Diet composition and biomarkers of one-carbon metabolism and B-vitamin status

Marie Kulseth

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Department of clinical science, Faculty of Medicine
University of Bergen

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Marie Kulseth,
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Summary

Background

One-carbon metabolism plays a central role in cellular energy metabolism, in part via regulation by the fatty acid-responsive nuclear receptor PPARα. Increased plasma concentrations of several one-carbon metabolites have been associated with increased risk of various chronic diseases. Diets high in fat or high in carbohydrates are popular approaches to induce weight loss, where these approaches have reported to yield similar weight loss but different responses in blood lipids. Dietary factors are known to affect one-carbon metabolism, where the regulation of diet composition has not been investigated. More recent research into effects of diet macronutrient composition on markers within one-carbon metabolism is needed, as it may help understand how the macronutrients differentially influence cellular metabolism and associated health effects.

Objective

The objective of this thesis was to determine the response of two isocaloric diets for fat loss that mainly contrast in fat and carbohydrate content on plasma one-carbon metabolites and related B-vitamin markers. We hypothesize greater effects on the plasma outcome metabolites by consuming a diet more concentrated in fat compared to a diet more concentrated in carbohydrates due to increased PPARα-activation by dietary fatty acids.

Materials and methods

Plasma samples of one-carbon metabolites and B-vitamin markers were measured in plasma from thirty-eight men with abdominal obesity during a 12-week randomized controlled trial (FATFUNC) conducted in January to May 2013 in Bergen, Norway. Participants were randomized to consuming a diet low in fat, high in carbohydrate (LFHC, n = 18), or a diet very high in fat and low in carbohydrates (VHFLC, n = 20). Blood samples were available at baseline, week 8, and week 12. The diets were similar in energy (8750 kJ/d) and protein content (17 E%) and differed mainly by carbohydrate and fat content. Body weight loss was on average similar for both groups (-12.2 kg LFHC, -11.8 kg VHFLC). Tendencies in plasma concentrations of outcome metabolites were presented by descriptive statistics and by direct graphic visualization, to assess potential non-linear trends. Between-group differences after 12 weeks were assessed by linear regression adjusted for baseline values. Standardized mean difference (SMD; Cohen's d) was calculated for each metabolite to provide effect estimates.

Results

At the end of study, the VHFLC diet lowered the mean plasma concentrations of methionine (-2.15 µmol/L [-4.29, -0.02]), total homocysteine (-1.05 µmol/L [-2.19, 0.1]), cystathionine (-0.08 µmol/L [-0.15, -0.01]) and raised the mean plasma concentrations of dimethylglycine (DMG) (0.45 µmol/L [-0.04, 0.94]) and serine (9.03 µmol/L [0.08, 17.99]) compared to the LFHC diet. No large between-group effect estimates were observed for the outcome metabolites, although the lower plasma concentrations of cystathionine were on the threshold (SMD = -0.74) of what is considered a large effect. For the other outcome metabolites, we observed large uncertainty in the estimates as evident by the wide 95 % compatibility intervals.

Conclusion

In conclusion, these data indicate that in the context of weight loss, following a VHFLC diet may lead to a small decrease in plasma methionine, total homocysteine and cystathionine, and slightly higher DMG and serine compared to following a LFHC diet. Although the groups differed sharply in fat and carbohydrate content, the observed differences were small, and largely inconclusive, likely due to the limited sample size. Thus, more research is needed to determine the effect of diets differing in carbohydrate and fat on these metabolic pathways, preferably in larger samples as well as outside the weight loss context. Further, the clinical relevance of these differences merit further study, to increase our knowledge regarding the health effects of these dietary approaches to weight loss.

Contents

List of abbreviations

BHMT	Betaine-homocysteine methyltransferase
BMI	Body mass index
CBS	Cystathionine-β-synthase
CGL	Cystathionine-γ-lyase
CI	Compatibility intervals
CVD	Cardiovascular disease
DMG	Dimethylglycine
DMGDH	Dimethylglycine dehydrogenase
E%	Energy %
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GC-MS/MS	Gas chromatography – tandem mass spectrometry
GCS	Glycine cleavage system
GNMT	Glycine N-methyltransferase
Hcy	Homocysteine
HDL	High-density lipoprotein
ICC	Intraclass correlation coefficients
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LFHC	Low fat, high carbohydrate
LDL	Low-density lipoprotein
MMA	Methylmalonic acid
mNAM	1-methylnicotinamide
mTHF	5-methyltetrahydrofolate

MTHF	5,10-methylenetetrahydrofolate
MTHFR	5,10-methylenetetrahydrofolate reductase
MTHFD1	Methylenetetrahydrofolate dehydrogenase 1
MS	Methionine synthase
MSR	Methionine synthase reductase
MUFA	Monounsaturated fatty acid
NAD	Nicotinamide adenine
NAM	Nicotinamide
PA	4-Pyridoxic acid
PC	Phosphatidylcholine
PEMT	Phosphatidylethanolamine methyltransferase
PL	Pyridoxal
PLP	Pyridoxal-5-phosphate
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acids
RCT	Randomized controlled trial
RER	Respiratory exchange ratio
sB12	Serum B12
SD	Standard deviation
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SARDH	Sarcosine dehydrogenase
SFA	Saturated fatty acid
SHMT	Serine hydroxymethyltransferase

SMD	Standardized mean difference
tCys	Total cysteine
tHcy	Total homocysteine
THF	Tetrahydrofolate
VHFLC	Very-high fat, low carbohydrate

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1. Introduction

1.1 One-carbon metabolism

One-carbon metabolism includes a range of complex biosynthetic reactions involving a cycle of one-carbon units. This cycle of one-carbon units is important for epigenetic regulation, amino acid synthesis, and synthesis of new molecules (1). Elevated blood concentrations of several metabolites within the one-carbon metabolism have been reported to increase the risk of several chronic diseases (1). As many components in the one-carbon metabolism are derived from the diet, what you eat may be important in regulating the one-carbon network and might therefore also play a potential role in disease prevention. This section will introduce the processes within one-carbon metabolism, divided into three subsections, namely the methionine-homocysteine cycle and the transsulfuration pathway, the folate cycle, and the choline oxidation pathway.

1.1.1 The methionine-homocysteine cycle and the transsulfuration pathway

Methionine is an essential amino acid obtained from dietary sources such as egg, fish, dairy products, nuts, and sesame seeds (2). Within one-carbon metabolism, methionine is the precursor for the global methyl donor S-adenosylmethionine (SAM) (**Figure 1)** (3). SAM is formed from methionine in a reaction catalyzed by methionine adenosyltransferase (EC 2.5.1.6) and donates its methyl group to different methyl acceptors in reactions catalyzed by different methyltransferases (2). This transfer of methyl groups to methyl acceptors is called *transmethylation*, which is essential for synthesis and modification of various molecules, such as creatine, phosphatidylcholine (PC), neurotransmitters, DNA, RNA, and other proteins (2,4). When SAM donates a methyl group to a target molecule, it forms a more stable bi-product called S-adenosylhomocysteine (SAH). As SAH inhibits further methylation, the ratio of SAM to SAH may be an index of methylation capacity (4,5). SAH will rapidly metabolize to adenosine and homocysteine (Hcy) by the enzyme adenosyl-homocysteinase (EC 3.3.1.1) (3). Hcy is an amino acid derived from all transmethylation reactions of SAM, which further proceeds into the circulation or metabolizes within the cell. Of the various SAM-dependent methyltransferases, synthesis of creatine and PC are believed to be major sources of Hcy production (3).
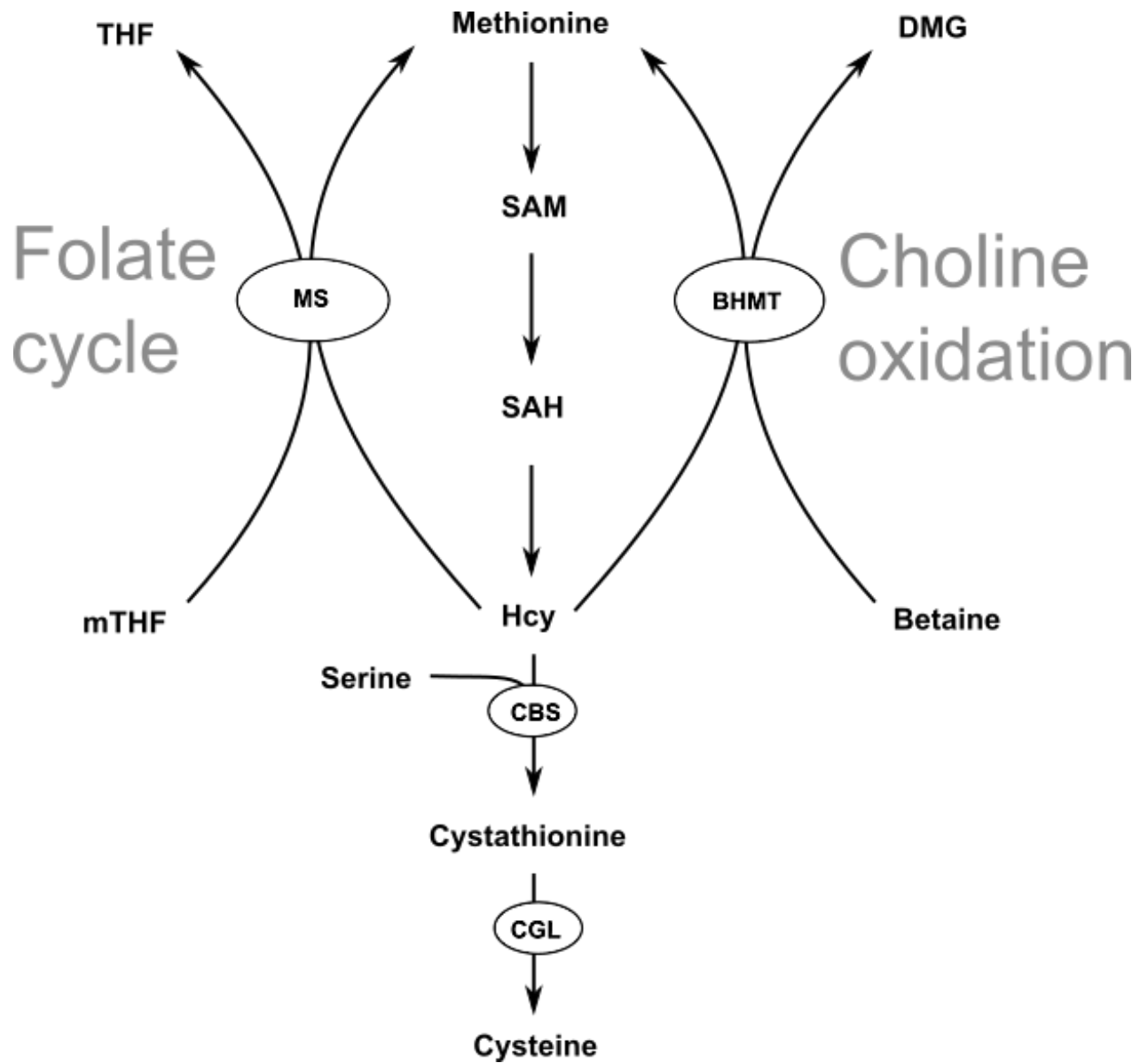
Figure 1. A simplified overview of the methionine-Hcy cycle and the transsulfuration pathway. Hcy is a product of all methylation reactions from SAM derived from methionine. Hcy may enter the transsulfuration pathway for cysteine formation or be remethylated to methionine by accepting a methyl group from the folate cycle or the choline oxidation pathway. BHMT indicates betaine-homocysteine methyltransferase; CBS, cystathionine beta-synthase; CGL, cystathionine γ-lyase; DMG, dimethylglycine; Hcy, homocysteine; mTHF, 5-methyltetrahydrofolate; MS, methionine synthase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; THF, tetrahydrofolate.

Hcy metabolism includes the processes where Hcy is being remethylated back to methionine or catabolized in the transsulfuration pathway, reactions located within the cytosol (6). Hcy can be remethylated through two distinct metabolic pathways by donating a methyl group from either the methyl donor betaine from the choline oxidation pathway, or the methyl donor 5-methyltetrahydrofolate (mTHF) from the folate cycle (5,6). Betaine derived from choline can donate a methyl group to Hcy by the enzyme betaine-homocysteine methyltransferase (BHMT; EC 2.1.1.5), a reaction confined mainly in the liver and kidneys. mTHF from the folate cycle donates a methyl group to Hcy by the cobalamin-dependent enzyme methionine synthase (MS; EC 2.1.1.13), a process occurring in all tissues. Betaine, choline, and folate can be obtained from the diet, leaving dietary intake of these compounds a factor influencing remethylation capacity. Based on the interconnection between these compounds through remethylation of Hcy, disturbances in one of these compounds may lead to changes in the other. However, under normal conditions, remethylation is evenly distributed between the two pathways (7).

Hcy is catabolized to the amino acid cysteine in the transsulfuration pathway. This reaction is performed by two pyridoxal- 5′-phosphate (PLP) dependent enzymes cystathionine β-synthase (CBS; EC 4.2.1.22) and cystathionine γ-lyase (CGL; EC 4.4.1.1), located mainly in the liver and kidneys (8). CBS catalyzes the irreversible reaction where Hcy and serine form the intermediate cystathionine, whereas CGL utilizes cystathionine to generate cysteine and α-ketobutyrate (6). Cysteine is a rate-limiting component of the antioxidant glutathione, an antioxidant with an array of functions within the body (9). Transsulfuration is favored in conditions of inflammation and oxidative stress to increase glutathione concentrations (8), and at adequate methionine levels, due to the reduced activity of remethylation enzymes, combined with increased activity of CBS (6). Besides one-carbon metabolism, cysteine can be obtained through endogenous breakdown of proteins or by consuming most dietary protein (10).

