Treatment with a Peroxisome Proliferator-Activated Receptor agonist influences one-carbon metabolism and markers of B-vitamin status in rats

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Summary

Introduction: Elevated plasma concentration of homocysteine (Hcy) has been considered as a candidate risk factor of cardiovascular disease (CVD), but lowering Hcy with B-vitamins has not been demonstrated to improve prognosis among CVD patients. It has thus been suggested that elevated Hcy serves only as a marker of underlying pathology, which prompts investigation into possible related mechanisms.

The choline oxidation pathway is directly linked to remethylation of Hcy to methionine, and increased systemic concentrations of metabolites along this pathway have previously been linked to major lifestyle diseases, including CVD. Choline-related remethylation of Hcy yields dimethylglycine (DMG). Notably, DMG has been demonstrated to be a strong predictor of CVD events and mortality, independent of other risk markers such as elevated Hcy levels.

The peroxisome proliferator-activated receptor (PPAR) α is a nuclear receptor and key regulator of energy metabolism which is activated by both dietary fatty acids and synthetic ligands such as fibrates. This receptor has recently been demonstrated to influence the genetic transcription of key enzymes of the choline oxidation pathway, and increased PPARα activity was thus suggested to be a possible underlying mechanism for the association between DMG and CVD.

Objective: The aim of the current study was to investigate the relationship between PPARα and one-carbon metabolism, with emphasis on the choline oxidation pathway. Most of the reactions in the metabolic pathways discussed depend on B-vitamins as cofactors, and hence we also assessed the associations between PPARα activation and systemic markers of B-vitamin status. A targeted metabolomic approach was implemented to investigate these relationships in a substudy of a long-term animal model.

Methods: During 50 weeks, 30 male Wistar rats were randomized to receive ad libitum of either a low fat control diet, a high fat (HF) diet or a HF diet supplemented with tetradecylthioacetic acid (TTA), a pan-PPAR agonist with pronounced affinity towards PPARα. At the end of the study, the animals were sacrificed under non-fasting conditions. Blood was drawn by cardiac puncture and urine collected directly from the urinary bladder. Metabolite concentrations were determined by gas and liquid chromatography coupled to tandem mass spectrometry. The groups were compared by one-way ANOVA, and planned

comparisons versus the control group were made for both intervention groups. Cohen's d effect sizes were calculated and reported for all comparisons, and d > 0.8 was considered a large effect. As the results were not adjusted for multiple comparisons, p-values < 0.01 were considered statistically significant.

Results: When comparing TTA-treated animals to the control group, TTA-treated animals had statistically significantly higher plasma DMG (d=5.05), glycine (d=1.3), serine (d=1.99), cystathionine (d=1.52), nicotinamide (d=6.4), methylnicotinamide (d=4.05), methylmalonic acid (d=3.98) and pyridoxal (d= 2.73), whereas plasma riboflavin (d=-1.6) and flavin mononucleotide (d=-2.22) were lower. Urinary concentrations of DMG (d=1.98), sarcosine (d=1.16) and methylmalonic acid (d=1.89) were higher among TTA treated rats.

When comparing HF to the control group, no statistically significant differences were observed on either plasma or urinary concentrations of one-carbon metabolites or markers of B-vitamin status. However, the small sample size may have impeded statistical significance, as some of the differences were considered large. No differences were observed in urine according to dietary fat intake.

Conclusion: Long-term TTA treatment was associated with altered blood and urinary concentrations of one-carbon metabolites and markers of B-vitamin status in male Wistar rats. Particular large differences were observed for plasma DMG, NAM, mNAM and MMA, which were all higher among TTA-treated rats. Based on current and previous results, these effects are probably mainly mediated through PPARα. Our findings add to the evidence that one-carbon metabolism may be regulated by PPARs, and these candidate biomarkers of PPARα activity may prove to supply useful information which could help identifying human subgroups who will have clinical benefit of certain nutritional advice.

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Tables and figures

Abbreviations

16:0/18:1-GPC	1-palmitoyl-2-oleyl-sn-glycerol-3-phosphocholine
ANOVA	analysis of variance
BHMT	betaine-homocysteine methyltransferase
Cbl	cobalamin
CBS	cystathionine-β-synthase
CGL	cystathionine-γ-lyase
CHD	coronary heart disease
CVD	cardiovascular disease
CoA	coenzyme A
DMG	dimethylglycine
DMGDH	dimethylglycine dehydrogenase
FMN	flavin mononucleotide
GC-MS/MS	gas chromatography – tandem mass spectrometry
Hcy	homocysteine
HF	high fat
LC-MS/MS	liquid chromatography – tandem mass spectrometry
Met	methionine
MMA	methylmalonic acid
MMACHC	methylmalonic aciduria combined with homocystinuria type C protein
mNAM	N^1-methylnicotinamide
mTHF	5-methyltetrahydrofolate
MTHF	5,10-methylenetetrahydrofolate
MTHFD1	methylenetetrahydrofolate dehydrogenase 1
MS	methionine synthase
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
NAM	nicotinamide

PA	pyridoxic acid
PL	pyridoxal
PLP	pyridoxal-5-phosphate
PPAR	peroxisome proliferator-activated receptor
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SARDH	sarcosine dehydrogenase
tHcy	total homocysteine concentration
TTA	tetradecylthioacetic acid

1 Introduction

1.1 One-carbon metabolism

1.1.1 Homocysteine metabolism and transmethylation

Homocysteine (Hcy) is a sulfur-containing amino acid formed by demethylation of the essential amino acid methionine (Met) [1]. In the cell, Hcy has two metabolic fates, namely remethylation back to Met and transsulfuration to form cysteine, and three metabolic pathways are involved in what is referred to as the Met-Hcy cycle (Figure 1). Remethylation of Hcy regenerates Met, and involves either the choline oxidation pathway or the folate cycle, respectively. Both remethylation pathways contribute about equal [2]. Folate-independent remethylation is catalyzed by betaine-homocysteine methyltransferase (BHMT; EC 2.1.1.5), which utilizes betaine, a choline oxidation metabolite, as the methyl donor [3-5], while 5-methyltetrahydrofolate (mTHF) is the methyl donor for the folate-dependent remethylation carried out by the vitamin B12-dependent Met synthase (MS; EC 2.1.1.13) [6-9]. Both BHMT and MS contain zinc, which is important for binding and activation of Hcy [10, 11]. Hcy may also be irreversibly catabolized through the transsulfuration pathway [12, 13], which consists of two vitamin B6-dependent enzymes. First, cystathionine-β-synthase (CBS; EC 4.2.1.22) converts Hcy to cystathionine [14-16]. Second, cystathionine-γ-lyase (CGL; EC 4.4.1.1) hydrolyzes cystathionine forming cysteine [17, 18], the limiting factor for synthesis of the antioxidant glutathione [19]. CBS is an iron-containing enzyme [20], and contains an autoinhibitory domain which needs to be relieved for the enzyme to be activated [21].

The synthesis of Hcy from Met takes place during transmethylation reactions (Figure 1). Transmethylation in general refers to the transfer of a methyl group from a methyl donor to a methyl acceptor, and these reactions are involved in both synthesis and modifications of a large variety of molecules throughout the body [22]. S-adenosylmethionine (SAM) is the main methyl donor for these reactions, and is formed by adenosylation of Met catalyzed by Met adenosyltransferases (EC 2.5.1.6). Transmethylation by various methyltransferases leaves S-adenosylhomocysteine (SAH) [23], which is subsequently hydrolyzed into Hcy [24]. The SAM:SAH ratio is often used as a measure of the intracellular methylation capacity [25]. It is generally believed that the synthesis of creatine and phosphatidylcholine are the major sources for Hcy production [26].

