.050	0.416	0.072	0.254	0.066	0.283
	Vitamin B_{12} intake	Low	0.338	0.496	-0.047	0.931	0.516	0.316	0.595	0.270	0.139	0.803	-0.239	0.661
		High	-0.293	0.550	-0.308	0.564	-0.039	0.939	-0.255	0.633	-0.587	0.288	-0.038	0.944
	Choline		0.010	0.783	-0.033	0.422	0.043	0.259	0.013	0.739	-0.012	0.772	-0.002	0.955
	Interaction	Low	-0.021	0.713	0.031	0.616	-0.060	0.303	-0.038	0.539	0.003	0.959	0.027	0.659
		High	0.048	0.387	0.050	0.404	0.017	0.764	0.034	0.573	0.065	0.297	0.013	0.825
	Serum folate	Low	-0.127	0.815	-0.186	0.751	0.173	0.757	-0.660	0.267	0.023	0.969	-0.177	0.755
		High	0.302	0.560	0.531	0.344	0.665	0.215	-0.768	0.176	-0.251	0.656	-0.327	0.545
	Choline		0.021	0.564	<0.001	0.998	0.051	0.173	-0.025	0.522	0.005	0.900	-0.011	0.776
	Interaction	Low	0.008	0.891	0.025	0.702	-0.041	0.515	0.059	0.373	0.014	0.835	0.038	0.546
		High	-0.030	0.617	-0.034	0.597	-0.084	0.169	0.082	0.206	0.021	0.744	0.046	0.458
	Serum vitamin B_{12}	Low	-0.283	0.567	-0.244	0.644	-0.458	0.372	0.779	0.146	0.047	0.931	-0.547	0.312
		High	-0.315	0.548	-0.517	0.355	-0.121	0.824	0.520	0.359	0.037	0.949	-0.847	0.139
	Choline		-0.002	0.956	-0.028	0.489	0.003	0.935	0.052	0.207	0.020	0.633	-0.033	0.427
	Interaction	Low	0.011	0.841	-0.007	0.906	0.042	0.641	-0.099	0.097	-0.014	0.823	0.064	0.288
		High	0.025	0.678	0.068	0.296	-0.012	0.849	-0.057	0.392	-0.019	0.778	0.106	0.114
Cysteine	Folate intake	Low	-1.209	0.173	-2.471	0.010*	-1.062	0.247	-0.120	0.901	0.379	0.704	1.209	0.212
		High	-0.323	0.697	0.021	0.982	-1.611	0.061	1.235	0.172	0.553	0.553	1.009	0.265
	Cysteine		-0.004	0.388	-0.010	0.068	-0.004	0.444	0.002	0.672	<0.001	0.981	0.006	0.298
	Interaction	Low	0.014	0.122	0.025	0.009#	0.011	0.237	0.003	0.733	-0.002	0.880	-0.009	0.347
		High	0.004	0.642	-0.001	0.949	0.017	0.052	-0.012	0.206	-0.004	0.643	-0.010	0.282
	Riboflavin intake	Low	0.116	0.893	-1.259	0.178	-0.119	0.894	0.857	0.363	1.573	0.103	1.164	0.220
		High	0.693	0.416	0.797	0.387	-0.195	0.825	1.427	0.125	1.400	0.142	-0.053	0.955
	Cysteine		0.002	0.710	-0.005	0.315	0.003	0.564	0.004	0.434	0.005	0.409	0.004	0.456
	Interaction	Low	-0.002	0.838	0.011	0.226	<0.001	0.984	-0.007	0.453	-0.014	0.153	-0.012	0.213
		High	-0.009	0.273	-0.010	0.279	<0.001	0.958	-0.014	0.137	-0.016	0.101	<0.001	0.960
	Vitamin B_6 intake	Low	-0.789	0.371	-1.036	0.279	-0.201	0.827	0.211	0.825	-1.606	0.105	-0.251	0.796
		High	0.821	0.301	1.152	0.181	-0.054	0.948	1.222	0.157	1.049	0.238	0.057	0.948
	Cysteine		-0.001	0.851	-0.005	0.376	0.001	0.797	0.003	0.622	-0.002	0.705	<0.001	0.977
	Interaction	Low	0.009	0.293	0.011	0.254	0.003	0.735	-0.001	0.917	0.017	0.084	0.004	0.702
		High	-0.009	0.257	-0.013	0.140	0.001	0.871	-0.015	0.082	-0.011	0.236	-0.002	0.826