Metabolites within the methionine-Hcy cycle and the transsulfuration pathway are transported in the circulation. Circulating Hcy is primarily bound to sulfur-containing protein compounds, where plasma measurements include all species of circulating Hcy, referred to as total Hcy (tHcy) (3). Elevated plasma tHcy, also called hyperhomocysteinemia, occurs in situations of excess Hcy production, reduced Hcy clearance, or a combination (6,11). Hyperhomocysteinemia has been linked to several diseases, such as cancer (12), neural tube

defects (13), neurological and cognitive conditions (14), insulin resistance (15), osteoporosis (16), and type 2 diabetes (17). Elevated plasma tHcy is also an established marker for increased risk for developing cardiovascular disease (CVD) (18). The role of lowering tHcy in disease prevention has been discussed, as therapeutic approach with folate, vitamin B6, and vitamin B12 supplementation has successfully decreased tHcy levels, however, it has failed to lower the risk of future CVD events in patients with heart disease (19,20). The role of B-vitamin approaches to lower tHcy in long-term disease prevention in questioned, as short-term treatment with folic acid was observed to be efficient for stroke prevention in healthy adults with hypertension (21). The clinical relevance of tHcy as a marker rather than a causative factor remains unclear (22).

The transsulfuration metabolites has been linked to chronic diseases, where increased plasma cystathionine concentrations have been linked to risk factors such as body max index (BMI) and unfavorable lipid profiles (23), as well as increased acute myocardial infarction and mortality risk (24). Increased cysteine concentrations have been associated with fat mass, obesity, and insulin resistance (25,26). The role of dietary intake of cysteine and methionine have also had its attention regarded disease prevention, as restriction of dietary sulfur amino acids has shown to be beneficial by increasing lifespan, reduce adiposity and improve insulin sensitivity in animal models (9). The role of methionine and cysteine restriction in disease prevention have also been suggested to beneficial in humans (27,28).

1.1.2   The folate cycle

The folate cycle refers to the cycle where folate is converted into different forms with tetrahydrofolate (THF) as a backbone. In one-carbon metabolism, the folate cycle is interconnected to Hcy remethylation by using mTHF as a methyl donor, forming methionine and THF (**Figure 2**). The cycle proceeds as THF may accept a methyl group from serine in a reversible reaction catalyzed by the PLP-dependent serine hydroxymethyltransferase (SHMT; EC 2.1.2.1), yielding 5,10-methylenetetrahydrofolate (MTHF) and glycine (29). Alternatively, THF may accept a methyl group derived from formate in a three-step reaction catalyzed by the niacin-dependent enzyme complex methylenetetrahydrofolate dehydrogenase 1 (MTHFD1; EC 6.3.4.3, 3.5.4.9, and 1.5.1.5), which also forms MTHF (1). MTHF may further be utilized for mTHF formation, a reaction catalyzed by the riboflavin- and niacin-dependent enzyme MTHF reductase (MTHFR; EC 1.5.1.20), completing the folate cycle as mTHF donates a methyl group to Hcy and regenerates THF. SAM regulates the activity of

MTHFR by binding at high intracellular concentrations and further inhibiting its activity, which further yields less mTHF for remethylation, while the opposite regulation is observed when the intracellular levels of SAM is low (29). The folate cycle exists in parallel in the mitochondria and cytosol, which are connected by formate as folate metabolites are not able to cross cell membranes. Formate carries one-carbon units derived from serine, glycine, and choline oxidation metabolites from the mitochondria into the cytosolic one-carbon pool (1).
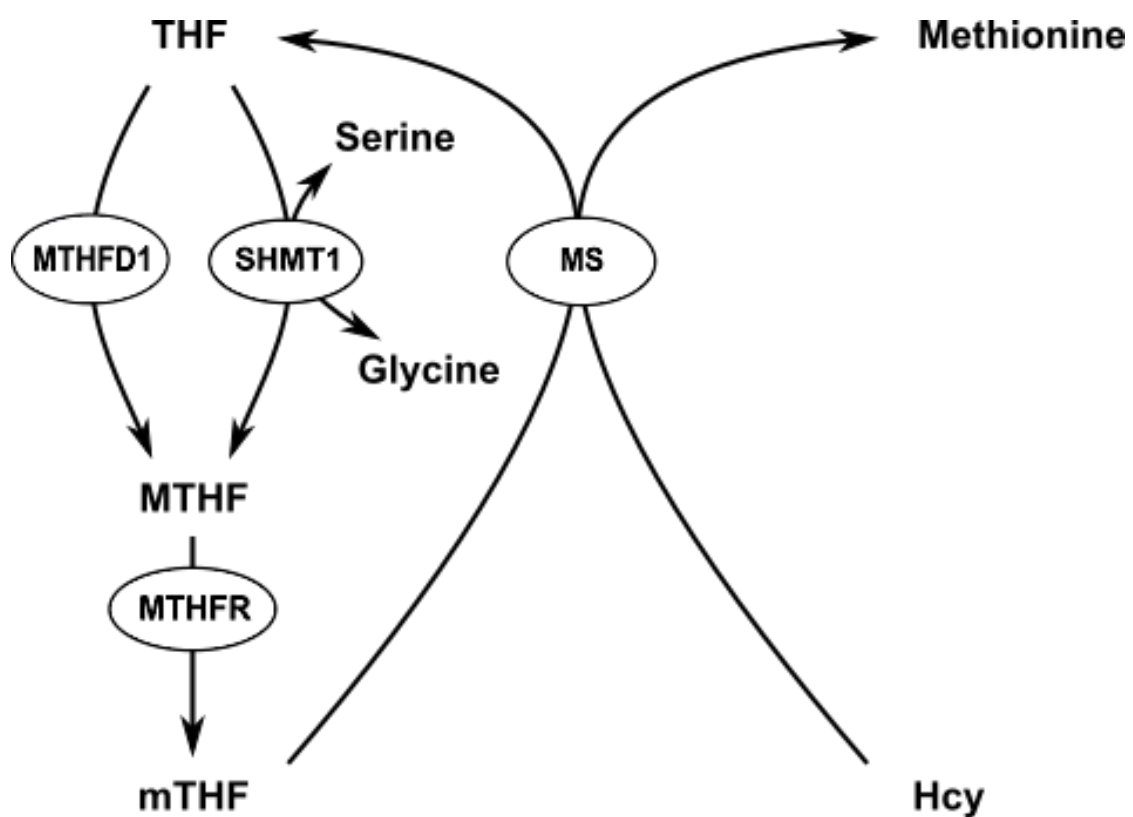
Figure 2. The role of folate in Hcy remethylation. THF is the biologically active form of folate which may accept a methyl group from either formate or serine forming MTHF. MTHF may further yield mTHF, which are utilized for Hcy remethylation forming methionine and THF. Abbreviations; Hcy, homocysteine; THF, tetrahydrofolate; mTHF, 5´methyl tetrahydrofolate; MTHF, 5, 10-methylene tetrahydrofolate; MTHFD1, methylenetetrahydrofolate dehydrogenase 1; SHMT1, serine hydroxymethyltransferase 1.

1.1.3 The choline oxidation pathway

The choline oxidation pathway covers the oxidating steps of choline to betaine, dimethylglycine (DMG), sarcosine, and glycine (**Figure 3**). Choline is a quaternary amine occurring both in water and lipid-soluble forms with various functions including cholinergic neurotransmission, lipid transport and membrane synthesis (30). Choline is synthesized *de novo* in the liver from phosphatidylethanolamine by the SAM-dependent enzyme phosphatidylethanolamine N-methyltransferase (PEMT; EC 2.1.1.17). However, this biosynthetic synthesis is not sufficient to meet choline requirements. The choline content is richest in foods of animal origin, including meat and meat products, eggs and milk, where less concentrated amounts are present in some plant-based food sources as pulses, nuts and beans (31). The main fate of both dietary and endogenous choline is the conversion to PC, accounting for ~95% of the total choline pool (32). For choline to enter the choline oxidation pathway, PC is converted to free choline by phospholipase D (EC 3.1.4.4). Free choline may be oxidized to betaine in a two-step reaction catalyzed by choline dehydrogenase (EC 1.1.99.1) and betaine aldehyde dehydrogenase (EC 1.2.1.8), reactions mainly located in the liver and kidneys (30,31). Betaine is an osmolyte in the kidneys regulating cell volume, which alternatively may diffuse to the cytosolic compartment to act as a methyl donor for Hcy remethylation (30). Betaine requirements can be met through adequate dietary choline intake or by consuming betaine-rich sources such as refined and whole grain wheat, or attributable sources as sugar beets, shellfish, and spinach (33,34). The remethylation is catalyzed by BHMT, which transfers a methyl group to Hcy forming methionine and DMG (30).

The choline oxidation pathway proceeds as DMG diffuses back into the mitochondria to yield sarcosine and glycine. These demethylation reactions are catalyzed by two riboflavin-dependent enzymes, namely DMG dehydrogenase (DMGDH; EC 1.5.8.4) and sarcosine dehydrogenase (SARDH; EC 1.5.8.3). Sarcosine is usually rapidly demethylated to form glycine and MTHF using THF as substrate (35). Glycine has several fates; conversion back to sarcosine by the SAM-dependent enzyme glycine N-methyltransferase (GNMT; EC 2.1.1.20), catabolism through the three-enzyme complex glycine cleavage system (GCS; EC 1.4.4.2, 2.1.2.10 and 1,.8.1.4), or conversion to serine by SHMT. GCS decarboxylate glycine to ammonia, carbon dioxide, the reduced form of nicotinamide adenine dinucleotide with a hydrogen atom (NADH), and a one-carbon group (36). GCS comprises three enzymes, each dependent niacin, vitamin B6, and folate as cofactors (37). The conversion to serine is through the enzyme SHMT. SHMT2, which is located within the mitochondria, appears to be

more active and distributed compared to the cytosolic SHMT1, which is mainly presented in the liver and kidneys (38). Cytosolic SHMT1 has been seen to operate in the direction of serine synthesis, while mitochondrial SHMT2 promotes glycine synthesis (39). Serine can be synthesized from glucose, connecting glucose metabolism to one-carbon metabolism (1). Other roles of glycine include the formation of creatine, purines, glutathione, and heme (1).
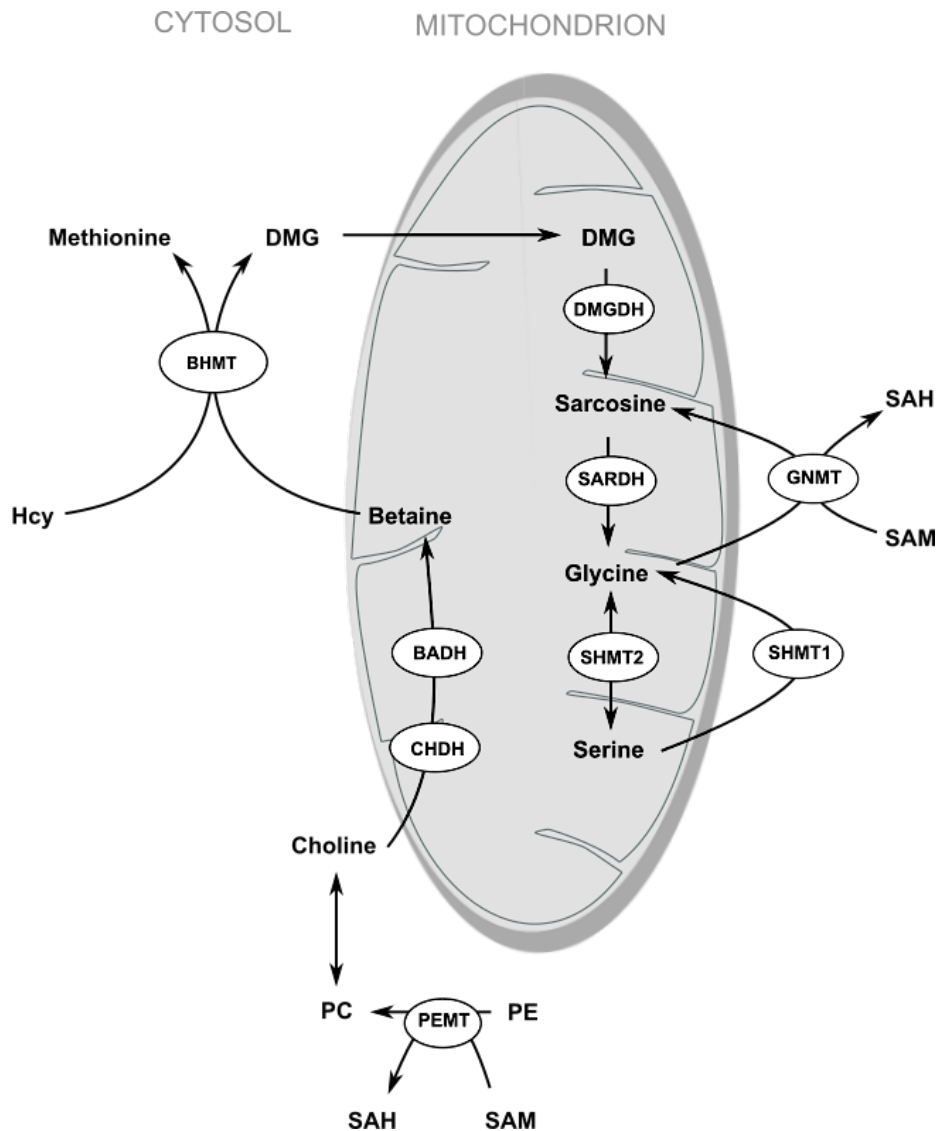
Figure 3. The role of choline in one-carbon metabolism. Endogenous or dietary choline may convert to betaine in a two-step reaction catalyzed by CHDH and BADH. Betaine serves as a methyl donor for Hcy remethylation forming methionine and DMG, where the latter diffuses into the mitochondria to be metabolized to sarcosine and glycine. BADH, betaine aldehyde dehydrogenase; BHMT, betaine homocysteine methyltransferase; CHDH, choline dehydrogenase; DMG, dimethylglycine; DMGDH, dimethylglycine dehydrogenase; GNMT, glycine N-methyltransferase; Hcy, homocysteine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine N-methyltransferase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SARDH; sarcosine dehydrogenase; SHMT, serine hydroxymethyltransferase.