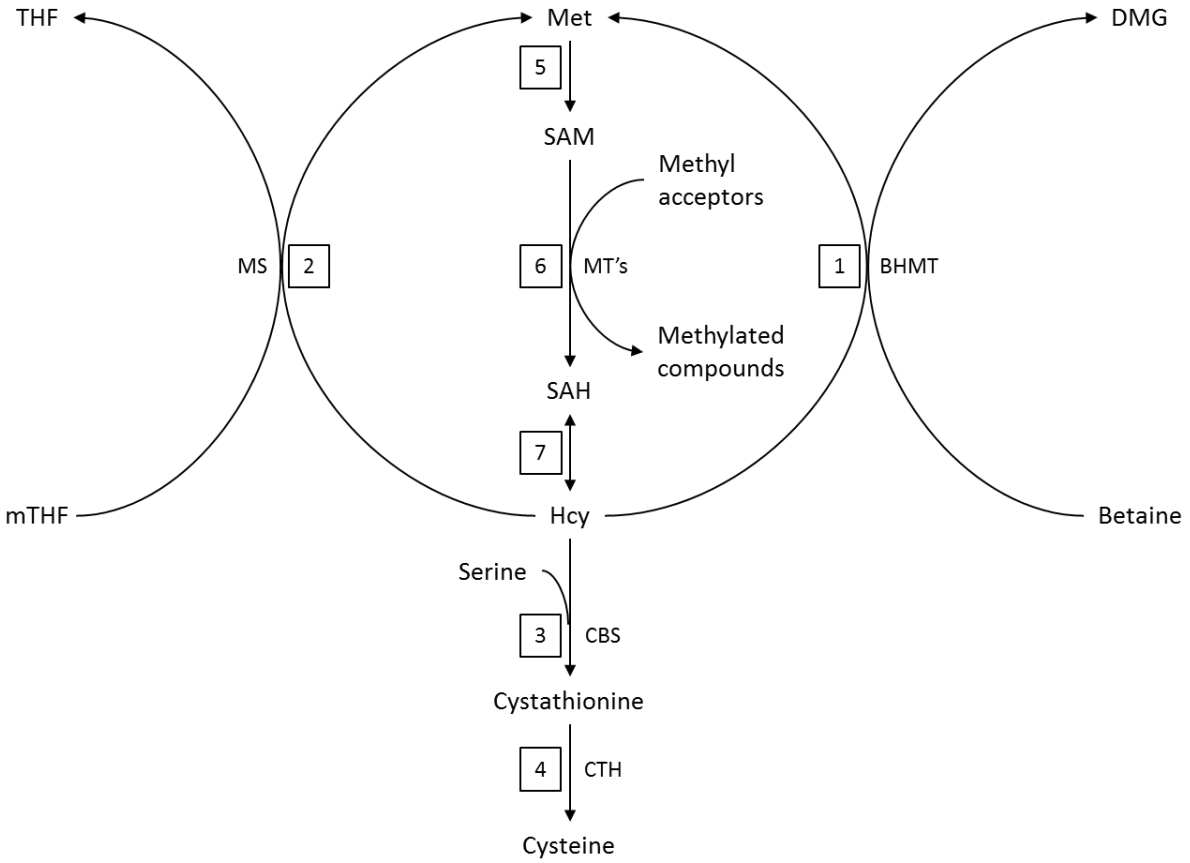

Figure 1 Reactions of the methionine-homocysteine cycle and transmethylation . Reaction 1 and 2 represents the two remethylation pathways, responsible for regeneration of methionine from homocysteine. They involve the choline (1) and folate (2) metabolism, respectively. Reaction 3 and 4 represents the transsulfuration pathway, responsible for catabolism of homocysteine. Reaction 5, 6 and 7 represents synthesis of the methyl donor S-adenosylmethionine, transmethylation and subsequent production of homocysteine. Abbreviations: BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine-β-synthase; CTH, cystathionine-γ-lyase; DMG, dimethylglycine; Hcy, homocysteine; Met, methionine; mTHF, 5-methyltetrahydrofolate; MT's, methyltransferases; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate.

The coordination between the three metabolic pathways using Hcy as a substrate is regulated by diet and the need for methionine conservation [2]. It is demonstrated that the dietary supply of methyl groups such as from Met, choline or betaine affects the partitioning between remethylation and transsulfuration, increasing remethylation when dietary supply is curtailed, such as during protein restriction [27, 28]. These metabolic pathways are also regulated by redox status, where increased oxidative stress by oxidation of the zinc and iron atoms of the enzymes inhibits remethylation [29, 30] and activates transsulfuration [19].

When intracellular concentrations of Hcy increases, excess Hcy is exported to the blood compartment. In blood, the majority of Hcy is found bound to protein, less as a disulfide with other sulfur compounds whereas only minor amounts are free Hcy. Total Hcy

(tHcy) includes all forms, and elevated tHcy in blood is referred to as hyperhomocysteinemia [31]. With increasing tHcy concentrations, two molecules of Hcy may condense and form homocystine, which can be excreted in the urine, a condition labeled homocystinuria [32]. Taken together, the intracellular concentration of Hcy is determined as a result of the rates of production, remethylation and transsulfuration, and the tHcy concentration in blood is dependent on cellular release and renal excretion.

Elevated plasma tHcy has consistently been linked to increased risk of cardiovascular disease (CVD) and coronary heart disease (CHD) [33]. Trials aiming to lower plasma tHcy with B-vitamins have, however, not yielded favorable results [34, 35], indicating that elevated tHcy should be regarded as a marker of underlying pathological mechanisms rather than a true causal risk factor [36]. This encourages investigation into alternative mechanisms which may explain the relationship between elevated tHcy and adverse prognosis. In addition to CVD including CHD, elevated tHcy has also been linked to a myriad of other adverse health outcomes like cancer [37], pregnancy complications [38], neural tube defects [39, 40], congenital defects of the heart [41], osteoporosis [42], Alzheimer's disease and dementia [43, 44], depression [45], cognitive decline [46], hyperinsulinemia [47] and type 2 diabetes [48].

1.1.2 The choline oxidation pathway

Choline is a water-soluble quaternary ammonium compound which can be obtained through diet or synthesized *de novo* by methylation of phosphatidyletanolamine catalyzed by the enzyme phosphatidyletanolamine N-methyltransferase (EC 2.1.1.17). In the body, choline is first and foremost found incorporated in phospholipids as phosphatidylcholine [49]. The dietary requirements for choline vary according to the capacity for *de novo* synthesis, and among dietary sources of choline are egg yolks, meat, soybeans, nuts and wheat [50]. Choline is oxidized through several steps (Figure 2), ultimately leading to the synthesis of glycine which can be interconverted with serine. The intermediate metabolites include betaine, dimethylglycine (DMG) and sarcosine [51]. The first metabolite, betaine, has two functions. Besides being the methyl donor for BHMT, it is an important osmolyte involved in the regulation of cell volume. Like choline, betaine is also found in the diet, and rich sources include wheat, spinach and vegetables of the beet family [52]. BHMT serves as a direct link between the choline oxidation pathway and Hcy remethylation, and accordingly, dietary intake of both choline and betaine has been demonstrated to lower plasma tHcy, as well as improving other CVD risk factors such as inflammation markers, although no significant

association between dietary choline/betaine and CVD incidence has been reported [53]. Induction of *BHMT* mRNA by betain-enriched diets has previously been demonstrated to be closely related to the production of hepatic apolipoprotein B and to increase the secretion of very-low-density lipoprotein from the liver, but with no subsequent alterations in circulating lipid levels [54].