	Vitamin B_{12} intake	Low	-0.42	0.496	0.324	0.706	-0.598	0.469	0.492	0.569	-1.003	0.260	-1.410	0.106
		High	-0.461	0.597	-0.330	0.727	-0.379	0.675	0.548	0.563	-0.455	0.641	-0.846	0.375
	Cysteine		-0.005	0.406	-0.007	0.282	-0.001	0.911	0.002	0.772	-0.007	0.293	-0.005	0.379
	Interaction	Low	0.007	0.366	-0.001	0.909	0.006	0.460	-0.002	0.804	0.012	0.180	0.014	0.103

Dependent variable	Model terms		Global cognition		Attention		Episodic Memory		Executive function		Location Learning		Working Memory	
			β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
Dimethylglycine[1]	Interaction	High	0.006	0.510	0.005	0.627	0.005	0.600	-0.005	0.577	0.004	0.660	0.009	0.325
	Serum folate	Low	-0.430	0.631	-0.248	0.798	-0.149	0.872	-1.314	0.181	-0.009	0.993	-0.127	0.892
		High	0.562	0.552	-0.111	0.913	1.098	0.261	-0.561	0.587	0.169	0.869	0.006	0.573
	Cysteine		-0.001	0.825	-0.006	0.252	0.004	0.466	-0.003	0.546	-0.001	0.860	<0.001	0.978
	Interaction	Low	0.004	0.679	0.003	0.767	-0.001	0.952	0.012	0.227	0.001	0.878	0.003	0.762
		High	-0.005	0.575	0.004	0.734	-0.012	0.232	0.005	0.638	-0.003	0.803	-0.005	0.609
	Serum vitamin B_{12}	Low	-1.078	0.218	-0.525	0.574	-1.271	0.160	-0.629	0.508	-0.512	0.597	-0.087	0.928
		High	0.272	0.751	-0.400	0.662	0.928	0.294	0.916	0.325	0.004	0.997	-1.213	0.198
	Cysteine		-0.003	0.526	-0.009	0.132	0.001	0.871	0.002	0.774	-0.003	0.591	-0.002	0.749
	Interaction	Low	0.009	0.305	0.002	0.803	0.012	0.192	0.005	0.564	0.004	0.647	0.001	0.913
		High	-0.004	0.642	0.004	0.632	-0.012	0.186	-0.009	0.332	-0.001	0.880	0.012	0.180
	Folate intake	Low	-0.123	0.651	-0.296	0.312	-0.298	0.291	0.136	0.646	0.177	0.563	0.469	0.112
		High	0.002	0.995	0.488	0.096	-0.323	0.252	0.082	0.782	0.147	0.632	-0.295	0.316
	Dimethylglycine		-0.037	0.945	0.022	0.969	-0.403	0.467	0.432	0.459	-0.027	0.964	0.492	0.394
	Interaction	Low	0.848	0.265	1.049	0.200	0.962	0.222	0.171	0.836	0.169	0.843	-0.576	0.484
		High	0.175	0.830	-1.857	0.045#	1.244	0.141	0.093	0.916	-0.081	0.929	1.205	0.172
	Riboflavin intake	Low	-0.074	0.784	-0.309	0.294	-0.196	0.484	0.387	0.191	0.343	0.259	0.071	0.810
		High	-0.383	0.156	0.095	0.745	-0.514	0.065	0.114	0.699	-0.431	0.154	-0.510	0.082
	Dimethylglycine		0.146	0.784	0.027	0.963	-0.085	0.876	0.836	0.150	-0.192	0.747	0.351	0.545
	Interaction	Low	-0.011	0.989	0.506	0.543	0.172	0.828	-0.835	0.318	-0.445	0.604	-0.331	0.692
		High	0.582	0.474	-1.058	0.230	1.000	0.233	-0.079	0.929	1.018	0.263	1.572	0.076
	Vitamin B_6 intake	Low	-0.204	0.469	-0.419	0.172	0.032	0.913	-0.022	0.942	-0.108	0.733	-0.213	0.487
		High	-0.242	0.385	-0.060	0.842	0.049	0.866	-0.873	0.004*	-0.051	0.870	-0.426	0.160
	Dimethylglycine		-0.049	0.915	-0.361	0.471	0.177	0.711	-0.065	0.895	-0.157	0.762	0.183	0.715
	Interaction	Low	1.091	0.193	1.515	0.098	0.250	0.774	0.432	0.632	0.648	0.493	1.073	0.240