Circulating concentrations of choline oxidation metabolites have been linked to the risk of several diseases. Increased levels of plasma concentrations of choline have been associated with an increased risk of CVD events in patients with established CVD (40). Additionally, both high and low plasma concentrations of betaine have been associated with an increased risk of cardiac events (41). Elevated plasma DMG has been associated with future acute myocardial infarction and increased mortality in patients with coronary heart disease (42), whereas plasma glycine has been associated with decreased risk of acute myocardial infarction in patients with suspected angina pectoris (43). Regarding cancer biology, higher plasma concentrations of choline and betaine may reduce the risk of colorectal cancer (44), where higher plasma sarcosine and glycine concentrations have been associated with reduced prostate cancer risk (45). Both serine and glycine have been at the center of attention as targets in cancer therapeutic interventions (46). Glycine has also been associated with lifestyle factors such as body weight, where low plasma levels have been observed in patients with obesity and type 2 diabetes (47). In addition, plasma glycine concentrations have been observed to increase following improvement in insulin sensitivity (47).

1.2 Role of B-vitamins in one-carbon metabolism

B-vitamins are essential water-soluble compounds required as cofactors in an array of biological processes, including one-carbon metabolism (**Figure 4).** The B-vitamins of importance relative to this thesis will be presented in this chapter.
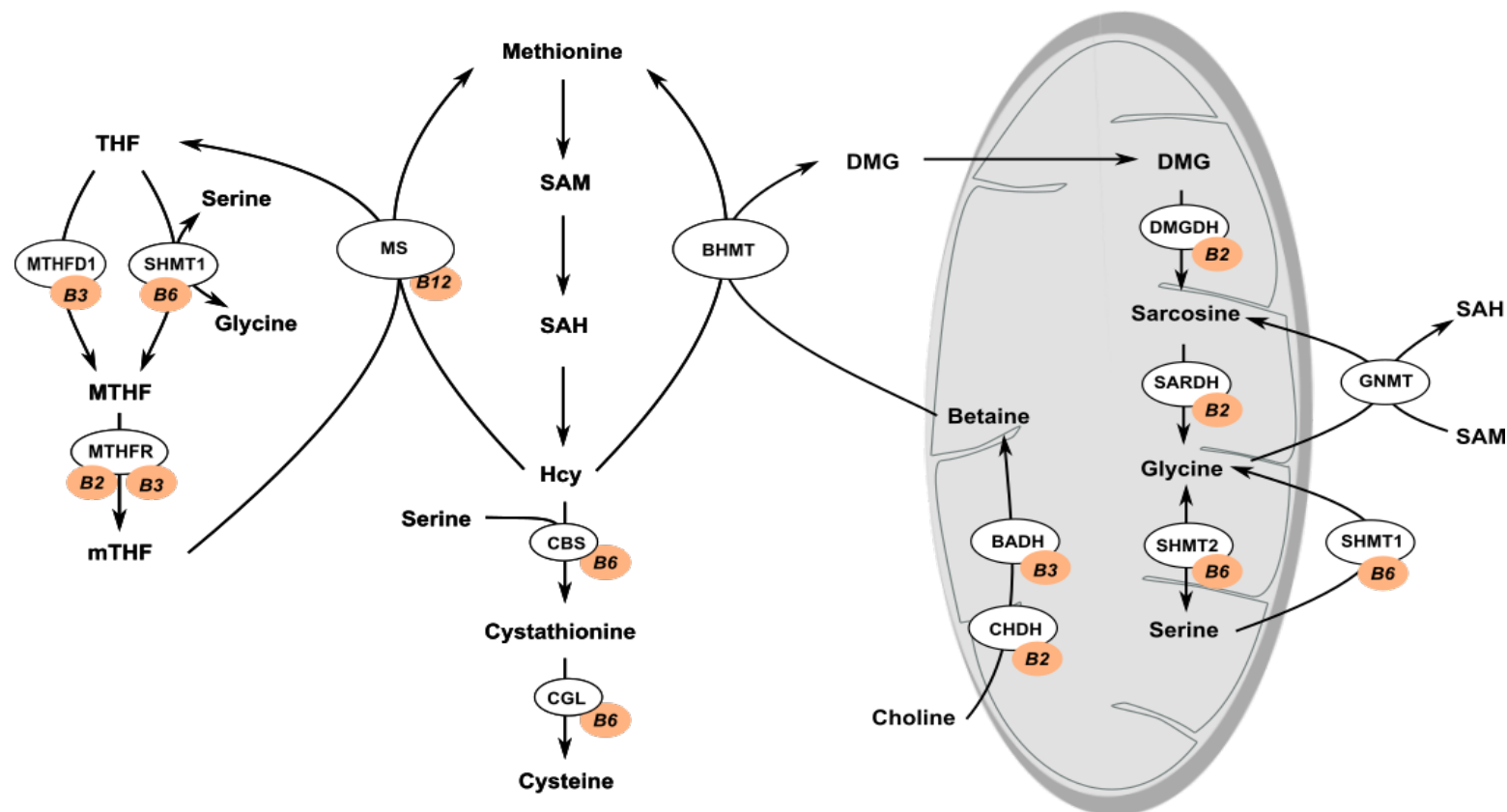
Figure 4. Overview of one-carbon metabolism and related B-vitamins. All B-vitamins utilized as cofactors in one-carbon metabolism are shown in orange circles**.** BADH, betaine aldehyde dehydrogenase; BHMT, betaine homocysteine methyltransferase; CBS, cystathionine beta-synthase; CGL, cystathionine γ-lyase; CHDH, choline dehydrogenase; DMG, dimethylglycine; DMGDH, dimethylglycine dehydrogenase; GNMT, glycine N-methyltransferase; Hcy, homocysteine; MS, methionine synthase; mTHF, 5´methyl tetrahydrofolate; MTHF, 5, 10-methylene tetrahydrofolate; MTHFD1, methylenetetrahydrofolate dehydrogenase 1; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine N-methyltransferase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SARDH; sarcosine dehydrogenase; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate.

1.2.1 Riboflavin

Riboflavin, known as vitamin B2, is a central B-vitamin in human metabolism, mainly through its derivatives flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (48). FMN and FAD are involved in redox-reactions by acting as cofactors for enzymes called flavoproteins (48). Flavoproteins have various functions, including energy metabolism and metabolism of other B-vitamins, such as folate, niacin, vitamin B6, and cobalamin. In one-carbon metabolism, riboflavin functions as a cofactor for MTHFR in the folate cycle and for several enzymes in the choline oxidation pathway (49–51). Riboflavin binds to choline dehydrogenase to synthesize betaine from choline. Further, riboflavin is needed as a cofactor

for DMGDH and SARDH, which forms glycine from DMG in the mitochondria (50). In the methionine-Hcy cycle, riboflavin is a cofactor for the enzyme methionine MS reductase (MSR; EC 1.16.1.8), which reactivates MS after oxidation in the folate-mediated Hcy remethylation (51). Riboflavin is present in food of animal origin like milk, cheese and eggs, and certain vegetables, legumes, fruits and cereals (48). In the blood, riboflavin circulates primarily as free riboflavin bound to albumin and specific immunoglobulins (48). Free riboflavin traverses the cell membrane where it is converted to its coenzyme forms FMN and FAD by flavokinase (EC 2.7.1.26) and FAD synthase (EC 2.7.7.2), both widely distributed in tissues (48). To assess riboflavin status, plasma concentrations of FMN and free riboflavin are considered useful biomarkers, while plasma concentrations of FAD may not reflect vitamin status as its seem to be tightly regulated and relatively unresponsive to changes in riboflavin intake (52).

1.2.2 Niacin

Niacin (vitamin B3) is a generic term for nicotinamide (NAM), nicotinic acid, and nicotinamide riboside (53,54). These compounds are naturally present in food, where NAM is more concentrated in meat and fish products while NA is more concentrated in plant foods. Within the cell, all the three vitamers synthesize nicotinamide adenine dinucleotide (NAD), which is utilized as cofactor to various enzymes primarily in redox reactions in pathways such as glycolysis, Krebs cycle, and fatty acid synthesis (53,54). Dietary tryptophan is also an attributable source of niacin, as tryptophan can be catabolized to NAD through the kynurenine pathway. This pathway requires riboflavin, vitamin B6, and iron as cofactors, and deficiency of these cofactors may therefore impair niacin synthesis from tryptophan (53). The requirement of niacin is given as niacin equivalents, which combine the total dietary intake of niacin metabolites including tryptophan (53). Niacin circulates in plasma mainly as NAM or nicotinic acid bound to plasma proteins. In one-carbon metabolism, NAD assists as a cofactor with riboflavin to the enzymes MTHFR and MSR (49,51). Niacin is also essential for the final conversion of choline to betaine (55). Niacin can be methylated to N1-methyl nicotinamide (mNAM), a SAM-dependent reaction that is the breakdown route of niacin (56). Higher levels of mNAM have been reported in cases with coronary artery disease and have suggested to have anti-inflammatory functions (56).

1.2.3 Vitamin B6

The term vitamin B6 covers six different compounds, including pyridoxine, pyridoxamine, pyridoxal (PL), and their derivatives pyridoxine 5′-phosphate, pyridoxamine 5′-phosphate, and PL 5′phosphate (PLP) (57). PLP is accounted as the active vitamer by serving as a cofactor in various reactions, the majority in amino acid metabolism (57). In one-carbon metabolism, PLP has a catalytic function in the two reactions in the transsulfuration pathway and in the conversion of glycine and serine through SHMT in the folate cycle. Vitamin B6 is connected to riboflavin, because the enzyme pyridoxine-5′phosphate oxidase (EC 1.4.3.5) uses FMN as a cofactor when converting pyridoxine 5′-phosphate and pyridoxamine 5′-phosphate to PLP. As mentioned previously, vitamin B6 is also essential in the synthesis of niacin from tryptophan. All B6-vitamers are widely distributed in foods, where good dietary sources include food of animal origin, beans, nuts, fruits, and vegetables (58). In plasma, vitamin B6 is present as PLP (70-90%), PL (8-30%,) and the excretion metabolite 4-pyridoxic acid (57). PLP is dephosphorylated by alkaline phosphatase (EC 3.1.3.1) to PL, which is the form that traverses cell membranes (57). Plasma PLP has been a standard assessment method for vitamin B6 status. However, plasma PLP is sensitive for factors such as smoking, inflammation, albumin concentrations, and alkaline phosphatase activity (57). Thus, assessment methods other than plasma PLP could be implemented in these cases, such as total B6, including both plasma PL and PLP (57).

1.2.4 Folate

Folate is a term covering all the reduced forms of vitamin B9. Folate is naturally present in foods such as dark green vegetables, mushrooms, legumes, peanuts, and certain fruits, mainly as mTHF (37). The oxidized form of the vitamin is folic acid, which is a synthetic form used in supplements and fortified foods because of its higher bioavailability compared to natural reduced folate. Fortification programs with folic acid are implemented in several countries, where flour, grains, cereals and juices are additional sources of the vitamin. Both dietary folate and folic acid are metabolized to dihydrofolate within cells and further reduced to THF, which is the active form of the vitamin. This reaction is catalyzed by the niacin-dependent enzyme dihydrofolate reductase (EC 1.5.1.3) (59).

Further, THF may be utilized for DNA synthesis, thymidylate synthesis, or purine synthesis. Relative to this thesis, the folate forms are involved in the remethylation of Hcy to methionine, as described in section 1.1.2. The remethylation links folate metabolism to cobalamin, as the

cobalamin-dependent MS is the only enzyme capable of convert mTHF to THF (60). Since folate and cobalamin are interconnected by Hcy-remethylation, plasma folate concentrations are affected by cobalamin status as cobalamin-deficiency causes lower plasma folate concentrations and vice versa (60). Low dietary folate intake has been reported to increase plasma tHcy concentrations, whereas supplementation has been seen to improve hyperhomocysteinemia in both healthy people and individuals with heart disease (37). In the circulation, the majority of folate is found as THF, mTHF and 10-formyl THF bound to proteins or in free form. Serum concentrations of folate have been shown to reflect recent dietary intake of the vitamin and further act as a nutritional biomarker. Additionally, folate concentrations in erythrocytes may function as an assessment of folate status as they attain folate during the erythropoiesis phase, reflecting tissue folate stores the last 2-3 months (37).

1.2.5 Cobalamin

Cobalamin is a generic term that covers all corrinoids with a corrin nucleus, where methylcobalamin and adenosylcobalamin are known as the biologically active forms of the vitamin. Cobalamin functions as a cofactor in two enzymatic reactions within the body, which are catalyzed by cytosolic MS and the mitochondrial methylmalonyl CoA mutase (EC 5.4.99.2) (61). MS has cobalamin as a prosthetic group, depending on a methyl group from mTHF to form methylcobalamin and THF (61). As methylcobalamin is formed, MS transfers the methyl group bound to methylcobalamin to Hcy forming methionine and free cobalamin (61). Cobalamin is easily oxidized, which leads to inactivation of MS until reactivation by the riboflavin- and niacin-dependent enzyme MSR. As cobalamin and folate are closely interconnected in remethylation, deficiency of either of the vitamins reduces the activity of MS, and thus, may lead to accumulation of Hcy (60). Cobalamin deficiency may also cause accumulation of methylmalonic acid (MMA), formed from hydrolysis of methylmalonyl CoA. Methylmalonyl CoA is an end product of oxidation of odd chain fatty acids and amino acids, which can be further converted to succinyl CoA by the adenosylcobalamin-dependent enzyme methylmalonyl CoA mutase (61). Accumulation of plasma MMA has been shown to be a more specific marker of cobalamin deficiency compared to accumulated plasma tHcy, due to tHcy levels being affected by folate status. However, plasma MMA has been suggested to be influenced by other factors than cobalamin deficiency, such as age, sex and kidney function (62), as well as genetic variation (63).

Nutritional deficiency of cobalamin may also arise by inadequate intakes of foods of animal origin, including meat, fish, milk, and eggs, which are primarily dietary sources of the vitamin. These products contain mostly the hydroxo- and adenosylcobalamin form, while vitamin preparations mainly contain the cyanocobalamin form (61). About 20-30 % of the circulating cobalamin are bound to transcobalamin (holotranscobalamin), whereas 80% are believed to be transported by haptocorrin. Holotranscobalamin can be recognized and taken up by target cells, where all forms of cobalamin are believed to be metabolized to the biologically active forms. Haptocorrin cannot be taken up by cells and may function as circulating storage (61). As circulating cobalamin mostly comprises cobalamin bound to haptocorrin, total serum cobalamin may not be reflective of status. Holotranscobalamin measurements may reflect vitamin activity, while the functional markers tHcy and MMA may reflect cobalamin function. Thus, assessment of cobalamin status could include the use of multiple indices, such as plasma tHcy and MMA combined with total serum cobalamin concentrations, including holotranscobalamin.