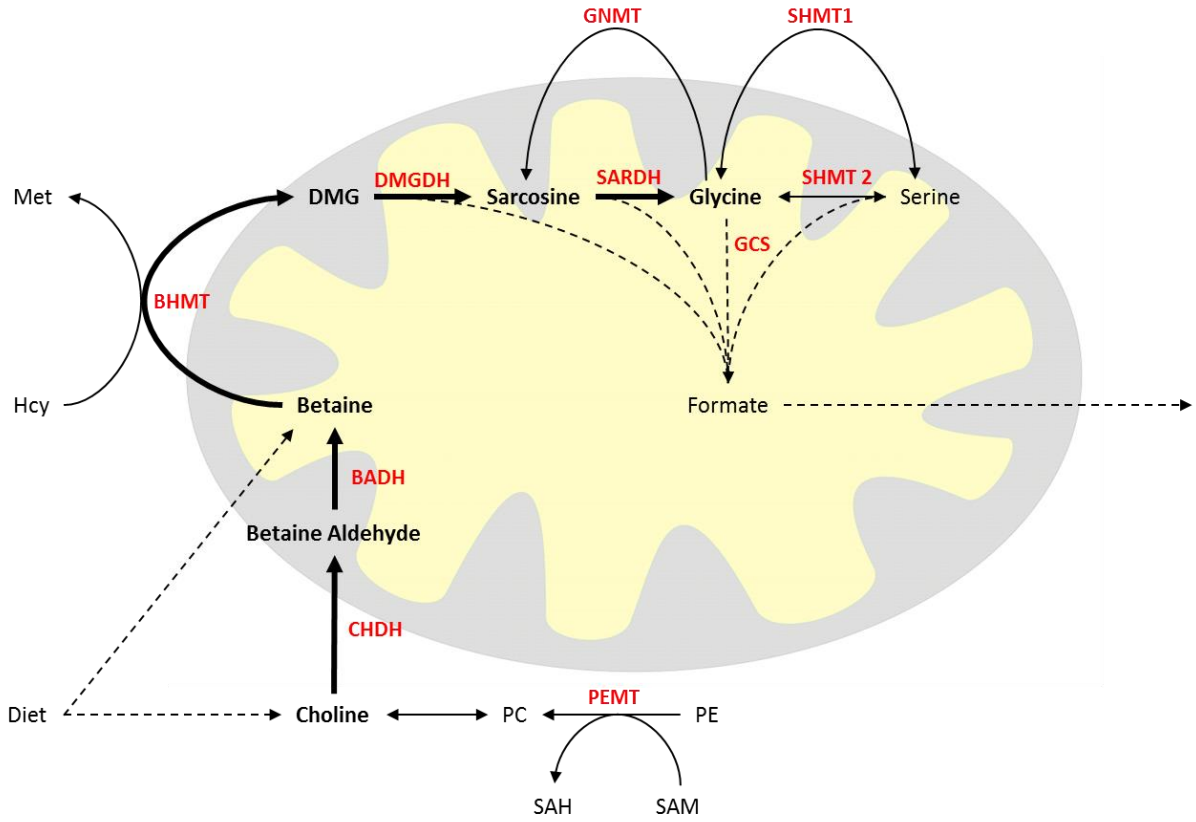

Figure 2 The choline oxidation pathway. Main metabolites are highlighted in bold text and enzymes in red color. Choline may be obtained from the diet or from endogenous synthesis. DMG, sarcosine, glycine and serine all donate a one-carbon unit which may leave the mitochondrion as formate. Abbreviations: BADH, betaine aldehyde dehydrogenase; BHMT, betaine-homocysteine methyltransferase; CHDH, choline dehydrogenase; DMG, dimethylglycine; DMGDH, dimethylglycine dehydrogenase; GCS, glycine cleavage system; GNMT, glycine N-methyltransferase; Hcy, homocysteine; Met, methionine; PC, phosphatidylcholine; PE, phosphatidyletanolamine; SAH, s-adenosylhomocysteine; SAM, s-adenosylmethionine; SARDH, sarcosine dehydrogenase; SHMT, serine hydroxymethyltransferase

Choline is actively transported into the mitochondrion and converted to betaine by two enzymes, choline dehydrogenase (EC 1.1.99.1) and betaine aldehyde dehydrogenase (EC 1.1.1.8) [55, 56]. Betaine diffuses to the cytosol and acts as the methyl donor in the BHMT-mediated remethylation of Hcy, forming Met and DMG [4]. DMG diffuses into the mitochondrion, where two subsequent demethylation reactions form sarcosine and glycine, catalyzed by DMG dehydrogenase (DMGDH; EC 1.5.8.4) and sarcosine dehydrogenase (SARDH; EC 1.5.8.3), respectively [57]. In the cytosolic compartment, glycine *N*-

methyltransferase (GNMT; EC 2.1.1.20) catalyzes a SAM-dependent methylation of glycine to form sarcosine [58]. This reaction is regarded an important mechanism in the regulation of methylation capacity, both by scavenging of excess methyl groups [59] and by producing SAH which inhibits methyltransferases [60]. Interconversion between glycine and serine, catalyzed by serine hydroxymethyltransferase (SHMT; EC 2.1.2.1) may also be regarded as a part of the choline oxidation pathway. Both a cytosolic [61] and mitochondrial [62] isoform of SHMT exist, referred to as SHMT1 and SHMT2, respectively. The methyl group derived from DMG, sarcosine and serine demethylation is transferred to folate and is further metabolized to formate which may leave the mitochondrion and enter the cytosolic one-carbon pool, as illustrated in Figure 2 [63]. Glycine may also be catabolized through the trifunctional glycine cleavage system (EC 1.4.4.2, 2.1.2.10, and 1.8.1.4), ultimately yielding one molecule of formate [64].

Circulating and urinary concentrations of metabolites in the choline oxidation pathway has previously been linked to major lifestyle diseases. Elevated whole blood or plasma choline concentrations were shown to be valuable predictors of cardiac events among patients with suspected acute cardiac syndrome [65, 66]. Furthermore, high concentrations of plasma choline was recently associated with incident acute myocardial infarction among patients with stabile angina pectoris, primarily non-smokers [67]. Both the highest and lowest quintile of urinary betaine concentrations, as well as high plasma concentrations have been linked to increased risk of secondary events among CVD patients [68], and elevated urinary excretion of betaine was associated with diabetes in patients with stabile angina pectoris [69]. High plasma betaine concentration has also been suggested to be related to lower risk of colorectal cancer [70]. In a nested case-control study of prostate cancer, high circulating sarcosine and glycine concentrations were found to be associated with a modestly reduced risk [71]. However, in a recent report, a higher plasma sarcosine concentration was found to be associated with increased prostate cancer risk [72]. Elevated plasma concentrations of DMG has been strongly associated with increased risk of acute myocardial infarction as well as both all-cause and cardiovascular mortality among CVD patients, independent of traditional risk factors like plasma tHcy, lipid levels, smoking, hypertension, diabetes and impaired kidney function [73, 74]. Also, the metabolism of glycine and serine and its relation to one-carbon metabolism is a hot topic in modern cancer research [75].

1.1.3 Folate-mediated one-carbon metabolism

Folate is a water-soluble vitamin which is present in the cell as several interconvertible coenzymes. The reduced form of the vitamin, tetrahydrofolate (THF), is the main intracellular form, which can bind activated one-carbon moieties [76, 77]. In the diet, folate is naturally occurring in green leafy vegetables and certain legumes such as beans, soy and peanuts, but in large parts of the world the main dietary source is grain products, due to mandatory fortification with folic acid [76]. The intracellular metabolism of folate, illustrated in Figure 3, is referred to as the folate cycle, and takes place in the mitochondrion, the nucleus and the cytosolic compartment, where distribution of the different cofactor forms differ [78, 79]. Impairment of folate metabolism or folate deficiency are linked to an increased risk of several diseases, including neural tube defects and congenital heart defects, megaloblastic anemia, various cancers and CVD [80, 81].