		High	0.589	0.448	0.001	0.999	0.056	0.945	1.941	0.020#	0.240	0.784	0.913	0.281
	Vitamin B_{12} intake	Low	0.063	0.811	0.033	0.908	0.187	0.495	0.262	0.358	-0.113	0.703	-0.394	0.172
		High	-0.163	0.562	0.179	0.559	-0.011	0.970	-0.553	0.069	-0.359	0.256	-0.333	0.278
	Dimethylglycine		0.036	0.940	-0.229	0.659	0.371	0.453	0.084	0.869	-0.488	0.362	0.004	0.993
	Interaction	Low	0.328	0.666	0.618	0.454	-0.601	0.446	0.053	0.949	-0.950	0.265	1.233	0.137
		High	0.879	0.287	-0.156	0.862	0.308	0.719	1.885	0.034#	1.065	0.250	1.347	0.135
	Serum folate	Low	-0.520	0.064	-0.564	0.064	-0.483	0.098	-0.516	0.094	0.014	0.964	0.176	0.548
		High	-0.091	0.765	0.080	0.807	-0.081	0.796	-0.178	0.591	0.159	0.630	-0.403	0.201
	Dimethylglycine		-0.246	0.612	-0.624	0.234	-0.043	0.932	0.080	0.881	-0.070	0.894	0.049	0.923
	Interaction	Low	1.473	0.065	1.899	0.028#	0.929	0.263	1.208	0.168	0.403	0.644	-0.058	0.944
		High	0.437	0.630	0.529	0.589	0.058	0.951	0.353	0.722	-0.815	0.411	1.566	0.098
	Serum vitamin B_{12}	Low	-0.127	0.645	-0.394	0.184	-0.246	0.393	0.179	0.550	0.378	0.215	0.262	0.385
		High	-0.097	0.720	0.002	0.994	-0.257	0.361	0.168	0.567	0.197	0.508	-0.159	0.590
	Dimethylglycine		0.219	0.669	-0.329	0.549	-0.102	0.849	0.854	0.124	0.707	0.211	0.627	0.262
	Interaction	Low	-0.190	0.811	0.301	0.723	0.487	0.553	-0.823	0.337	-1.447	0.097	-0.808	0.349
		High	-0.014	0.987	0.130	0.880	0.122	0.885	-0.402	0.646	-1.069	0.231	0.739	0.402
Glycine[1]	Folate intake	Low	-0.952	0.717	-1.513	0.598	-0.675	0.804	1.007	0.726	-1.057	0.719	-0.131	0.964

Dependent variable	Model terms		Global cognition		Attention		Episodic Memory		Executive function		Location Learning		Working Memory	
			β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
Homocysteine[1]	Folate intake	High	-2.149	0.383	-3.079	0.253	-2.531	0.321	-2.421	0.370	2.350	0.394	1.808	0.503
	Glycine		0.865	0.247	0.419	0.608	0.889	0.251	0.071	0.931	1.518	0.070	-0.206	0.802
	Interaction	Low	0.486	0.669	0.676	0.586	0.307	0.795	-0.342	0.784	0.558	0.661	0.186	0.882
		High	0.953	0.373	1.312	0.262	1.116	0.314	1.097	0.349	-0.973	0.417	-0.763	0.516
	Riboflavin intake	Low	0.337	0.891	-2.450	0.362	1.358	0.593	1.611	0.552	-1.470	0.594	2.835	0.296
		High	0.966	0.710	0.323	0.909	0.737	0.784	0.977	0.733	1.030	0.724	4.687	0.103
	Glycine		1.464	0.048*	0.738	0.359	1.474	0.054	0.648	0.425	1.277	0.123	0.413	0.612
	Interaction	Low	-0.169	0.874	0.996	0.391	-0.638	0.562	-0.630	0.591	0.727	0.542	-1.235	0.293
		High	-0.524	0.643	-0.227	0.864	0.212	0.855	-0.401	0.747	-0.512	0.686	-2.078	0.096
	Vitamin B_6 intake	Low	2.927	0.248	2.653	0.341	2.193	0.403	-0.358	0.898	2.674	0.350	2.036	0.469
		High	4.660	0.059	2.026	0.455	5.438	0.034*	1.561	0.566	4.254	0.127	0.181	0.947