1.3 Diet

1.3.1 Different diets and circulating biomarkers

Previous studies indicate that consumption of a low fat, high carbohydrate (LFHC) diet have similar effects on weight loss and improvements of body composition compared to a very high fat, low carbohydrate (VHFLC) diet (64–66). However, individual variation has been observed in the response to diets differing in fat and carbohydrate content on blood lipids (64,67). Individuals replacing ~6.5 energy % (E%) saturated fatty acids (SFA) with poly-unsaturated fatty acids (PUFA) were reported to improve both low-density lipoprotein (LDL) cholesterol and high-density lipoprotein (HDL) cholesterol after 8 weeks compared to the control group (68). Studies where individuals consumed a VHFLC diet found that women trended to respond with decreasing mean changes of LDL-cholesterol concentrations, while men showed the opposite response (64). Different responses to diets differing in fat and carbohydrate content by gender have been observed elsewhere, suggesting a role of genetic traits in diet response (69). In addition, in individuals with overweight, more favorable changes in LDL cholesterol were observed in the group consuming a diet low in fat for 1 year, while more favorable changes in HDL cholesterol and triglycerides were observed for the group consuming a diet low in carbohydrates (66). Since restriction of either fat or carbohydrates are popular strategies for managing the increasing prevalence of overweight and obesity, there is a need to increase the knowledge of diet composition response on other circulating markers.

1.3.2 Dietary regulation of one-carbon metabolism

There is also increasing evidence on the importance of dietary habits in regulating one-carbon metabolism as diet provide substrates, methyl groups, and B-vitamins. Intake of complex carbohydrates and proteins has been associated with lower plasma tHcy, while the intake of fat, processed meat, and sugar-rich foods has been associated with higher plasma tHcy (70). Smoking (71), coffee, and caffeine (72) have been reported to increase plasma tHcy, while high intakes of fish and eggs may have a lowering effect (73). Dietary intake of B-vitamins influences plasma concentrations of tHcy, such as folate and cobalamin, as mentioned in previous sections (20). Intake of whole grains rich in betaine has been associated with elevated plasma betaine (33,74). Preliminary data from the Western Norway B-vitamin intervention trial (Lysne, V et al, unpublished) observed that an increase in protein intake was strongly associated with higher plasma levels of vitamin B6, folate, riboflavin, niacin, and cobalamin and lower plasma levels of tHcy and MMA in patients with diagnosed stable angina pectoris, where no such associations were found for the intake of fat and carbohydrate. However, a randomized controlled trial replacing ~6.5 E% dietary SFA with PUFA observed higher concentrations of glycine, serine, cystathionine, and riboflavin, and lower concentrations of cysteine and cobalamin compared to the control group, suggesting a role of fatty acid composition (68). The role of fatty acid composition has been investigated in several studies. Krill oil rich in n-3 PUFAs and PC was reported to reduce plasma tHcy and further increase the concentration of choline oxidation metabolites, such as fasting plasma choline, betaine, DMG, and sarcosine (75). In healthy individuals, a plant-based diet high in PUFAs and naturally low in methionine and cysteine was reported to increase plasma tHcy levels compared to a plant-based diet high in SFA, methionine, and cysteine (28). Similar results were reported in individuals consuming a vegan-based diet restricted in dietary methionine and cysteine, compared to a vegan diet rich in methionine and cysteine (27).

A higher dietary fat intake has been reported in rodents to increase plasma cysteine and induce gene expression of *BHMT* and *GNMT* (76), and lower levels of hepatic CBS and CGL (77). High intake of dietary protein showed to increase hepatic concentrations of CBS (78), whereas rats fed a glycine-supplemented diet responded with increased activity of SHMT and GCS, which respectively convert glycine to serine and catabolize glycine (79). Excessive intake of dietary methionine has reported decreasing plasma betaine and serine concentrations, and further decrease flux in their respective reactions (80). Dietary fatty acids

have been shown to active the nuclear receptor peroxisome proliferator-activated receptor (PPAR)α, a key regulator of energy metabolism (81). Animal studies of PPARα-activation have reported effects on one-carbon metabolites and B-vitamins (82–84), providing a potential mechanism through which dietary fatty acid may influence these metabolic pathways. To summarize, dietary composition has been clearly shown to influence one-carbon metabolism, resulting in altered circulating concentrations of one-carbon metabolites and markers of B-vitamin status.

1.4 Aim and hypothesis

Plasma metabolites within the methionine-Hcy pathway and choline oxidation pathway are associated with risk of major lifestyle diseases. Supported by evidence implicating a role of diet in regulation of the one-carbon metabolism, more study is a needed to elucidate the effects of diet composition on plasma concentrations of one-carbon metabolites. As previous studies observe changes in many circulating markers in response to diets differing in fat and carbohydrate composition, we hypothesize that this may also be true for metabolites related to one-carbon metabolism. Thus, this present thesis aims to explore how two isocaloric diets, contrasting in fat and carbohydrate content, affects plasma one-carbon metabolites and related B-vitamin markers. Specifically, we hypothesize that participants consuming a diet with high fat content shows greater changes in levels of these metabolites and markers, which may be mediated by PPARα activation in response to fatty acid exposure.

2. Materials and methods

2.1 Study design

The population under study comprised participants who completed the 12-week diet intervention study FATFUNC (ClinicalTrials.gov Identifier: NCT01750021) performed in Bergen, Norway, from January to May 2013. FATFUNC was a randomized controlled trial aiming to observe the metabolic effects of two isocaloric weight loss diets differing in fat and carbohydrate composition (67). The trial was conducted according to the guidelines of the Declaration of Helsinki and approved by The Regional Ethics Committee. Details regarding study design, recruitment of participants, and data collection procedures were described in more detail in the original trial (67).

2.2 Participants

Men with abdominal obesity aged 30-50 years were recruited to FATFUNC by a newspaper ad (67). Regarding body composition, the inclusion criteria were BMI > 29 kg/m^2 or ≥25% body fat, waist circumference > 98 cm, and stable body weight (<5% change) before intervention startup. Other inclusion criteria were normal blood glucose levels defined as fasting blood glucose < 7mmol/L. Participants with any form of severe disease, food allergies, used regular medication (alkalizing gastric buffers were accepted), consumed alcohol >2 units per week, or weight reduction attempts the previous 6 months were excluded. The participants had two prescreening meetings before the intervention startup, where the randomization was conducted in the latter. Information on group allocation was announced to the participants after the baseline measurements. All participants provided written informed consent on potential risks and benefits. Of the 46 participants who met the inclusion criteria and completed the study, plasma analysis of one-carbon metabolites and related B-vitamin markers were available for n = 18 in the LFHC diet group and n = 20 in the VHFLC diet group. **Figure 5** provides an overview of the complete selection process.
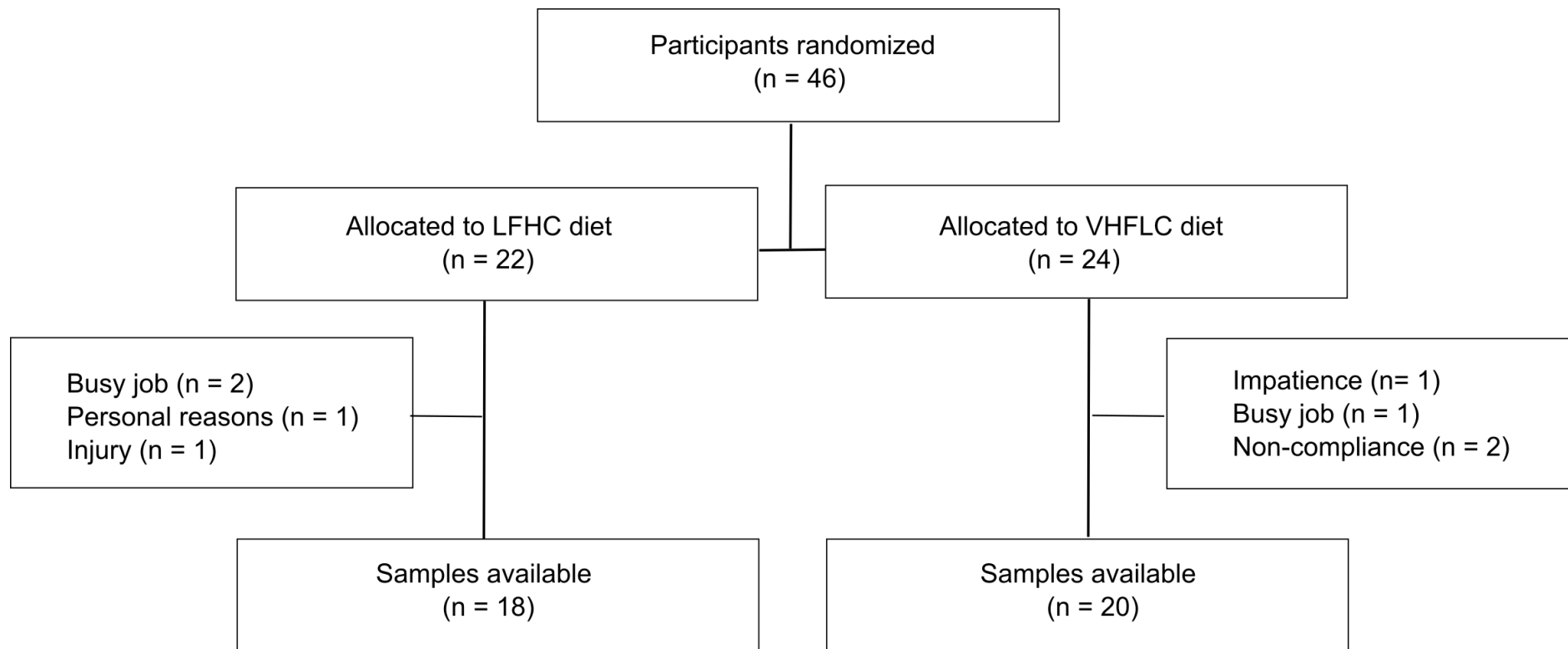
Figure 5 Flow diagram of the study selection. Of the 46 participants included for randomization, n = 18 in the LFHC diet group and n = 20 in the VHFLC diet group met the inclusion criteria for analysis of one-carbon metabolites and B-vitamin markers. LFHC, low fat, high carbohydrate, VHFLC, very high fat, low carbohydrate.

2.3 Diet interventions

2.3.1 Diet composition

For the intervention period of 12 weeks, 18 participants in the LFHC diet group consumed on average 51 E% from carbohydrate, 17 E% from protein, and 29 E% from fat, where 20 participants in the VHFLC diet group consumed on average 11 E% from carbohydrate, 17 E% from protein, and 71 E% from fat. Both diet groups had the same food profile and consumed the same primary sources of fats, carbohydrates, and proteins. The diets were similar in energy content (8750 kJ/d) and energy protein (17 E%), allowing to compare responses of two diets contrasting only in carbohydrate and fat. In both groups, the most consumed fat sources were butter, extra virgin olive oil, and cream, while the most consumed sources of carbohydrates were bread/baguettes, vegetables, rice, berries, and juices without added sugar. Regarding protein sources, the most consumed sources were meat and meat products, eggs and egg products, and cheeses. The contribution of PUFAs was on average equal in both groups (4.6 E% LFHC, 5.2 E% VHFLC). In general, the diets contained minimal highly processed foods and plant oils with high amounts of ω-6 fatty acids. They were recommended to eat two fish meals per week and avoid foods with poor food quality, such as hydrogenated vegetable fat, sugar, and to consume $\geq$500 g of fruits, berries, and vegetables every day. 8 weeks before the intervention startup, they were instructed to use vitamin and mineral supplements daily (Solaray Spektro without iron, provided by Au Naturel (UK)). They had a limited consumption of alcohol (0.3 E% LFHC, 0.2 E% VHFLC) and a limited intake of foods added with sugar (0.8 E% LFHC, 0.1 E% VHFLC).

The diets differed in the volume of food consumed, as the LFHC group consumed twice the volume of food as the VHFLC group (2126 g/day compared with 1234 g/day) to meet the energy requirements. The fat composition did also differ between the groups, where the contribution of SFA was 12 E% and 34 E% for the LFHC and VHFLC, respectively. The intake of monounsaturated fatty acids (MUFA) was also higher in the VHFLC group compared to the LFHC group, 22.9 E%, and 8.1 E%, respectively. Regarding carbohydrates, the LFHC group had a higher consumption of fiber (2.8 E%) compared to the VHFLC group (1.6 E%). Overall, the diet groups consumed the same food products throughout the intervention, only varying in the quantity of carbohydrates and fat.

2.3.2 Dietary adherence

The dietary data were assessed by using self-reported food records by using www.diett.no (operated by Dietika). They got a recipe booklet with information of the nutrient content and instructions for meal preparation for all meals (breakfast, lunch, dinner, supper, snacks), where the meals were designed according to the macronutrient profile of each diet group. The participants collected diet records for 5 consecutive days (including weekends) at baseline and 5 days including weekends each month the entire intervention. They also recorded the physical activity level (frequency, duration, intensity) throughout the study, which reflected their habitual physical activity level. The physical activity level throughout the study were reported to be on average similar in both groups (67).

The dietary adherence was verified using respiratory exchange ratio (RER), a tool to assess the substrate utilized for energy fuel. Complete carbohydrate oxidation was referred to as RER = 1, where the ratio of oxygen consumed and carbon dioxide molecules produced are equal, while an RER = 0.69-0.73 was referred to as complete fatty acid oxidation. The dietary adherence were considered as good (67), and the RER for the VHFLC group and for the LFHC group were 0.85 and 0.84 at baseline, and 0.80 and 0.82 after 12 weeks, respectively.

2.4 Biochemical Assays

All circulating one-carbon metabolites and B-vitamin markers assessed in FATFUNC were of interest in this thesis. Blood samples were conducted at four study visits: at baseline and after 4, 8, and 12 weeks. However, circulating one-carbon metabolites and B-vitamin markers were unfortunately not assessed at week 4, leaving samples from baseline, week 8, and week 12 eligible for analysis.

