One Carbon Metabolism in Older Adults

A comparison of the postprandial responses of plasma compounds involved in one carbon metabolism to different mixed-meals in healthy young and healthy older adults: A preliminary randomised control trial.

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Abstract

Ageing is associated with altered diet and metabolism, including altered one-carbon metabolism (OCM). Disturbances of OCM, namely elevated homocysteine (Hcy), are associated with age-related diseases. Many substrates and cofactors of OCM are essential nutrients, yet, how ageing affects postprandial responses of one-carbon (OC) compounds to meals is largely unknown. This is important if acute responses affect long-term health outcomes. This study aimed to establish if plasma OC-compounds in healthy young and older adults respond differently to the intake of nutrient-dense (ND) and energy-dense (ED) meals.

Healthy young and older adults (n=15 each) consumed two different breakfast meals on separate occasions in a randomised cross-over trial. One meal was ND (oats, milk, fruit, cottage cheese, toast and peanut butter); and one was ED (egg and sausage English muffins with hash browns). Plasma samples, collected at fasting and hourly for five hours postprandially, were analysed for 13 OC-compounds using high-performance liquid chromatography with tandem mass spectrometry.

Postprandial concentrations of OC-compounds were not simultaneously dependent on meal type and age. Across all timepoints, older adults had similar concentrations of OC-compounds for the two meals, while young adults had higher betaine, choline, glycine and taurine for the ED meal compared to the ND meal. Fasting concentrations of some OC-compounds differed between age-groups; compared to young adults, homocysteine (Hcy) was lower at baseline and across all time points for the ED meal in older adults. Time-course responses of OC-compounds after eating were similar between the age-groups; only the ratio for dimethylglycine-to-betaine increased for older adults. Serine, methionine, S-adenosylmethionine, choline and dimethylglycine increased after consuming the ED meal for both age-groups. Serine, methionine and S-adenosylmethionine also increased for all subjects after eating the ND meal; the ED meal delayed postprandial increases of serine and methionine.

Postprandial responses of plasma OC-compounds to a meal are not dependent on ageing alone, and may depend more on meal composition. Older adults may have less divergent responses to different meals, with health implications of this yet to be established. Additional postprandial research in less-healthy cohorts of older adults is justified, given the link between age-related disease and altered OCM.

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Table of Contributions

Researcher	Contribution
Hannah Corke	Author of this thesis. Sample preparation, data preparation and processing including extraction of sample data and integration of standard curves. Conducted statistical analyses. Interpreting, graphing and presenting results.
Dr Amber Milan (Academic supervisor)	Guidance interpreting results, feedback and editing on write-up of this thesis. Study design, recruitment, ethical approval and trial procedures were carried out previously by Dr Amber Milan with contributions inclusive of her team at that time. The study itself was undertaken by by Milan et al, at the Maurice and Agnes Paykel Clinical Research Unit (MAPCRU) at the Liggins Institute, University of Auckland, Auckland, New Zealand between October 2012 and July 2013.
Nicola Gillies (PhD candidate)	Carried out laboratory proceedures using UHPLC-MS/MS on samples with assistance in sample preparation and data extraction from Hannh Corke. Supervised Hannah Corke through data prepartion for statitistical analysis and taught Hannah Corke to use the statistical software used to conduct statistics for this thesis.
Pankaja Sharma (PhD candidate)	Assisted in defining and calculating nutrient density and energy density of the test meals.
Stephanie Andraos (PhD candidate)	Developing the UHPLC-MS/MS methods that were used to quantify the one-carbon compounds in these samples.

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BHMT	betaine-homocysteine methyltransferase
BMI	body mass index
CBS	cystathionine beta synthase
DMG	dimethylglycine
ED	energy-dense
Hcy	homocysteine
hHcy	hyperhomocysteinemia
HOMA-IR	homeostatic model assessment of insulin resistance
MTHFR	methylenetetrahydrofolate reductase
ND	nutrient-dense
OC	one-carbon
OCM	one-carbon metabolism
QC	quality control
SAH	s-adenosylhomocysteine
SAM	s-adenosylmethionine
THF	tetrahydrofolate
UHPLC-MS/MS	ultra-high-performance liquid chromatography coupled with tandem mass spectrometry
5MTHF	5-methyl-THF

Chapter 1 Introduction & Literature Review

1.1 Introduction

Many developed and developing nations now face an ageing population. Improvements to health care, sanitation, medicine and medical technologically have enabled us to live longer, resulting in a shift from infectious disease to chronic disease as the leading cause of death globally (1). Nutritional intake and micronutrient status can have profound effects on chronic disease pathogenesis. Low intake and status of nutrients involved in one-carbon metabolism (OCM) have been linked to increased homocysteine (Hcy) levels, and correlated with age-related disorders including cognitive decline and cardiovascular disease (2–6). Good nutrition supports healthy metabolism, which is paramount to healthy ageing. This importance is acknowledged by dietary guidelines developed specifically for older adults, by regulatory bodies such as the Ministry of Health (NZ) and the World Health Organisation (7,8). Yet, age-specific considerations that may account for differences in digestion, absorption and ultimately postprandial metabolism, are still not well defined (9). The global aged population (people ≥ 60 years) is projected to double by 2050 (10) and this will incur large economic and health implications for nations (11,12). Better understanding of the mechanisms behind healthy ageing is essential, including how nutrition supports vital metabolic processes, such as OCM.

The literature surrounding altered OCM is heavily focused on elevated concentrations of circulating Hcy, known as hyperhomocysteinemia (hHcy), as this is linked to poor health outcomes. Studies looking to improve health outcomes by lowering Hcy through supplemental nutrients are yet to show clinical significance of longer-term micronutrient supplementation. Hence, the mechanisms behind nutrient intake, hHcy and age-related disorders remain largely unexplained. Optimising nutrient supply of one-carbon (OC) compounds, to allow appropriate functioning of OCM, requires better understanding of how OCM is regulated by nutrient intake. Few have studied what happens to OCM in the postprandial period and few have considered the circulating concentrations of OC-compounds other than Hcy. These factors prompt exploration into the effect of food-based interventions on OCM in older adults, in the acute postprandial period, to better understand the impact of nutrient intake on OCM in older adults. This review of the literature will explore the interplay between ageing, nutrition and OCM, with an emphasis on how these factors may influence postprandial circulating concentrations of OC-compounds.

1.2 One carbon metabolism

Metabolic pathways such as OCM play a key role in maintaining whole-body homeostasis (13). OCM is the universal metabolic process in the body of supplying single methyl moieties to biological reactions (14,15). Multiple physiological processes in the body are supported by this, including biosynthesis of nucleotides and histones (16), deoxyribonucleic acid (DNA) methylation (17), cell-proliferation (15), amino-acid homeostasis (15) and redox balance (18). Many OC-compounds are dietary nutrients including choline, betaine, methionine, serine, folate and numerous other B-vitamins (13,19). These contribute to OCM as either methyl-group supplying substrates or as enzyme cofactors (13,15,19). Sufficient availability and balance of these compounds within OCM pathways is essential and is often assessed by circulating levels of key compounds (6). The health effects of many nutrients involved in OCM are imparted through the outputs of OCM pathways (6,13). Altered intake or status of OC-compounds may redirect pathways to accommodate compound excesses or deficiencies (20,21) however, altered OCM, largely assessed as hHcy, has also been linked to chronic age-related pathologies, including cancer, cardiovascular and neurodegenerative disorders (6,13).

1.2.1 Functions and regulation of OCM

Multiple interlinked metabolic pathways comprise OCM. These are the methionine cycle, the folate cycle, the transsulfuration pathway and the betaine-homocysteine methyltransferase (BHMT) pathway (13,22). These pathways interlink through the metabolism of Hcy (Figure 1.1) which has two main fates: transsulfuration or remethylation (19). Remethylation of Hcy back to methionine can occur via demethylation of 5,10-methylene-tetrahydrofolate to tetrahydrofolate (THF) in the folate cycle (13) or, via the conversion of betaine to dimethylglycine (DMG) catalysed by the enzyme BHMT (20) in the BHMT pathway. Methionine, produced through Hcy remethylation, is adenylated to S-adenosylmethionine (SAM) which is the predominant methyl donor for methylation reactions in the cell (20). S-adenosylhomocysteine (SAH) is produced when SAM donates a methyl group in these methylation reactions, and Hcy can be regenerated from SAH in a reversible reaction (13). Hcy regenerated in the methionine cycle can also be used to produced cysteine in the transsulfuration pathway, which is ultimately converted to taurine or glutathione (19).

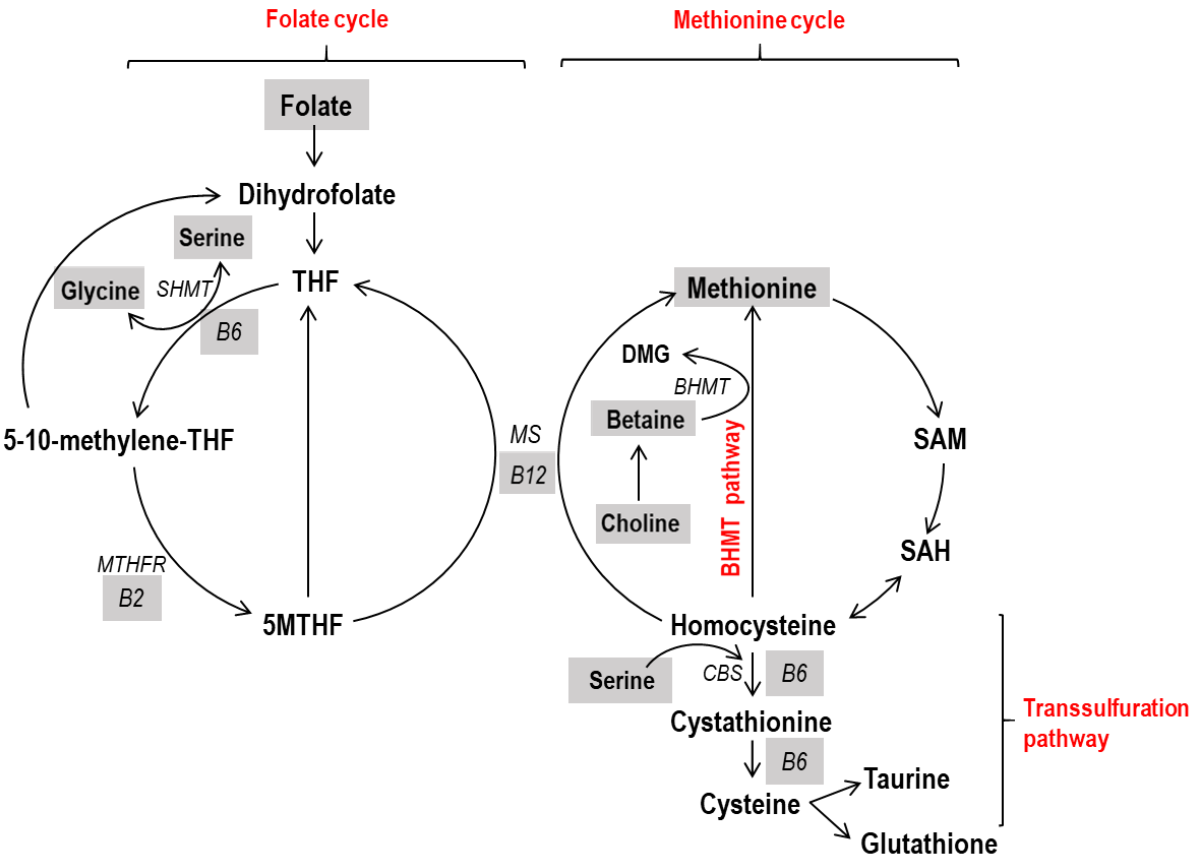

Figure 1.1 One Carbon Metabolism overview.

In One Carbon Metabolism, homocysteine is metabolised to methionine through coupling of the methionine cycle with the transsulfuration pathway and either the folate cycle or the betaine-homocysteine methyltransferase (BHMT) pathway. The folate cycle involves the conversion of folate, present in cells as the biologically active form tetrahydrofolate (THF), to 5,10-methylene-THF and subsequently to 5-methyl-THF (5MTHF), via the enzymes serine hydroxymethyl transferase (SHMT) and methylenetetrahydrofolate reductase (MTHFR), respectively. Most folate in the body is present as 5MTHF, which is demethylated to THF in the folate cycle by the enzyme methionine synthase (MS), requiring vitamin B12 as a cofactor (15). The methyl group from 5MTHF is donated to homocysteine to produce methionine, which is converted to s-adenosylmethionine (SAM) and subsequently to S-adenosylhomocysteine (SAH) when SAM donates a methyl group in methylation reactions. Homocysteine can also be remethylated to methionine via the BHMT-dependent conversion of betaine, derived from the diet or choline, to dimethylglycine (DMG) (20). The methionine cycle is also connected to the transsulfuration pathway through the intermediary metabolite homocysteine, which combines with serine to generate cysteine and ultimately glutathione and taurine (13). Compounds that can be obtained from the diet are highlighted; pathway names are indicated by red text; italicised text indicates an enzyme or cofactor. Figure adapted from Suh, Choi & Friso (2016) Figure 36.1, One-carbon metabolism, p. 514-515 (13).

Pathways of OCM output compounds that have important biological functions in the body. For example, a major role of OCM is in producing SAM through the methionine cycle (16,18). SAM is a key methyl donor throughout the cell (20), methylating many substrates including DNA, RNA, histones and phospholipids (16,18,19). The synthesis of purines requires 10-formyl-THF, which can be produced from the reduction of 5,10-methylene-THF (generated in

the folate cycle) to 5,10-methyl-THF and subsequently to 10-formyl-THF (13,20). Additionally, the input and output of amino acids in OCM, such as serine, glycine and methionine, assists amino acid homeostasis within the body (15). Furthermore, OCM has a role in regulating redox status. This mostly occurs through the reduction of NADPH and oxidation of $NADP^+$ occurring throughout OCM (18) however, the production of glutathione in the transsulfuration pathway also contributes to redox status as one of the major antioxidants influencing the NADPH:$NAPH^+$ ratio in cells (15,18,23). All of these important outputs of OCM rely on the input and appropriate balance of numerous compounds which contribute to OCM (Table 1.1).

Table 1.1 The key contributions of one-carbon compounds to OCM pathways.

Compound	Role(s) in OCM	Pathway(s)
Choline	Betaine synthesis (13).	BHMT pathway
Betaine	Methyl-donor in the remethylation of Hcy to methionine, producing DMG (22,24,25).	BHMT pathway
DMG	The product of betaine metabolism and therefore marker of the BHMT pathway in Hcy remethylation (6). Can be metabolised to sarcosine (26) or degraded to glycine (22).	BHMT pathway
Vitamin B2	Enzyme cofactor for MTHFR (13). Also involved in converting B6 to its bioactive form for use in OCM (27).	Folate cycle
Vitamin B6	Enzyme cofactor for CBS and the conversion of cystathionine to cysteine (16). Also influences the metabolism of 5,10-methylene-THF in the first step of the folate cycle (18).	Transsulfuration pathway & folate cycle
Vitamin B12	Enzyme cofactor for MS (Figure 1.1).	Methionine & folate cycles
Folate derivatives[1]	5MTHF is a methyl donor for Hcy to produce methionine and THF (13). THF accepts a methyl group from serine to produce 5,10-methylene-THF, which can then be irreversibly reduced by MTHFR to form 5MTHF (13).	Folate cycle coupled to methionine cycle
Hcy	Accepts a methyl group from either 5MTHF or betaine to form methionine. Or, condenses with serine to form cystathionine (Figure 1.1) via CBS activity.	Methionine & folate cycles, BHMT & transsulfuration pathways
Methionine	Adenylated to produce SAM (15). Methionine is the only nutrient precursor of Hcy (28).	Methionine cycle
SAM	The metabolite of methionine, SAM is a universal methyl donor throughout the body (6,13,16). SAM becomes SAH upon donation of a methyl group (16).	Methionine cycle
SAH	The product of SAM donating a methyl group. SAH is hydrolysed back to Hcy by SAH hydrolase (16) and can then be used to metabolise Hcy.	Methionine cycle
Serine	Methyl donor for the reversible conversion of THF to 5,10-methylene-THF and glycine (18), catalysed by SHMT (15). Additionally, serine combines with Hcy in the transsulfuration pathway to produce cysteine (29).	Folate cycle & transsulfuration pathway
Glycine	Product of serine donating a methyl group to THF.	Folate cycle
Cystathionine	Product of Hcy and serine condensing, which requires B6 as a cofactor.	Transsulfuration pathway
Cysteine	Product of cystathionine cleavage which also requires B6 as a cofactor.	Transsulfuration pathway

[1]THF, 5,10-methylene-THF, 5MTHF.

Abbreviations: BHMT (betaine-homocysteine methyltransferase); CBS (cystathionine beta synthase); DMG (dimethylglycine); Hcy (homocysteine); MS (methionine synthase); MTHFR (methylenetetrahydrofolate reductase); OCM (one carbon metabolism); SAH (S-adenosylhomocysteine); SAM (S-adenosylmethionine); SHMT (serine hydroxy methyltransferase); THF (tetrahydrofolate); 5MTHF (5-methyl-tetrahydrofolate).

1.2.2 Homocysteine metabolism

In addition to the inputs and outputs of OCM described above, one of the major functions of OCM is in regulating Hcy levels within the body. Hcy serves as an intermediary metabolite of OCM where the pathways involved (the methionine cycle, folate cycle, transsulfuration pathway and BHMT pathway) converge (Figure 1.1) (30). Hcy is a sulphur-containing, non-essential amino-acid, which is produced in the body as a requisite by-product of methionine metabolism (30). Hcy interacts with each pathway in OCM which can metabolise it such that intracellular concentrations do not rise to detrimental levels, as Hcy is understood to be toxic to the cell (13,30). Hcy produced in the cell can either undergo transsulfuration or remethylation within the cell, or be removed from the cell and exported into circulation (16,29,30). As such, appropriate regulation of OCM and balance within the pathways is often inferred through circulating levels of Hcy (19,31).

Metabolism of Hcy through OCM is influenced by both endogenous and exogenous factors (30). For example, endogenous regulation of OCM includes enzymatic efficiency, which can be influenced by genetic deficiencies of enzymes regulating OCM pathways (23,30,32–35). The most well-known of these is the C677T single-nucleotide polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene (13,27,30), the enzyme required to produce 5-methyl-THF (5MTHF) for Hcy remethylation via the folate cycle. This polymorphism has been associated with hHcy (34) as it reduces the activity of MTHFR, reducing its capacity to remethylate Hcy via 5MTHF in the folate cycle (6,27,33–36). This demonstrates how alterations in the enzymic efficiency of OCM can affect plasma Hcy levels. The primary exogenous factor influencing Hcy metabolism is the influence of dietary intake over OCM (30). Many substrates, enzymes and cofactors of OCM are dietary nutrients (Table 1.3). When there is altered intake or status of nutrients involved in OCM, this may result in either a compensatory shift in these pathways to accommodate altered compound availability, or lead to inappropriately high circulating concentrations of the disease-associated compound, Hcy (6,21,37).

1.2.3 Compensatory shifts in OCM

Compounds of OCM can be obtained from the diet and/or synthesised endogenously (Table 1.2). Because many of these compounds are essential nutrients (Table 1.2), with important regulatory roles over OCM, adequate intake of these is necessary to ensure appropriate functioning of OCM. To some extent, OCM can shift to accommodate altered intake or status

of a nutrient involved in regulating OCM (21). This is largely apparent through the two alternative pathways of Hcy remethylation; the BHMT-dependent pathway and the vitamin B12-dependent conversion of 5MTHF to THF via methionine synthase in the folate cycle (Figure 1.1).