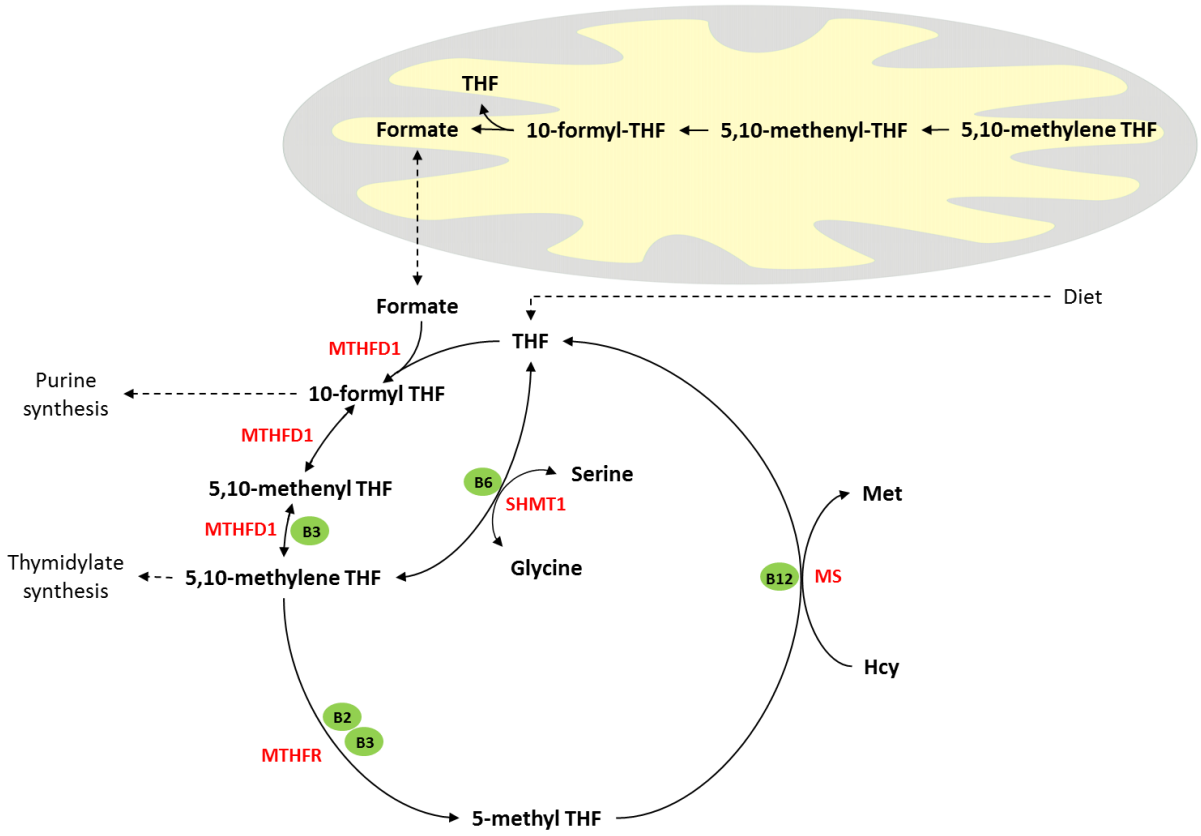

Figure 3 The cytosolic and mitochondrial folate cycle. Formate may cross the mitochondrial membrane and connects the cytosolic and mitochondrial folate metabolism. The different folate cofactors serve as one-carbon donors in purine and thymidylate synthesis, as well as in the remethylation of homocysteine. Abbreviations: Hcy, homocysteine; Met, methionine; MS, methionine synthase; MTHFD1, methylenetetrahydrofolate dehydrogenase 1; MTHFR, methylenetetrahydrofolate reductase; SHMT1, cytosolic serine hydroxymethyltransferase; THF, tetrahydrofolate.

The predominant circulating form of folate is mTHF [82], but even though plasma folate is believed to reflect intracellular concentrations, plasma mTHF has not been demonstrated to correlate very well with neither tissue nor liver mTHF [83].

The folate cycle involves several enzymes which provide the different folate cofactor forms. A trifunctional enzyme complex, methylenetetrahydrofolate dehydrogenase 1 (MTHFD1; EC 6.3.4.3, 3.5.4.9 and 1.5.1.5), catalyze three enzymatic reactions ultimately forming 5,10-methylenetetrahydrofolate (MTHF) from THF, using formate as the one-carbon source [84, 85]. Alternatively, MTHF may be formed from THF by the cytosolic SHMT1, using a one-carbon group from serine, a reversible reaction which also yields glycine [61, 86, 87]. Mainly, two enzymes compete for MTHF as substrate. Thymidylate synthase use MTHF as a one-carbon source for thymidylate, and hence nucleotide, synthesis, whereas MTHF reductase (MTHFR) irreversibly reduces MTHF to form mTHF for Hcy remethylation [88-90]. It has been demonstrated that MTHF derived from SHMT1 is preferentially used for thymidylate synthesis and not remethylation [91, 92]. The folate cycle is completed when mTHF donates its methyl group to Hcy, regenerating free THF [93]. Formate, provided by the mitochondrial folate cycle from sources such as the choline oxidation pathway, conversion of serine to glycine and glycine catabolism through the glycine cleavage system (Figure 2), is able to cross the mitochondrial membrane [63], and thus connects the mitochondrial and cytosolic folate metabolism.

1.2 B-vitamins

B-vitamins are water-soluble essential nutrients with a myriad of physiological functions as cofactors in enzymatic reactions. Some of these vitamins are of importance of this thesis, and will be presented in this chapter.

1.2.1 Vitamin B2

Vitamin B2, also known as riboflavin, is obtained from the diet mainly from eggs, dairy products such as milk or cheese, meat and some vegetables. In blood, riboflavin is transported bound to albumin and globulins [94]. However, after uptake to the cells, the vitamin is mainly found in its two cofactor forms; flavin mononucleotide (FMN) and flavin adenine dinucleotide, formed from riboflavin by the enzymes riboflavin kinase (EC 2.7.1.26) and flavin adenine dinucleotide synthetase (EC 2.7.7.2), respectively [95, 96]. These compounds act as coenzymes for numerous flavoproteins, and clinical signs of deficiency include growth retardation, hair loss, dermatitis and normocytic anemia [94].

1.2.2 Vitamin B3

Vitamin B3 is known as niacin or nicotinic acid, and may be derived from the diet or from catabolism of the essential amino acid tryptophan, and dietary sources of niacin include meats and fish, cereals, vegetables and peanuts [97]. In its cofactor forms, nicotinamide dinucleotide (NAD) and NAD phosphate (NADP), vitamin B3 is essential for a vast number of enzymatic redox reactions [97]. Both β-oxidation of fatty acids and substrate oxidation in the Krebs cycle is dependent on NAD(P) as an electron carrier. NAD is formed from its precursors; nicotinamide (NAM) and nicotinic acid (NA), and the breakdown metabolite is N^1-methylnicotinamide (mNAM). Because half-life of plasma NAD is only a few seconds, measurement of the other vitamers and metabolites are used as a proxy for vitamin B3 status [97]. Deficiency of vitamin B3 is well known to cause Pellagra, which manifest as diarrhea, dermatitis and dementia. Also, pharmacological doses of niacin have been used to treat dyslipidemia [97].

1.2.3 Vitamin B6

Vitamin B6 is a vitamin family which by acting as cofactors is involved in wide variety of enzymatic processes. Vitamin B6 is present in a number of foods, both of plant and animal origin, and among the main sources are meat, especially organ meat, fish and whole-grains. The different vitamin forms are pyridoxal (PL), pyridoxine and pyridoxamine, and the vitamin is mainly excreted in urine as pyridoxic acid (PA) [98]. The only active cofactor form is 5'-phosphorylated PL, pyridoxal-5'-phosphate (PLP). Several enzymes are involved in the regulation and interconversion of the different vitamin forms [99]. Notably, the production of PLP seems to be dependent on sufficient vitamin B2 status [100]. The most common marker of vitamin B6 status is plasma PLP concentration, but the total amount of circulating B6 vitamers is frequently regarded a more precise measurement of B6 status [98]. Systemic vitamin B6 status has been inversely associated with both increased oxidative stress [101] and inflammation [102], which may be due to an increased demand for PLP during inflammation [103].

1.2.4 Vitamin B12

Vitamin B12, or cobalamin (Cbl), is a micronutrient primarily found in foods of animal origin, such as meat, fish, dairy and eggs [104]. The only known biological function of cobalamin is to serve as a cofactor for MS and methylmalonyl coenzyme A (CoA) mutase

(mut; EC 5.4.99.2) [6, 105-107], and deficiency of vitamin B12 thus leads to impaired enzyme activity.

Cbl may exist in three different oxidation states, Cbl(I), Cbl(II) and Cbl(III), and once inside the cell, free Cbl(II) is necessary for cofactor formation. To produce its active cofactors, the free Cbl(II) needs to be reduced to the highly reactive Cbl(I) before oxidative alkylation with a methyl (methylcobalamin, MeCbl) or adenosyl (adenosylcobalamin, AdoCbl) group [108]. The intracellular processing of Cbl (Figure 4) is complex, and involves several chaperones [108, 109]. One of the central chaperones, the methylmalonic aciduria combined with homocystinuria type C (MMACHC) protein, is responsible for making free Cbl(II) available to the cell, a process that involves both vitamin B2 and glutathione [110-113].

Mut is catalyzing the catabolism of methylmalonyl-CoA, an end product in the metabolism of odd chained fatty acids, branched-chained amino acids and cholesterol, to succinyl-CoA which can enter the citric acid cycle [104]. During cobalamin deficiency methylmalonyl-CoA is metabolized to methylmalonic acid (MMA).