2.4.1 Blood samples

All analyses were performed according to standardized procedures at the Laboratory of Clinical Biochemistry and the Hormone laboratory at Haukeland University Hospital, Bergen. Blood samples were taken in venous blood and stored at -80 degrees Celsius after preparation. All blood samples, including whole blood, plasma, and serum, were collected in a fasting state (overnight or ≥ 10 h) between 08.00 am, and 11.30 am, with only small amounts of water allowed. All blood samples collected were measured simultaneously (67).

2.4.2 Analysis of metabolites

As one-carbon metabolites and B-vitamin markers are transported in the circulation, plasma or serum measurements are standard assessment methods. In FATFUNC, the analysis of all plasma and serum metabolites was done by trained personnel at Bevital A/S (Bevital, Bergen, Norway, www.bevital.no). Plasma one-carbon metabolites and B-vitamin markers assessed in FATFUNC and quantification methods are listed in **Table 1**, which also provides intraclass correlation coefficients (ICC) and an overview of defined reference ranges of normal plasma and serum values for the metabolites (www.bevital.no). However, it should me mentioned that the reference ranges are based on reported measurements from various cohorts and may not reflect all populations.

Table 1. Overview of plasma one-carbon metabolites and B-vitamin markers assessed in FATFUNC, analytic methods, and reference ranges in plasma/serum levels.

Plasma metabolite	Analytic method	ICC	Values in plasma/serum*
Methionine-Homocysteine cycle			
Methionine	GC-MS/MS	0.33	18-50 µmol/L
Total homocysteine (tHcy)	GC-MS/MS	0.72	5-15 µmol/L
Cystathionine	GC-MS/MS	0.63	<0,4 µmol/L
Total cysteine (tCys)	GC-MS/MS		150-350 µmol/L
Choline oxidation pathway			
Free choline	LC-MS/MS	0.36	5-12 µmol/L
Betaine	LC-MS/MS	0.65	20-60 µmol/L
Dimethylglycine (DMG)	LC-MS/MS	0.64	1,5-5 µmol/L
Sarcosine	GC-MS/MS	0.68	0,7-2,3 µmol/L**
Serine	GC-MS/MS	0.71	95-125 µmol/L
Glycine	GC-MS/MS	0.81	200-300 µmol/L
B-vitamin markers			
Riboflavin	LC-MS/MS	0.79	5-100 nmol/L
Flavin mononucleotide (FMN)	LC-MS/MS	0.69	3-30 nmol/L
Nicotinamide (NAM)	LC-MS/MS	N/A	100-600 nmol/L
1-methylnicotinamide (mNAM)	LC-MS/MS	N/A	20-250 nmol/L
Pyridoxal 5´-phosphate (PLP)	LC-MS/MS	0.70	15-150 nmol/L
Pyridoxal (PL)	LC-MS/MS	0.62	5-150 nmol/L
4-Pyridoxic acid (PA)	LC-MS/MS	0.58	10-200 nmol/L
5-Methyl-tetrahydrofolate (mTHF)	LC-MS/MS	N/A	>7,5 nmol/L
Cobalamin (sB12)	Microbiological assay	0.82	>150 pmol/L
Methylmalonic acid (MMA)	GC-MS/MS	0.81	<0,26 µmol/L

Source: www.bevital.no
*Plasma/serum values in this table are based on plasma concentrations observed or reported in various cohorts and should not directly be referred to as normal reference ranges, as population characteristics such as gender, age, ethnicity vary across study populations.
**Sarcosine cannot be measured by GC-MS/MS by all suppliers because sarcosine may be present in EDTA-tubes. Abbreviations; GC-MS/MS, Gas Chromatography-Tandem mass spectrometry; ICC, intraclass correlation coefficient; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry.

Quantification of metabolites was performed at Bevital AS (www.bevital.no) (85–89). Methionine, tHcy, cystathionine, total cysteine (tCys), serine, glycine, and sarcosine were quantified by gas chromatography-tandem mass spectrometry (GC-MS/MS) (85). Free choline, betaine, and DMG were quantified by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (86). LC-MS/MS was also used to quantify B-vitamins, such as riboflavin, FMN, NAM, mNAM, PLP, PL, PA and mTHF (87,88). Regarding cobalamin, GC-MS/MS were used to quantify plasma MMA, while serum B12 (sB12) was quantified by microbiological assays (89). The within-person reproducibility of the plasma measurements is expressed as ICC. In other words, it means how a single measure may represent long-term exposures within a subject, whereas ICC < 0.40 indicated poor reproducibility, 0.40-0.75 indicates fair to good reproducibility, while ICC > 0.75 indicates excellent reproducibility (57).

2.5 Statistical Analyses and Presentation of Data

Plasma concentrations of outcome metabolites and baseline characteristics are presented as means (standard deviation, SD) for continuous variables and counts (%) for categorical variables. We chose to use Pearson correlation analysis at baseline to evaluate potential relationships between the outcome metabolites. As we aim to explore the response of two contrasting diets on plasma concentrations metabolites in a 12-week intervention, our main results are presented graphically. We used linear regression adjusted for baseline to assess the between-group differences in plasma concentrations of the outcome metabolites after 12 weeks. The results are given as mean differences (95 % compatibility intervals) with a p-value. In line with the recently highlighted misconceptions regarding the interpretation of p-values and statistical significance (90,91), the p-values are reported and interpreted as a continuous measure of compatibility rather than dichotomized intro the arbitrary categories "significant" and "non-significant". In line with recent research, we emphasize using effect sizes to estimate statistical inference and 95% compatibility intervals (CI) to express variation and uncertainty in the data. As separate analyses of changes from baseline within each group may be misleading, we have focused on the direct between-group comparisons of the follow-up at 12 weeks (92). At last, we calculated the standardized mean difference (SMD) for each metabolite where the effects were regarded as small, medium, or large according to the conventional cutoffs of 0.2, 0.5, and 0.8 (93). All statistical analyses and data presentation were performed using R (94), version 4.0.2 (https://www.R-project.org), using the packages and codes within *"tidyverse"* (95), *"xlsx", "broom", "effsize" and "corrr"*.

3. Results

3.1 Baseline characteristics

Of the 46 randomized participants in the FATFUNC study, plasma samples of the outcome metabolites were available for 18 participants in the LFHC diet group (mean ± SD age 40.2 ± 4.5 years) and 20 in the VHFLC diet group (40.2 ± 5.5 years). 12 participants were classified as smokers based on plasma cotinine-levels ≥ 85 nmol/L, 6 participants (33.3%) and 6 participants (30.0%) in the LCHF diet group and VHFLC diet group, respectively. As reported in the original study (67), the BMI (-3.7 kg/m^2 LFHC, -3.6 kg/m^2 VHFLC), body weight (-12.2 kg vs. -11.8 kg) and waist circumference (-12.18 cm vs. -10.6 cm) decreased on average equally from baseline to intervention end in both groups. The mean plasma metabolite concentrations were within normal reference ranges defined in **Table 1** throughout the study except for NAM which were > 600 nmol/L in both diet groups. Descriptive characteristics of the study participants and mean plasma concentrations of the outcome metabolites at baseline, week 8, and week 12 is presented in **Table 2**. The correlation analysis between the metabolites at baseline is presented in **Figure 6.** An overview of the mean plasma concentrations of the outcome metabolites throughout the intervention are visualized in **Figure 7.** In this figure, some extreme values were excluded due to large variation in plasma values. However, it should be noted that all values are included for statistical analysis and that this exclusion were just for visualization purposes. Between-group differences and SMD are presented in in **Table 3**. An overview of the main effects of two diets contrasting in fat and carbohydrate content on plasma one-carbon metabolites is presented in **Figure 8**.

Table 2. Baseline and follow-up characteristics and plasma values for one-carbon metabolites and B-vitamin markers in participants from the FATFUNC study[1].

	Total population n = 38			LFHC n = 18			VHFLC n = 20		
	Baseline	Week 8	Week 12	Baseline	Week 8	Week 12	Baseline	Week 8	Week 12
Population characteristics									
Age, y	40.2 (5.00)			40.2 (4.50)			40.2 (5.53)		
Weight, kg	112 (11.6)	103 (10.5)	100 (9.90)	111 (13.8)	101 (12.5)	98.8 (11.8)	114 (9.47)	104 (8.36)	102 (7.93)
BMI, kg/m^2	33.9 (2.99)	31.0 (2.78)	30.3 (2.64)	33.6 (3.62)	30.7 (3.39)	29.9 (3.29)	34.1 (2.35)	31.3 (2.13)	30.6 (1.90)
Waist circumference, cm	116 (8.58)	107 (8.93)	104 (8.96)	116 (10.4)	107 (10.1)	104 (10.4)	116 (6.84)	108 (7.93)	105 (7.65)
Smokers[2], n (%)	12 (31.5%)			6 (33.3%)			6 (30.0%)		
One-carbon metabolites									
Met, μmol/L	28.0 (6.62)	26.5 (5.26)	25.0 (3.81)	28.7 (4.33)	27.4 (4.41)	26.3 (3.71)	27.4 (8.23)	25.6 (5.91)	23.8 (3.59)
tHcy, μmol/L	9.89 (1.99)	10.8 (2.15)	10.5 (2.67)	9.82 (2.09)	11.2 (2.48)	11.0 (3.26)	9.96 (1.95)	10.5 (1.79)	10.1 (1.99)
Cysta, μmol/L	0.22 (0.14)	0.20 (0.12)	0.18 (0.11)	0.20 (0.048)	0.24 (0.14)	0.22 (0.14)	0.23 (0.20)	0.17 (0.099)	0.15 (0.047)
tCys, μmol/L	320 (31.8)	320 (30.3)	318 (28.6)	326 (36.1)	330 (26.6)	324 (28.4)	315 (27.2)	310 (30.9)	313 (28.3)
Choline, μmol/L	10.1 (1.57)	9.40 (1.55)	9.19 (1.69)	10.3 (1.76)	9.35 (1.67)	9.17 (1.69)	9.86 (1.40)	9.43 (1.47)	9.21 (1.73)
Betaine, μmol/L	34.3 (6.57)	40.1 (8.97)	40.3 (9.48)	35.0 (6.45)	40.4 (8.54)	39.7 (8.13)	33.7 (6.78)	39.7 (9.55)	40.8 (10.7)
DMG, μmol/L	4.13 (1.23)	4.62 (1.29)	4.46 (1.09)	4.06 (0.76)	4.49 (1.02)	4.18 (0.74)	4.20 (1.56)	4.74 (1.50)	4.72 (1.30)
Sarc, μmol/L	2.81 (0.46)	2.80 (0.47)	2.72 (0.38)	2.89 (0.45)	2.86 (0.52)	2.80 (0.33)	2.73 (0.47)	2.75 (0.42)	2.66 (0.41)
Ser, μmol/L	105 (16.7)	123 (23.2)	118 (15.6)	107 (18.9)	119 (21.9)	114 (14.9)	102 (14.7)	126 (24.5)	121 (15.8)
Gly, μmol/L	201 (31.3)	227 (36.4)	218 (33.2)	211 (28.1)	235 (33.7)	228 (33.9)	192 (31.9)	219 (37.9)	210 (30.8)

B-vitamin markers

Riboflavin, nmol/L	32.6 (20.0)	20.9 (23.6)	18.2 (14.2)	37.2 (24.8)	18.3 (14.5)	18.9 (18.2)	28.4 (13.9)	23.4 (29.8)	17.7 (9.73)
FMN, nmol/L	18.2 (7.78)	17.0 (8.02)	14.5 (5.42)	19.7 (9.31)	16.9 (10.6)	15.1 (6.53)	16.9 (6.02)	17.0 (4.88)	14.1 (4.31)
NAM, nmol/L	791 (172)	886 (221)	641 (139)	821 (176)	883 (260)	643 (139)	764 (168)	889 (187)	639 (143)
mNAM, nmol/L	200 (72.4)	115 (54.0)	139 (46.9)	202 (56.6)	119 (63.0)	145 (57.8)	198 (85.7)	112 (45.9)	133 (34.9)
PLP, nmol/L	109 (56.0)	102 (54.2)	80.8 (35.9)	113 (61.3)	88.1 (42.0)	75.8 (34.5)	105 (52.2)	114 (61.7)	85.3 (37.4)
PL, nmol/L	26.1 (19.1)	20.8 (17.3)	14.7 (8.01)	30.5 (24.1)	20.1 (16.0)	16.4 (10.3)	22.1 (12.3)	21.4 (18.7)	13.1 (4.98)
PA, nmol/L	66.5 (55.8)	40.0 (30.0)	31.2 (17.3)	80.0 (70.1)	39.4 (25.9)	34.1 (19.5)	54.3 (36.6)	40.5 (33.9)	28.6 (15.2)
mTHF, nmol/L	15.3 (6.75)	14.6 (7.04)	15.8 (7.11)	15.5 (4.77)	15.2 (6.23)	16.0 (7.40)	15.1 (8.26)	14.0 (7.82)	15.6 (7.03)
sB12, pmol/L	527 (121)	506 (118)	460 (108)	566 (136)	511 (127)	455 (103)	496 (99.4)	501 (113)	464 (114)
MMA, µmol/L	0.14 (0.049)	0.13 (0.029)	0.13 (0.031)	0.14 (0.054)	0.13 (0.031)	0.13 (0.036)	0.14 (0.045)	0.13 (0.026)	0.12 (0.025)

[1]Variables are given as mean (standard deviation) except of smoking, which is given as n (%)

[2]Smokers are identified by plasma cotinine > 85 nmol/L

BMI, body mass index; Cysta, cystathionine; DMG, dimethylglycine; FMN, flavin mononucleotide; Gly, glycine; Met, methionine; MMA, methylmalonic acid; mNAM, 1-methylnicotinamide; mTHF, 5-methyl-tetrahydrofolate; NAM, nicotinamide; PA, 4-pyridoxic acid; PL, pyridoxal; PLP, pyridoxal 5´phosphate; sB12, serum B12; Sarc, sarcosine; Ser, serine; tCys, total cysteine; tHcy, total homocysteine.