The remethylation of Hcy via the folate cycle relies on adequate dietary intake of both folate and vitamin B12 since the folate-derivative, 5MTHF, donates a methyl group to Hcy and vitamin B12 is the enzyme-cofactor to this reaction (20,38). Conversely, the BHMT pathway of Hcy remethylation relies on betaine as the methyl-group donor for Hcy remethylation (39). Betaine is either sourced from the diet or produced endogenously from choline oxidation (24) - which itself is an essential nutrient that can be sourced from the diet (Table 1.2). Although both pathways function simultaneously in the body to remethylate Hcy (30), altered intake or status of nutrients involved in either of these pathways may result in compensatory shifts to rely more heavily on the alternate pathway of Hcy metabolism .

Compensatory shifts in OCM were shown in one study demonstrating how a seasonal switch between the two pathways of Hcy remethylation may occur with seasonal shifts in folate, vitamin B12, choline and betaine intakes (21). Moreover, in vitamin B12 or folate deficient individuals, there is a reduced capacity to remethylate Hcy via the folate cycle, because these deficiencies can trap folate as 5MTHF or reduce the availability of 5MTHF respectively (38). In such circumstances, betaine becomes the primary methyl donor for Hcy remethylation and as such, dietary requirement for choline, the precursor for betaine, is known to increase (6,37). Similarly, greater demand for dietary folate and vitamin B12 occurs in choline-deficient individuals, to accommodate a reduced capacity to methylate Hcy via the BHMT-mediated pathway (37). These shifts represent some degree of metabolic flexibility within OCM to accommodate shifts in nutrient intake and status (6,20,21,37,40). Whether these shifts are apparent in the acute postprandial timeframe has not been established.

Table 1.2 Dietary-derived one-carbon compounds.

Compound	Key dietary sources of compound	Essential nutrient	Can be synthesised endogenously
Choline	Eggs, meat, liver and legumes, including soy beans and peanuts (27,37,39,41). Dairy products also contain choline but are not as rich sources comparably to meat and egg.	Yes[1]	Yes
Betaine	Spinach, wheat products, beets, some seafood (22,39,42).	No	Yes
Vitamin B2	Milk, milk products, fortified cereals (27).	Yes	No
Vitamin B6	Wide variety of foods including meat, fruits, vegetables, whole grains, breakfast cereals (27).	Yes	No
Vitamin B12	Animal-based foods including organ meat, red meat, egg, dairy products, fish (27,43).	Yes	No
Folate[2]	Green leafy vegetables, legumes, cereal products, liver, nuts and fruits (27,44,45). Uncooked sources of these foods contain higher amounts of folate (45).	Yes	No[4]
Cysteine	Protein-based foods with particularly high bioavailability in animal-based proteins; good sources include meat, poultry, eggs, dairy products, legumes and fish (7).	No[3]	Yes
Serine		No[3]	Yes
Glycine		No	Yes
Methionine	As above for cysteine, serine and glycine. Has also been associated with a diet high in refined grains and processed meat, as well as vegetarian diets and diets high in fish (46).	Yes[1]	Yes

[1]Endogenous choline can be produced in the liver and methionine can be synthesised from remethylation of homocysteine, however, these are both essential nutrients because endogenous production is not sufficient to meet metabolic demands for these compounds (27,47).
[2]Including foods fortified with folic acid.
[3]Conditionally essential in the diet under certain, extreme physiological conditions (27).
[4]Metabolism of folate derivatives occurs in the folate cycle, however, this must be replenished with dietary-derived folate or folic acid which cannot be synthesised endogenously (48).

1.2.4 Consequence of nutrient deficiency for OCM

Although altered or inadequate intake of a nutrient involved in OCM may be partly compensated for by other nutrients, deficiencies of these are linked to disturbed OCM and diseased states (6,13,20,30). Circulating Hcy acts as a biomarker of OCM regulation (19) and metabolic disturbances of OCM may manifest as hHcy (13). The reference range for plasma Hcy is between 5 - 15 μM (49) and under normal conditions, fasting plasma concentrations typically range between 5 – 10 μM (30,42). hHcy is generally considered as concentrations greater than 15 μM (13,30,50,51) and is an established risk factor for age-related diseases including cancer, cardiovascular disease and neurological disorders (6,13,30).

Appropriate metabolism of Hcy relies on many nutrients to act as substrates or coenzymes for the conversion of Hcy back to methionine, or to cystathionine and cysteine (6,27,28). As such, much of the understanding about hHcy and poor health outcomes has been established through observed deficiencies of nutrients involved in OCM. For example, hHcy is associated with deficiencies of folate and vitamins B12, B6 and, to some extent, B2 (2,6,28,51); these are all substrates or cofactors in OCM (Figure 1.1). Inadequate intake (32,44,52,53) or circulating status (3,54,55) of these B-vitamins, have been associated with poor health outcomes including increased cancer risk (44,52), cognitive decline (3,32,53,54) and cardiovascular disease risk (6,55). To a lesser extent, other nutrients that metabolise Hcy, including betaine and choline, have also been associated with hHcy and linked to poor health outcomes (20). Moreover, excessive intake of methionine, the sole nutrient precursor to Hcy production (28), or methionine-rich proteins are also associated with elevated Hcy levels (13,20,56).

Despite well-recognised associations between individual nutrients of OCM, hHcy, ageing and age-related disease, the mechanisms underlying altered OCM in age-related disease are not well established. Although hHcy appears to be associated with ageing (6,13,28,30,51), it is not yet established whether altered OCM is a result of inherent ageing, or a cause or consequence of the comorbidities associated with ageing (6,13). Nevertheless, older adults represent a high-risk group for hHcy since the prevalence of this increases with age (6,28,51) and may be present in up to one third of older adults aged 60 years and over (51,57).

1.3 Ageing, nutrition and OCM

Older people have different nutritional considerations to younger people. Although dietary guidelines across nutrients remain similar as for younger adults (27), there is increasing recognition that older people likely have different nutrient needs to younger people (7). The New Zealand Ministry of Health recommend an increased intake of protein, calcium and vitamins B6 and D for adults as they advance past ages 50 – 71 years (27). Excluding protein, these recommendations are similarly reflected by the US National Institute of Medicine Dietary Reference Intakes guidelines (58,59). Compared with other adult populations, nutritional status among elderly populations is more likely to be compromised (60). Malnutrition, whether clinical or subclinical, has been reported to be highly prevalent in older adult populations (61–63) and contributes to ill-health in a number of ways. These health consequences are relatively well-explored with regards to the role of malnutrition in age-related muscle wasting (sarcopenia), which is a principle component of frailty syndrome and loss of independence in older adults (63–65). The consequences of subclinical micronutrient deficiencies however are less well-established, but are likely important to OCM homeostasis since many OC-compounds are nutrients.

1.3.1 Changes to nutritional adequacy in ageing

Ageing involves a reduction in lean body mass and relative increase in fat mass (7). Alongside reduced physical activity, these factors result in reduced daily energy requirements for older adults, despite micronutrient requirements remaining relatively stable for this age group (6–8,62). This means older adults require fewer calories than younger adults, but the same, if not greater (27), amount of micronutrients. Moreover, the requirement for some nutrients are thought to increase with age, including protein, calcium, vitamins B6 and D (27,58,59); due to the role of oestrogen in synthesising endogenous choline, postmenopausal women may also have increased requirements for dietary choline intake (24).

Nutritional adequacy in older adults is multifaceted and can depend on physiological, pathological, environmental, social, psychological, and pharmacological factors (7,62,63). Combinations of these factors may increase malnutrition risk, with commonly reported reasons for malnutrition and micronutrient insufficiency in older adults relating to one or more of; inadequate intake of food and/or nutrient-density of foods, poor nutrient digestion and absorption, pharmaceutical drug-nutrient interactions, and increased nutrient losses and/or requirements due to comorbidity (66). Prevalence of malnutrition among older adults varies

widely depending on the subset observed, the measures used to assess nutritional status and the type of malnutrition reported. Reports vary from less than 10 % among community-dwelling, healthy older adults, to between 13.8 - 70 % across residential aged-care, rehabilitation and hospital settings (61–63). Importantly, the proportion of older adults *at-risk* of malnutrition across settings is significant, with reports ranging between 22 - 83 % (61–63). This is consequential since older adults *at-risk* of malnutrition, who do not meet common malnutrition thresholds to cause overt symptoms (67), may still have changes to diet or nutrient status that affect health (subclinical deficiencies).

Compared to more overt signs of malnutrition, such as unintentional weight loss in sarcopenia (63), subclinical micronutrient deficiencies exist for older adults at less visible, but still consequential levels (67–70). Since many dietary-derived nutrients feed into OCM, including B-vitamins, choline, betaine and essential amino acids like methionine (Table 1.3), the risk of subclinical deficiencies in older adults makes them a particularly important population group to study in regards to nutrient intake and OCM. Moreover, the ability to identify subclinical nutrient deficiencies through non-invasive measures is likely poor (70). For example, vitamin B12 deficiency is suggested to increase in prevalence with increasing age (6,12,51,71), and inadequacies of other B-vitamins involved in OCM have also been associated with ageing, including vitamins B2, B6 and folate (6,27,28,45,72). Yet, common measures of nutritional status, including malnutrition screening tools and body-mass index (BMI), may poorly identify these subclinical deficiencies (70,71). Therefore, the efficiency of identifying subclinical deficiencies of nutrients involved in OCM, such as the B-vitamins, in older adults is likely poor. Contributing to this may be a lack of screening for nutrient status in older adults, due to the common assumption that nutritional deficiencies are inevitable with ageing (66), and/or ineffective screening tests for nutrient status (71). In any case, this tells us that apparently healthy older adults may have, or be at increased risk of, subclinical nutrient deficiencies (70) – including of nutrients involved in OCM. The risk that older adults have over other age groups for poor nutrient status of OC-compounds is further heightened by age-related factors influencing the circulating status of these compounds.

1.3.2 Age-associated confounders to circulating status of OC-compounds

Ageing is associated with physical and behavioural changes that can influence the circulating status of nutrients, including OC-compounds. For example, reduced nutrient intake has already been eluded to above as a contributing factor to malnutrition and subclinical deficiency in older

adults. Poor nutrient intake in ageing is multifaceted, and may relate to reduced caloric requirements (7), poor appetite, depression, dysphagia, taste changes and/or dentition (62). These factors place older adults more at-risk of inadequate intake, and therefore poor circulating status (69), of micronutrients involved in OCM over younger age groups. In addition, gastrointestinal changes impacting nutrient digestion and absorption, declining renal function, insulin resistance and a high prevalence of polypharmacy among older adults, may also confound the circulating status of some OC-compounds. Specific examples of these age-related factors largely relate to the well-studied vitamin B12, but the possibility that other OC-compounds are affected by similar mechanisms cannot be ruled out. The influence of age-specific changes on the status of all OC-compounds is not yet synthesised in the literature, requiring additional research as to how this might affect OCM in older adults.

i. Gastrointestinal changes with ageing

Ageing is associated with a general decline in overall bodily function, with all tissues and organ systems impacted (7). This includes changes to gut morphology and function (73) that can impair nutrient digestion and absorption (74), and ultimately impact circulating concentrations of nutrients. The impacts of ageing gut physiology are exemplified by well-studied alterations in protein and B-vitamin digestion and metabolism. For example, sarcopenia (63) is caused by unresponsiveness of the muscle to amino acids (75), and older adults have altered protein digestion and amino acid absorption (9,76). Similarly, altered gastrointestinal function in the elderly is a known contributor to vitamin B12 deficiencies (7,32).

Older adults have altered digestion and absorption of protein (9,76). Findings suggest that older adults benefit from an increased intake of high-quality protein throughout the day in order to digest, absorb and utilise the same amount of protein and its amino acids as their younger-counterparts (9,75–77). This is reflected by a 25% higher recommended intake of protein for older adults compared to younger adults by the Ministry of Health (NZ) (7,27). These recommendations were predominantly implemented to prevent protein-energy malnutrition and delay sarcopenia in older adults (7,27). Glycine, serine and methionine are amino acids which have integral roles in OCM. Although absorption and postprandial circulating concentrations of these may be altered in ageing, little information exists yet to demonstrate whether differences in protein digestion and amino acid absorption for older adults influence OCM pathways.

Furthermore, a high prevalence of chronic atrophic gastritis exists among older adults, increasing with age (78) and ranging from 10 - 30% (8,27) to 50% or higher (43) in the oldest adults. Atrophic gastritis reduces the availability of stomach acid, pepsin and intrinsic factor, which are requisites for the cleavage of vitamin B12 from food proteins and the subsequent absorption of vitamin B12 in the ileum (7,43,79,80). Hence, atrophic gastritis can reduce the bioavailability and absorption of vitamin B12, likely contributing to the high rates of vitamin B12 deficiency in older adults (7,32). The association between poor B12 status and elevated Hcy is well established (28,51), and age-related gastrointestinal changes impacting circulating B12 status pose an increased risk for hHcy in older adults compared to younger counterparts.

ii. Renal function

The increased prevalence of hHcy with increasing age (6,28,51) may in part relate to the decline in renal function that occurs with ageing (51,81,82), which can impair renal metabolism of circulating Hcy (6,28,51). As clearance of circulating Hcy occurs via the kidneys (13), impaired renal function can result in improper clearance of excess Hcy (6,28,83), contributing to detrimental levels of circulating Hcy (6,13,28). The circulating concentrations of other OC-compounds that regulate Hcy may also accumulate in renal insufficiency. For example, DMG normally feeds-back on OCM to inhibit the BHMT-dependent remethylation of Hcy; when it accumulates in uraemia, this negative feedback on BHMT can result in elevated Hcy levels (83). Due to a lack of research on the postprandial responses of other OC-compounds, we do not know the full extent of age-related renal function decline on circulating concentrations of all OC-compounds, particularly in the postprandial period. Nevertheless, declining renal function in older adults is a potential confounder to circulating concentrations of OC-compounds.

iii. Insulin resistance

Insulin resistance is a metabolic change associated with ageing (84,85). Insulin is the primary anabolic hormone regulating postprandial uptake of glucose from the blood (85). Reduced glucose tolerance has been associated with ageing, such that both reduced secretion and sensitivity to insulin is thought to occur in ageing (7,86). Moreover, age-related factors, including reduced lean muscle mass (skeletal muscle is responsible for most glucose uptake), abdominal obesity, physical inactivity and diet, are also associated with declining insulin sensitivity (9). In terms of OC-compounds, fasting insulin and

insulin resistance have been inversely correlated with low intakes of choline and betaine (87) and low circulating concentrations of glycine (88). Given that older adults are at increased risk of insulin resistance compared to younger adults (84), insulin resistance may be a confounding factor to age-differences in fasting concentrations of some OC-compounds between young and older adults. Moreover, in the postprandial period, insulin resistance has known impacts on lipid metabolism (89) and amino acid metabolism (90), with similar alterations in postprandial lipid and amino acid metabolism demonstrated also in healthy older adults (91). Although healthy older adults may have poorer insulin sensitivity and alterations to postprandial metabolism influenced by insulin, there is little OCM-specific evidence to date to predict an impact of age on postprandial OC-compounds.

iv. Polypharmacy

The availability of micronutrients to contribute to metabolic pathways in older adults may be further reduced through drug-nutrient interactions (32,72). The high prevalence of disease and disorder among older adults causes a large range of pharmaceuticals to be taken by this age group. Drug-nutrient interactions, including of OC-compounds, exist for medications of age-related chronic conditions that may contribute to nutritional status (11). For example, antacids and proton-pump inhibitors, commonly administered to older adults, supress gastric acid which is required for appropriate absorption of vitamin B12 (11,32). Moreover, folate bioavailability can be rendered less available by methotrexate use (32). As for many other nutrients, the full extent of drug-nutrient interactions on the postprandial availability of nutrients involved in OCM is not well established (92) but likely exist, and there should be better understanding of drug-nutrient interactions for drugs that are commonly administered to older adults (92).

Overall, numerous factors associated with ageing may alter the availability or processing and regulation of OC-compounds, which may be further complicated by drug-nutrient interactions. These factors contribute to increased risk of subclinical nutrient deficiencies in older adults, including of OC-compounds. In the long term, subtle micronutrient inadequacies may contribute to increased risk and accelerated onset of age-related degenerative diseases, including neurocognitive disease, cardiovascular disease and cancer (68,69). These diseases are associated with insufficiencies of OC-compounds, including folate, vitamins B12 and B6, betaine and choline (2,6,20,93,94), and are typically characterised by perturbations of the OC-

compound, Hcy (6,42,94). However, these associations remain largely unexplained, which has led to research about nutritional intervention as tool to avoid or improve subclinical deficiency in older adults.

1.3.3 Improving health outcomes associated with altered OCM in older adults

The associations between nutrition, hHcy and age-related diseases have led to trials looking at supplementing nutrients implicated in hHcy, as Hcy-lowering interventions to improve the diseased state. In this regard, there has been a heavy focus on supplementing the B-vitamins involved in OCM and by in large, these trials have been in older adults (Table 1.3). Results from these studies have shown that OCM-related B-vitamin supplementation is an effective treatment at lowering Hcy levels (54,95–98). However, despite a reduction in Hcy levels, few trials have been able to reduce the risk, or improve the clinical outcomes, of these conditions (Table 1.3); namely, osteoporotic fracture risk (95), cardiovascular event risk (97,99), and cognitive function (5,96,98). Moreover, despite the role that other nutrients play in Hcy metabolism (Figure 1.1), only a few studies have considered supplementation of nutrients outside of the OCM-related B-vitamins. Both choline and betaine have important roles regulating plasma Hcy levels (20,94,100,101) with both dietary intake (94) and supplemental intake (24,42,100) of these inversely associated with circulating Hcy levels. However, similar to B-vitamin supplementation, the supplementation of choline (24) and betaine (42) can lower Hcy levels but the clinical significance of these results on improving health outcomes has also not been established (42). Moreover, betaine supplementation studies (42) have largely been in the context of healthy young adults, which does not lend insight as to how these results might translate to older populations. Although the association between Hcy and age-related diseases are nowadays well-established (13), as is the role of OCM nutrients in maintaining appropriate circulating levels of Hcy (6,20), the mechanisms underlying the relationship between altered OCM, nutrition and age-related disease remain unclear. In light of this, Suh, Choi & Friso (13) suggested that a paradigm shift in the way we explore OCM in ageing should be made. In this regard, clear gaps in the literature surrounding mechanisms of nutrient intake, altered OCM in ageing and age-related disease relate to the lack of exploration of OC-compounds other than Hcy as biomarkers of disease and a lack of understanding as to the acute mechanisms of nutrient intake and OCM regulation in the postprandial time period. Understanding how OCM responds postprandially, particularly in older adults (a high-risk group for altered OCM), may lend insight as to how hHcy is implicated in age-related disease.

Table 1.3 Evidence of B-vitamin supplementation† efficacy to lower Hcy but not improve health outcomes.