MS is the only known enzyme to accept the methyl group of mTHF, thus uniquely linking folate and Cbl metabolism [93]. MS reductase (MSR; EC 1.16.1.8) is an enzyme crucial for the initial methylation forming MS bound MeCbl [114]. The methyl group is then transferred to Hcy, leaving MS-bound Cbl(I) ready to accept another methyl group from mTHF [115, 116]. Because of the reactive nature of Cbl(I), spontaneous oxidation to Cbl(II) occurs in about one out of every 2000 catalytic cycles through MS [117]. MSR is then necessary to reactivate the MS-Cbl(II) complex [114]. MSR, in combination with the reducing factor NADP, is shown to be sufficient to reactivate the MS complex after such oxidation [118].

Both blood or urinary MMAand elevated tHcy can be used as clinical markers of Cbl deficiency [104]. Circulating cobalamin is not necessarily a good marker of B12 status; a study among patients with diabetes demonstrated this by showing that circulating cobalamin did not reflect the intracellular status as measured by red blood-cell cobalamin, serum MMA and methylation status in these patients, [119]. The authors suggested this to be due to a metabolic B12 deficiency or B12 resistance, indicating that a functional Cbl deficiency may

be caused by disturbances in the intracellular Cbl metabolism and not related to circulating Cbl concentrations.

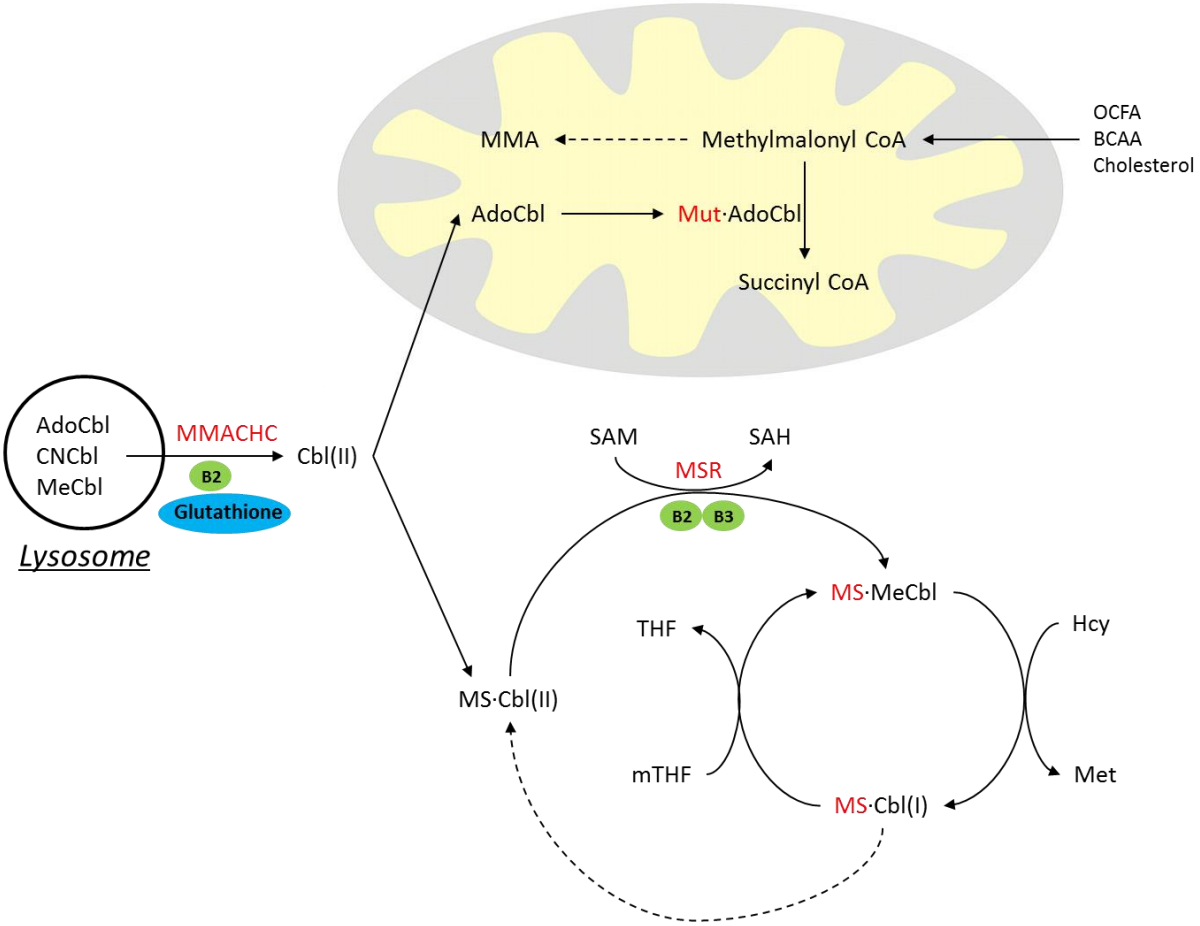

Figure 4 Intracellular cobalamin metabolism and cofactor generation. Abbreviations: AdoCbl, Adenosylcobalamin; ATR, Adenosyl transferase; BCAA, Branched-chained amino acids; Cbl, Cobalamin; CNCbl, Cyanocobalamin; Hcy, Homocysteine; MeCbl, Methylcobalamin; Met, Methionine; MMA, methylmalonic acid; MMACHC, methylmalonic aciduria combined with homocystinuria type C protein; MS, Methionine Synthase; MSR, Methionine Synthase Reductase; MS·MeCbl, Ms-bound MeCbl; MS·Cbl, MS-bound cobalamin; mTHF, Methyltetrahydrofolate; OCFA, Odd-chained fatty acids; SAH, S-Adenosyl Homocysteine; SAM, S-Adenosyl Methionine; THF, Tetrahydrofolate

1.2.5 B-vitamins and one-carbon metabolism

Folate, B6 and B12 status is known to influence plasma tHcy [120], but other B-vitamins are also involved as cofactors for reactions in the metabolic pathways related to one-carbon metabolism. Related to folate-dependent remethylation, vitamin B2 and B3 are important cofactors for both MSR [114, 118] and MTHFR [89]. In the transsulfuration pathway, both CBS and CGL are dependent on PLP [121, 122]. In the synthesis of betaine from choline, CHDH contains B2 [123] whereas BADH utilizes B3 [56]. Subsequently, during DMG catabolism, both DMGDH and SARDH are flavoproteins dependent on vitamin B2 [124], which also transfer a one-carbon unit from their substrates to folate [125]. Both cytosolic and

mitochondrial SHMT are dependent on vitamin B6 and also transfers a one-carbon unit from serine to folate [61, 62]. An illustration of all discussed metabolic pathways and the B-vitamin cofactors are provided in Figure 5.