	Glycine		2.151	0.004*	1.522	0.059	2.202	0.004*	0.536	0.505	2.094	0.012*	-0.190	0.815
	Interaction	Low	-1.216	0.268	-1.135	0.347	-0.905	0.425	0.203	0.867	-1.124	0.365	-0.833	0.494
		High	-2.051	0.056	-0.913	0.438	-2.335	0.036#	-0.786	0.504	-1.841	0.128	-0.138	0.908
	Vitamin B_{12} intake	Low	0.073	0.978	-0.872	0.758	-3.079	0.252	-0.042	0.988	2.077	0.292	7.861	0.006*
		High	1.382	0.574	2.428	0.365	-1.200	0.637	2.972	0.269	0.314	0.910	2.733	0.310
	Glycine		1.431	0.057	1.161	0.155	0.864	0.265	0.638	0.435	1.623	0.054	0.769	0.348
	Interaction	Low	0.038	0.973	0.469	0.701	1.333	0.251	0.136	0.912	-1.255	0.320	-3.340	0.006#
		High	-0.548	0.609	-0.997	0.393	-0.570	0.608	-1.284	0.273	-1.421	0.906	-1.159	0.322
	Serum folate	Low	-0.801	0.761	0.727	0.801	-1.324	0.627	0.681	0.816	-0.032	0.991	-2.708	0.330
		High	-1.847	0.472	1.347	0.630	-4.149	0.118	0.938	0.741	-1.050	0.708	-0.950	0.725
	Glycine		1.035	0.197	1.244	0.155	0.762	0.357	0.555	0.532	1.141	0.192	-0.707	0.401
	Interaction	Low	0.311	0.786	-0.312	0.803	0.481	0.685	-0.361	0.776	0.065	0.959	1.246	0.301
		High	0.819	0.426	-0.479	0.693	1.773	0.124	-0.441	0.721	0.419	0.730	0.438	0.708
	Serum vitamin B_{12}	Low	-0.958	0.732	-2.589	0.388	0.428	0.883	-4.470	0.144	-0.602	0.846	3.765	0.222
		High	-1.935	0.444	-3.653	0.178	-2.270	0.386	-4.072	0.141	1.242	0.657	6.050	0.030*
	Glycine		0.999	0.232	0.212	0.813	1.246	0.151	-0.961	0.292	1.542	0.097	0.905	0.325
	Interaction	Low	0.331	0.786	0.994	0.447	-0.234	0.853	1.912	0.152	0.226	0.867	-1.635	0.223
		High	0.784	0.472	1.597	0.173	0.879	0.437	1.776	0.137	-0.598	0.621	-2.603	0.031#
	Folate intake	Low	-0.026	0.934	-0.176	0.610	-0.155	0.654	0.043	0.898	0.192	0.595	0.421	0.239
		High	-0.146	0.669	-0.207	0.573	-0.093	0.800	-0.259	0.465	0.186	0.629	-0.035	0.927
	Homocysteine		-0.807	0.179	-1.193	0.067	-0.138	0.832	-0.979	0.118	-0.396	0.560	-0.226	0.736
	Interaction	Low	0.852	0.337	0.971	0.310	0.723	0.450	0.702	0.447	0.243	0.808	-0.308	0.756
		High	0.933	0.319	1.050	0.298	0.599	0.553	1.390	0.154	-0.232	0.827	0.107	0.918
	Riboflavin intake	Low	0.347	0.278	-0.005	0.989	0.276	0.421	0.286	0.394	0.692	0.055	0.166	0.642
		High	-0.251	0.498	-0.363	0.365	-0.003	0.994	-0.266	0.495	0.299	0.472	-0.643	0.121
	Homocysteine		0.098	0.867	-0.658	0.298	0.816	0.193	-0.438	0.475	0.363	0.580	-0.357	0.584
	Interaction	Low	-1.311	0.157	-0.441	0.658	-1.255	0.206	-0.576	0.552	-1.324	0.202	-0.825	0.424
		High	0.095	0.924	0.752	0.480	-0.724	0.494	0.949	0.359	-1.291	0.244	1.347	0.222
	Vitamin B_6 intake	Low	0.198	0.578	-0.018	0.962	0.387	0.313	0.064	0.860	0.142	0.724	-0.177	0.656
		High	-0.242	0.487	-0.221	0.555	-0.108	0.773	-0.079	0.825	0.093	0.812	-0.568	0.144
	Homocysteine		-0.352	0.523	-0.747	0.208	0.384	0.517	-0.310	0.582	-0.357	0.567	-0.815	0.186