Study	Folic acid	B12	B6	Improved disease outcome	Reduced mean plasma Hcy	Population group
B-PROOF RCT on the effect of B-vitamin supplementation and osteoporotic fracture risk (95)	+	+	n/a	No: osteoporotic fracture risk	Yes: Δ[Hcy] -4.4 μmol/L compared to -0.2 μmol/L for treatment vs. placebo	2919 participants ≥ 65 years with elevated Hcy levels[1]
B-vitamin supplementation to prevent cardiovascular events after acute MI (97)	+	+	+	No: risk of MI, stroke or death related to CAD	Yes Folic acid + B12 supplementation lowered mean [Hcy] by 27%	3749 participants aged 30 - 85 years that had had an ACI within 7 days of treatment
B-vitamin supplementation (in combination) on improving cognitive function in women with AD (98)	+	+	+	No: cognitive function tests did not improve	Yes: –2.25 μmol/L in [Hcy] for treatment group compared to placebo	89 participants with mild – moderate AD and normal B-vitamin status. Mean age 75 ± 7.3 years
Meta-analysis of RCTs on efficacy of B-vitamin supplementation in cognitive decline (96)	+	+	+	No: MMSE score did not improve	Yes: Mean difference in serum [Hcy] -3.625 μmol/L between intervention and placebo	679 patients with dementia (Alzheimer's disease or vascular dementia) aged between 74.6 - 79.1 years
Cochrane review of B-vitamin supplementation to lower Hcy & prevent cardiovascular events (99)	+	+	+	No: effect on risk of cardiovascular event Yes: small risk reduction for stroke	Yes	71,422 adults with CVD or at risk of a cardiovascular event
RCT of a combined B-vitamin supplementation in subjects with MCI (5)	+	+	+	Yes: improved some cognitive tests for subjects who had above-median Hcy levels[2]	Yes: Δ[Hcy] -2.6 μmol/L compared to +0.8 μmol/L for treatment vs. placebo	223 participants with MCI (pre onset of AD) aged ≥ 70 years

†of B12, B6, folate - alone or in combination.
[1]Defined as plasma Hcy 12-50 μM.
[2]Defined as plasma Hcy concentration >11.3 μM.
Abbreviations: ACI (acute myocardial infarction); AD (Alzheimer's disease); CAD (coronary artery disease); CVD (cardiovascular disease); Hcy (homocysteine); hHcy (hyperhomocysteinemia); MCI (mild cognitive impairment); MI (myocardial infarction); MMSE (Mini-Mental State Examination); n/a (not applicable as not included in study); OCM (one-carbon metabolism); RCT (randomised controlled trial). Symbols: + (included in supplement in this study).

1.4 Food intake and OCM in the postprandial period

OCM outcomes are often discussed in a long-term timeframe, yet the influx of key nutrients from the diet occurs firstly in a postprandial time period. As many OC-compounds with important regulatory roles in OCM pathways are essential nutrients (Table 1.2), appropriate OCM regulation relies on nutrients from food. Yet, OCM in the acute postprandial setting has been minimally researched. There is a gap in our understanding around how the profile of OC-compounds are influenced postprandially, particularly in response to realistic feeding scenarios (29,36,101,102) and certainly in older adults (36). Since disorders related to altered OCM are predominantly age-related (13), with higher prevalence of disrupted OCM (measured as elevated Hcy) in older adults (6,28,51), older adults are an important population group in which to understand the postprandial responses of OC-compounds. Age-related confounders, discussed above, can reduce post-absorptive nutrient availability (9), including that of B-vitamins and amino-acids involved in OCM. However, availability of these nutrients in circulation can also be modified by meal composition (29,50,56) and the concentrations of those nutrients in foods (41,50). Factors that can make postprandial responses of dietary-derived OC-compounds difficult to interpret, in regards to their role in OCM, include the contribution of endogenous production to circulating concentrations, and the metabolism of these compounds in biochemical pathways other than OCM. Because many OC-compounds are dietary nutrients (Table 1.2), and given that dietary intake occurs in the acute setting, multiple times throughout the day, the postprandial period is relevant to understanding the mechanisms by which dietary intake influences these outcomes.

1.4.1 Confounders to postprandial circulating OC-compound concentrations

Compounds of OCM may be endogenously produced or exogenously sourced through dietary intake (Table 1.2). Yet, the relative contribution of exogenous intake and endogenous production of circulating OC-compounds is not well established. One study looking at postprandial responses of methionine (41) was able to show that circulating increases in postprandial methionine concentrations were proportionally greater than the content of methionine in that food (41), suggesting some degree of endogenous production influences circulating methionine concentrations in the postprandial time period (41). However, few others have studied the circulating responses of OC-compounds that can be both dietary-derived and endogenously produced (101). As such, the relative contribution between dietary

intake and endogenous production of circulating OC-compounds in the postprandial period remains a complex relationship that is not well defined in the literature (103,104).

Additionally, many OC-compounds are not solely involved in OCM, and participate in numerous other biochemical reactions. For example, choline is the precursor for betaine in OCM (Figure 1.1) however, through other pathways, choline also modulates important cellular functions such as cell membrane structural integrity and signalling, lipid transport and metabolism, and neurotransmission (37,47). Choline is also required for synthesis of phosphatidylcholine, a major lipid membrane component, and is a precursor of the neurotransmitter acetylcholine (25,47). Similarly, the B-vitamins involved in OCM also act as cofactors in many other metabolic pathways (38), with particularly extensive roles in neurocognitive function (38,53). For example, vitamins B2, B6, B12 and folate, are required for energy production pathways in the brain and the synthesis of the neurotransmitters noradrenaline and dopamine (12,53); while vitamin B6 also has a role in the neurotransmitter binding of glycine, glutamate and glutamic acid (12). The interdependent roles of most OC-compounds convey the difficulty in interpreting how acute shifts in circulating concentrations of these compounds might reflect their involvement in OCM. Nevertheless, it is important to explore the mechanisms behind nutrient-intake and OCM in the short-term, since this has been minimally studied despite explanations for altered OCM and poor health outcomes remaining largely unexplained.

1.4.2 Evidence of postprandial OCM responsiveness

Postprandial responses of circulating concentrations of a subset of OC-compounds, namely, Hcy (29,34,36,50,56,101), methionine, DMG, betaine, choline (41,101,105), SAM (105) and SAH (41,105), have been studied. These have largely been in response to the ingestion of isolated nutrients (50,105), fortified meals (29,56) and/or individual food items (41,50). For the most part, circulating concentrations of these compounds have shown to increase after eating (29,34,36,41,50,56,101), however there is conflicting data on the response of Hcy in the postprandial period (29,36,101,102).

Multiple routes for Hcy metabolism exist in OCM (Figure 1.1). Observational data suggests that remethylation of Hcy through either the folate-dependent or BHMT-dependent pathways (6,37) may shift depending on the relative availability of methyl donors (folate or choline) in the diet (21). Whether these shifts translate to the acute setting is yet to be determined, particularly since studies measuring postprandial responses of Hcy to a meal have shown

conflicting outcomes (29,36,101,102). Two of these studies have shown circulating Hcy concentrations increase in response to a meal (29,36), while two others have shown that Hcy concentrations decrease following a meal (101,102). Hcy is metabolised from methionine in OCM (28), so dietary methionine has important regulatory control over Hcy levels (29,36). This has been established through studies of Hcy responses to oral methionine loading, whereby acute high doses of oral methionine raise circulating Hcy levels (34,50) as this acutely overburdens the cells capacity to dispose of Hcy through transsulfuration (29). Where studies have shown postprandial increases in Hcy, these findings were attributed to the methionine content of the test foods or meals (29,50,56). Of these studies, only one has shown this effect in the context of a non-fortified mixed-meal (29). However, the meal used in that study contained a very high amount of dietary protein (91 g; inclusive of animal sources) and therefore methionine (2221 mg) (29). Where studies have used lower, more generalisable, amounts of protein (and therefore methionine) in their test meals (36,102), these have not resulted in postprandial increases in Hcy in healthy adults.

Contrary to the effect that high loads of dietary methionine appear to have on postprandial concentrations of Hcy, meals designed to be high in dietary betaine and choline can lower circulating Hcy acutely (101). As with methionine, the link between orally-administered betaine and acute reductions in Hcy has been previously established (42,100,101) in supplementary studies. Since betaine is a methyl donor for Hcy remethylation (24,94,101,106), and betaine is metabolised from choline (13), the Hcy-lowering effects of dietary betaine and choline in the acute setting have been attributed to likely upregulation of the BHMT-dependent pathway of Hcy remethylation (42,101).

Overall, postprandial studies considering OC-compounds have thus far focussed heavily on the responses of Hcy. In a study that measured the postprandial responses of choline, betaine, methionine and DMG (41) these were in the context of consuming individual foods (egg, beef or fish) so it is unclear how these findings translate to consumption of a meal. Moreover, in the studies showing postprandial changes to circulating Hcy, these have largely been in response to either fortified meals, or meals contrived to be high in a specific nutrients (29,50,56,101). Since nutrients are most often consumed in combination as part of a meal (107), rather than in isolation, it is important to consider how OCM responds in the postprandial period in the context of generalisable mixed-meals.

1.4.3 How meal composition may influence postprandial OC-compound responses

Once digested and absorbed, nutrient-derived compounds appear in the blood stream for use in metabolic pathways (9). However, the concentration of nutrients in the blood stream following a meal can be influenced by the food matrix and the composition of a meal (41,46,77). Few studies have measured postprandial responses of OC-compounds to mixed-meals (29,34,36,101). Yet, this is important since consuming nutrients as part of a mixed-meal is known to elicit different postprandial responses compared to consuming nutrients as supplements, fortified foods or individual foods (29,41,50,108). Moreover, combining nutrients can slow postprandial increases of compounds, such that adding fat, protein or fibre into a carbohydrate-dense meal delays postprandial blood glucose spikes (109,110). In regards to OCM, this has been demonstrated for the Hcy-raising effect of methionine, where studies have shown how ingested methionine causes postprandial rises in circulating Hcy (34,36,50,56) in a manner dependent on meal composition (29,50,56). For example, the Hcy-raising effect of methionine was dampened when methionine was consumed from whole-foods, rather than as free-methionine (29), and the presence of other amino-acids in a meal ameliorated the Hcy-raising effect of fortified methionine (29,56). Moreover, consuming methionine as part of a mixed-meal dampened the postprandial elevations in Hcy compared to ingesting oral free-methionine (50). These studies demonstrate how the effects of methionine on raising Hcy postprandially are dampened when dietary methionine is consumed as part of a meal rather than in isolation as a supplement. Since few studies have researched postprandial responses of other OC-compounds (41) there is little understanding of how consuming mixed-meals might influence other OC-compounds postprandially.

1.4.4 Postprandial OCM and older adults

Cross-sectional observations show altered OCM, as measured through hHcy, appears to largely affect the aged (6,51); however, test subjects in most studies of postprandial OC-compound responses appear to be younger adults. The studies discussed above were performed in healthy adults with mean ages ranging from 25 to 45 years (29,34,41,50,56,101,102), with one exception (mean age of 65 years) in a group of patients with major depressive melancholia (36). It appears that no study of postprandial OC-compound responses has compared these between healthy young and healthy older adults. Yet, older adults might be more prone to the effects of the food matrix of OC-compound availability. For example, amino acid digestion and absorption is slower for older adults compared to young adults in response to a high-protein

meal (77). Since the amino-acids methionine, serine, cysteine and glycine are heavily involved in OCM (Figure 1.1), age-differences in time-to-appearance of peak concentrations of amino acids in the blood stream (77) may have implications for postprandial responses of OCM. Moreover, older adults may be more prone to subclinical nutrient deficiencies (69,111) due to factors inherent in ageing. As such, older adults comprise a particularly important group to understand how consuming a meal influences the postprandial profile of OC-compounds in circulation.

1.4.5 Nutrient-dense and energy-dense foods and diets

There is a need for nutrient-dense (ND) foods in the diets of older adults (27,69) to cater for the reduced caloric requirements, yet stable, and sometimes increased, micronutrient requirements that occur with ageing (6–8,62). In other words, older adults require more nutrients per calorie of food, with implications for optimal food selection. Moreover, dietary guidelines from the New Zealander Ministry of Health recommend that older adults aim to incorporate more ND foods into their diets (7); the USDA similarly recommends that all age-groups, not just older adults, aim to make ND food choices (112). Despite this, the modern food supply is becoming increasingly energy-dense (ED) and nutrient-poor (113). The term ND can be used to describe foods that are high in beneficial nutrients, such as essential vitamins, minerals and amino-acids, healthy fats and dietary fibre, relative to their energy content (114–116). Conversely, the term ED describes foods that are high energy relative to these beneficial nutrients, which are typically foods that are high in fat, particularly unhealthy fats, highly refined and high in added-sugars and/or sodium (7,117,118). These sorts of foods are increasingly common in modern Western diets (113) and excessive intakes have been associated with chronic disease (7,118). In relation to OC-compounds, observational data suggests circulating betaine concentrations may be reduced by ED diets (39), while methionine-rich diets have been linked to diets high in processed meats and refined grains (46). How these observations translate to the postprandial period, and what the impact of a single meal (ED or ND) is for these and other OC-compounds is not yet known. Given that ND foods are suggested to be a priority in the diets of older adults (7), while nutrient poor and ED foods should be limited (112,119) for all age-groups, these are relevant test meal conditions to consider in postprandial studies involving older adults.

1.5 Conclusions

Whether altered OCM, as measured through circulating Hcy, is inherent in the ageing process or is a cause or consequence of disease remains undetermined (13,20,30). Despite associations between poor nutrient intake and status, abnormal concentrations of biomarkers of OCM (largely hHcy), and age-related disorders, the mechanisms underlying nutrition, OCM regulation and age-related disease remain unclear. This limits interpretation of what comes first - altered OCM, or age-related pathologies that alter OCM. This uncertainty is further confounded by clinical trials of supplemental nutrients involved in OCM, which can lower circulating Hcy but, for the most part, not improve health outcomes. Since underlying mechanisms of altered OCM in ageing remain unexplained by observational and cross-sectional data, and trials of nutritional supplements, this prompts exploration into less-studied avenues of OCM, including postprandial regulation of OCM. This is relevant as many OC-compounds are essential nutrients and their intake and influx occurs firstly in the postprandial period. Moreover, the acute fluxes in OC-compounds might be important if they influence health outcomes, such as those linked to altered OCM (2–6,51), or plasma concentrations of Hcy, in the long-term.

A few trials have considered OCM in the postprandial setting. Most of these trials have measured responses of Hcy, few have used realistic and generalisable feeding scenarios, and none appear to have compared these responses between healthy young and older adults. Exploring the responses of OC-compounds to a ND meal, versus a highly refined ED meal, may be important given that the New Zealand Ministry of Health (7,119) and the USDA (112) recommend eating patterns that aim for nutrient-density and limit energy-density in the diet. A better understanding of whether age-related differences exist for circulating concentrations of OC-compounds could support nutritional interventions that target healthy metabolism in ageing.

Therefore, it would be useful to measure known circulating biomarkers of OCM and other OC-compounds to see how these compare between healthy young and healthy older adults, in the acute setting of consuming mixed-meals. Comparing healthy young to healthy older adults may provide insight as to whether postprandial responses are altered inherently due to age and may lend insight as to whether food-based interventions could optimise circulating concentrations of OC-compounds in older adults.

1.6 Aims

1. To compare postprandial plasma concentrations of compounds involved in OCM between younger and older healthy adults to determine how acute feeding affects plasma compound concentrations of OC-compounds in older adults.

2. To determine the impact of meal type on the acute dynamics of plasma concentrations of OC-compounds in older adults, and to determine whether this differs from younger adult meal responses.

1.7 Hypotheses

We hypothesise that due to age-related factors, including altered intake and status of nutrients and subtle digestive and metabolic alterations inherent in ageing, older adults will have a different response of plasma OC-compound concentrations to feeding than younger adults.

For the same reasons, we expect older adults to have a different circulating profile at baseline (for these compounds) compared to the younger adult group. Where older adults have alterations in OCM, we expect this will result in an overall different postprandial profile of all compounds involved in OCM, compared to younger adults.

Due to nutrient-nutrient interactions that exist in the context of mixed-meals and the subtle digestive and metabolic changes inherent in ageing, it is also postulated that for older adults, the response of circulating OC-compound concentrations will be different for the two meal types compared to younger adults.

Chapter 2 Materials and Methods

2.1 Study design

The analyses described in this thesis used samples derived from a randomised postprandial clinical trial with a cross-over design and took place between October 2012 and July 2013 at the Maurice and Agnes Paykel Clinical Research Unit (MAPCRU) at the Liggins Institute, The University of Auckland, Auckland, New Zealand. The study was firstly designed to look at older adult chylomicron and inflammatory responses to low- and high-fat meals in the acute feeding time frame (120). All participants (*n*=30) were their own control and consumed an ED and an ND breakfast meal, on two separate occasions.

2.2 Ethical considerations

All participants of this study gave written informed consent and the study was performed in accordance to guidelines set out in the Declaration of Helsinki. The University of Auckland Human Participants and Ethics Committee (reference no. 8026) approved all procedures involving human subjects. This study was registered prospectively with the Australian New Zealand Clinical Trials Registry at anzctr.org.au (ID: ACTRN12612000515897).

2.3 Participant recruitment

For the original trial, thirty healthy, community-dwelling subjects (*n*=8 young males; *n*=7 young females; *n*=6 older males; *n*=9 older females; Figure 2.1) from the Auckland region were recruited through the university community and local newspaper advertising (120). To be eligible, subjects were required to be between the ages of 20–25 years and 60–75 years, have a BMI between 18 and 30 kg/m^2 and be non-smokers. Additional requirements excluded individuals with a history of metabolic or cardiovascular conditions (including cardiovascular disease, diabetes and thyroid conditions), and/or those on medications that may have interfered with the original study's endpoints (such as statins and non-steroidal anti-inflammatory drugs) (91). One subject was also excluded from participation for a gastrointestinal condition that may have confounded normal gut functioning. The original study protocol aimed to recruit older adults between 70–75 years. Owing to difficulty identifying older subjects in this age bracket who satisfied the eligibility criteria, the older adult age bracket was amended during recruitment (91). Screening, enrolment and allocation of participants to breakfast meals are outlined below (Figure 2.1).