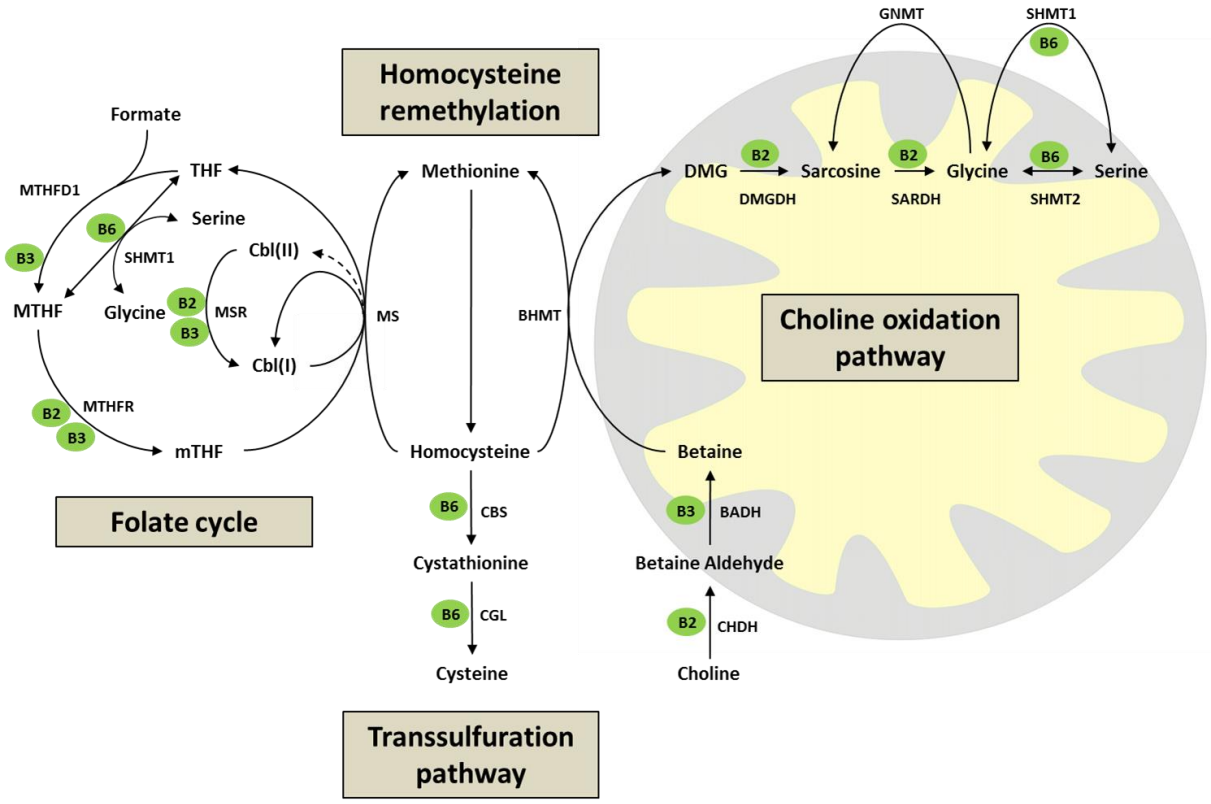

Figure 5 Illustration of relevant metabolic pathways, enzymes and B-vitamin cofactors. Abbreviations: BADH, betaine aldehyde dehydrogenase; BHMT, betaine-homocysteine methyltransferase; Cbl, cobalamin; CBS, Cystathionine-β-synthase; CGL, cystathionine-γ-lyase; CHDH, choline dehydrogenase; DMG, dimethylglycine; DMGDH, dimethylglycine dehydrogenase; GNMT, glycine N-methyltransferase; MS, methionine synthase; MSR, methionine synthase reductase; mTHF, 5-methyltetrahydrofolate; MTHF, 5,10-methylenetetrahydrofolate; MTHFD1, methylenetetrahydrofolate dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; SARDH, sarcosine dehydrogenase; SHMT, serine hydroxymethyltransferase

1.3 Peroxisome proliferator-activated Receptors

1.3.1 PPARs are nuclear receptors

Nuclear receptors are ligand-activated transcription factors which connect the cellular environment to the genome. The nuclear receptors are classified into three groups, the endocrine receptors, the orphan receptors and the adopted orphan receptors. These receptors share a common structure, and respond to hormones, vitamins or other signal molecules, which act as ligands. The NR-ligand-complex then binds to specific sequences on DNA called hormone responsive elements, and this mechanism is involved in regulation of gene

expression [126]. Peroxisome proliferator-activated receptors (PPARs) belong to the adopted orphan receptors [127], and their ability to become activated by peroxisome proliferators, hypolipidemic drugs known to result in proliferation of peroxisomes and liver hyperplasia in rodent models, was the basis for their identification in 1990 [128]. The PPARs exists either as the α, β/δ or γ subclass, respectively, being encoded by different genes [129], and the subtypes are activated by a large variety of natural and synthetic ligands [130]. Common for all PPAR subtypes is the requirement to form a heterodimer with another nuclear receptor, the retinoid-X-receptor, to be able to bind the PPAR responsive elements on target genes [131].

The PPARs are involved in a variety of metabolic functions, mainly fatty acid and glucose metabolism, as well as inflammation and other cellular processes like differentiation [129]. Because of this, synthetic ligands for PPARs have frequently been utilized in the treatment of metabolic conditions such as dyslipidemia, atherosclerosis, insulin resistance and diabetes mellitus [126]. Even though the three subtypes share a high degree of homology, their differences in ligand specificity and tissue distribution constitute the basis for subtype-specific functions. PPARα augments fatty acid catabolism and apolipoprotein synthesis, and is highly expressed in liver, heart, muscle and kidneys [126]. PPARγ stimulates lipid storage and improves insulin sensitivity, and is mainly expressed in adipose tissue [126]. PPARβ/δ promotes fatty acid oxidation in the mitochondrion [126]. Among PPARs, the main focus of this thesis is on PPARα, which is suggested to have a regulatory role in one-carbon metabolism related pathways, which will be discussed further.

1.3.2 PPARα

PPARα is regarded a key regulator of all aspects of energy metabolism [132], with a vast amount of identified target genes [133]. It is activated by natural ligands such as fatty acids and their derivatives [134], and is well known for its role in lipid metabolism, where it upregulates the expression of genes encoding important enzymes involved in both peroxisomal and mitochondrial β-oxidation [135]. PPARα also has ramifications to glucose metabolism, by activation of gluconeogenetic and suppression of glycolytic genes in the liver [136]. Mice who are PPARα-deficient develop metabolic abnormalities like hypoglycemia, hypoketonemia and fatty liver when fasted, an observation underlining the role of PPARα in managing of energy stores and adaptations to the fasting state [136, 137]. Recently, a role of PPARα in amino acid metabolism has been demonstrated, and PPARα may thus act as a regulator of energy metabolism by coordinating the utilization of all the three different energy

substrates [138]. In addition to affecting energy metabolism, PPARα is known to carry anti-inflammatory [139, 140] and anti-oxidative properties [141-143].

PPARα mRNA is demonstrated to increase throughout the day, closely following the diurnal rhythm of the corticosterone, a glucocorticoid shown to increase the genetic expression of hepatic *PPARα* [144]. However, even though the amount of PPARα protein correlated strongly with *PPARα* expression, no increase in target gene expression was seen unless the animals were simultaneously treated with a PPARα ligand [144]. In rats, maternal protein restriction has been demonstrated to increase *PPARα* expression in the offspring, which was followed by increased expression of PPARα target genes [145]. Interestingly, the flux through BHMT also seems to influence the transcription of PPARα. Studies in mice have demonstrated that betaine supplementation, while decreasing the hepatic concentrations of betaine, increase the genetic transcription of PPARα by demethylation of the promoter region on the *PPARα* gene [146]. Also, HF feeding among mice has been demonstrated to stimulate flux through BHMT, and this also increased PPARα expression [147].

Interestingly, it was suggested that only fatty acids synthesized in the liver or derived from the diet, but not those derived from the fat stores throughout the body, activate hepatic PPARα [136]. A recent investigation has identified the endogenously synthesized phospholipid 1-palmitoyl-2-oleyl-sn-glycerol-3-phosphocholine (16:0/18:1-GPC), produced by the enzyme fatty acid synthase (EC 2.3.1.85), to be a physiological relevant ligand for PPARα [148]. Besides the natural ligands, PPARα is activated by several synthetic ligands like fibrates, a class of drugs used to treat dyslipidemia, and WY14,643, which specifically activate PPARα [134]. Finally, PPARα may be activated by modified fatty acids like the pan-PPAR agonist tetradecylthioacetic acid (TTA), the ligand being administered in the current investigation [134].

1.3.3 Tetradecylthioacetic acid, a pan-PPAR agonist

TTA is a modified 16-carbon saturated fatty acid, with a sulfur atom on the third carbon of the fatty acid chain [149]. TTA is a pan-PPAR agonist shown to stimulate all PPAR subtypes, but seems to have a particular high affinity towards PPARα [134, 150, 151]. TTA inhibits fatty-acyl-CoA dehydrogenase (EC 1.3.8.7) which catalyzes the first step in mitochondrial β-oxidation of fatty acids [134, 152]. Thus, it was suggested that TTA in addition to act as a ligand, activates PPARα as a metabolic inhibitor, facilitating accumulation of an endogenous ligand [134]. Such a ligand might be 16:0/18:1-GPC. Previous findings associated with TTA

treatment include reduced weight gain despite higher feed intake [153], reduced markers of oxidative damage [149] and inflammation [154], altered plasma levels of amino acids [155] and tissue-specific alterations in lipids and fatty acid composition [156].

1.3.4 PPARα and one-carbon metabolism

Several studies have suggested a role of PPARα in the regulation of one-carbon metabolism. Lipid lowering therapy with fibrates is consistently associated with elevated tHcy among patients with the metabolic syndrome [157], but the underlying mechanisms for this observation has not been fully elucidated. The association between betaine supplementation and *PPARα* expression is another noticeable link between the receptor and the choline oxidation pathway [146].