	Interaction	Low	-0.354	0.708	0.137	0.893	-0.976	0.338	-0.041	0.966	-0.062	0.954	0.646	0.541

Dependent variable	Model terms		Global cognition		Attention		Episodic Memory		Executive function		Location Learning		Working Memory	
			β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
Methionine	Interaction	High	0.186	0.849	0.087	0.934	0.321	0.760	-1.134	0.258	-0.311	0.778	1.319	0.227
	Vitamin B_{12} intake	Low	0.098	0.784	0.796	0.037*	-0.195	0.613	0.319	0.389	-0.610	0.128	-0.257	0.524
		High	-0.715	0.028*	-0.489	0.157	-0.442	0.207	-0.587	0.081	-0.869	0.017*	-0.220	0.548
	Homocysteine		-0.898	0.103	-0.658	0.262	-0.248	0.676	-0.783	0.171	-1.468	0.018*	-0.644	0.300
	Interaction	Low	-0.004	0.997	-1.891	0.049#	0.472	0.665	-0.191	0.855	2.170	0.056	0.576	0.613
		High	2.134	0.018#	1.492	0.119	1.441	0.137	1.488	0.110	2.195	0.030#	0.984	0.376
	Serum folate	Low	0.353	0.384	0.710	0.100	-0.001	0.999	0.334	0.430	0.190	0.664	-0.038	0.927
		High	0.169	0.615	0.509	0.153	0.068	0.849	0.061	0.861	-0.511	0.158	0.119	0.733
	Homocysteine		0.218	0.739	0.190	0.784	0.851	0.224	-0.015	0.982	-1.041	0.141	-0.579	0.396
	Interaction	Low	-1.028	0.305	-1.564	0.142	-0.600	0.575	-1.089	0.299	-0.051	0.963	0.403	0.699
		High	-0.552	0.595	-0.951	0.388	-0.302	0.786	-0.704	0.516	0.943	0.399	-0.435	0.687
	Serum vitamin B_{12}	Low	0.267	0.487	0.067	0.871	0.015	0.971	0.351	0.379	0.726	0.093	0.227	0.597
		High	0.160	0.624	0.153	0.660	0.313	0.372	0.166	0.624	-0.200	0.585	-0.292	0.422
	Homocysteine		0.194	0.743	-0.172	0.786	0.701	0.272	-0.077	0.901	-0.081	0.904	-0.416	0.528
	Interaction	Low	-1.163	0.230	-0.778	0.450	-0.501	0.630	-0.790	0.431	-1.908	0.079	-0.693	0.519
		High	-0.862	0.422	-0.060	0.958	-1.845	0.110	-0.485	0.663	0.093	0.938	1.020	0.392
	Folate intake	Low	-0.843	0.269	-1.530	0.063	-0.607	0.441	-0.144	0.863	0.104	0.903	0.292	0.725
		High	0.025	0.975	0.954	0.276	-1.601	0.057	0.304	0.732	1.561	0.086	0.985	0.311
	Methionine		-0.022	0.359	-0.026	0.318	-0.027	0.287	0.007	0.799	0.015	0.573	-0.014	0.599
	Interaction	Low	0.047	0.185	0.074	0.055	0.029	0.425	0.016	0.674	0.006	0.882	0.001	0.977
		High	0.001	0.976	-0.048	0.241	0.078	0.048#	-0.010	0.817	-0.068	0.109	0.040	0.336
	Riboflavin intake	Low	-0.310	0.679	-1.581	0.052	0.488	0.527	0.411	0.615	0.248	0.767	-0.474	0.563
		High	-0.266	0.745	-0.478	0.590	-0.680	0.420	0.198	0.825	0.981	0.284	0.302	0.736
	Methionine		-0.009	0.732	-0.039	0.148	0.009	0.724	0.018	0.507	0.016	0.558	-0.025	0.352
	Interaction	Low	0.012	0.737	0.069	0.074	-0.030	0.411	-0.012	0.749	-0.002	0.964	0.023	0.552
		High	0.002	0.965	0.013	0.751	0.021	0.593	-0.007	0.874	-0.053	0.214	-0.019	0.654
	Vitamin B_6 intake	Low	-1.069	0.150	-1.513	0.061	-0.521	0.500	0.216	0.790	0.256	0.760	-1.276	0.115