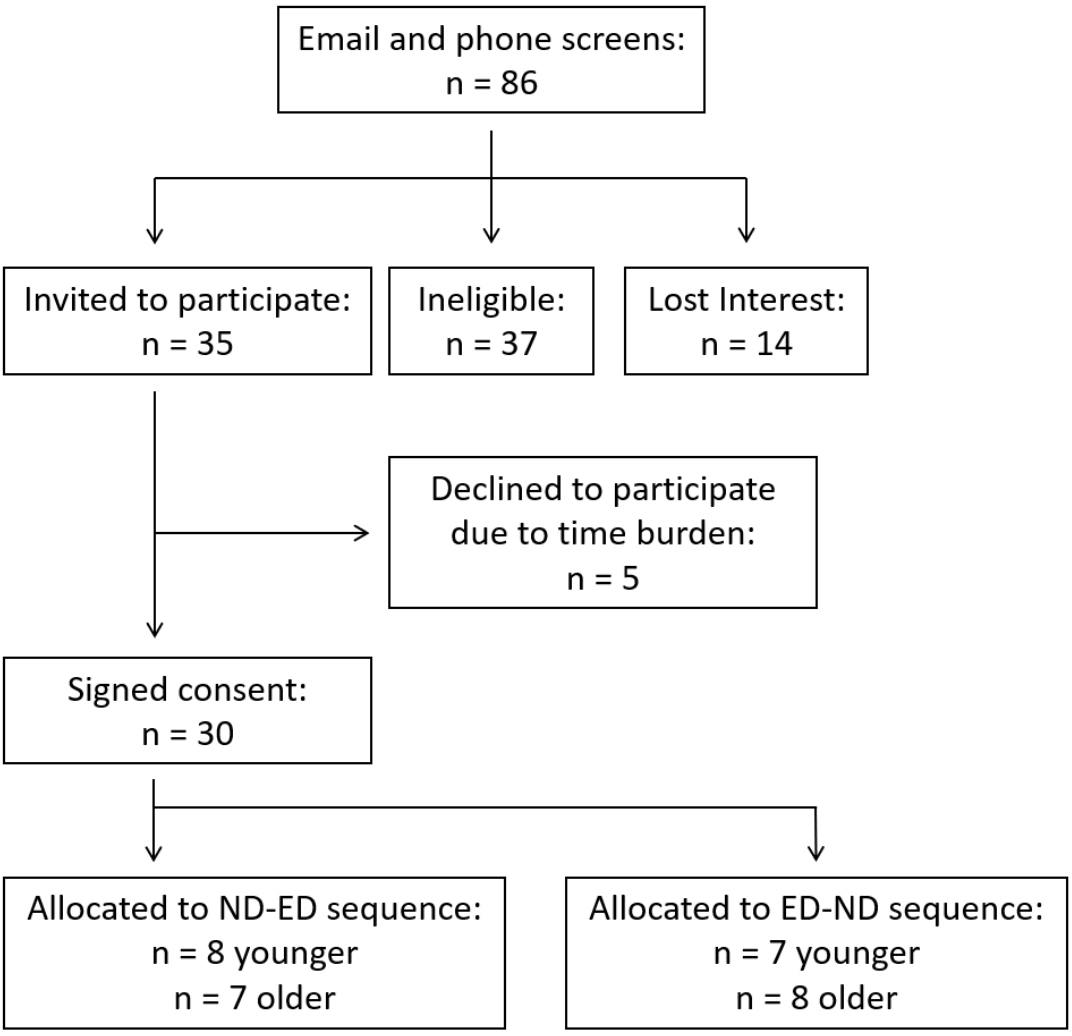

Figure 2.1 Participant screening, enrolment and randomisation to breakfast meal sequence.

Adapted from Milan et al (91). Abbreviations: ND (nutrient-dense meal); ED (energy-dense meal).

2.4 Study procedures

Stratified (by age group) random assignment for the sequence of breakfast meals was carried out using www.random.org (121). Subjects were not blinded to meal type and received this sequence via concealed envelopes before their first visit. Participants consumed each test meal in the morning from a fasted state and consumption of the two meals were separated by a wash-out period of at least 14 days. On the day prior to each visit, participants were asked to refrain from potential confounders to postprandial responses relevant to both the original and present studies outcomes; including the ingestion of high-fat foods, supplements, anti-inflammatory medication and strenuous physical activity. Baseline anthropometric measurements for all participants were collected on arrival to the research unit. Blood samples for ethylenediaminetetraacetic acid (EDTA) plasma were collected prior to test meal ingestion,

then hourly for five hours in the postprandial state. Subjects remained sedentary during this time. Plasma supernatants were collected after centrifuging the blood samples at 1500 x g for 15 minutes; chylomicron-rich fractions were separated by ultracentrifugation (for 10 minutes at 117 000 x g) for use in prior studies with the remaining chylomicron-free plasma samples frozen at -80°C until analysis (77).

2.5 Test meals

2.5.1 Preparation & composition of test meals

The ED meal was purchased from McDonald's restaurants in Auckland. This meal had been used previously as a standard high-fat test meal for study outcomes measuring postprandial inflammatory, endotoxaemic and lipaemic responses (122), which was relevant to primary and secondary outcome studies using these samples of macronutrient metabolism (91,120). The ED meal consisted of two sausage and egg English muffin sandwiches (each comprising one English muffin, egg, sausage patty, and cheese slice) and two hash browns. The ND breakfast meal had a low-fat content and matched the protein and carbohydrate loads of the ED meal (Table 2.1). The ND meal was formulated following the Australian Guide to Healthy Eating (123) and comprised of rolled oats prepared with trim milk, fresh peach, light cottage cheese, multigrain and wholemeal bread (toasted) and reduced-fat, smooth peanut butter. This meal was carefully prepared (items weighed twice) by researchers at the MAPCRU on trial days.

Table 2.1 Macronutrient composition, nutrient density and energy density of test meals.

	Energy		Macronutrients (g)			Total weight (g)	Nutrient density (%)	Energy density (kcal/g)
Meal	**(kJ)**	**(Kcal)**	**Carbohydrate**	**Protein**	**Fat**			
ED	4640	1108	77.4	49.8	62.2	189.4	24.9	2.3
ND	2860	685	77.4	49.8	16.6	143.8	67.7	0.9

Macronutrient composition data was obtained from analysis of meals using Foodworks 8 Professional (Xyris Software PTY LTD, Brisbane, Australia) and the New Zealand Food Composition Database (FOODfiles™ 2016 Version 1). Nutrient-density and energy density scores were determined following the Darmon et al (2005) naturally nutrient rich score method (115). Abbreviations: ED (energy-dense); ND (nutrient-dense).

2.5.2 Nutrient and energy densities of test meals

The ND meal had higher nutrient-density and lower energy-density scores relative to the ED meal (Table 2.1). Methods developed by Darmon et al (115) and presented by Sharma et al (108), were used to calculate energy density and nutrient density scores of test meals in the present study. This method can be used to assess and compare nutrient and energy densities of single foods or meals (115). In short, the method involved calculating energy density and nutrient adequacy scores for each meal in order to determine nutrient density for those meals.

(1) Nutrient adequacy score = $(\Sigma\ (\text{nutrient}_{(\text{amount in test meal})} / \text{DV}_{2000\text{kcal}}) \times 100) / 15$

(2) Energy density (kcal/g) = energy (kcal) of test meal / weight of test meal (g)

(3) Nutrient density score = nutrient adequacy score / energy density score

Nutrient density for test meals was determined by dividing nutrient adequacy score by energy density (Equations (1), (2) & (3)). Nutrient adequacy score was originally determined by calculating the mean of the percentage of Daily Values (DVs) for 16 predetermined nutrients that a food/meal provides (115). These DVs were based on French recommended daily intake targets for those nutrients based on a 2000 kcal diet. Notably, only 15 of the 16 nutrients originally used by Darmon et al (115) were included to calculate nutrient adequacy scores for the test meals, due to limitations of the nutrient database used which does not have data for the vitamin B5 content in some foods (108).

Table 2.2 Micronutrient content of relevant OCM nutrients in each test meal.

Nutrient (unit)	ED breakfast meal	ND breakfast meal
Vitamin B2 (mg)	0.79	1.28
Vitamin B6 (mg)	0.51	0.52
Vitamin B12 (µg)	1.98	1.60
Folate - total (µg)	124.53	298.29
Folate (µg)	124.53	236.76
Folic acid (µg)	0.00	61.53
DFE (µg)	124.53	339.52

Nutrients shown are those relevant to OCM, which could be appropriately analysed using the New Zealand Food Composition Database (FOODfiles™ 2016 Version 1) and Foodworks 8 Professional (Xyris Software PTY LTD, Brisbane, Australia). Values for some one-carbon compounds have not been presented as data for choline, betaine, methionine, serine, glycine and cysteine were not available from the database for all food items analysed. Abbreviations: OCM (one-carbon metabolism); DFE (dietary folate equivalents); ED (energy-dense); ND (nutrient-dense).

2.6 Primary and secondary outcome measures

In this study, we were interested in whether older adults responded differently, in their circulating concentrations of OC-compounds, to young adults after consuming single mixed-meals that represent either high nutrient-density or high energy-density.

Primary outcomes:

1. The primary outcome of interest in this study is whether older adults have a different response in the circulating concentration of Hcy, an overall biomarker of OCM, to young adults following the acute intake of a single meal. We hypothesise that the change in Hcy concentration after eating for healthy older adults will be dampened compared to young adults, due to age-related factors altering nutrient digestion and absorption in older adults.

Secondary outcomes:

2. Our secondary outcome measures of interest are postprandial changes in the circulating concentrations of other compounds involved in OCM (namely, cysteine, cystathionine, SAM, SAH, betaine, choline, DMG, Taurine, Carnitine, glycine, serine and methionine) that differ between younger and older adults or between meal-types.

3. Moreover, we are interested in whether the OC-compound responses are influenced differently between young and older adults in the acute postprandial period in response to two different mixed-meals.

4. We are also interested in whether baseline differences in circulating concentrations of these OC-compounds differ between young and older adults.

2.7 Biochemical analyses

For biochemical analysis of OC-compounds in this study, plasma samples were selected for use based on availability of a full set of samples with sufficient sample left and that had been minimally freeze-thawed. The available samples had been removed of chylomicrons for previous analysis of chylomicrons in the primary outcomes study (120). Chylomicron-rich fraction isolation from plasma samples has been previously described (91). The samples went through one freeze-thaw cycle prior to analysis for use in another study (108). During this

study, samples were kept on ice and at 4°C when in use, in order to preserve concentration and stability of plasma compounds.

2.7.1 Sample preparation and processing

Samples were removed from storage at -80°C and arranged onto five separate plates at 4°C and kept on ice during preparation. Samples were randomised by age, meal type and time for order of analysis. Subsequent sample preparation and processing steps involving use of an Eppendorf robot (EpMotion 5075vt, Germany) were carried out following protocol outlined in more detail by Andraos et al (124). In brief, this involved preparation of blanks, labelled (internal) and unlabelled standards, calibration standards and quality control (QC) samples; then automated solid-phase extraction, protein precipitation of plasma samples, collection of extracted compounds through vacuum extraction, reduction of disulphide bonds, mixing and dilution stages (124). Blanks were positioned at the start of each plate, followed by eight calibration standards (S1-S8) which each contained increasing concentrations of the unlabelled standard (a 1:1 mixture of OC-compounds and amino-acids). The internal standard (also a mixture of labelled OC-compounds and amino-acids mixed at a 1:1 ratio) was added to the calibration standards and to all plasma samples. QC samples were included in each plate to measure compound recoveries and stability across each run (Equations (4) and (5)). In short, three sets of three QC samples were included in each plate, positioned at the start, middle and end of each run. QC1 contained stripped plasma, QC2 contained stripped plasma plus 70µL of the internal standard (S7 concentration) and QC3 stripped plasma plus 70µL internal standard (S8 concentration) (124).

2.7.2 Quantitation of plasma concentrations of compounds

The above sample preparation phases using robotic automation were followed by ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) to quantify 13 OC-compounds and amino acids in the present study (Table 2.3) using the validated analytic method developed at the Liggins Institute (124). Briefly, a Vanquish™ UHPLC system with a 1.7µm Kinetex™ EVO 100Å C18 column 150 x 2.1mm (Phenomenex, CA, USA) was used to conduct reversed-phase UHPLC; a TSQ Quantiva mass spectrometer (Thermo Scientific, USA) was coupled to this for mass spectrometry, using positive ionisation mode for heated electrospray ionisation (124). This method was capable of quantifying 37 compounds involved in a number of interlinked metabolic pathways, including those central to OCM (124). Of these 37 compounds, the 13 compounds selected for analysis in the present

study were the relevant compounds to OCM pathways (Table 2.3). Most of these compounds have been reported in previous studies on OCM, including in the acute postprandial timeframe (in response to single foods) (41) and in longer-term dietary intake studies (21). The integral role of compounds less commonly reported on, such as cysteine, cystathionine, taurine, serine and glycine in these pathways has been outlined in reviews of OCM (6,15,18).

The data acquisition and interpretation software, Xcalibur^{TM} version 4.1 (Thermo Fisher Scientific Inc, USA) was used to generate data from the UHPLC-MS/MS process and subsequently, to identify and quantify peaks corresponding to those 13 OC-compounds of interest in our samples.

Table 2.3 Compound acquisition and success rates from UHPLC-MS/MS.

Compound†	CV average[1]	No. of plates[2]	Comments[3]	% Data acquired
Carnitine	11.3	5/5	Data from all five plates were included in statistical analysis.	98.1
Betaine	4.41			
Choline	9.89			
DMG	7.54			
Cysteine	6.73			
Hcy	16.6			
Serine	9.76			
Glycine	8.91			
Methionine	3.48			
SAM	15.89	4/5	One plate failed both reproducibility (CV=32.8) and recovery (>20%).	78.3
Taurine	38.67	3/5	Two plates did not pass both reproducibility (CV's = 97.4 & 56.7 each) and recovery (>20% each).	59.4
Cystathionine	16.13	2/5	One plate failed reproducibility (CV=32.7) & recovery (>20%), one plate failed just recovery and one plate was unable to be appropriately integrated.	40.0
SAH	56.10	0/5	All plates failed reproducibility CV (all >20%) and/or recovery average (>20%).	0.0

†Compounds as measured in plasma samples.
[1]The average of the reproducibility CVs from all five plates for each compound; includes plates that failed.
[2]Number of plates where data from those plates were included in statistical analysis because they passed quality control checks. Samples were divided evenly between the five plates, such that each plate held 72 samples. For a plate to pass quality control checks, the threshold for both reproducibility CV and recovery were set to 20%. That is, an individual plate 'passed' if the average of all three recoveries within that plate were within 20% of what we expected of them and, the reproducibility CV was ≤ 20%.
[3]CVs presented here are the individual CV's calculated for each plate.
Abbreviations: CV (reproducibility coefficient of variation); DMG (dimethylglycine); Hcy (homocysteine); SAH (s-adenosylhomocysteine); SAM (s-adenosylmethionine); UHPLC-MS/MS (ultra-high-performance liquid chromatography coupled with tandem mass-spectrometry); %Data acquired (percentage of data that passed quality control, to ensure data integrity, and was thereafter included in statistical analyses).

2.7.3 Quality control

Three sets of recoveries (using the three sets of QC samples) were calculated for each compound of each plate as outlined below (Equations (4) i. & ii.). Recovery was deemed

acceptable if the average of three recoveries fell within 20% of expected recovery (100%). To determine reproducibility, the coefficient of variation (CV) was calculated using the mean and standard deviation of the three QC3 samples in each plate for each compound (Equation (5)). Reproducibility CV's less than or equal to 20% were deemed acceptable. Although we sought to quantify all 13 compounds for statistical analyses, we were unable to appropriately integrate SAH, and not all plates passed quality control tests for taurine, SAM and cystathionine (Table 2.3).

(4) i. $$\textbf{Expected recovery} = \frac{(\text{Volume of internal standard (70µL) x [internal standard S7])}}{(\text{Total volume (430µL)})} + [\text{QC1}]$$

ii. $$\textbf{Recovery (\%)} = ([\text{QC2}] / (\text{expected recovery})) \times 100$$

(5) $$\textbf{Reproducibility CV} = (\text{Reproducibility SD} / \text{Reproducibility mean}) \times 100$$

2.8 Statistical analysis

R software, including R Project (R Core Team; version 3.6.1) and RStudio (RStudio Inc., MA, USA, version 1.2.5019), were used to conduct all statistical analyses. Data are expressed as means ± standard error of the mean (SEM) except for baseline variables which are represented as means ± standard deviation (SD). Alpha was set to $p < 0.05$. Figures were created using GraphPad Prism (GraphPad Prism Software, Inc., CA, USA, version 8.2.1).

Welch's two-sample *t*-test was used to determine baseline differences in plasma concentration of OC-compounds between age groups. This method was considered appropriate given some compounds had skewed distributions, and there were unequal numbers of female-to-male participants, overall and within age groups. Baseline differences between females and males were not assessed due to small sample size; however, compared to Student's *t*-test, Welch's *t*-test loses little statistical robustness even when there is equal sample size and assumptions of equal variance between the groups are met (125).

The Shapiro-Wilk normality test was used to determine if data was normally distributed. For data that was not normally distributed, log transformations were used to approximate normality prior to mixed-model statistical analyses. No outliers were identified through use of box and

whisker plots. A generalised linear mixed model was used to determine three-way interactions for meal, time and age. However, as no three-factor interaction existed, the three-factor model was run using only two-factor interactions between time and age, time and meal and meal and age. For repeated measures, each participant and plate were considered random factors (to account for repeated sampling) while age group, meal type and time were considered fixed factors. Sidak-corrected post hoc pairwise comparisons were then used for compounds with two-factor significant interactions. Due to small sample size (n=14 males and n=16 females, unequally distributed within age groups) sex was not included as a factor for analyses. The generalised linear mixed-model could account for missing values in our data set which were missing completely at random or as outlined by excluded plates (Table 2.3). Successful acquisition of at least one plate (out of five plates) was required for statistical analyses to be conducted on each compound.

The ratios of plasma betaine-to-choline, DMG-to-betaine and glycine-to-serine concentrations were included in analyses as indices of their product-precursor metabolism in OCM. Plasma betaine-to-choline and DMG-to-betaine ratios have been used in other studies to indicate the oxidation of choline to betaine for the BHMT-dependent remethylation of Hcy, producing DMG (21,83,105,126). Serine is a precursor of glycine and the glycine-to-serine ratio has been used previously as an indicator of glycine availability, particularly in the context of certain disease states (schizophrenia, prostate cancer) (26,127).

2.8.1 Sample size

The sample size of this study (n=15 older and n=15 young adults) was calculated for the original study outcomes of lipaemic response in older adults and deemed necessary to measure statistical significance of $p<0.05$ and statistical power of 80% for the primary outcome (120). At present, there is minimal literature on postprandial differences of plasma Hcy and other OC-compounds between young and older adults, which would enable us to appropriate whether this sample size had enough statistical power for the measures we were conducting. Postprandial responses of many OC-compounds have not been described, so it is possible that this study was underpowered to detect changes in all OC-compounds after each meal and in each age-group. However, the sample size was likely appropriate to measure postprandial responses of circulating Hcy in both young and older adults after consuming a meal, which have shown to differ (29,36). Healthy young adults have shown to respond to consuming a mixed-meal with a 2.1 ± 0.4 $\mu molL^{-1}$ increase in circulating Hcy concentrations (29).

Conversely, healthy older adults may have a dampened response, with postprandial Hcy concentration decreasing by 0.3 ± 1.5 $\mu molL^{-1}$ after consuming a mixed-meal (36). Assuming a mean difference in fasting versus postprandial concentrations of circulating Hcy in young and older adults to be 2.1 and -0.3 ± 1.5 $\mu molL^{-1}$, respectively, we would expect a sample size of n=7 per group (younger adults versus older adults) would be required for an alpha of 5% and statistical power of 80%.

These mean concentration changes, -0.3 ± 1.5 for older adults and 2.1 ± 0.4 $\mu molL^{-1}$ for young adults, were taken at different timepoints; 2 hours and 7 hours after eating, respectively (29,36). Differences in shorter timeframes have not been reported as thoroughly. Information regarding postprandial Hcy concentrations between young and older adults at other time points is limited, and there appears to be no studies comparing young to old within the same study.

Additionally, plasma Hcy has been shown to respond differently to different types of meals in the acute postprandial period (56), with the effect size of a different meal greater than the effect-size of difference in age outlined above. A previous study showed that a high-protein meal elicits a mean increase of 2.1 ± 1.6 $\mu molL^{-1}$, while a low-protein meal elicits a mean decrease of 1.0 ± 1.3 $\mu molL^{-1}$, in circulating concentrations of Hcy postprandially. To elicit a mean change in plasma Hcy levels of 2.1 and -0.1 ± 1.6 $\mu molL^{-1}$ after eating a high-protein meal versus a low-protein meal, respectively, a sample size of n=5 per group would be required to reach statistical significance at the 5% level of significance with 80% statistical power.