3.2 Correlation between plasma metabolites

To evaluate the intercorrelations between the metabolites of interest, Pearson correlation coefficients between the metabolite concentrations at baseline are reported in **Figure 6**. Strongest correlations were those for methionine and cystathionine ($r = 0.80$), riboflavin and PA ($r = 0.73$), and PL and PA ($r = 0.86$), riboflavin and PL ($r = 0.66$), PLP and PL ($r = 0.65$), PLP and PA ($r = 0.61$), cystathionine and MMA ($r = 0.53$). Other prominent correlations were for serine and glycine ($r = 0.48$), tHcy and tCys ($r = 0.48$), choline and betaine ($r = 0.43$), glycine and NAM ($r = 0.43$) and riboflavin and PLP ($r = 0.38$).
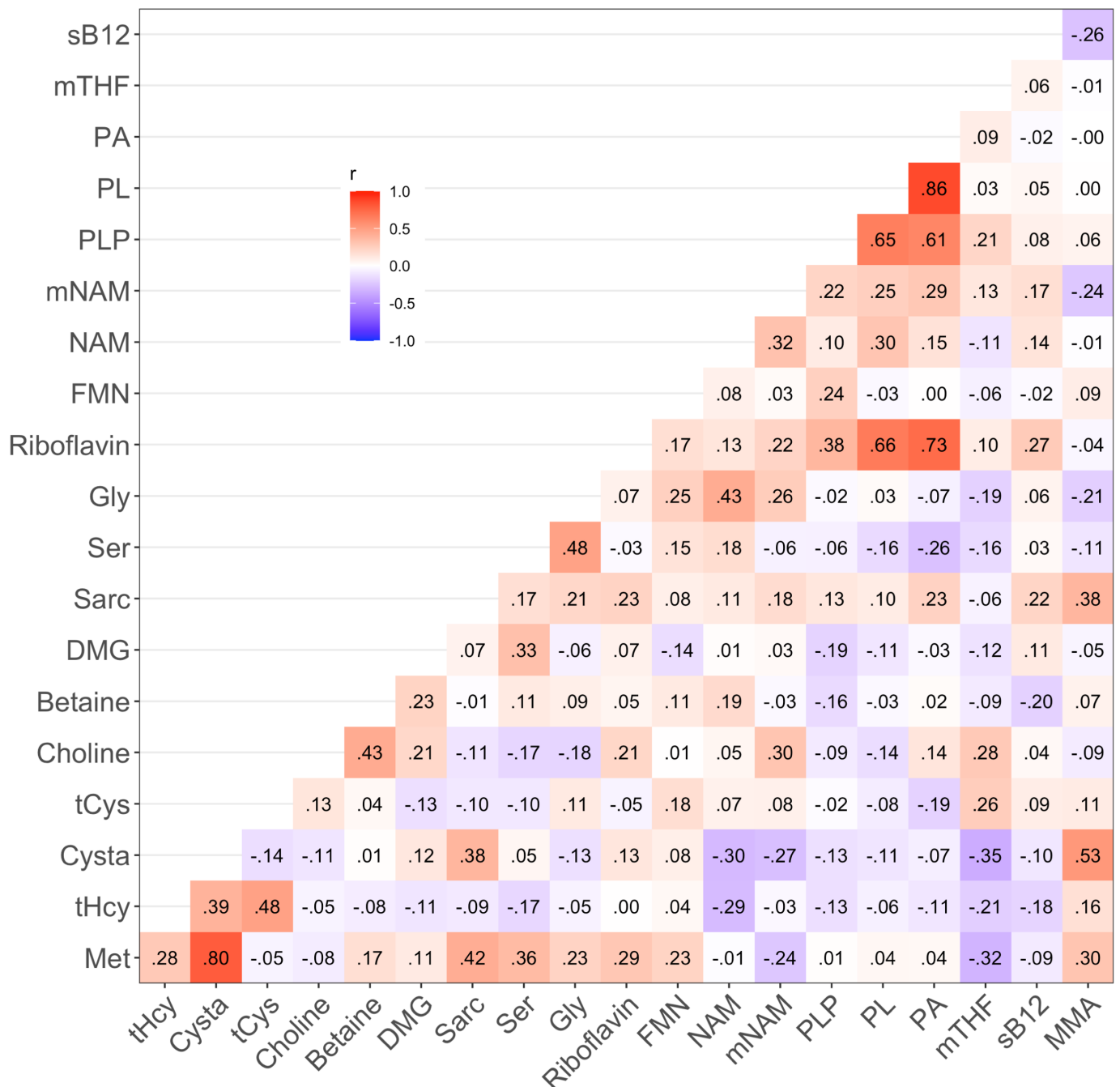
Figure 6. Correlation matrix of plasma one-carbon metabolites and related B-vitamin markers measured at baseline in the FATFUNC-study. Red color reflects positive correlation while blue color reflects negative correlation. Cysta, cystathionine; DMG, dimethylglycine; FMN, flavin mononucleotide; Gly, glycine; LFHC, low fat, high carbohydrate Met, methionine; MMA, methylmalonic acid; mNAM, 1-methylnicotinamide; mTHF, 5-methyl-tetrahydrofolate; NAM, nicotinamide; PA, 4-pyridoxic acid; PL, pyridoxal; PLP, pyridoxal 5´phosphate; sB12, serum B12; Sarc, sarcosine; Ser, serine; tCys, total cysteine; tHcy, total homocysteine.

3.3 Plasma concentration of metabolites

3.2.1 One-carbon metabolites

At baseline, the mean plasma concentrations of tHcy, cystathionine, choline, betaine, DMG, and serine were on average similar in both diet groups, while the mean levels of methionine (~1.3 µmol/L), tCys (~11 µmol/L), sarcosine (~0.16 µmol/L), and glycine (~19 µmol/L) were slightly higher in the LFHC group (**Table 2)**. From baseline toward 12 weeks, parallel trends in the groups were observed for decreasing mean plasma methionine, choline, and sarcosine, and increasing trends of plasma tHcy, betaine, DMG, serine, glycine (**Figure 7).** Divergent trends were observed in the groups for plasma cystathionine and tCys. At the end of study, the VHFLC group had lower levels of methionine (-2.5 µmol/L), tHcy (-0.9 µmol/L), cystathionine (0.07 µmol/L), tCys (-11 µmol/L), sarcosine (-0.14 µmol/L) and glycine (-18 µmol/L), while higher levels of choline (0.04 µmol/L), betaine (1.1 µmol/L), DMG (0.54 µmol/L) and serine (7 µmol/L).

Results from our linear regression analysis indicated the strongest response toward decreasing plasma methionine (-2.15 µmol/L, 95% CI [-4.29, -0.02]), tHcy (-1.05 µmol/L, [-2.19, 0.1]), cystathionine (-0.08 µmol/L, [-0.15, -0.01]) while increasing plasma DMG (0.45 µmol/L, [-0.04, 0.94]), and serine (9.03 µmol/L, [0.08, 17.99])) in the VHFLC group compared to the LCHF group at 12 weeks (**Table 3).** Based on the wide compatibility intervals, the trends for the other one-carbon metabolites were more uncertain. The SMD (Cohen´s d) estimates indicated no large between-group effects (SMD > 0.8), whereof plasma concentrations of cystathionine were on the threshold (SMD = -0.74). Medium effect estimates were indicated for plasma methionine (SMD =-0.68), glycine (SMD = -0.57), and DMG (SMD = 0.51).
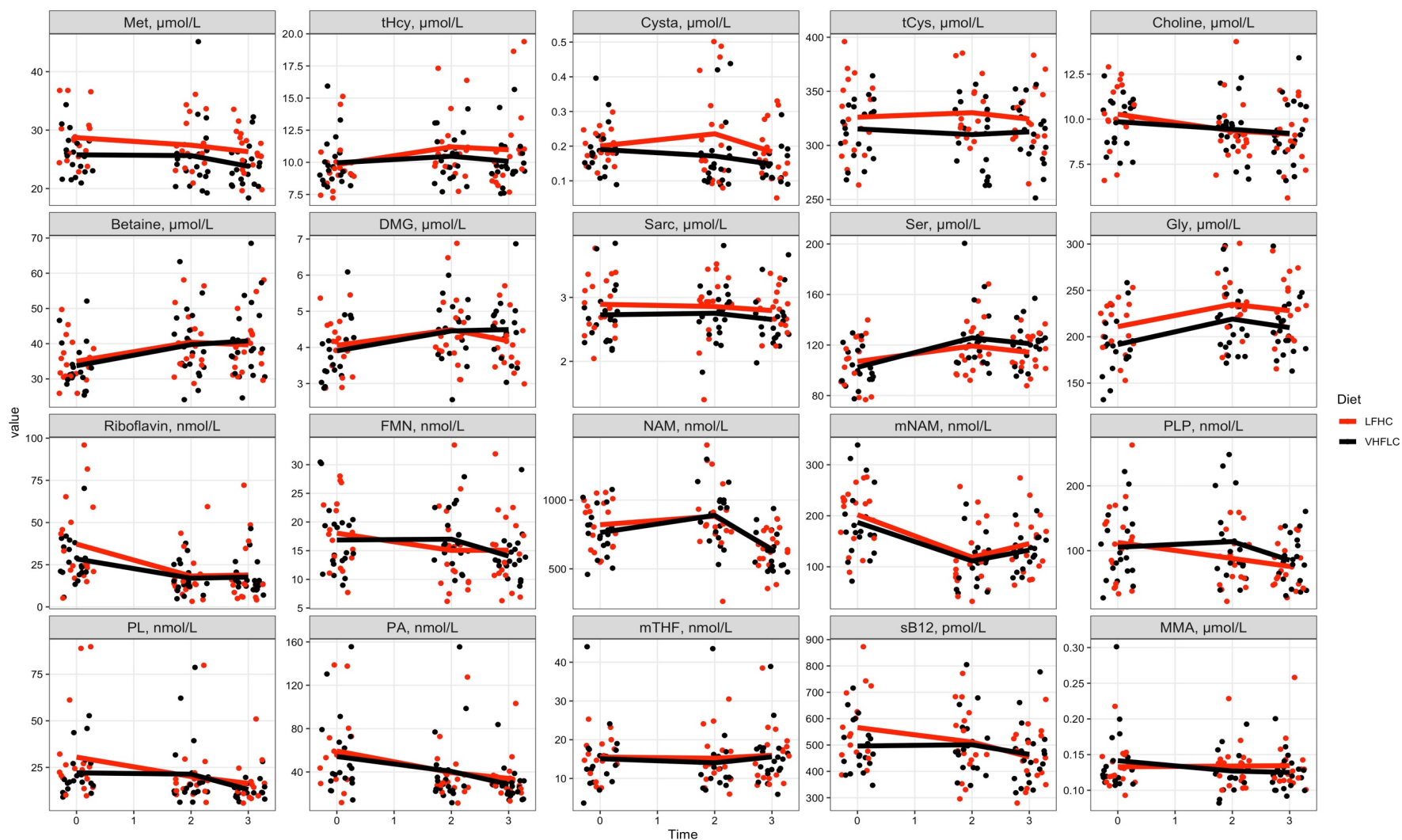
Figure 7. Plasma concentrations of biomarkers within the one-carbon metabolism and related B-vitamins in participants from the FATFUNC study.
Points are the observed values, and the lines reflect the mean trend within the two diet groups. Red lines reflect the plasma metabolite trends for the LFHC diet group, while the black lines for the VHFLC diet group. Cysta, cystathionine; DMG, dimethylglycine; FMN, flavin mononucleotide; Gly, glycine; Met, methionine; MMA, methylmalonic acid; mNAM, 1-methylnicotinamide; mTHF, 5-methyl-tetrahydrofolate; NAM, nicotinamide; PA, 4-pyridoxic acid; PL, pyridoxal; PLP, pyridoxal 5´phosphate; sB12, serum B12; Sarc, sarcosine; Ser, serine; tCys, total cysteine; tHcy, total homocysteine.

Table 3 Changes in plasma metabolites after 12-weeks diet intervention

	LFHC	VHFLC	VHFLC vs. LFHC (95% CI)[1]	P	SMD[2] (95% CI)
Met, µmol/L	26.3 (3.71)	23.8 (3.59)	-2.15 (-4.29, -0.02)	0.05	-0.68 (-1.36, -0.01)
tHcy, µmol/L	11.0 (3.26)	10.1 (1.99)	-1.05 (-2.19, 0.1)	0.07	-0.34 (-1, 0.33)
Cysta, µmol/L	0.22 (0.14)	0.15 (0.047)	-0.08 (-0.15, -0.01)	0.02	-0.74 (-1.42, -0.06)
tCys, µmol/L	324 (28.4)	313 (28.3)	-5.39 (-19.94, 9.17)	0.46	-0.41 (-1.08, 0.25)
Choline, µmol/L	9.17 (1.69)	9.21 (1.73)	0.31 (-0.6, 1.23)	0.49	0.02 (-0.64, 0.68)
Betaine, µmol/L	39.7 (8.13)	40.8 (10.7)	2.2 (-3.07, 7.47)	0.4	0.12 (-0.54, 0.78)
DMG, µmol/L	4.18 (0.74)	4.72 (1.30)	0.45 (-0.04, 0.94)	0.07	0.51 (-0.16, 1.18)
Sarc, µmol/L	2.80 (0.33)	2.66 (0.41)	-0.09 (-0.32, 0.15)	0.45	-0.37 (-1.04, 0.29)
Ser, µmol/L	114 (14.9)	121 (15.8)	9.03 (0.08, 17.99)	0.05	0.45 (-0.22, 1.11)
Gly, µmol/L	228 (33.9)	210 (30.8)	-8.73 (-28.92, 11.47)	0.39	-0.57 (-1.24, 0.11)
Riboflavin, nmol/L	18.9 (18.2)	17.7 (9.73)	2.59 (-5.35, 10.52)	0.51	-0.09 (-0.74, 0.57)
FMN, nmol/L	15.1 (6.53)	14.1 (4.31)	0.49 (-1.99, 2.97)	0.69	-0.19 (-0.85, 0.47)
NAM, nmol/L	643 (139)	639 (143)	22.47 (-55.72, 100.67)	0.56	-0.03 (-0.69, 0.63)
mNAM, nmol/L	145 (57.8)	133 (34.9)	-12.6 (-44.11, 18.9)	0.42	-0.27 (-0.93, 0.39)
PLP, nmol/L	75.8 (34.5)	85.3 (37.4)	12.46 (-6.28, 31.19)	0.19	0.26 (-0.4, 0.93)
PL, nmol/L	16.4 (10.3)	13.1 (4.98)	-2.23 (-7.44, 2.97)	0.39	-0.41 (-1.08, 0.25)
PA, nmol/L	34.1 (19.5)	28.6 (15.2)	-1.43 (-11.75, 8.89)	0.78	-0.32 (-0.98, 0.35)
mTHF, nmol/L	16.0 (7.40)	15.6 (7.03)	-0.11 (-3.87, 3,64)	0.95	-0.05 (-0.71, 0.6)
sB12, pmol/L	455 (103)	464 (114)	36.93 (-13.23, 87.09)	0.14	0.09 (-0.57, 0.74)
MMA, µmol/L	0.13 (0.036)	0.12 (0.025)	-0.01 (-0.03, 0.01)	0.33	-0.3 (-0.97, 0.36)

Variables are given as mean plasma concentration (SD).
[1]Model adjusted for baseline metabolite concentration.
[2]Standardized mean difference (SMD) is estimated by Cohen´s d
95% CI, 95% compatibility interval; Cysta, cystathionine; DMG, dimethylglycine; FMN, flavin mononucleotide; Gly, glycine; LFHC, low fat, high carbohydrate Met, methionine; MMA, methylmalonic acid; mNAM, 1-methylnicotinamide; mTHF, 5-methyl-tetrahydrofolate; NAM, nicotinamide; PA, 4-pyridoxic acid; PL, pyridoxal; PLP, pyridoxal 5´phosphate; sB12, serum B12; Sarc, sarcosine; Ser, serine; tCys, total cysteine; tHcy, total homocysteine; VHFLC, very-high fat, low carbohydrate.