		High	0.282	0.740	-0.001	0.999	0.160	0.857	-0.721	0.436	1.631	0.089	0.197	0.831
	Methionine		-0.021	0.358	-0.036	0.147	-0.006	0.802	-0.001	0.981	0.020	0.423	-0.042	0.086
	Interaction	Low	0.056	0.103	0.073	0.053	0.030	0.405	0.016	0.677	-0.008	0.837	0.065	0.083
		High	-0.017	0.671	-0.005	0.916	-0.005	0.913	0.023	0.602	-0.096	0.089	-0.018	0.686
	Vitamin B_{12} intake	Low	-0.599	0.451	-1.095	0.20	-1.013	0.217	0.234	0.789	0.776	0.386	0.854	0.327
		High	1.111	0.163	-0.040	0.963	1.451	0.078	-0.481	0.579	1.218	0.174	1.295	0.138
	Methionine		-0.002	0.935	-0.026	0.254	0.005	0.819	0.007	0.749	0.022	0.336	0.001	0.973
	Interaction	Low	0.037	0.326	0.063	0.123	0.049	0.205	0.002	0.957	-0.028	0.502	-0.041	0.318
		High	-0.047	0.200	0.008	0.845	-0.064	0.093	0.024	0.552	-0.059	0.157	-0.057	0.156
	Serum folate	Low	-1.184	0.137	-1.843	0.032*	-0.725	0.381	-0.136	0.876	-0.235	0.786	-0.262	0.752
		High	0.350	0.692	-0.549	0.565	0.149	0.871	1.058	0.276	1.008	0.296	0.591	0.522
	Methionine		-0.022	0.370	-0.048	0.070	-0.009	0.735	0.025	0.364	<0.001	0.986	-0.020	0.436
	Interaction	Low	0.052	0.154	0.087	0.028#	0.025	0.517	<0.001	0.991	0.017	0.663	0.019	0.611
		High	-0.017	0.695	0.036	0.427	-0.011	0.802	-0.055	0.243	-0.054	0.243	-0.028	0.531
	Serum vitamin B_{12}	Low	0.729	0.368	0.223	0.796	0.647	0.442	0.242	0.783	1.103	0.218	0.369	0.679
		High	0.482	0.581	-1.143	0.220	0.870	0.338	0.808	0.395	1.136	0.240	0.599	0.532

Dependent variable	Model terms		Global cognition		Attention		Episodic Memory		Executive function		Location Learning		Working Memory	
			β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
S-adenosyl-methionine	Methionine		0.012	0.608	-0.018	0.468	0.021	0.363	0.023	0.343	0.025	0.312	-0.009	0.725
	Interaction	Low	-0.043	0.252	-0.024	0.538	-0.034	0.373	-0.015	0.711	-0.055	0.184	-0.017	0.683
		High	-0.028	0.499	0.057	0.196	-0.052	0.228	-0.037	0.412	-0.060	0.188	-0.028	0.542
	Folate intake	Low	-0.017	0.955	-0.259	0.409	0.109	0.718	-0.111	0.727	0.355	0.276	-0.104	0.743
		High	-0.409	0.175	-0.747	0.022*	-0.272	0.385	-0.154	0.639	0.492	0.146	-0.256	0.435
	S-adenosylmethionine		-0.002	0.192	-0.004	0.024*	-0.001	0.727	<0.001	0.983	<0.001	0.970	-0.001	0.346
	Interaction	Low	0.0001	0.546	0.002	0.346	-0.001	0.702	0.002	0.253	-0.001	0.657	0.003	0.147
		High	0.003	0.097	0.005	0.022#	0.002	0.272	0.002	0.371	-0.003	0.223	0.002	0.299
	Riboflavin intake	Low	0.524	0.059	0.059	0.844	0.385	0.181	0.518	0.090	0.521	0.096	0.624	0.041*
		High	0.002	0.995	-0.294	0.398	-0.041	0.900	0.407	0.244	0.243	0.497	0.095	0.787
	S-adenosylmethionine		0.001	0.668	-0.002	0.315	0.001	0.423	0.002	0.201	<0.001	0.096	0.001	0.433
	Interaction	Low	-0.004	0.018#	-0.001	0.448	-0.004	0.045#	-.003	0.170	-0.002	0.252	-0.004	0.022#