It should be noted that we are comparing meals matched for protein content, as well as carbohydrate content, but differing in fat and micronutrient contents (Tables 2.1 & 2.2). Although the protein in each of our test meals were derived from different food sources (and therefore exact amino-acid content likely differs between the meals), we might expect the effect size of the two study meals on postprandial Hcy concentration to be more similar than this previous study (56). In saying this, we know that foods and meals containing other OC-compounds can also influence circulating concentration of Hcy postprandially (29,41), so we expect the difference in meal composition between our study meals will influence postprandial Hcy concentrations differently.

Chapter 3 Results

3.1 Subject characteristics

The two age groups had similar BMIs (Table 3.1) and other baseline characteristics that have been presented elsewhere, including fasting plasma insulin, glucose, homeostatic assessment of insulin resistance (HOMA-IR) and triacylglyceride (91). No significant differences existed between treatment days for baseline variables. In total, 30 subjects (n=15 young and n=15 older adults) participated in this trial. The older group was on average 45 years older than the young adult group (mean ± SD = 67.3 ± 5.8 and 22.7 ± 1.8 years respectively, $p<0.001$; Table 3.1). The young adult group had seven female and eight male participants while the older adult group had nine female and six male participants (n=16 female and n=14 male combined).

Table 3.1 Baseline characteristics and fasting concentrations of one-carbon compounds.

Subject characteristic	Young (n=15)	Older (n=15)
	Mean ± SD	**Mean ± SD**
Age (years)	22.7 ± 1.8	67.3 ± 5.8***
BMI (kg/m^2)	23.8 ± 3.1	24.4 ± 4.0
Fasting plasma		
Betaine (µM)	31.80 ± 12.85	33.18 ± 9.48
Carnitine (µM)	54.17 ± 16.48	62.08 ± 12.67***
Choline (µM)	9.79 ± 3.24	11.93 ± 2.78***
Cystathionine (nM)	173.88 ± 112.82	370.61 ± 491.42
Cysteine (µM)	47.34 ± 17.91	20.48 ± 15.10***
DMG (µM)	3.12 ± 1.33	3.08 ± 0.77
Glycine (µM)	159.42 ±35.36	204.73 ± 68.11***
Homocysteine (µM)	0.88 ±0.63	0.56 ± 0.55***
Methionine (µM)	24.80 ±4.26	16.4 ± 4.43***
Serine (µM)	88.33 ±19.98	89.24 ± 16.64
Taurine (µM)	18.16 ± 4.34	23.93 ± 7.38**
SAM (nM)	72.72 ± 34.40	79.82 ± 34.80
Betaine:Choline ratio	3.40 ± 1.24	2.89 ± 0.92***
DMG:Betaine ratio	0.11 ± 0.08	0.10 ± 0.04
Glycine:Serine ratio	1.88 ± 0.55	2.30 ± 0.60***

Welch's two sample *t*-test was used to determine significance for baseline characteristics (age and BMI) and differences in fasting plasma concentration of compounds between the age groups. Significant difference for older adults compared to young adults is indicated by **p<0.01 and ***p< 0.001. Values represent means ± SD. Abbreviations: BMI (body mass index); DMG (dimethylglycine); SAM (s-adenosylmethionine).

3.2 Fasting plasma OC-compound concentrations

Older adults had greater fasting plasma concentrations of carnitine, choline, glycine, taurine and glycine-to-serine ratio compared to young adults ($p<0.001$ for all except taurine where $p=0.002$; Table 3.1 & Figure 3.1). In contrast, older adults had lower fasting plasma concentrations of cysteine, Hcy, methionine and betaine-to-choline ratio compared to young adults ($p<0.001$ for all).

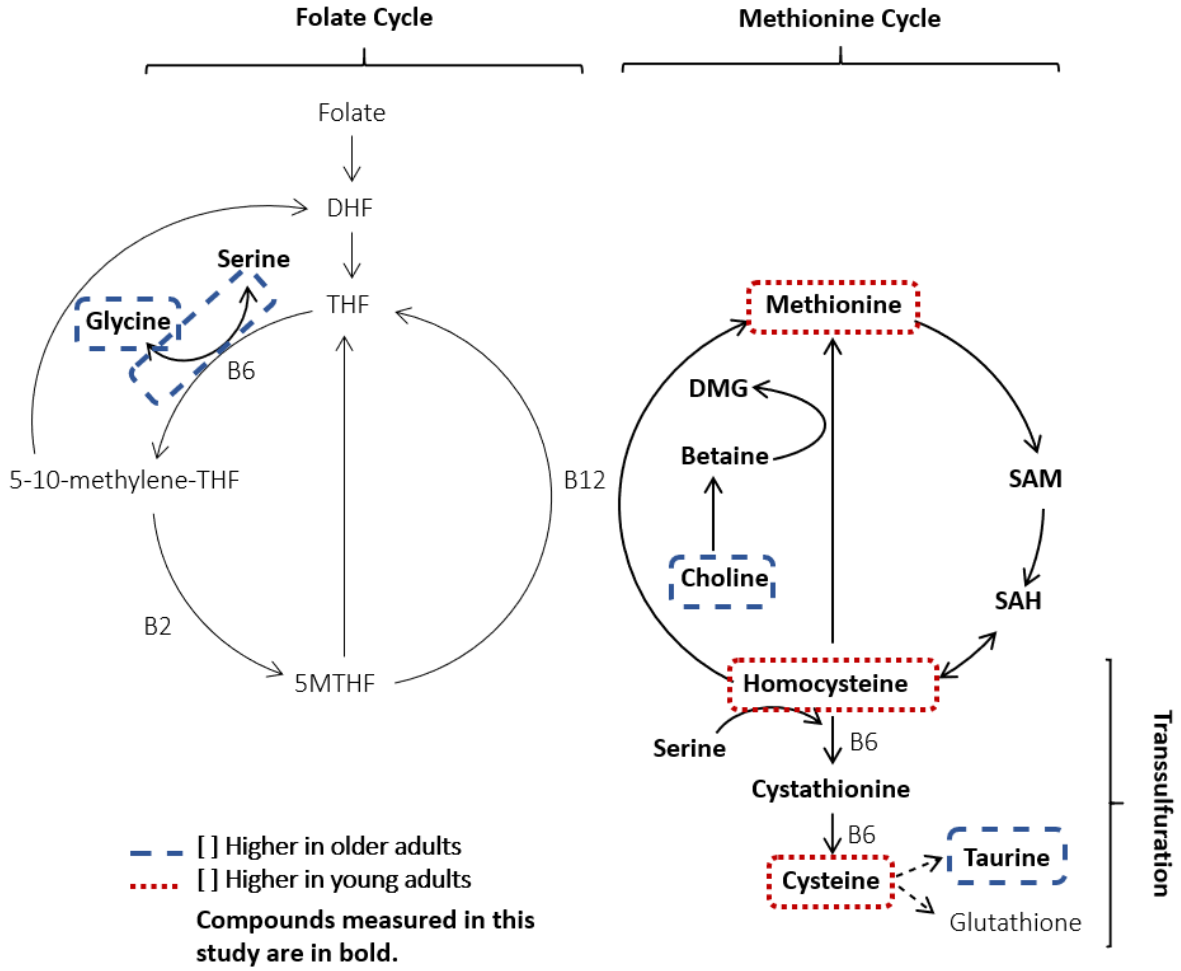

Figure 3.1 OCM pathways with baseline differences between young and older adults.

Differences ($p<0.05$) in fasting plasma concentration of one-carbon compounds between the age groups, where blue dashed lines represent higher fasting concentrations for older adults compared to younger adults and red dotted lines represent higher fasting concentrations in younger adults compared to older adults. Abbreviations: DHF (dihydrofolate); DMG (dimethylglycine); OCM (one-carbon metabolism); SAH (s-adenosylhomocysteine); SAM (s-adenosylmethionine); THF (tetrahydrofolate); 5-methyl-tetrahydrafolate (5MTHF).

3.3 Postprandial plasma OC-compound responses

The change in plasma concentrations of compounds after eating were not simultaneously dependent on both age and meal type for any compound (age x meal x time interaction $p>0.05$ for all compounds). However, plasma concentrations of compounds did show two-factor interactions between meal and time, age and time and age and meal. These two-factor interactions are firstly presented using Figure 3.2 as a visual overview showing which compounds measured in this study displayed two-factor interactions.

The pairwise comparison for the age x time and meal x time interactions are then discussed using time-series figures. For completeness, time-series graphs display three-factor information of age, meal type and time (Figures 3.3, 3.4, 3.5 & 3.6). These figures are grouped based on compounds that are involved in the same pathways within OCM; namely the folate cycle, methionine cycle, transsulfuration pathway and BHMT-dependent pathway of Hcy remethylation.

For completeness, meal x age interactions are symbolised on the time-series figures however, these are re-presented and discussed later using column graphs (Figure 3.7). The column graphs allow the average concentration across all timepoints to be pooled and clearly visualised for the meal x age interactions.

3.3.1 An overview of the two-factor interactions

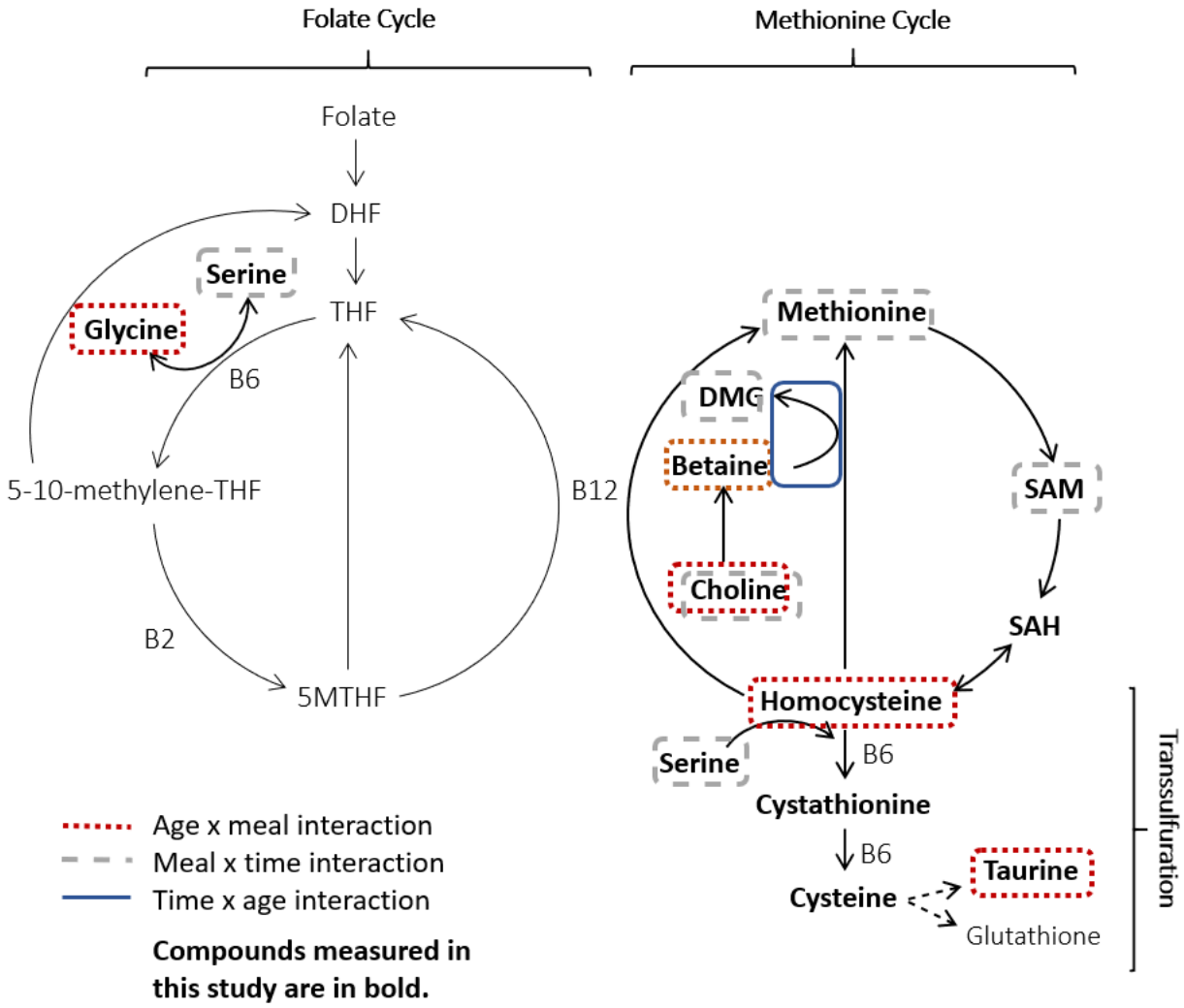

Figure 3.2 OCM pathways showing compounds that had two-factor interactions between age, meal and time.

A visual depiction of the OCM pathways highlighting the OC-compounds that had significant two-factor interaction(s) between age and meal type, meal type and time and/or age and time. Compounds in bold are those measured in the present study, compounds in grey were not measured in this study. Abbreviations: DHF (dihydrofolate); DMG (dimethylglycine); OCM (one-carbon metabolism); SAM (s-adenosylmethionine); SAH (s-adenosylhomocysteine); THF (tetrahydrofolate); 5-methyl-tetrahydrafolate (5MTHF).

3.3.2 Folate cycle meal x time interactions

A significant interaction between meal type and time existed for serine (interaction meal x time p=0.038, Figure 3.3 A). Plasma concentrations of serine from baseline increased later for the ED meal (p<0.001 at 4 hours from baseline) compared to the ND meal (p=0.004 at 2 hours from baseline). There was no effect of age or meal type on the postprandial concentrations of glycine or the glycine-to-serine ratio (p>0.05 for age x time and meal x time interactions; Figure 3.3 B & C).

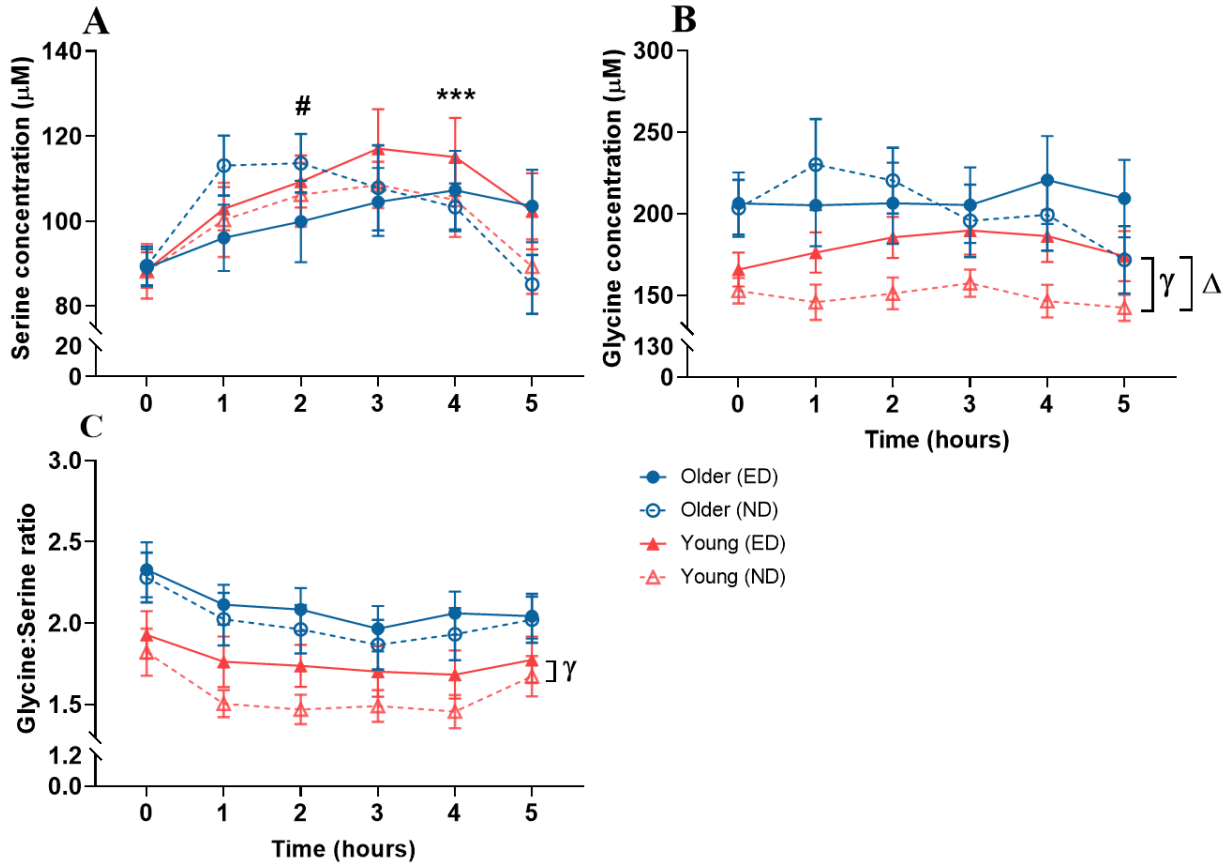

Figure 3.3 Postprandial plasma response of one-carbon compounds involved in the folate cycle to mixed meals in young and older adults.

Postprandial plasma response of serine (A) and glycine (B) concentrations, and glycine-to-serine ratio (C) to mixed meals in young and older adults. Data presented as means ± SEM. Significance indicated by ***p<0.001 for the ED meal and # p< 0.05 for the ND meal for change in compound concentration from baseline; Δ p<0.05 for a difference, in mean concentration of all time points pooled, between age groups for the ND meal; and γ p<0.001 for a difference, in mean concentration of all time points pooled, between meal types for younger adults. Abbreviations: ED (energy-dense); ND (nutrient-dense).

3.3.3 Methionine cycle meal x time interactions

There were no differences between the age groups for the effect of time on postprandial compound concentrations of Hcy, methionine, SAM or carnitine (age x time interaction p>0.05 each; Figure 3.4 A, B, C & D, respectively).