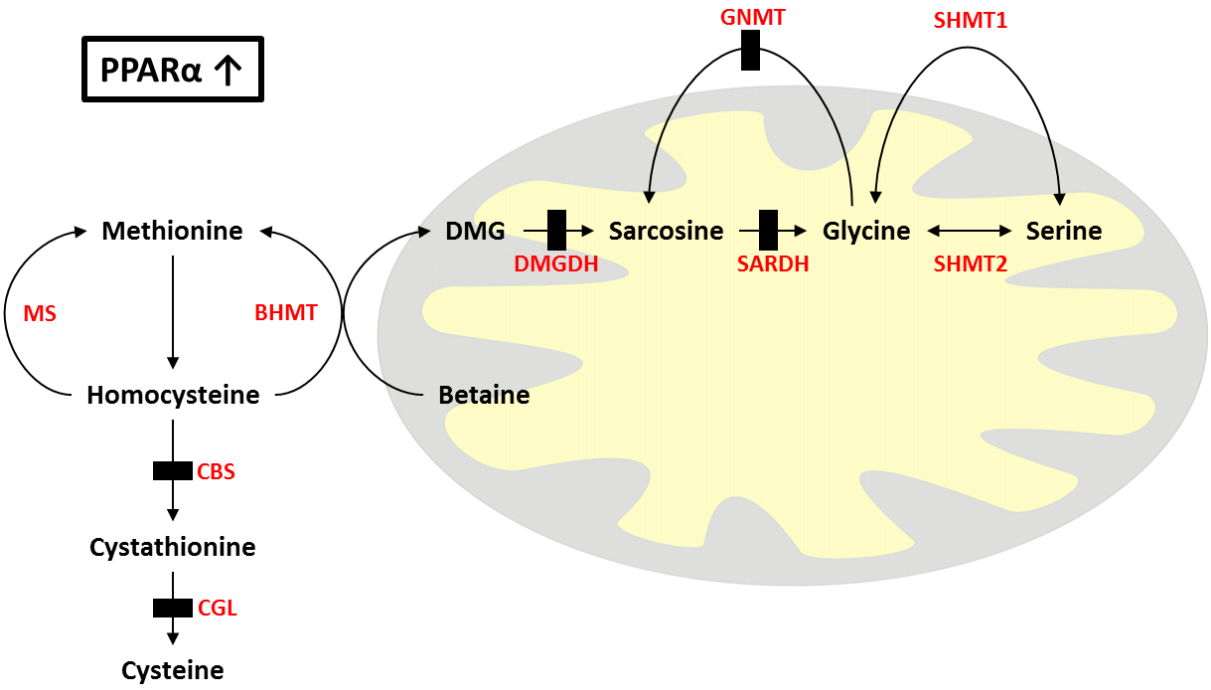

Figure 6 Enzymes of one-carbon metabolism downregulated by PPARα. Animal models have suggested PPARα activity to reduce the genetic transcription of several enzymes in the transsulfuration and the choline oxidation pathway. Abbreviations: BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine-β-synthase; CGL, cystathionine-γ-lyase; DMG, dimethylglycine; DMGDH, dimethylglycine dehydrogenase; GNMT, glycine N-methyltransferase; MS, methionine synthase; PPARα, peroxisome proliferator-activated receptor α; SHMT, serine hydroxymethyltransferase; SARDH, sarcosine dehydrogenase.

In a rat model, using the highly specific PPARα agonist WY 14.643, Sheikh et al demonstrated reduced genetic transcription of genes encoding central enzymes in both the transsulfuration and the choline oxidation pathways, namely *CBS*, *CGL*, *DMGDH*, *SARDH* and *GNMT* [158]. Treatment with WY14.643 was associated with lower protein levels of

SARDH and CGL in mice, as well [159]. Findings from a recent proteomic investigation among rats from the same study as those in the current thesis were in support the suggested relationship between PPARs and one-carbon and choline metabolism. Rats treated with TTA had lower protein levels of DMGDH and SARDH compared to control [160], which is in accordance with what is seen when more specific PPARα agonists are administered, suggesting this to be a PPARα effect. Moreover, the association between increased plasma DMG concentrations and adverse cardiovascular prognosis was suggested to partly depend on PPARα mediated interference with DMG catabolism [73, 74]. Figure 6 summarizes these previously demonstrated links between PPARα and one-carbon metabolism.

Due to its close relation to the flux through the choline oxidation pathway as well as the glycolysis, another source of serine and glycine [161], it is reasonable to suggest that PPARα also influences the production of formate by these two pathways and thereby has an impact on folate metabolism. However, so far no studies have reported any potential direct or indirect regulatory role of PPARα in the folate cycle.

1.4 Aims of the investigation

Circulating and urinary concentrations of several one-carbon metabolites, both belonging to Hcy metabolism and the choline oxidation pathway, have been associated with disease risk. However, determinants of systemic one-carbon metabolites are not fully elucidated, but as suggested, a role of PPARs, and PPARα in particular, seems plausible. Thus, the aim was to investigate the association of PPAR activation and circulating and urinary concentrations of one-carbon and choline oxidation metabolites as well as markers of B-vitamin status. We explored this in male Wistar rats treated with a low-fat control diet or a high-fat (HF) diet with or without the pan-PPAR agonist TTA.

2 Methods

2.1 Animals

Male Wistar rats, 8-10 weeks old at arrival, were obtained from Taconic Europe A/S (Bomholt, Denmark). The animals were housed five per cage and maintained at a constant 12 h light/dark cycle at a temperature of 22± 1°C and relative humidity of 55% ± 10%. After arrival, the rats were acclimatized for one week, with free access to standard chow and water.

After 50 weeks, 4-6 h into the light cycle, the animals were sacrificed under non-fasting conditions, anaesthetized by Isofluorane (Forane, Abbott Laboratories, Abbott Park, IL,USA) inhalation. Blood was drawn by cardiac puncture and collected in BD Vacutainer tubes containing EDTA (Becton-Dickinson, Plymouth, UK). Urine samples were collected directly from the urinary bladder.

The current investigation is a substudy from a long-term study of male Wistar rats which originally set out to explore the effect of TTA and/or fish oil on incidence ventricular cancer [162].

2.2 Diets

For the study period of 50 weeks, 30 rats were randomly attributed one of three diets: 1) A low fat control diet (LF) with 7% fat (5% lard, 2% soybean oil, w/w); 2) A HF diet with 25% fat (23% lard, 2% soybean oil, w/w); 3) A HF diet with 25% fat supplemented with TTA (22.6% lard, 2% soybean oil, 0.4% TTA, w/w). All diets were isoenergetic and isonitrogenous (20% protein, w/w), and the rats ate ad libitum. Casein (Tine BA, Oslo, Norway) was used as the protein source. Fat sources were lard (Ten Kate Vetten BV, Musselkanaal, Netherlands) and soybean oil (Dyets Inc., Bethlehem, Pa, USA). TTA was provided by the Lipid Research Laboratory, Section of Medical Biochemistry, University of Bergen, Bergen, Norway. The rest of the ingredients (cornstarch, sucrose, fiber, AIN-93 G mineral mix, AIN-93 vitamin mix, L-cysteine and choline bitartrate) were obtained from Dyets Inc. (Betlehem, Pa, USA), with the exception of Tert-butyl-hydroquinon (Sigma-Aldrich, St. Louis, MO, USA). The pellets were made by Nofima Ingredients, Bergen, Norway, and the diets are described in detail in Table 1.

Table 1 Composition of diets.

Ingredient (g/kg)	Low fat	High fat	TTA
Lard	50	230	226.3
Soybean Oil	20	20	20
TTA	-	-	3.75
Casein	156.0	196.9	196.9
Cornstarch	572	397	397
Sucrose	100	100	100
Fiber	50	50	50
AIN-93G mineral mix	35	35	35
AIN-93 vitamin mix	10	10	10
L-cysteine	3	3	3
Choline bitartrate	2.5	2.5	2.5
Tert-butyl-hydroquinone	0.014	0.014	0.014
KH_2PO_4, monobasic	1.3	1.3	1.3
Energy content (kcal/kg diet)	3841	5569	5569
Fat E%	16	40	40
Protein E%	14	24	24
Carbohydrate E%	70	36	36

Abbreviation: TTA, tetradecylthioacetic acid.