		High	-0.002	0.403	0.001	0.806	-0.001	0.508	-0.002	0.284	-0.003	0.225	-0.001	0.543
	Vitamin B_6 intake	Low	0.352	0.222	-0.145	0.645	0.429	0.150	0.267	0.395	0.607	0.062	0.147	0.642
		High	-0.331	0.262	-0.299	0.352	-0.308	0.312	-0.211	0.510	0.123	0.710	-0.230	0.478
	S-adenosylmethionine		-0.001	0.423	-0.003	0.081	<0.001	0.821	0.001	0.525	<0.001	0.981	-0.001	0.650
	Interaction	Low	-0.002	0.402	0.001	0.511	-0.002	0.246	-0.001	0.581	-0.004	0.077	<0.001	0.923
		High	0.002	0.291	0.002	0.442	0.003	0.158	<0.001	0.910	-0.001	0.759	0.001	0.741
	Vitamin B_{12} intake	Low	0.269	0.383	-0.181	0.588	0.291	0.364	0.647	0.054	0.384	0.267	0.009	0.979
		High	0.078	0.795	-0.065	0.841	0.124	0.689	0.158	0.625	0.265	0.427	-0.206	0.530
	S-adenosylmethionine		-0.001	0.483	-0.029	0.058	<0.001	0.887	0.001	0.393	<0.001	0.764	-0.001	0.573
	Interaction	Low	-0.001	0.728	0.003	0.178	-0.002	0.332	-0.003	0.227	-0.001	0.513	<0.001	0.973
		High	<0.001	0.933	0.001	0.530	<0.001	0.890	-0.001	0.659	-0.002	0.314	0.002	0.348
	Serum folate	Low	0.133	0.672	0.141	0.679	-0.213	0.515	-0.037	0.914	0.495	0.146	0.589	0.072
		High	0.030	0.921	0.163	0.618	-0.207	0.510	-0.380	0.251	0.435	0.183	0.358	0.255
	S-adenosylmethionine		-0.001	0.618	-0.002	0.253	<0.001	0.829	0.001	0.699	<0.001	0.977	<0.001	0.952
	Interaction	Low	0.001	0.504	-0.001	0.734	<0.001	0.941	-0.001	0.759	-0.002	0.239	-0.003	0.143
		High	<0.001	0.977	0.001	0.799	0.001	0.612	0.002	0.307	-0.004	0.076	-0.002	0.276
	Serum vitamin B_{12}	Low	-0.226	0.463	-0.441	0.179	0.030	0.926	0.072	0.831	-0.080	0.814	-0.402	0.235
		High	-0.087	0.774	-0.289	0.371	0.029	0.928	0.243	0.462	-0.152	0.651	-0.053	0.873
	S-adenosylmethionine		-0.001	0.359	-0.004	0.035*	<0.001	1.000	0.002	0.221	-0.001	0.496	-0.002	0.385
	Interaction	Low	<0.001	0.897	0.001	0.633	-0.001	0.657	-0.001	0.615	<0.001	0.984	0.003	0.183
		High	<0.001	0.916	0.002	0.272	-0.002	0.382	-0.001	0.493	<0.001	0.954	0.001	0.805
Serine	Folate intake	Low	0.502	0.474	-0.113	0.882	0.802	0.268	-0.378	0.619	0.888	0.256	0.196	0.797
		High	-0.061	0.822	-0.024	0.972	-0.434	0.501	-0.157	0.818	0.936	0.180	0.186	0.784
	Serine		0.001	0.913	-0.001	0.854	0.002	0.699	-0.002	0.621	0.006	0.247	-0.004	0.431
	Interaction	Low	-0.004	0.610	0.002	0.853	-0.010	0.267	0.007	0.436	-0.008	0.392	0.001	0.884
		High	0.002	0.848	<0.001	0.956	0.006	0.434	0.003	0.703	-0.010	0.242	-0.002	0.816
	Riboflavin intake	Low	1.160	0.085	0.375	0.610	0.969	0.165	0.560	0.449	1.324	0.080	0.962	0.194
		High	0.164	0.790	-0.441	0.510	0.124	0.845	-0.096	0.887	0.827	0.230	0.721	0.285
	Serine		0.005	0.331	<0.001	0.947	0.006	0.253	<0.001	0.932	0.008	0.152	0.002	0.771
	Interaction	Low	-0.015	0.065	-0.006	0.71	-0.013	0.107	-0.005	0.585	-0.014	0.124	-0.012	0.185
		High	-0.005	0.512	0.003	0.709	-0.005	0.559	0.002	0.814	-0.012	0.150	-0.010	0.217