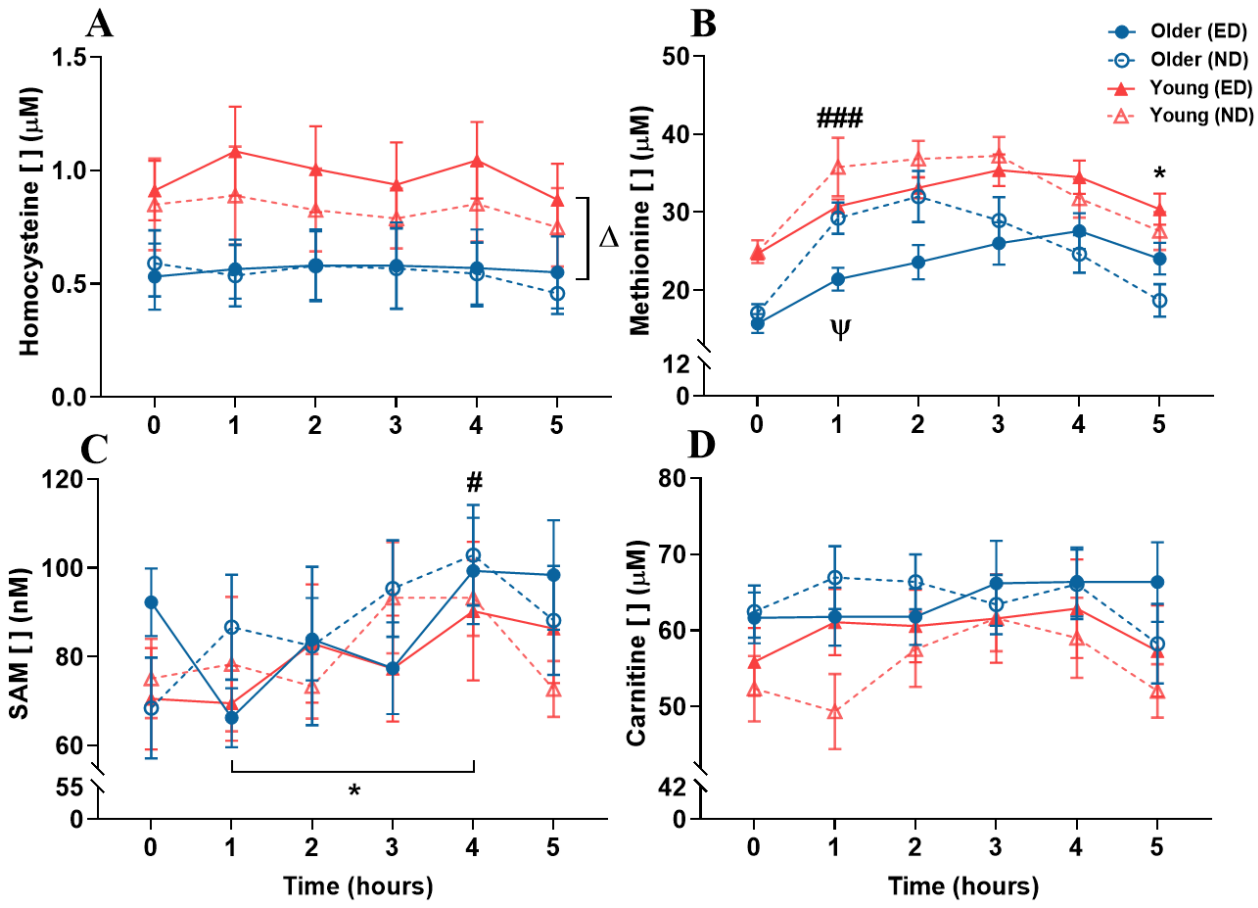

Figure 3.4 Postprandial plasma response of compounds involved in the methionine cycle and carnitine to mixed meals in young and older adults.

Postprandial plasma response of homocysteine (A), methionine (B), SAM (C) (all part of the methionine cycle) and carnitine (D) (which can be produced from SAM) concentrations to mixed meals. Data presented as means ± SEM. Significance is indicated by * p<0.05 for a change in concentration from baseline for the ED meal (or as otherwise indicated by a bar between two timepoints); # p<0.05, and ### p<0.001 for a change in concentration from baseline for the ND meal; Ψ p<0.05 for a difference between ED and ND meals at a single time point; and Δ p<0.05 for a difference in mean concentrations pooled from all time points for that meal type between young and older adults. Abbreviations: ED (energy-dense); ND (nutrient-dense); SAM (s-adenosylmethionine).

Meal type had no effect on postprandial concentration of Hcy (meal x time interaction, $p>0.05$; Figure 3.4 A). Conversely, there were significant interactions between time and meal type for methionine and SAM (interaction meal x time $p<0.001$ and $p=0.022$ respectively; Figure 3.4 B & C). Methionine concentrations increased 1 hour after eating the ND meal ($p<0.001$) and were higher for the ND meal compared to the ED meal (mean ± SD: 32.62 ± 11.95 vs 26.08 ± 6.63 μM) at this time point ($p=0.037$ at 1 hour). In contrast, methionine concentrations increased later after eating the ED meal ($p=0.011$, 5 hours after eating). SAM concentrations increased after eating the ND meal at 4 hours ($p=0.034$) and for the ED meal increased at 4 hours from 1 hour ($p=0.024$). As with Hcy, there was no effect of meal-type on the postprandial concentrations of carnitine (meal x time interaction $p>0.05$; Figure 3.4 D).

3.3.4 BHMT pathway compounds meal x time and age x time interactions

An effect of meal type on postprandial concentrations was seen for both choline and DMG (meal x time interaction $p=0.003$ and $p=0.038$ respectively; Figures 3.5 A & B). Postprandial increases in choline and DMG concentrations were seen for the ED meal only, at 3 hours ($p=0.029$) and 4 hours ($p=0.009$) from baseline for choline (Figure 3.5 A) and 5 hours ($p<0.001$) from baseline for DMG (Figure 3.5 B). There was no effect of meal type on the postprandial concentrations of betaine, the betaine-to-choline ratio and the DMG-to-betaine ratio (meal x time interaction $p>0.05$ each; Figures 3.5 C, D & E, respectively). Of the compounds and ratios analysed, the ratio of DMG-to-betaine was the only one to show an interaction between time and age (age x time interaction $p=0.042$; Figure 3.5 E). Older adults had an increase in the DMG-to-betaine concentrations at 4 and 5 hours after eating ($p=0.002$ for both), which was not apparent for young adults (Figure 3.5 E).

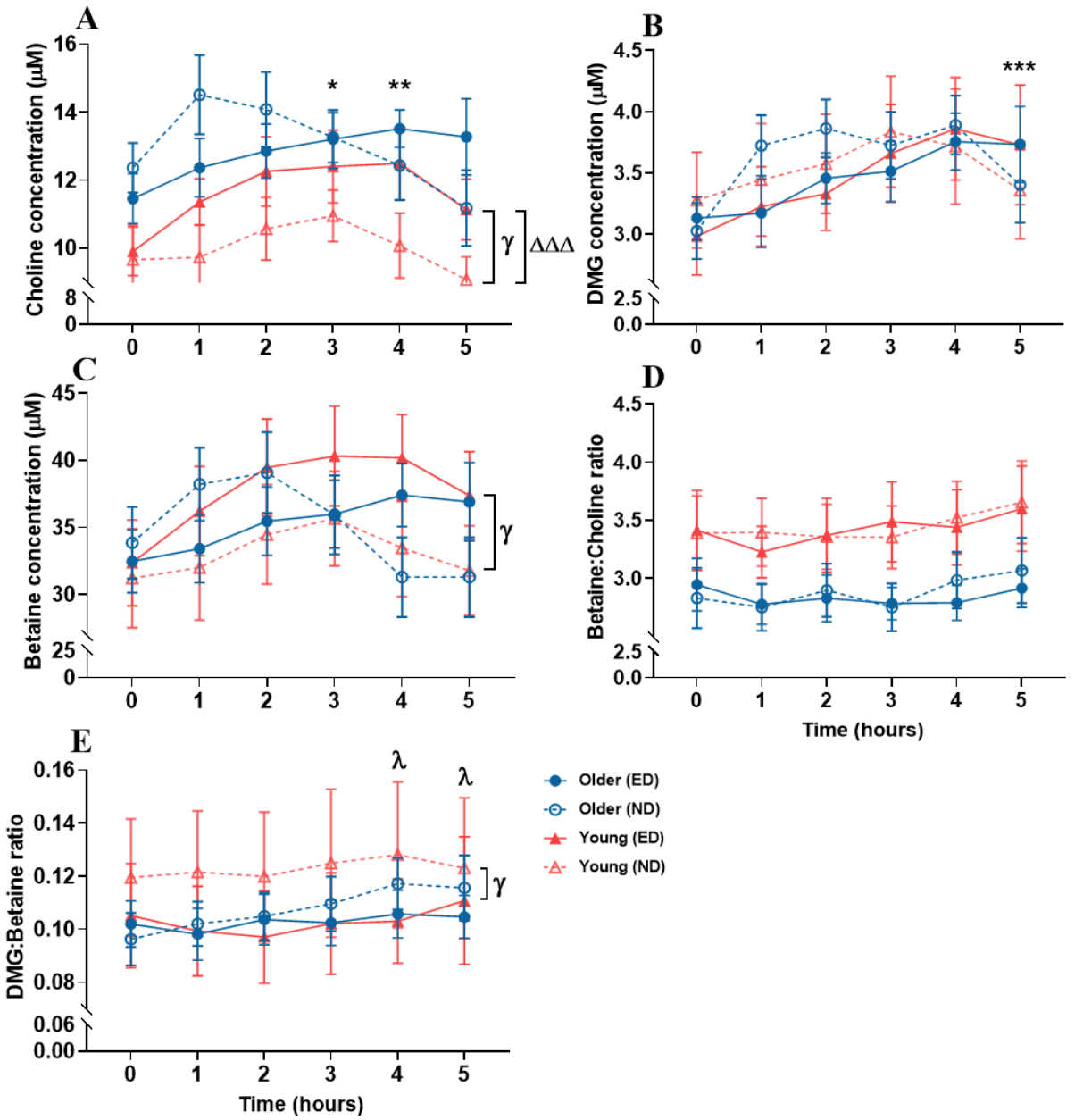

Figure 3.5 Postprandial plasma response of choline compounds in the BHMT-dependent pathway to mixed meals in young and older adults.

Postprandial plasma responses of choline (A), DMG (B), betaine (C) and the ratios of betaine-to-choline (D) and DMG-to-betaine (E) to mixed meals in young and older adults. Data presented as means ± SEM. Significance is indicated by *p<0.05, **p<0.01 and ***p<0.001 for a change in plasma compound concentrations from baseline in the ED meal; λ p<0.01 for a change in compound concentrations from baseline for older adults; ΔΔΔ p<0.001 for a difference, in the mean concentration of all timepoints pooled, between young and older adults in the ND meal; γ p<0.001 for a difference between meal type within young adults. Abbreviations: BHMT (betaine-homocysteine methyltransferase); DMG (dimethylglycine); ED (energy-dense); ND (nutrient-dense).

3.3.5 Transsulfuration pathway compounds

Neither age group nor meal type had an effect on postprandial concentrations of cystathionine, cysteine or taurine ($p>0.05$ for interactions of meal x time and age x time for all compounds; Figure 3.6).

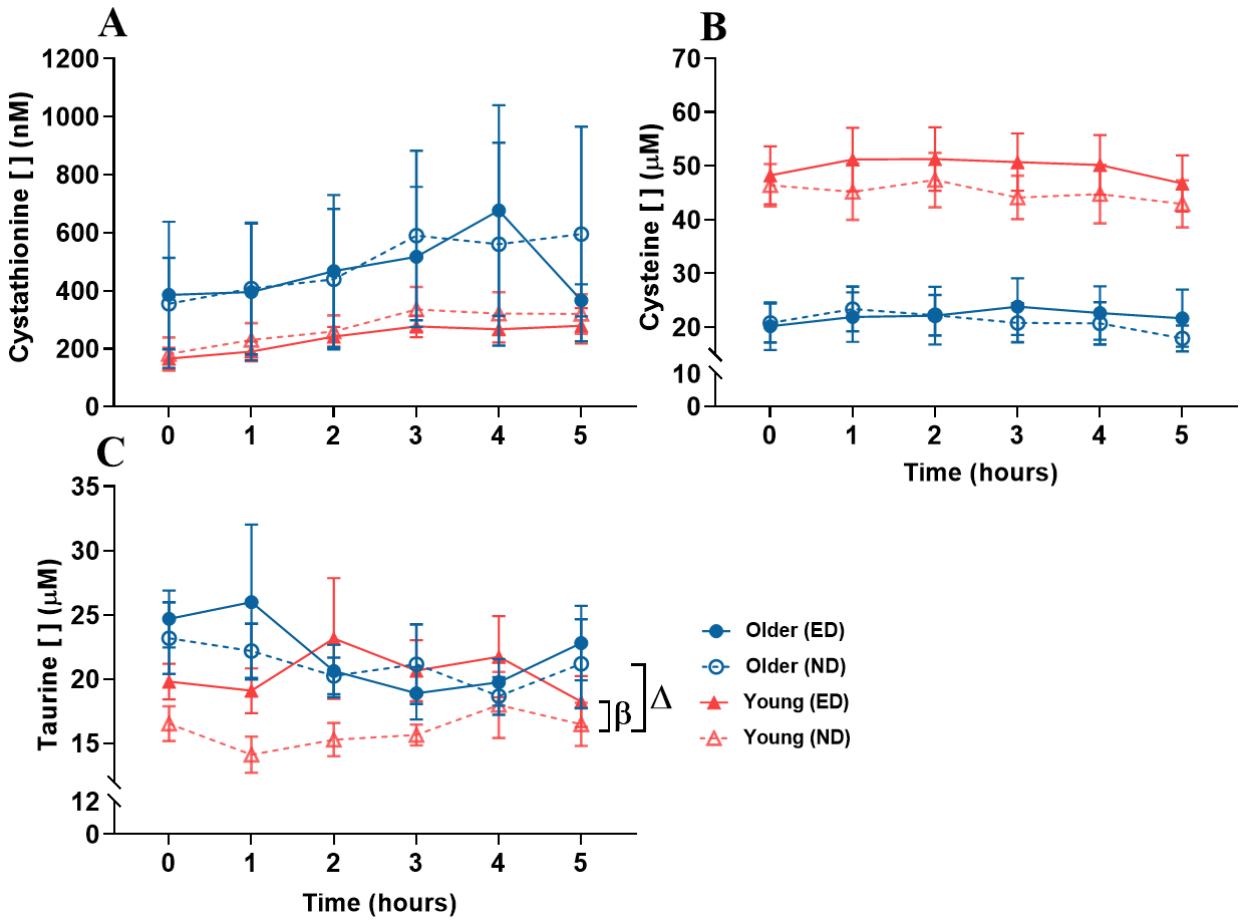

Figure 3.6 Postprandial plasma response of compounds involved in the transsulfuration pathway to mixed meals in young and older adults.

Postprandial plasma responses of cystathionine (A), cysteine (B) and taurine (C) to mixed meals in young and older adults. Data presented as means ± SEM. Significant differences between meal and age are indicated by Δ $p<0.05$ for a difference between age groups for the ND meal; and β $p<0.01$ for a difference between meal type within young adults. Abbreviations: ED (energy-dense); ND (nutrient-dense).

3.3.6 Meal x age interactions

Pairwise comparisons between age and meal type are presented with time pooled from all timepoints and presented in column graphs (Figure 3.7). There were no significant interactions between age group and meal type (age x meal interaction p>0.05 each) for plasma carnitine, cystathionine, cysteine, serine, DMG, SAM, methionine and the betaine-to-choline ratio (data not shown, refer to three-factor time series graphs; Figures 3.3, 3.4, 3.5 & 3.6). In contrast, there were interactions between age group and meal type for Hcy (p=0.034), the DMG-to-betaine ratio, betaine, choline (p<0.001 each), glycine (p=0.001), taurine and the glycine-to-serine ratio (p=0.046 each; meal x age interaction; Figure 3.7).

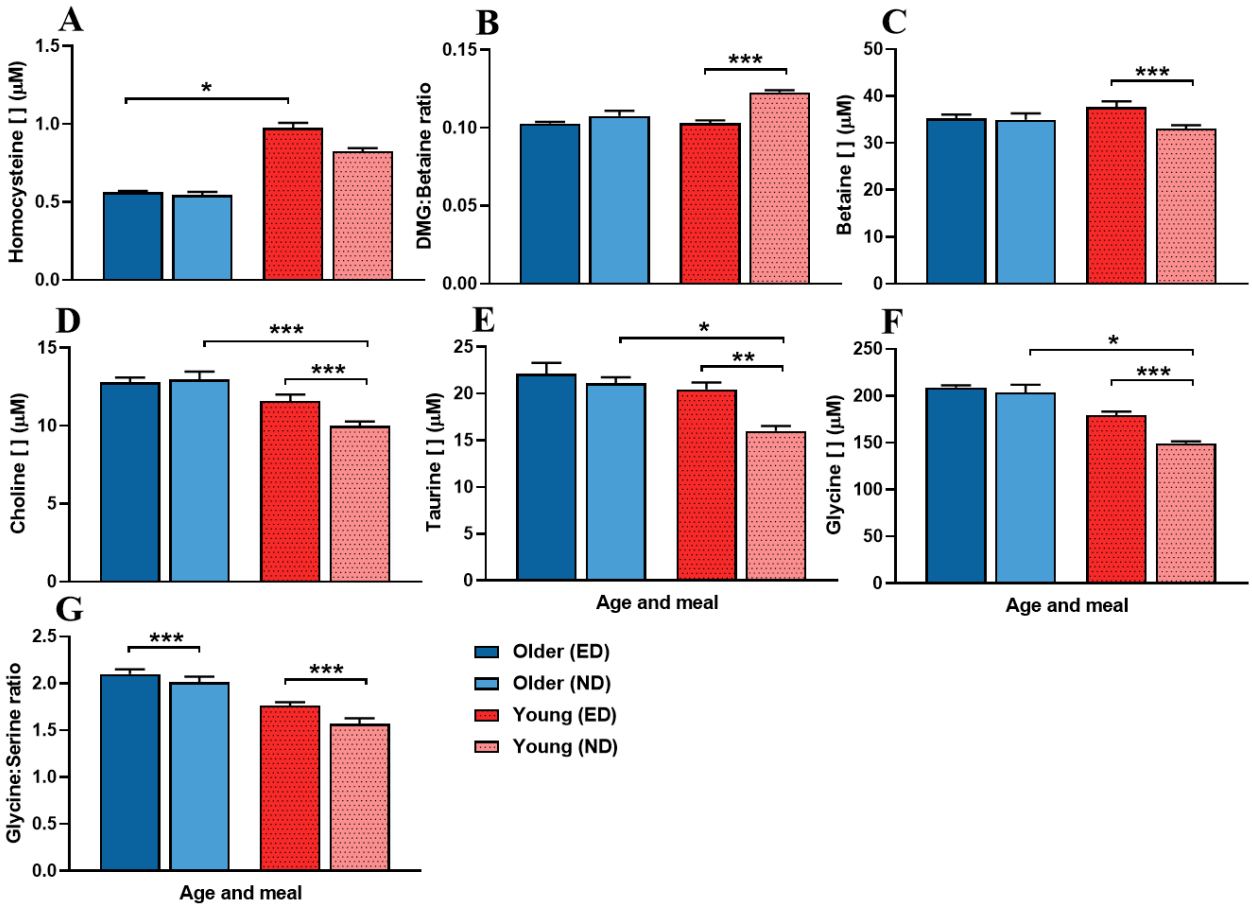

Figure 3.7 Column graphs of OC-compounds with responses differing by meal and age.

Pairwise comparisons of interactions between age and meal type for mean plasma compound concentrations pooled from all time points (including baseline). Data presented as means ± SEM. Significance is indicated by *p< 0.05; **p< 0.001 and ***p< 0.001 for pairwise comparisons between meal types and within the same age group or, between age groups and within the same meal type. Abbreviations: ED (energy-dense); ND (nutrient-dense).

Mean plasma Hcy concentrations were nearly twice as high in young adults compared to older adults for the ED meal ($p=0.016$, Figure 3.7 A). In young adults, the ratio of DMG-to-betaine concentrations was higher for the ND meal compared to the ED meal ($p<0.001$, Figure 3.7 B). Young adults had higher plasma concentrations of betaine, choline, taurine, glycine ($p<0.001$ each) and the glycine-to-serine ratio ($p=0.002$) for the ED meal compared to the ND meal (Figure 3.7 C, D, E, F & G, respectively). Older adults had greater plasma choline, taurine and glycine concentrations than young adults ($p<0.001$, $p=0.037$ and $p=0.044$, respectively) in response to the ND meal (Figure 3.7 D, E & F, respectively). There was no difference between age groups in the ratio of glycine-to-serine; however, for both young ($p<0.001$) and older ($p=0.026$) adults, this ratio was greater in response to the ED meal compared to the ND meal (Figure 3.7 G).