3.2.2 B-vitamin markers

At baseline, the LFHC group had slightly greater levels of the mean concentrations of all the B-vitamins compared to the VHFLC group **(Table 2).** From baseline toward week 12, there was an overall decreasing response in mean plasma concentrations of all the B-vitamin markers in both groups, expect of mTHF and MMA which were rather stable. Parallel trends are observed for most of the markers, while some different tendencies in the groups are observed for plasma riboflavin, FMN, PL, PLP and sB12 **(Figure 7)**. At the end of study, the VHFLC group had higher levels of PLP (9.5 nmol/L) and sB12 (9.0 pmol/L) and slightly lower levels of riboflavin (-1.2 nmol/L), FMN (-1 nmol/L), NAM (-4 nmol/L), mNAM (-12 nmol/L), PL (-3.3 nmol/L), PA (-5.5 nmol/L), mTHF (-0.4 nmol/L) and MMA (-0.01 µmol/L) compared to the LFHC group.

Results from the linear regression indicated largest between-group differences for higher plasma PLP (12.46 nmol/L, 95% CI [-6.28, 31.19]), sB12 (36.93 [-13.23, 87.09]) and lower plasma PL (-2.23 nmol/L [-7.44, 2.97] and mNAM (-12.6 nmol/L [-44.11, 18.9]) in the VHFLC group compared to the LFHC group **(Table 3).** As evident by wide 95% compatibility intervals, large uncertainty was observed for all B-vitamins. The SMD estimates indicated no between-group effects for the B-vitamin markers, where plasma PL were on the threshold to what is considered a medium effect (SMD = -0.41). The most pronounced between-group effects in diet response were for the one-carbon metabolites, which is visualized in **Figure 8.**
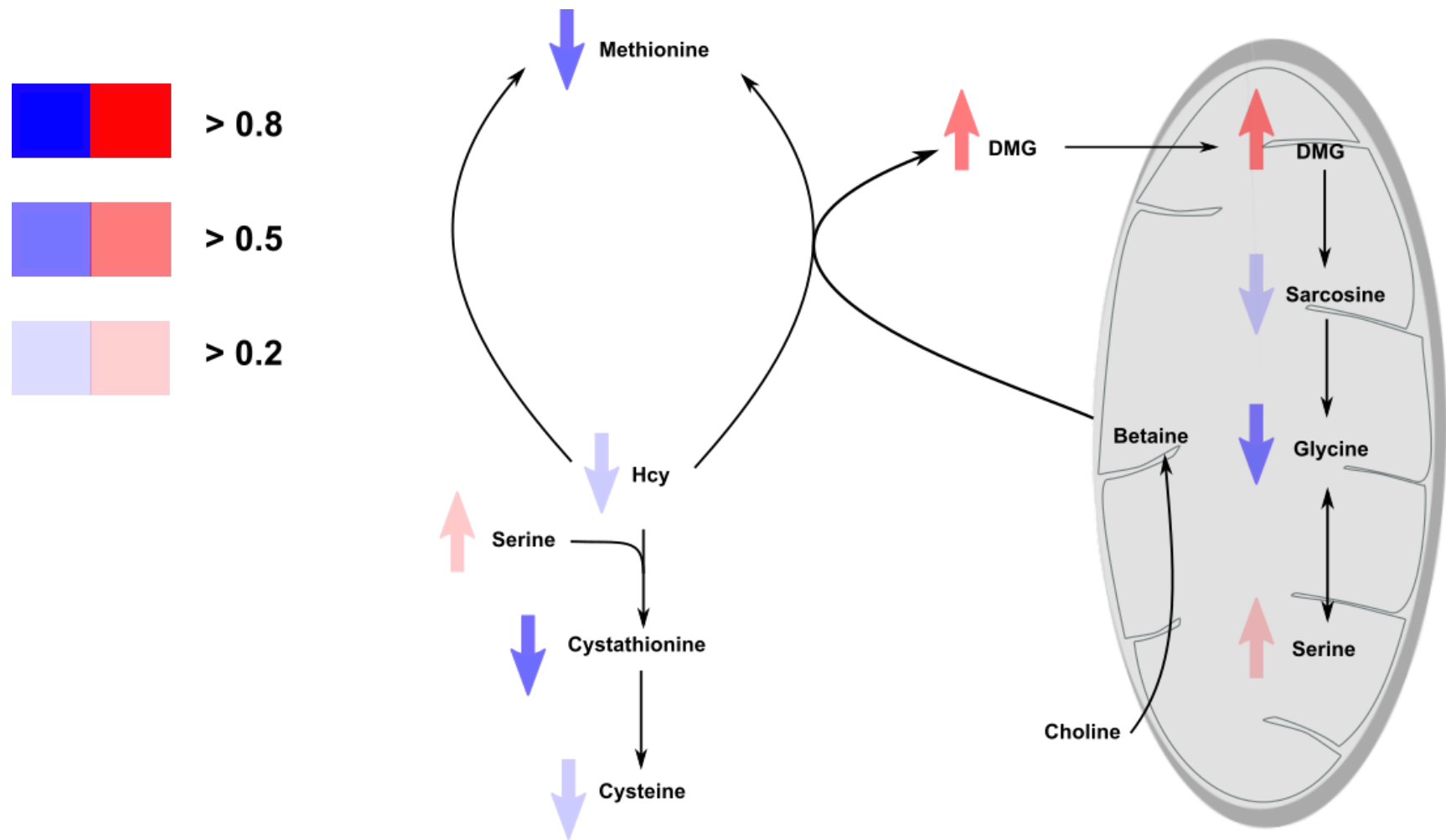
Figure 8. Observed changes in plasma concentrations of one-carbon metabolites by consuming a VHFLC diet compared to a LFHC diet for 12 weeks. The red arrows indicate higher while the blue arrows indicate lower plasma concentrations in the VHFLC group compared to the LFHC group. Effect sizes are visualized by opacity where the opaque red and blue arrows reflect strong effect estimates > 0.8, brighter red and blue arrows reflect medium effect estimates > 0.5, and the transparent arrows reflect small > 0.2. No arrow reflects no effect (choline and betaine). DMG; dimethylglycine; tHcy, total homocysteine.

5. Discussion

5.1 Principal findings

In this 12-week randomized controlled trial among men with abdominal obesity, we observed lower mean plasma concentrations of methionine, tHcy, cystathionine and higher mean plasma concentrations of DMG and serine in participants consuming a VHFLC diet compared to a LFHC diet. We observed lower plasma concentrations of cystathionine on the threshold of what is considered a large effect, while the observed effect on the other metabolites were small. The 95 % compatibility intervals were wide and pointed both directions causing some uncertainty in the estimates.

5.2 Methodological aspects and considerations

5.2.1 Study design

This study was a randomized controlled trial, of where the participants were randomized to diets sharply contrasting in fat and carbohydrate content. Randomization, a procedure where participants have the same probability to be assigned to an intervention (96), balances both known and unknown prognostic factors with the purpose to distribute factors other than the exposure of interest equally. This minimizes the effect of potential *confounder* variables, which are variables affecting the exposure or outcome of interest without being under study, as they are equally distributed in both diet groups. By using this study design, it allows us to better investigate the causal effects of test interventions on outcomes of interest, and not just associations (97). Notably, when testing effects of dietary interventions that deviate substantially from the habitual diet, the time of exposure may need to be limited to a short period of time to ensure sufficient adherence (97). Thus, the present results reflect short-term, more acute responses to diet exposure, and not necessarily long-term responses.

Also previous comparable randomized controlled trials reporting effects of diet composition on plasma one-carbon metabolites were of similarly short duration (27,68,98). A general limitation in these kinds of diet studies is the lack of blinding or double blinding, referring to when neither the participants nor the researchers know which of the participants receives the intervention (96). Blinding helps minimize bias, in particular *researcher bias*, which is present when the researchers may unknowingly or with intention influences the research, such as increasing the likelihood of a positive outcome in a group expected or hoped to have such effects (99). It opens

for bias introduced by differential treatment of groups or when assessing outcomes (99). Studies not implementing blinding have found to report greater effects of the treatment than those studies reporting to implement blinding (99). However, blinding is by nature a challenge in nutritional research, as placebo does not exist for diet composition and that participants obviously see what foods they consume (100). Although blinding is more difficult in nutritional research, it is not impossible and measures can be taken to reduce the associated bias. Ideally, all individuals involved in the study could possibly be blinded to some extent (99). First, randomization can be implemented by someone not involved in the study rather than the investigators. Furthermore, blinding when collecting data and samples could also reduce the risk of researcher bias by reducing ascertainment of the outcomes (99). This also implies to the current study, as the researchers were not blinded when performing statistical analyses and the interpretation of the results. Participants can also be blinded by certain ways, such as obtaining less information about the group they are allocated to, or less information about the other diet intervention group. This could prevent bias, as awareness of group assignment and knowledge of the intervention may affect behavior and responses to the trial (99). For instance, a participant aware of losing weight in a trial may probably lose more weight than he would when not being aware that this is an outcome of interest, which may bias the efficacy of the intervention.

Another consideration in nutrition research is the use of self-reported dietary data. In the current study, the participants reported their diet intake by using food records. This dietary assessment instrument is considered a *reactive tool*, meaning that the usage of food records itself influence changes in dietary habits (100), where this awareness of being observed may reduce the energy intake and further may deviate the diet intake from the true intake (101). However, in the context of a dietary intervention trial, this can be utilized to remind the participants to follow their diets and further increase the compliance, which should be considered a strength. Indeed, the compliance were considered as good in the original trial (67), as only 2 of the 38 included participants did not report their food intake, further supported by the expected differences in RER estimates which reflect the rate of fat oxidation.

5.2.2 Diets

An important feature in the current trial was to reduce potentially dietary factors influencing circulating markers. A limitation in nutritional research is that we are always exposed to diet where different dietary habits and the nutritional status may be an influencing factor on the

outcome of interest (100). The strength in the current trial is the control of factors in the diet that potentially could lead to between-group differences in plasma concentrations of the outcome metabolites. The diets were similar in protein (E%) and energy content (8750 kJ), only varying in quantity of fat and carbohydrates. Another feature was the consumption of the same food products. Health effects depend partly by types and sources of carbohydrates and fat, where an intake of different sources of fat and carbohydrates varying in quality could lead to differences. Thus, as these factors affect both groups equally, there is unlikely that these factors contribute to differences in plasma concentrations of the outcome metabolites. Another important feature is that the LFHC diet reflect an average Norwegian diet (18% protein, 34% fat and 43-44% carbohydrates) (102), allowing to control the effects of a VHFLC diet to a diet that is considered as normal in the Norwegian population. However, consuming different quantity of the same food products may lead to differences in the micronutrient intake, which possibly could be a factor explaining the observed between-group differences.

5.2.3 Outcomes

The outcome metabolites studied were quantified at three measurements points, allowing us to address short-term acute changes. When interpreting markers in blood samples, some considerations need addressing. First, the outcome metabolites were assessed in a fasting state (>10 h). Plasma concentrations has reported to vary in response to diets, where for instance variation in plasma concentrations of cystathionine has been reported in response to meal intake (103), and decreasing plasma concentrations of PLP have been reported within hours after a high carbohydrate intake (57). Thus, the most reliable measurements are believed to be in a fasting state. However, variation in plasma concentrations have still been detected even after long-term fasting where metabolites show different response to the fasting duration (104), which may be a contributing feature explaining some of the observed variation in the plasma estimates. To reduce this variation, the most optimal would be to ensure that the fasting state, the time since the last meal to the sampling, are equal for each participant. Secondly, intraindividual variation may also be a factor influencing plasma metabolites. However, as indicated by the good to excellent ICC for the outcome metabolites, the within-variation in the plasma measurements may not be explained by random fluctuations, but rather other influencing factors such as weight loss and effects of diet exposure.

Another aspect when interpreting circulating markers is the uncertainty of what the plasma concentrations reflect. For instance, plasma concentrations of choline have previously not reported to be affected by dietary choline intake (105). Accordingly, we did not observe any between-group differences in plasma choline. However, diet may still activate metabolic responses within the body that are not detectable in plasma, where a higher intake of choline may compensate by reducing the endogenous production. Changes in plasma might reflect changes in tissue distribution, cellular release to the circulation, renal excretion, production, expenditure, and storage, and not necessarily increased or decreased flux through one-carbon metabolism. We cannot precisely know what metabolic process or distribution of the metabolite the observed plasma concentration reflects. Decreasing methionine levels may not necessarily mean increased uptake in tissues, it might also reflect reduced availability. Metabolites are also distributed in different metabolic tissues, where an increased uptake in one tissue may lead to a decrease in another. The complexity increases as the demand of metabolites are determined by an interaction of various factors such as genetics, environmental factors, stress level, biological factors, and gut microbes. Thus, the role of diet exposure on the *metabolic phenotype* may be different between individuals and across populations.