Dependent variable	Model terms		Global cognition		Attention		Episodic Memory		Executive function		Location Learning		Working Memory	
			β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
	Vitamin B_6 intake	Low	1.251	0.060	0.280	0.700	1.211	0.079	0.390	0.589	1.588	0.034*	0.876	0.229
		High	-0.338	0.585	-0.824	0.224	0.049	0.939	-1.013	0.134	0.510	0.464	0.193	0.776
	Serine		0.002	0.711	-0.003	0.614	0.004	0.396	-0.003	0.538	0.008	0.168	-0.001	0.853
	Interaction	Low	-0.014	0.085	-0.003	0.730	-0.014	0.104	-0.003	0.692	-0.019	0.041#	-0.009	0.290
		High	0.004	0.625	0.010	0.251	<0.001	0.954	0.010	0.245	-0.006	0.492	-0.004	0.619
	Vitamin B_{12} intake	Low	0.397	0.540	-0.017	0.980	-0.131	0.845	-0.078	0.911	2.109	0.003*	0.582	0.411
		High	0.036	0.955	-0.241	0.723	-0.031	0.962	-0.467	0.492	0.75	0.265	0.472	0.491
	Serine		0.001	0.879	-0.002	0.711	0.001	0.921	-0.003	0.559	0.011	0.052	-0.001	0.909
	Interaction	Low	-0.003	0.717	0.003	0.728	0.002	0.833	0.004	0.605	-0.024	0.006#	-0.007	0.391
		High	0.001	0.905	0.005	0.580	0.002	0.851	0.006	0.464	-0.010	0.250	-0.005	0.550
	Serum folate	Low	0.571	0.404	0.864	0.244	-0.057	0.936	0.526	0.483	1.564	0.035*	-0.526	0.460
		High	-0.015	0.983	0.381	0.596	-0.503	0.466	-0.209	0.774	0.789	0.272	<0.001	0.999
	Serine		0.002	0.763	0.003	0.545	<0.001	0.952	0.001	0.856	0.007	0.182	-0.007	0.161
	Interaction	Low	-0.008	0.346	-0.010	0.251	-0.002	0.840	-0.008	0.365	-0.018	0.051	0.009	0.323
		High	0.001	0.925	-0.002	0.857	0.006	0.510	0.002	0.849	-0.011	0.221	0.001	0.952
	Serum vitamin B_{12}	Low	1.048	0.145	1.282	0.095	0.625	0.404	0.339	0.665	1.246	0.118	-0.223	0.778
		High	0.680	0.293	0.389	0.572	0.405	0.546	0.713	0.311	0.173	0.808	0.873	0.218
	Serine		0.006	0.203	0.006	0.274	0.005	0.315	0.003	0.638	0.006	0.271	-0.001	0.925
	Interaction	Low	-0.015	0.080	-0.020	0.037#	-0.009	0.325	-0.005	0.583	-0.016	0.092	0.003	0.765
		High	-0.010	0.214	-0.004	0.612	-0.008	0.344	-0.009	0.320	-0.004	0.666	-0.011	0.224

β estimate and p values presented for models fit with one-carbon metabolite as continuous variables and B vitamin intake or status as categorical variables (low – quartile 1, high – quartile 4, reference – quartiles 2 and 3), and an interaction term between these main effects for each cognitive domain fitted as the dependent variable. All models are adjusted for age, sex, education, batch effects, energy intake, physical activity, history of anxiety/depression, and supplement use. [1]Fit as log-transformed variables in models. A significant association ($p < 0.05$) between the main effects and dependent variable is indicated by *for main effects, and #for the interaction term.

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