Chapter 4 Discussion

As far as I am are aware, this is the first study to directly compare the acute postprandial responses of circulating OC-compounds between healthy young and healthy older adults. To our knowledge, only a handful of studies have researched plasma Hcy and a few other OC-compounds in the postprandial time period (29,34,36,41,50,56,101,102). Only some of these studies have used realistic (non-fortified, non-supplemental) mixed-meals (29,36,101,102) and few have been in the context of healthy older adults (36). It appears that no trial has directly compared healthy young to healthy older adults and equally, no trial has measured as many compounds as the present study simultaneously. Moreover, this is the first study to compare these responses between a ND and an ED meal.

Overall, younger and older adults had similar time-course responses of OC-compounds to a single mixed-meal. Contrary to what we hypothesised (that the response of all OC-compounds would differ between old and young), only one postprandial response differed between age groups, being the ratio of plasma DMG-to-betaine concentrations. Conversely, meal-dependent postprandial responses were more common; these existed for some, namely choline, DMG, methionine, serine and SAM, but not all, OC-compounds. Interestingly, older adults had similar cumulative concentrations of OC-compounds across all timepoints for the ED and ND meals, whereas young adults had divergent concentrations of choline, betaine, taurine and glycine for the two different meals. As expected, some fasting concentrations of OC-compounds differed between the age groups and importantly, older adults had lower Hcy levels than young adults at baseline. There were four main findings of this study; firstly, that older adults did not have different responses in circulating Hcy, however older adults had lower fasting concentrations of Hcy compared to young adults. Secondly, older adults generally had the same time course response to young adults in circulating concentrations of OC-compounds following the consumption of a mixed-meal. Thirdly, meal effects existed for OC-compounds and were such that the ED meal generally delayed postprandial increases compared to the ND meal. Fourthly, young adults appeared to have more divergent responses to two different meals than older adults.

4.1 Homocysteine concentrations did not respond to acute feeding

Circulating Hcy concentrations did not change postprandially in response to intake of an ED meal or a ND meal. Moreover, neither young nor older adults had changes in Hcy

concentrations after eating a meal. In contrast, studies have previously shown that plasma Hcy concentrations change in response to acute feeding of a meal (29,36,101,102). Some of these studies have shown that plasma Hcy decreases following a meal (101,102), while others have shown Hcy increases following a meal (29,36). Reasons for these conflicting results on Hcy may relate to the composition of our test meals (29,56,101); the overall healthiness of our study group (36); and/or the duration of time considered the postprandial period (29,56). In the present study, nutrient contents of methionine, choline and betaine in our test meals were unknown, protein contents were matched between the meals, subjects (young and old) were generally healthy and the optimal time to measure peak postprandial shifts in plasma Hcy is not yet established. These factors may lend insight as to why no postprandial changes in Hcy concentrations were seen for either age group in response to a meal (29,36,56,101).

4.1.1 Composition of the test meals

It is possible that circulating Hcy did not respond to feeding in our study due to the (unknown) amounts of methionine, betaine and choline in the test meals. Dietary methionine has a regulatory role over Hcy levels (29,36) as it is the sole nutrient precursor to Hcy (28). Studies of oral methionine loading show that this increases circulating Hcy in the acute setting (34,50). In studies of healthy adults that have shown Hcy increases postprandially, these results were attributed to methionine-rich foods or meals (29,50,56). Only one of these studies measured Hcy responses in the context of a non-fortified, non-supplemental mixed-meal (29). This study used high amounts of protein (~90 g) and methionine (2.2 g) in their test meals (29). Conversely, our study used less protein (49.8 g), and therefore likely less methionine; although, the protein used was animal sourced and unlikely to be low in methionine (Table 1.2) (7). Since the present study did not elicit postprandial increases in Hcy, it is possible that higher protein (and methionine) loads in a single meal are required to elicit postprandial increases in Hcy. Nevertheless, since our study meals met 60 - 105 % of the daily recommended protein intakes for older adults (7), the use of protein loads greater than this in a single meal, as used in (29), draws into question the generalisability of such loads, particularly for older people who are known to have reduced energy and protein intakes (9,76).

In a study which showed plasma Hcy concentrations reduced after eating, the results were attributed to the betaine and choline contents of those test meals (101). The meals in that study were contrived to be high in dietary betaine and the betaine precursor, choline (101), as betaine is a methyl donor for Hcy remethylation (24,94,101,106). In our study, we were unable to

measure the betaine and choline contents in the test meals due to limitations of the food composition database. Nevertheless, neither meal was designed to be specifically high in either of these nutrients and we did not see a reduction in postprandial Hcy. It is possible that reductions of plasma Hcy following a meal may relate to high dietary betaine and choline contents in mixed-meals (101).

In healthy adults, a meal high in dietary methionine (29) appears to have the opposite effect on postprandial circulating Hcy levels to that of a meal high in dietary betaine or its precursor choline (101). A meal that contains a variety of nutrients and is not contrived to be particularly high in methionine, betaine or choline, including that which was used in a previous study (102) and the present study, appears to have no effect on postprandial concentrations of Hcy in healthy adults (102). Together, these findings suggest that postprandial plasma Hcy concentrations are maintained within a fasting range in response to mixed-meals containing all three of these nutrients, among other nutrients, in amounts generalisable to common food sources and meals. However, no study has been able to report the nutrient content of all three of these nutrients in their test foods/meals, nor been designed to measure the effect of all three simultaneously on circulating Hcy in the postprandial period. Given that our study was also limited in reporting these nutrients, further research is needed to confirm the physiological control of Hcy in the postprandial period in response to mixed-meals containing all of these nutrients in amounts typically found in standard mixed-meals.

4.1.2 Postprandial Hcy increases may relate to ill-health

In addition to consuming a very high protein meal (29), postprandial increases in Hcy concentration following a mixed-meal might relate to a state of ill-health (36). A study of Hcy responses to a balanced mixed-meal in patients with depression (36) showed that only the patient group had postprandial increases in Hcy, while the healthy controls maintained fasting Hcy levels. In caveat, postprandial plasma increases of Hcy have previously been seen in healthy individuals (29,50,56); however, these were in response to high doses of free-methionine (50), protein fortification (56) and/or high-protein food items (29), rather than realistic mixed-meals. Since all participants of the present study were generally healthy, this may account for Hcy concentrations remaining stable following a meal, and may have contributed to the lack of age-related differences in postprandial responses observed between young and older adults. Our results suggest that, when generally healthy, older adults retain the ability to maintain circulating Hcy levels within a fasting range in response to a meal. However,

this may not be true for older adults with comorbidity, who may have dysregulation of this homeostatic control (36). Additional research comparing healthy older adults to older adults with comorbidity is required to understand if this hypothesis is true, and may be important since the mechanisms underlying the associations between hHcy age-related disease (30) remain unclear.

4.1.3 Adequacy of the five-hour time period

A postprandial time period of five hours was used for this study. Although some studies have captured postprandial increases in Hcy within two hours of consuming a meal (34,36), others have shown it may take as long as seven (29) and nine (56) hours for Hcy to reach peak concentrations following a meal. This indicates data on the appropriate time period to measure postprandial changes in Hcy concentration is conflicting and it is possible a study period longer than five hours may be required to capture peak changes in postprandial Hcy concentrations (29,56). Studies on the postprandial responses of other OC-compounds is sparse, but one study showed choline, betaine, DMG and methionine (41) reached their peak concentrations within one to two hours of eating, while SAH concentrations increased by the end of the six hour study period (41). The five hour time period was considered suitable to measure postprandial shifts in other nutrients, including amino acids involved in OCM (77,120,128). Because few studies have measured postprandial responses of OC-compounds to a meal, the appropriate time frame to capture circulating shifts in all OC-compounds is yet to be fully established. Since we found increases in methionine, choline, DMG, SAM and serine concentrations within five hours of consuming a meal, a five-hour time period may be appropriate to measure postprandial shifts in these compounds in future studies.

4.2 Baseline age-differences

Although the study objectives were to determine postprandial differences for plasma OC-compounds, some baseline age-differences existed. Differences in baseline characteristics between the age groups included higher measures of total cholesterol and low-density lipoprotein for the older adult group (120), as well as age-differences for fasting concentrations of some OC-compounds. Older adults had higher concentrations of carnitine, choline, glycine and taurine, but lower concentrations of cysteine, methionine and Hcy, relative to young adults in the present study. These baseline differences may have been influenced by factors previously shown to influence fasting concentrations of OC-compounds, including circadian variability (21,56,129,130), seasonality of nutrient intake (21) or genetic polymorphisms of the enzymes

metabolising these compounds (34). Although fasting and postprandial concentrations have shown to be interrelated and associated with health outcomes for other compounds, such as fasting and postprandial blood glucose and type two diabetes outcomes (128,131), few studies have measured fasting and postprandial responses of OC-compounds. Little is known as to whether fasting and postprandial concentrations of OC-compounds are interrelated, including whether factors known to affect fasting concentrations may have also influenced postprandial plasma concentrations in the present study.

Importantly, fasting Hcy concentrations in our study were lower in older adults compared to young, which is contrary to suggestions that circulating Hcy increases with increasing age (28,51). The reference range for fasting plasma Hcy is between 5 - 15 μM (30,42,49) and hHcy is generally considered as concentrations greater than 15 μM (13,30,50,51). We observed much lower Hcy concentrations than this for both age groups (0.56 ± 0.55 and 0.88 ± 0.63 μM; mean ± SD; old vs young respectively). This was possibly due to the methods we used to analyse Hcy concentration (124). Although these were based on validated methods (124), and have been peer-reviewed and published, the range detected for Hcy concentrations was much lower than other methods using liquid-chromatography coupled with mass-spectrometry (21,50,56,102). It should also be noted that concentrations of methionine, serine, glycine and taurine in the present study were lower than that which was previously detected using ultra performance liquid chromatography (77). The disparity in Hcy concentrations may relate to the forms of Hcy we measured, which were free and oxidised (bound to cysteine) Hcy (124). Although total Hcy in plasma includes both free and oxidised Hcy, the predominant portion (80-90%) of plasma Hcy exists as protein-bound (30,49). Without further detailed investigations for possible low detection reasons (such as incomplete detection of protein-bound Hcy (30,49)) this discrepancy is difficult to explain. Since the current findings fall outside of established references ranges and previously reported fasting and postprandial values for Hcy (29,34,49,102,132), it is difficult to establish whether subjects in this study had concentrations of Hcy within normal reference ranges at baseline. Nevertheless, when comparing age groups *within* our study, baseline circulating Hcy concentrations were lower for older adults compared to young. This finding does not conclusively lend support to the suggestion that Hcy concentrations may inherently increase with age (28,51).

4.3 Age had little influence over postprandial compound responses

Contrary to what we expected, healthy older and younger adults had similar postprandial responses of OC-compounds after consuming a mixed-meal. Only the ratio of DMG-to-betaine differed postprandially between young and older adults. Age therefore had little influence over postprandial shifts in circuiting concentrations of OC-compounds. This is contrary to our hypothesis that the responses of OC-compounds to feeding would be different for older adults compared to young. Although we know age-differences exist for the postprandial responses of other compounds (77), it is difficult to ascertain why few age-differences existed in the current study, particularly since no previous studies have compared these responses between healthy young and older subjects. A plausible explanation for this may relate to the general healthiness of the older subjects in this study, such that postprandial responses of OC-compounds are not inherently altered with age but likely relate to comorbidities of ageing (92).

4.3.1 Older adults had increased DMG-to-betaine concentrations after eating

The only response that differed between young and older adults was for the ratio of DMG-to-betaine. In older adults, there was increased DMG-to-betaine concentrations at four and five hours after eating. This indicates more DMG and less betaine in plasma postprandially for older adults. This may suggest that, relative to the younger group, older adults upregulate the BHMT pathway after eating, since the DMG-to-betaine ratio suggests flux of betaine through BHMT (21,83,126). However, although the ratio of DMG-to-betaine was increased for older adults, there were no corresponding age differences in the individual compounds of the BHMT pathway; namely, betaine, DMG, Hcy and methionine (20). Because there has been minimal postprandial studies of OCM, it appears that this ratio has previously only been used cross-sectionally (21,83,126). As such, it remains unclear whether acute regulation of the BHMT pathway occurs postprandially, and whether this is reflected by postprandial changes to the DMG-to-betaine ratio. Further research utilising this ratio postprandially is needed to understand these data.

The ratio of DMG-to-betaine may be influenced by basal micronutrient status. One study suggests this ratio is increased when there is a seasonal switch from folate to betaine as the primary methyl donor for Hcy remethylation in OCM, thus increasing DMG concentrations and the DMG-to-betaine ratio (21). A coordinated switch from the folate-dependent, to the BHMT-dependent, pathway of Hcy remethylation can occur when folate and/or vitamin B12 status is poor (6,21,94), indicating the increased DMG-to-betaine ratio in older participants

could relate to low folate and/or B12 status. Although plasma measures showed no indication of folate or other B-vitamin deficiencies in our subjects (108), some measures of B-vitamin status were not assessed, including methylmalonic acid for B12 status; the full extent of B-vitamin status in our subjects was unknown. In a more generalised subset of older adults, where vitamin B12 deficiency may be more common (7,71), the DMG-to-betaine ratio might be further increased postprandially than in this study. However, it is not clear from this study whether this switch (between the methyl donors' folate and betaine) occurred transiently in the postprandial time period (105) and more studies investigating this ratio in postprandially would be needed to draw this conclusion. Moreover, since folate and vitamin B12 status are potential confounders to the flux of choline and betaine through the BHMT-pathway (94), future studies might consider adequate assessment of the status of these nutrients in their participants.

4.3.2 Why few postprandial responses differed by age

One question raised by our results was why such few age-differences existed in the time-course responses of OC-compounds? The similarity in postprandial responses between the age groups might relate to the overall healthiness of the subjects, since it was hypothesised that subtle age-related confounders to nutrient digestion and absorption might contribute to age-differences in OC-compound responses to feeding. Although we know that the processing of other nutrients, including amino-acids (77) and lipids (120) may be altered in ageing, age-associated alterations to nutrient digestion, absorption and metabolism in older adults are typically confounded by comorbidities of ageing (9). It is possible that our cohort of healthy older adults had gastrointestinal structure and functioning that were equally capable of processing (digesting, absorbing and subsequently metabolising) OC-compounds as the younger participants in this study. We cannot be sure of this since controlling for all aspects of digestive and metabolic health would likely have been difficult and infeasible. However, across several parameters of health, including fasting plasma glucose, insulin, triglyceride, HOMA-IR (data presented elsewhere (91)) and BMI, the two age groups were similar, lending support to the relative healthiness of this study group. This hypothesis relates to progressive understanding that malabsorption of nutrients in older adults is a consequence of age-related comorbidities, rather than inherent ageing (92). This conclusion in the context of postprandial OCM requires further investigation, including through comparison of these responses in healthy older adults and older adults with comorbidity.

4.4 The impact of different meals on postprandial concentrations of OC-compounds

Irrespective of age, meal type influenced postprandial responses of choline, DMG, methionine, serine and SAM. Meal effects were most evident for compounds involved in the methionine cycle and BHMT-dependent pathway of Hcy remethylation. Less responsiveness was seen for compounds involved in the transsulfuration pathway and we cannot infer responsiveness of compounds in the folate cycle, which would have required measurement of the folate derivatives (THF, 5MTHF, 5,10-methyleneTHF) and vitamins B12 and B6 for a complete pathway analysis (Figure 1.1). Overall, where meal differences existed for the postprandial responses of OC-compounds, the ED meal tended to delay postprandial increases relative to the ND meal. Reasons for this observation may relate to the relative fat contents of the two meals (77,133), the divergent insulin responses to the two meals (84), and/or the potential contribution of endogenous production pathways for compounds that can be both derived from the diet or endogenously produced (41).

4.4.1 Metabolites of choline and the BHMT-pathway

Irrespective of age, plasma choline and DMG concentrations increased in response to the ED meal. This is consistent with other studies showing that choline and its metabolites increase in response to a meal or individual foods (41,134). Although this could suggest that the conversion of choline through to DMG (via betaine) was occurring in this timeframe (41), there was no corresponding increase in betaine concentrations; this is inconsistent with other findings showing betaine increases alongside circulating increases of circulating choline and DMG (41,134). As such, it is not clear whether the conversion of choline through to DMG was occurring in our study. Moreover, a trial looking at the effect of consuming single food items (egg, beef or fish) on the postprandial responses of choline metabolites found rapid increases in choline and DMG within two hours (41). The responses of choline and DMG in our study of mixed-meals were delayed, increasing at three and five hours respectively after eating. These differences might suggest that consuming nutrients as part of a mixed-meal may delay postprandial increases in choline and DMG concentrations, compared to consuming individual food items. Although we know this is true for other nutrients, for example amino-acid digestion and absorption is slowed by the presence of other macronutrients, such as fat, in a meal (77,133), evidence comparing mixed-meals to individual food items within the same study would be needed to confirm this for choline and its metabolite DMG.

Postprandial increases in DMG and choline concentrations were only observed in response to the ED, and not the ND, meal. Although we were unable to measure choline concentration in our meals, it is reasonable to assume the ED meal likely contained a greater amount of choline than the ND meal. This is because the ED meal contained good sources of dietary choline, including egg and meat, whereas the ND meal contained fewer high-choline foods (27,37,39,41). Postprandial increases in circulating choline concentrations have previously shown to be positively correlated with the choline content of foods (41). This lends support to greater postprandial increases in plasma choline concentrations for the ED meal relative to the ND meal in our study. However, this does not explain why postprandial choline concentrations were unaffected by the ND meal since this meal still contained some dietary sources of choline, such as the dairy products (27,37,39,41). Whether there is a minimum threshold of dietary choline required in mixed-meals to elicit postprandial increases in plasma choline concentrations cannot be determined from our study, in part, due to a lack of nutritional information of choline content of the test meals. Further studies, with known content of nutrients including choline in their test foods, would be required to confirm this.

4.4.2 Compounds of the methionine cycle

An effect of meal type on postprandial concentrations was also seen for methionine and SAM, whereby both compounds increased in plasma after consuming the ND meal at one and four hours, respectively. The timing of these increases were consistent with another study (41) that showed the intake of methionine-containing food items caused postprandial increases in plasma methionine, followed by increases in SAH concentrations (the product of SAM) by the end of the six hour study period. These postprandial increases might indicate the adenylation of methionine to SAM during this timeframe (41). However, studies looking at the mechanism behind the conversion of methionine through to SAH, via SAM, would be required to confirm these observations, including whether plasma concentrations of these compounds correspond to this intracellular metabolism. Moreover, compared to the ND meal, increases in methionine concentrations were delayed, increasing at five hours postprandially, in response to the ED meal. Since amino-acid digestion and absorption can be slowed by the presence of other macronutrients, such as fat, in a mixed-meal (77,133), the greater fat content of the ED meal relative to the ND meal may have slowed amino-acid absorption in our study. This likely contributed to the delayed appearance of methionine, as well as serine, for the ED meal compared to the ND meal.

4.4.3 Compounds of the transsulfuration pathway and folate cycle

No differences in the responses of cystathionine, cysteine, the glycine-to-serine ratio or taurine concentrations were seen between the meals or between the age groups. For cystathionine and taurine, it is possible that these results may be due to a lack of statistical power owing to the large number of samples that were not recovered on these plates (Table 2.3). Serine was the only compound of the transsulfuration pathway that showed a difference in timeseries responses between the two meals. Serine increased sooner, at two and four hours respectively, after eating the ND meal compared to the ED meal. As above for methionine, the delayed response of serine to the ED meal (relative to the ND meal) was likely affected by the higher fat content of the ED meal (77,133). Serine has multiple metabolic fates within OCM (13,29) and is involved in both the transsulfuration and folate cycles (Figure 1.1). Because of this dual role, and since the other transsulfuration compounds were unaltered in our study, to establish how the response of serine in the present study might implicate OCM, metabolites of the folate cycle, which were not measured in this study, would be required.