5.2.4 Bias

Selection Bias

Bias is unavoidable in clinical research, which is any deviation leading to conclusions that necessarily is not correct, whereof the errors arising when selecting or allocating the study participants in a trial is referred to as *selection bias* (106). Due to the randomization, the risk of *allocation bias* is minimized, meaning that the results should not be influenced by systematic differences in who was assigned to which group (96). However, an aspect of selection bias under consideration is *attrition bias*, referring to the bias induced by the loss of study participants after the randomization process (96). In the current study, n = 8 participants withdrew after randomization (n = 4 in LFHC group, n = 4 in VHFLC group). This could potentially bias the results as the characteristics of the 8 participants that withdrew could have differed compared to participants completing the study. Another phenomenon under consideration is a variant of nonresponse bias called the *healthy volunteer effect* (107). Participants volunteering to trials are more likely to be health-conscious (*e.g.,* not smoking, consume healthier food, higher level of exercise). If all included participants are generally more health-conscious, they may not represent other populations consisting of less health-conscious

people nor be representative toward the source population. If selection bias is present, it may decrease the *external validity*, where the results observed in the population under study may be less generalized into other populations (96).

Information bias

Information bias is the error occurring when collecting data and measuring the exposure or outcome of interest, which may deviate the results from the truth (106). Regarding the current study, information bias may be of concern when analyzing the outcome metabolites in form of random measurement error. Random measurement error are errors fluctuating both positively and negatively around the true value to different extent, that combined are expected to give an average of zero (96). Such errors would reduce the precision resulting in wider compatibility intervals surrounding the effect estimates. However, Bevital has reported great within- and between-day reproducibility (www.bevital.no), limiting the potential uncertainty related to the analytic variation. In addition, the uncertainty related to intraindividual fluctuations in metabolite concentrations is not regarded a major issue in the current study, as indicated by the good to excellent ICC.

5.2.4 Statistical methods

As we mainly wanted to explore the response of diet exposure on plasma concentrations of the outcome metabolites, descriptive statistics and graphic presentation of data were considered as useful approaches to observe the direct changes and potential between-group differences (108). For simplicity, we used arithmetic means to characterize the tendencies and standard deviations (SD) to describe the variance. As metabolites typically follows a log-normal distribution (108), we could have selected other parameters, such as geometric mean (geometric SD) or median (percentiles). This could better represent the true distribution of the plasma metabolites, but the interpretation is more complicated. Nevertheless, future studies could beneficially consider the log-normal distribution approach (108).

Results are presented as mean difference (95% CI). In line with recent recommendations from American Statistical Association (91) and others (90), we interpret p-values as a continuous measure of compatibility between the data and the corresponding null hypothesis of no between-group differences. Accordingly, we did not dichotomize the results into categories of statistical significance. Interpreting results in terms of the presence or absence of statistical significance have some major limitations, such as erroneously interpreting two results to be in

conflict when one is significant while the other not, when they may be largely consistent. In the current thesis, this dichotomization would declare the between-group differences for plasma methionine (p = 0.05) as significant, while for tHcy (p = 0.07) as not significant, which would be misleading as they are trending the same direction. We emphasized the use of CI, which contains a range of parameter values more compatible with the data compared to the values outside the CI, and thus provide information on both the magnitude and direction of any potential difference, not just whether the difference is different from zero (90). The calculations of SMD were also considered as a good approach, as it allows to compare the effects on a common scale. The conventional cutoffs of what is considered a small, medium, and large effects should, however, be interpreted with caution. SMD is a continuous effect measure, and the cutoffs should be used as a starting point when interpreting results, and not should be interpreted with caution, as an effect may still be large even though it is not a bright line rule.

An important feature in our linear regression analysis was the adjustments of the end values at 12 weeks for baseline values. When analyzing continuous outcomes such as plasma biomarkers, the baseline concentrations is a major predictor of the follow-up concentrations. An extreme value is likely to be closer to the mean in the next measure, a phenomenon referred to as *regression to the mean* (109). Unadjusted comparisons between groups may therefore be biased, and the recommended approach is to include the baseline value as a covariate (109). However, in the context of being an RCT, it should not necessarily make a big difference, as we could expect the groups to be similar at baseline. However, in smaller studies we could always expect some differences, which we see for some metabolites such as sB12 (566 pmol/L in LFHC, 496 pmol/L in VHFLC), PA (80 nmol/L vs. 54 nmol/L) and NAM (821 nmol/L vs. 764 nmol/L), where the adjustment for baseline was considered a good approach. The main objective of this study was to compare the two groups, and hence we did not include any analysis of within-group change from baseline, as this has previously been shown to be a misleading approach to analyze RCT-data (92).

A major limitation in the current study is the limited sample size, where the smaller data provide less precision when estimating parameter estimates such as mean difference, as the CI is constructed using the standard error of the mean, which is directly influenced by sample size. Therefore, the large uncertainty in the observed differences may be related to the relatively small sample size, leaving the data largely inconclusive as evident by the p-values

and the wide 95% CIs. However, we cannot rule out the possibility of there being an effect of the diets, but a larger population would be needed to make more definitive inferences. Indeed, our effect estimates provided information of the magnitude on the between-group differences, which could be meaningful and should aid future large-scale studies to provide more insight into the role of diet composition.

5.3 Discussion of main findings

The most obvious explanation of the observed between-group differences of lower plasma methionine, tHcy, cystathionine and greater DMG and serine levels in the VHFLC group compared to the LFHC group may be due to the different amount of food consumed, which could provide different quantities of methyl groups, substrates, and B-vitamins. Both groups had on average the same protein (17 E%) and energy (8750 kJ/d) intake throughout the study. However, the VHFLC group consumed twice the amount of energy derived from animal products compared to the LFHC group, such as meat and meat products (24 E% vs. 9.8 E%), fish (2.8 E% vs. 1.4 E%), and eggs (6.6 E% vs. 3.3 E%). Animal products are in general high in amino acids, riboflavin, niacin, vitamin B6, and cobalamin, and we would expect that a higher intake of these metabolites in the VHFLC group. A higher intake of methionine would provide more methionine available for SAM synthesis, and thus yield Hcy more favored to enter the transsulfuration pathway. As we observed a greater decrease in plasma methionine (-2.15 µmol/L) in the VHFLC group compared to the LFHC group, it might be indicative of larger Hcy production. We observed increasing levels of plasma tHcy through the intervention in both groups, which might be indicative of increased Hcy production, of where the slightly lower plasma tHcy levels (-1.05 µmol/L) in the VHFLC group compared to the LFHC group may be reflective of greater flux through the transsulfuration pathway. This may also explain the observed trends in plasma cystathionine. Cystathionine is produced in a condensation of Hcy and serine catalyzed by CBS (6). The higher intake of animal products in the VHFLC group, and thus higher dietary intake of methionine and serine, may have contributed to a higher availability of serine and Hcy to condense to cystathionine. Since we observed a greater decrease in plasma cystathionine (-0.08 µmol/L) in the VHFLC group compared to the LFHC group, it might suggest a greater flux in the transsulfuration pathway in the first group compared to the latter. This may also be supported by the higher plasma levels of serine (9.03 µmol/L) in VHFLC diet compared to the LFHC diet at the end of study,

where the greater serine levels may be reflective of the higher serine intake during the intervention.

The observed between-group difference for plasma tHcy may also be due to differences in B-vitamin intake, as plasma tHcy are inversely associated with folate, vitamin B6, and cobalamin. Folate and vitamin B6 are widely distributed in foods, while cobalamin is mainly present in foods of animal origin. Thus, the higher intake of cobalamin-rich animal products in the VHFLC group are most likely to be a contributing factor for the lower plasma concentrations of tHcy observed in this group compared to the LFHC group. This may be supported by the more delayed decrease in sB12 in the VHFLC group compared to the LFHC group, which may be due to the higher intake of animal products. Another influencing factor of plasma tHcy is smoking, as a dose-response relationship between smoking cigarettes and plasma tHcy has been implicated (110), suggesting that there might be between-group differences in the number of cigarettes smoked. Although the smokers were equally distributed in both diet intervention groups (30% in VHFLC group, 33.3% in LFHC group), the LFHC group had higher plasma cotinine levels at all measurement points, where the greatest difference was observed at 8 weeks (409 nmol/L in the LFHC group, 259 nmol/L in the VHFLC group, data not shown). Thus, increasing level of plasma tHcy in both diet groups may be influenced by smoking, whereof the higher levels observed in the LFHC group may at least partially result from higher recent smoking exposure as reflected by the higher cotinine levels.

The higher levels of DMG (0.45 µmol/L) observed in the VHFLC group may be due to the higher dietary intake of fat compared to the LFHC group. Short-term and long-term PPARα-activation has reported to decrease DMG catabolism by downregulating DMGDH and SARDH and further increase plasma concentrations of DMG (82,83,111). As dietary fatty acids are reported to bind and activate PPARα (81), the higher intake of fat in the VHFLC diet group may increase the activity of PPARα, and may further explain the higher plasma levels of DMG observed in this group compared to the LFHC group. Notably, we observed similar increasing trends of plasma DMG concentrations in both diet groups in the first interval (0-8 weeks), where it declined for the LFHC group while stabilized in the VHFLC group in the last (8-12 weeks). As plasma DMG increased similar in both diet groups toward week 8, PPARα-activation by dietary fat might not fully explain the observed alterations in this interval, as we would expect a stronger activation in the VHFLC group and further higher

plasma DMG. However, as PPARα are involved in energy metabolism and essential in starvation (112), it is plausible that the increase in both groups are mediated by some PPARα-activation as a response to weight loss, of where the observed between-group differences from week 8 toward week 12 are due to different PPARα-mediated adaptions to diet. Thus, it may be likely that other metabolites also are affected different PPARα-mediated adaptions to diet such as plasma serine, since plasma serine have reported to increase in animals treated with PPARα-agonists (113). However, since we did not measure PPARα- expression or activity, this remains speculation. In addition, the higher increase of plasma DMG may also be due to the lower plasma concentrations of riboflavin and FMN observed in both groups, as both DMGDH and SARDH are flavoproteins (50).

Other diet interventions have observed higher plasma cystathionine and serine by replacing ~6.5 E% dietary SFA with n-6 PUFAs in healthy subjects compared to control group after 8 weeks (68). This is consistent with the observed increase in plasma serine in the current study, but in contrast with the observed decrease in plasma cystathionine. They observed no between-group differences by improving fat quality on plasma tHcy (68), while another study reported alterations in plasma tHcy in a context of weight loss by consuming a diet composed of 32E% from fat, 17E% from protein, and 47E% from carbohydrates in women with overweight (98). Plasma tHcy were reported to increase toward 8 weeks and further flattened toward 16 weeks, compared to a similar diet supplemented with betaine (98). As we also observed an increase of plasma tHcy toward week 8 in both groups, which were rather stable toward week 12, this trend may be consistent to our findings. However, tHcy has reported to increase in the context of weight loss (114,115). Decreasing plasma concentrations of methionine and cystathionine has also been reported in response to weight loss (114), which is consistent to the observed tendencies in current study. We observed a positive correlation between methionine and cystathionine at baseline (r = 0.80), which may suggest that the metabolites respond similarly to metabolic changes. Lower plasma concentrations of serine has been associated with visceral obesity (116), and thus, increased circulating levels of serine may be a response to weight loss. The weight loss may be likely to explain the similar tendencies observed for the metabolites in both groups. However, as the weight loss were on average similar in each group (-12.2 kg LFHC, -11.8 kg VHFLC), the weight loss is not likely to explain the observed between-group differences in plasma concentrations.

In addition to the context of weight loss, the participants under study had metabolic syndrome which also could be a contributing factor for the observed tendencies. Individuals with metabolic syndrome have been observed to have increased risk of oxidative stress and low-grade inflammation (117), and metabolic markers related to oxidative stress and inflammation were found to improve in response to weight loss (117). Regarding one-carbon metabolism, increased oxidative stress has been reported to inhibit remethylation and activate transsulfuration (118,119). Thus, improvements of oxidative stress may influence the flux of Hcy being remethylated or catabolized in the transsulfuration pathway, and further be a factor influencing the plasma concentrations of metabolites. PPARα activation are believed to increase in response to weight loss (112), and reported to reduce both inflammation and oxidative stress (120,121). Thus, these regulations may partially explain the observed alterations in plasma concentrations of the metabolites, such as for plasma DMG (119).

Another factor that may influence the plasma concentrations is the usage of vitamin supplements. The participants used vitamin supplements containing B-vitamins 8 weeks prior the intervention, which may explain the overall decreasing mean trend for the B-vitamin markers from baseline toward week 12 in both groups. The decreasing trends in plasma concentrations may reflect a natural decrease as a response to the discontinuation of vitamin supplements. As mentioned, the decreasing trend of plasma concentrations of the B-vitamins may also be a contributing factor for the observed increase in plasma tHcy in both groups due to the inverse association with folate, vitamin B6, and cobalamin. This natural decrease may also indicate that the plasma concentrations were higher than it would have been in participants not using supplements. Further, if the participants used supplements during the trial, it might also explain some of the observed variation in plasma concentrations. However, the usage was not reported. Overall, as both groups received the same vitamin supplements, it is unlikely to explain between-group differences.

6. Conclusion and future perspectives

In this exploratory study among men with abdominal obesity, we observed decreasing plasma levels of methionine, tHcy, and cystathionine while increasing DMG and serine by consuming a diet more concentrated in fat compared to a diet more concentrated in carbohydrates for 12 weeks. However, the observed between-group differences were considered small. Uncertainty in the data is mainly believed to be caused by the small sample size, leaving the findings inconclusive. The observed direction and tendencies of most plasma concentrations are suggested to be mainly a response to weight loss. Thus, future studies should preferably include a larger sample size and investigate the effect on the metabolites outside the weight loss context. Further, dietary effects should be explored in both sexes, collecting both genetic information as well as repeated blood samples, to disentangle the dietary regulation of one-carbon metabolism. The clinical relevance of the findings at this point is uncertain. Since higher concentrations of several one-carbon metabolites have been associated with increased risk of chronic diseases, future studies on the effect of dietary composition are warranted.

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