The methodology used to quantify OC-compounds does not reliably detect 5MTHF (124); this is more appropriately analysed, along with other folate derivates in plasma, as a separate panel. This method was also not specific to B-vitamins (124), including those involved in the folate cycle. Although it might be considered a limitation of the present study that more metabolites of the folate cycle were not measured, there is limited understanding around what postprandial changes of these would actually mean for OCM.

4.4.4 Insulin effects

Insulin may have influenced postprandial concentrations of OC-compounds differently for each meal. Insulin responses to a meal are known to differ depending on the type of food or meal composition, such as higher fat contents leading to increased plasma insulin following the digestion and absorption of a meal (135,136). The composition of the two study meals, which varied greatly by fat content, may have differently affected gastrointestinal responses and release of hormones such as insulin. This appeared to be evident in the present study since postprandial insulin responses tended to be higher after the ED meal than the ND meal (data presented elsewhere) (84). Insulin is the primary anabolic hormone in the body and stimulates the uptake of glucose from the circulation into tissues (85), as well as can influence tissue uptake of some amino acids (137). Thus, circulating insulin is known to influence the circulating concentrations of dietary compounds such as glucose and amino-acids (85,137)

Moreover, intracellularly, insulin has known effects on glucose, lipid and protein metabolisms (137). In the context of OCM, there is some suggestion that intracellular Hcy levels may be modulated by insulin effects downregulating the transsulfuration pathway and thereby increasing intracellular Hcy (56,138). How this translates to circulating Hcy levels, and the effects of insulin on concentrations of other OC-compounds, has not been established (56). Insulin effects on circulating OC-compounds, particularly postprandially and in healthy older adults, have been minimally studied (138) and we are yet to understand how each of the compounds measured in the present study behave postprandially. Further studies researching the relationship between postprandial insulin and OC-compound responses would be needed to understand if insulin influences circulating concentrations of OC-compounds as it does for other nutrients (85,137).

4.4.5 Confounders to circulating concentrations of OC-compounds

When exploring what the responses in our study might mean for OCM processes, it is important to caveat that we measured peripheral circulating concentrations of OC-compounds. However, OCM occurs intracellularly, within various tissues throughout the body (15,22). Although plasma concentrations may be an appropriate marker of intracellular concentrations for some OC-compounds, including plasma DMG as a marker of betaine utilisation in the BHMT pathway (20), we do not know how peripheral circulating concentrations correlate to intracellular concentrations for all OC-compounds. Nevertheless, the objectives of this study were simply to determine whether postprandial concentrations of OC-compounds were dependent on meal type and age. Although minimal age effects were observed, we have shown that OC-compounds, including methionine, serine, SAM, choline and DMG are responsive to consuming a meal and these responses differ by meal composition.

Some of these compounds, including serine (18), methionine (20) and choline (47), can be both exogenously sourced from the diet or endogenously produced. However, the relative contribution of exogenous intake and endogenous production to circulating concentrations of nutrients involved in OCM is not well established. Thus, postprandial studies such as the present, should consider this confounding factor when interpreting postprandial concentrations of OC-compounds. We cannot say for certain, particularly as the nutritional content information for each of these compounds in our study meals was unavailable, whether postprandial plasma increases in these compounds were solely due to exogenous dietary intake of these from the study meals. Further research is needed to understand how circulating

increases in these compounds correspond to dietary intake versus endogenous production in the postprandial setting.

4.5 Young adults had more divergent responses to meals than older adults

The concentrations of choline, glycine and taurine were, on average across all time points, higher for older adults compared to young for the ND meal. However, these compounds were already higher in older adults compared to young at baseline. Likewise, the average concentration of Hcy across all time points was lower in older adults compared to young for the ED meal but, as already mentioned, older adults in this study started out with lower fasting Hcy concentrations. Hence, baseline age differences likely contributed to these interactions between meal type and age. The interactions between age and meal type (age x meal) for each compound were an average across all timepoints, including the baseline data (time = 0 hours). Since these interactions were not specific to any timepoint, and since baseline data was included, baseline age-differences are a potential confounder to these interactions. However, as most of the data included in this interaction (five out of six) were postprandial measures, it could also be argued that postprandial concentrations were having a greater influence over this interaction (age x meal) than baseline data. Compared with older adults, who appeared to have similar OC-compound responses to different meals, young adults had higher average concentrations of betaine, choline, glycine and taurine for the ED meal compared to the ND meal, while the DMG-to-betaine ratio was higher for the ND meal compared with the ED meal. Thus, young adults appeared to have more divergent responses to two different meals than older adults. This is contrary to what we hypothesised, that the response of OC-compounds between the two meals would be different for older adults compared to young. Although baseline age-differences may be confounding these results, this data suggests that the ability for healthy adults to respond differently to two divergent meals, as measured through circulating OC-compound responses, may be dampened with ageing.

This hypothesis, that older adults may have reduced capacity to respond differently to two divergent meals, relates to the concept of ‘metabolic’ or ‘phenotypic’ flexibility (40,139); this refers to the flexibility of metabolic systems to adapt to external challenges, such as a meal, or disruptions to the homeostatic state (40). To my knowledge, there have been no studies to suggest that postprandial regulation of OCM in response to different meals is dampened with ageing, particularly as a result of loss of phenotypic flexibility. In fact, to the best of my knowledge, this is the first study to compare postprandial responses of OC-compounds between

healthy young and older adults. Nevertheless, it has been hypothesised that maintaining flexibility in the ability for metabolic processes to respond to different external challenges, such as meals, may be important for the maintenance of health (40,139); moreover, that the ability to adapt to different dietary challenges is a key feature of metabolic health (140). Importantly, it has been suggested that nutritional interventions might be a successful tool in restoring metabolic flexibility in older adults (139). However, since older adults responded similarly to both meal types in our study, dietary intervention as a tool to manipulate OC-compound responses might not be as effective for healthy older adults as for younger adults. More research is required to understand the 'optimal' postprandial responses of OC-compounds since this has been infrequently studied (29,41,50,56,101).

4.6 Strengths, limitations and future directions

This study had many strengths, but inevitably, some limitations. For example, the cross-over design meant that participants acted as their own controls, thus reducing intragroup confounding covariates more so than a parallel design. This allowed stronger conclusions around postprandial meal differences to be made as this design accounts for variation in fasting concentrations between the two treatment days. Moreover, although these samples were free from chylomicrons (due to separation of these for previous study outcomes (120)) it is unlikely that removing these implicated our study findings, as most compounds of interest to this thesis were water-soluble, rather than lipophilic, compounds. Additional considerations as to strengths and limitations of this study include; the prolonged storage and freeze-thaw cycle of the samples, the uneven sex distribution between the age groups, and the composition of the study meals – which were not matched for all dietary-derived OC-compounds. These are discussed below to lend insight as to the generalisability of our study findings.

4.6.1 Sample storage time and prior use

The samples used in this thesis had been stored for an extended period of time (six years old at the time of analysis) and had been used once prior to our study (108), thereby subjecting them to one freeze-thaw cycle and light exposure. It is possible that these factors may have affected compound stability and concentration, as some amino-acids, including taurine, have shown to be unstable with increasing freeze-thaw cycles, under increased storage time and even when kept on ice (141). Conversely, other OC-compounds measured in this study have shown to be stable in plasma under some of these conditions (25,102,141,142). For example, plasma concentrations of choline are preserved when stored at -80°C for a prolonged period of time

(five years) (142), while choline, betaine and DMG concentrations have shown to be stable through multiple freeze-thaw cycles (25). Moreover, Hcy has shown to be stable in plasma, including through freeze-thaw cycling (143) and if kept at temperatures of 4°C or lower (102), as our samples were when being used. Although measures were taken to ensure compound stability in these samples, including storage at -80°C, minimal handling, and handling on ice and at low temperatures (4°C), it is possible that the stability of some compounds, namely the amino-acids, but not others, such as the metabolites of choline, were affected by the age of the samples and the single freeze-thaw cycle. In caveat, all samples were equally subjected to these measures.

4.6.2 The imbalance of female-to-male participants

There is a level of reporting bias in trials looking at OCM where females are either not included (29,41,56) or are included to a lesser degree than males (34,50,83). Metabolically, males and females differ across a number of factors that may influence postprandial responses; including, postprandial glycaemic responses and postmenopausal hormonal variation (110,144), fasting differences in circulating amino-acids (usually higher in young males) (77,84), and altered lipid profiles between men and women (with men having a higher CVD risk profile) (145). Given these differences, it is important to include both sexes in postprandial studies of OCM, to understand these responses in both females and males. Hence, a strength of our study is the inclusion of both female and male participants.

However, the uneven sex distribution in each age group may have increased the variability of responses in this study. Postmenopausal females may have more variable postprandial responses than premenopausal females (144) due to greater hormonal variation. Therefore, the more female-weighted older group, compared to the more male-weighted younger group, may have impacted the variability of responses within each age-group. Although it is known that postprandial metabolites, like amino-acids, differ according to sex (84), sex-specific variation in postprandial responses of all other OC-compounds has not yet been reported. Moreover, although we included both females and males, the sample size was too small for subgroup analysis by sex. Hence, the study could have been improved by including a greater number of participants as well as an equal number of females-to-males in both age groups.

4.6.3 The study meals

Carbohydrate and protein contents were matched, while total fat, energy and micronutrient contents differed between the two study meals. The meals were not matched for micronutrient content, including nutrients involved in OCM, as controlling for these was not necessary to the primary outcome of the original trial (120). Importantly, as with our study, most OCM postprandial studies have not previously controlled for OCM nutrients, in terms of matching these like for like in each test meal / food item (34,36,41,50,56). This may be because matching for all nutrients of interest in a study such as this would have required matching at least six compounds across different meals, which is likely to result in a contrived combination of foods. However, contrary to the present study, previous studies have been able to report the content of nutrients of interest in their test foods or meals; including, methionine (29,34,36,41,50,56), serine, cysteine (29), choline and betaine (41). This has helped interpret whether postprandial increases in these circulating compounds were likely reflective of nutrient intake or, when postprandial increases were comparably larger than that elicited by other study foods with similar nutrient content, were suggestive of endogenous production (41). Hence, the interpretation of our study findings, including deducing whether circulating responses of OC-compounds were more likely the result of endogenous or exogenous influence (41), may have been improved had we been able to measure all OCM nutrients of interest in our test meals.

Nevertheless, a major benefit of our study meals was their generalisability. Many of the aforementioned studies have been unable to supply mixed-meals reflective of real-world diets, and have often used individual food items (41,50), very high intakes of protein (29,50), and/or supplemental nutrients (29,56), which may be poorly reflective of the usual diet for older adults (9,76). Since consuming OC-compounds as part of a mixed-meal has been shown to influence circulating concentrations differently than consuming nutrients from individual foods, fortified foods or supplements (29,41,50,108), understanding postprandial responses in the context of realistic meals is important for generalisability of study findings. A strength of this study was therefore the real-world applicability of the study meals; the ED meal represented a highly processed and high-fat meal, while the ND meal represented a leaner, less refined, and more ND meal. Importantly, the Ministry of Health (NZ) and USDA recommend we limit and aim for these sorts of food choices respectively (7,112,119).

4.6.4 Generalisability of the study

Aside from the generalisability of our study meals, additional factors to consider when generalising our findings include the lack of postprandial data for which to base effect-size on for all compounds of interest in this study, and whether the healthiness of the older adult group is typical of healthy ageing. There were 13 compounds plus three ratios of compounds analysed in this study. Of these OC-compounds, postprandial descriptions of these have been reported for Hcy (29,34,36,50,56,102), methionine, choline, betaine and DMG (41,101). Because most of these studies have been specific to Hcy (29,34,36,50,56,102), and because Hcy is generally considered an overall biomarker of OCM, with links to poor health outcomes (6), our study was powered to measure postprandial increases in Hcy following a meal in young and old adults. The postprandial responses, in addition to the known effects of meal type or age, are limited for other OC-compounds, so it was difficult to obtain both expected distributions and expected effect sizes for all 13 OC-compounds analysed. Hence, it is possible that some compounds analysed were underpowered to detect differences between age group or meal type where they existed. This could partly account for the lack of age differences in our study, or the large variation that some OC-compound responses, for example SAM, exhibited.

Moreover, since older adults were generally healthy in our study, our findings may not be generalisable to other populations of older adults, including those with age-related disorders associated with altered OCM; namely, cardiovascular disease, cancer, chronic renal failure, depression and cognitive decline (2,6,28,36,51,53,83,99,111). Postprandial responses have been minimally investigated in older adults with these disorders (36), and the mechanisms behind nutrient intake and hHcy in these conditions remains largely unexplained. Since we found no effect of age on the time-course responses of OC-compounds in healthy adults, while another study has shown postprandial Hcy responses might relate to comorbidity in older adults (36); continuing postprandial research in older adult cohorts with comorbidities associated with altered OCM may help to understand the relationship between nutrition, hHcy and age-related disease.

Although there is good cross-sectional data for Hcy (6,28,57,146,147) we do not know the normal ageing distribution for Hcy and the other OC-compounds measured, particularly in the postprandial period. Since this appears to be the first postprandial study comparing OCM in healthy young and older adults, we are unable to ascertain whether age-differences (or more, similarities) are reflective of normal ageing. A strength of this preliminary study is that it adds

to the handful of trials (29,34,36,41,50,101,102) that have researched postprandial responses of OC-compounds in the acute feeding time-frame. Moreover, in contrast to these previous studies measuring the response of just one (29,34,36,50,56,101,102) or a handful (41) of OC-compounds, we were able to simultaneously acquire data on the postprandial concentrations of 12 OC-compounds. Initially, it was thought that measuring a large number of OC-compounds might lend insight as to how OCM as a whole was responding postprandially. However, this proved difficult to determine since the postprandial profiles of all OC-compounds are yet to fully be established. The ability to measure this many compounds within the same pathway simultaneously, may be more beneficial once reference ranges and factors (other than nutrient intake) influencing plasma concentrations are better understood. With further research to confirm what we have found, measuring this number of compounds simultaneously may better inform how OCM pathways as a whole are responding postprandially.

4.6.5 Future directions and implications

Future directions resulting from this study have been outlined throughout the discussion, including additional research required to draw some of the aforementioned conclusions. Briefly, these include whether fasting and postprandial Hcy elevations are a result of ageing or age-related disease (which requires comparison of healthy older adults to less-healthy older adults); consideration for nutrients of the folate cycle and for underlying B-vitamin status; better understanding of product/precursor compound ratios in postprandial contexts; the relative effects of betaine, choline, methionine and other Hcy-regulating nutrients on postprandial Hcy concentrations; the relative contribution of endogenous production and exogenous intake to acute fluctuations in circulating OC-compounds; and, the influence of endogenous responses to different meal compositions (such as insulin secretion) on postprandial concentrations of OC-compounds. Due to the preliminary nature of this study, two key questions have arisen and need to be addressed before the implications of this study can be fully established. Namely;

1. What nutritional recommendations should be made for older adults to support appropriate regulation of OCM and prevent or ameliorate OCM-related disease?

Before this question can be answered and nutritional strategies recommended (such as ED versus ND meals), we first need to understand what ‘optimal’ postprandial responses of OC-compounds are; is it good, bad or neutral that more OC-compounds increased after eating the ED meal, and that the ED meal delayed postprandial increases of some OC-compounds

compared to the ND meal? Understanding this requires establishing reference ranges for postprandial responses of OC-compounds, which could be achieved through a series of studies on postprandial responses of OC-compounds to simple oral nutrient tests, such as oral betaine, choline and methionine loading, and oral glucose and fat tolerance tests. A handful of studies have begun to establish these; including, the responses of circulating betaine to orally administered betaine (39,100), circulating Hcy to orally administered betaine (100) and methionine (34,50), and circulating choline metabolites (choline, betaine, DMG, methionine) to oral glucose and fat tolerance tests (105). To fully determine the reference ranges of these compounds in circulation, studies in a wider cohort of older adults, both healthy and with known impairments of OCM would be required (including cardiovascular and neurocognitive diseases, cancer, polymorphisms such as in the MTFHR gene, and in those with low B-vitamin status). Once these ranges are established, simple food-based trials could be conducted to determine how different foods affect postprandial responses, followed by mixed-meal challenges to establish eating patterns that could optimally support OCM in older adults. Longer-term studies could then be implemented to establish how altered short-term (acute) postprandial responses of OC-compounds implicate health outcomes associated with OCM.

2. Are food-based interventions in healthy older adults, who appeared to lose the ability to respond differently to two different meals, likely to be effective?

The cumulative concentrations of OC-compounds in older adults for the two different meals were similar, whereas young adults had different concentrations of OC-compounds (glycine, taurine, choline and betaine) for the divergent meals. This could suggest a loss of phenotypic flexibility in ageing (139,140). A loss of flexibility or adaptability to different external challenges (in this case, meals) with ageing, could limit the efficacy of nutritional intervention as a tool to optimise circulating nutrient status in older adults. However, the effect of ageing on phenotypic flexibility is still an emerging concept (148) and there is need for further understanding of this in the context of healthy ageing (148) and in a range of metabolic processes such as OCM. Studies such as the simple oral nutrient tests mentioned above, could help establish whether a loss of phenotypic flexibility might be implicated in ageing. Continuing the comparison of older adults to younger adults in studies of OCM would help to determine whether older adults lose the ability to respond fluidly to different nutritional interventions. Although this topic is still emerging, it may have important implications for designing nutritional interventions targeting older adult health.

Overall, potential health implications from the present findings requires additional research before dietary recommendations tailored toward older adults could be confidently made. Nevertheless, this study provides potential directions that future studies might consider in postprandial studies of OCM in older adults.

4.7 Conclusion

In conclusion, this appears to be the first known study to compare the postprandial responses of OC-compounds to two different meals between healthy young and healthy older adults. The ED and ND meals had differential effects on postprandial concentrations of some OC-compounds; namely, choline, DMG, methionine, serine and SAM. These five compounds increased in circulation after consumption of the ED meal, while only serine, methionine and SAM increased after consumption of the ND meal. For compounds that were influenced by both meals, the ED meal tended to delay postprandial increases in circulating concentrations. It is unclear whether these meal effects were attributable to compositional differences between the two meals, including differences in fat content, nutrient density and the unknown nutrient contents of some OC-compounds in each meal. Or, whether endogenous factors, such as insulin responses and endogenous production of OC-compounds, were also influential. Both younger and older adults had similar time-course responses of circulating OC-compounds to a single meal. However, when both meals were considered, as with the interaction between age and meal, older adults appeared to have less-divergent responses of OC-compounds to the ED and ND meals. The mechanisms explaining this remain unclear but may indicate a loss of phenotypic flexibility with ageing. This could indicate that nutritional intervention as a tool to optimise postprandial OCM may be less effective in older adults. However, ‘optimal’ postprandial responses of OC-compounds, including reference ranges for appropriate postprandial shifts in these compounds and how these relate to health outcomes implicating OCM, are yet to be established. Given the associations between hHcy, ageing and age-related disease, the lower Hcy concentrations in older adults compared to young suggests that the results from subjects of the current study (who were generally healthy) may differ from other cohorts of older adults with diseases associated with altered OCM. Additional postprandial research in less-healthy cohorts of older adults (particularly with diseases relating to hHcy) is justified. Longer-term studies would be needed to establish whether these acute postprandial fluctuations of OC-compounds are implicated in long-term changes to OCM and health.

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