2.3 Quantification of metabolites

All one-carbon metabolites and B-vitamin analyses were performed at Bevital A/S, Bergen, Norway (http://www.bevital.no). The blood samples were put on ice immediately, and within 2 hours the samples were stored at -80°C until analysis, which for most metabolites were conducted in 2010. Separation of plasma from red blood cells, protein precipitation and extraction were done using a robotic workstation (Hamilton Microlab AT Plus, Reno, NV, USA), and the metabolites were quantified using gas chromatography or liquid chromatography coupled to tandem mass spectrometry (GC-MS/MS, LC-MS/MS) or microbiological assays which will be described in the following paragraphs. Table 2 provides an overview of analytical platforms and metabolites.

Table 2 Analytical platforms, methods and analytes

Platform	Method	Plasma metabolites	Urine Metabolites
B	GC-MS/MS	tHcy Glycine Serine MMA	Cysteine Cystathionine Serine Glycine MMA Sarcosine
C	LC-MS/MS	Choline Betaine DMG Methionine Cysteine Cystathionine	Methionine Choline Betaine DMG
D	LC-MS/MS	Riboflavin Flavin mononucleotide Nicotinamide N^1-Methylnicotinamide Nicotinic Acid Pyridoxal Pyridoxal-5-phosphate 4-Pyridoxic acid	
F	Microbiological Assays	Folate Cobalamin	

Abbreviations: DMG, dimethylglycine; MMA, methylmalonic acid

2.3.1 Platform B: Gas Chromatography-Tandem mass spectrometry

Platform B is developed from an automated isotope-dilution GC-MS assay (Platform A), originally developed for the simultaneous determination of MMA, tHcy and related amino acids [163]. Platform B is distinguished form platform A by the use of MS/MS [164], which allows for a more precise quantification of an extended repertoire of metabolites, and all metabolites previously determined on platform A is now determined on platform B.

On the microtiter plates, 100 µL of plasma samples were mixed with D,L-dithioerytritol (25 µL) and incubated for 20 minutes. To deproteinize the samples, ethanol (450 µL), containing deuterated internal standards, was added. Samples were then centrifuged for 3 min at 5800 g and moved to an empty microtiter plate. 300 mL water, 50 µL pyridine and 250 µL of toluene (200 mL/L) were added by repeated pipetting. The samples were incubated for 6 minutes, before an aliquot of the toluene layer was analyzed by GC-MS/MS (a

Thermo Finnigan trace GC ultra-system coupled to a Fisons MD800 mass spectrometer used in electron ionization mode).

Samples were injected through a CP Sil 24-CB low-bleed/MS capillary column (Varian) at a temperature of 75°C, which was increased to 85°C (1 min) and then 290°C (2 min). Helium was used as carrier gas, and infused at a rate of 1.1-2.2 mL/min. Analytes were quantified using the area ratios of analytes to the deuterated internal standards and calibrator samples with known concentrations. Within- and between-day CV ranged from 0.7-8.1% for all analytes.

2.3.2 Platform C: Liquid chromatography – Tandem mass spectrometry

Platform C is a modified version of a previously published method based on LC-MS/MS [165]. A robotic device (Plato 7; RoSyst Anthos) mixed 30 µL of the samples with three volumes of acetonitrile which contained a mixture with 10 µL of all internal standards. Samples were centrifuged at 5800 g for two minutes, and the supernatant transferred to sealed autosampler glass vials (Chromacol) or microtiter plates (Costar).

A HPLC system (Agilent Technologies, CA, USA) with a thermostated autosampler and degasser introduced the samples to an ether-linked phenyl reversed-phase column, which replaced the silica column used in the original method. A triple quadrupole tandem mass spectrometer (API 3000, Applied Biosystems, MDS SCIEX, CA, USA) with Turbo Ion Stray interface was used in the positive-ion mode for detection of analytes. Within- and between-day CV was 2.1-8.8% for all analytes.

2.3.3 Platform D: Liquid chromatography – Tandem mass spectrometry

Platform D is a LC-MS/MS based method for detection of vitamin B2, B3 and B6, plus metabolites in the tryptophan-niacin pathway [166]. Plasma (60 µL) was first deproteinized by mixing with trichloroacetic acid (60 g/L) in water, which also contained all labeled internal standards. The solution was, after 60 min incubation on ice, centrifuged (5796 g at 4°C for 15 min), before 60 µL of supernatant was transferred to a cooled autosampler (8°C) protected from light.

Separation was performed by a HPLC system (Agilent Technologies, CA, USA), where 50 µL of deproteinized plasma is injected into a stable-bond C8 reverse-phase column (Zorbax) at 40°C. Concentrated acetic acid (650 mmol/L) was present in the mobile phase. A triple-quadrupole tandem mass spectrometer (API 4000, Applied Biosystems, MDS SCIEX)

with electrospray ionization source was used in multiple reaction positive ion mode for detection of analytes. Within- day CV was <10% for all analytes, and slightly higher values were obtained for between-day CV's.

Platform D is later developed to also determine Vitamin B3 metabolites, which were analyzed in 2013.

2.3.4 Platform F: Microbiological assays

Serum folate and cobalamin were measured by microbiological assays [167, 168]. Cobalamin was estimated using a resistant strain of bacteria (Lactobacillus leichmannii, NCIB 12519) [167]. Serum was first diluted (1:10) with a buffer containing 8.3 mmol/L of sodium hydroxide, 20.7 mmol/L of acetic acid and 0.45 mmol/L of sodium cyanide, with a pH of 4.5. Samples were mixed, autoclaved (115°C for 10 minutes) and centrifuged at 1000 g for 10 minutes. The supernatant were transferred to the microtiter plates. After mixing with the culture broth containing the resistant bacteria and incubation for 20 hours at 37°C, the plates were determined by spectrophotometry at 595 nm. The determination of serum folate is built on the same method, using a different strain of resistant bacteria (Lactobacillus casei, NCIB 10463). The microtiter plates were incubated at 27°C for 42 hours, and then read at 590 nm on the plate reader [168].

2.4 Statistical analysis and presentation of data

The data was explored according to intervention groups, and tested for normality using the Kolmogorov-Smirnov test. The results are presented as means (standard deviations; SD) for each group, and the groups were compared by one-way analysis of variance (ANOVA) . The assumption of homogeneity of variance was tested using Levene's test, and where heteroscedasticity was observed, Brown-Forsythe F statistic were reported. A statistically significant F-statistic would indicate a between-group difference for the actual metabolite. To further investigate which group differed significantly from the others, planned comparisons were performed where both intervention groups were compared towards the control group. Post hoc tests were also performed to verify the planned comparisons where all groups were compared to each other. Dunnet's test was chosen for the metabolites with homogeneity of variance, whereas Dunnet's T3 test was performed where heteroscedastic variance across the groups was observed.

To measure the size of the effect, Cohen's d effect sizes were calculated for all planned comparisons. Cohen's d was calculated using Equation 1, and a d > 0.8 was considered a large effect [169]. Cohen's d represents the difference between two groups expressed in pooled SD units. The pooled SD was calculated by Equation 2.

(Eq. 1) $$d = \frac{\bar{x}_1 - \bar{x}_2}{s_p}$$

(Eq. 2) $$s_p = \sqrt{\frac{(n_1-1)s_1^2+(n_2-1)s_2^2}{n_1+n_2-2}}$$

The concentration of urinary metabolites was corrected for urine concentrations of creatinine, to adjust for any potential dilution. To evaluate the relationship between blood and urinary concentrations of metabolites, Pearson's correlation coefficients were calculated, and bootstrapped 95% confidence intervals were provided as these are robust for any violation of the assumption of normality [170].

Because the results are not adjusted for multiple comparisons and the planned comparisons are non-orthogonal, the level of statistical significance was set to $p<0.01$. Statistics were performed by using IBM SPSS Statistics for Windows, version 21 (SPSS Inc., Chicago, IL, USA).

2.5 Ethical statement

The animal experiments were designed to comply with the Guidelines for the Care and Use of Experimental Animals and the study protocol was approved by the Norwegian State Board for Biological Experiments with Living animals.

3 Results

All plasma metabolites were available for all 10 animals in each group. However, in the case of urinary samples, the groups were not completely equal. In the control group, 9 results were available for urine concentrations of methionine, choline, betaine and DMG, while 8 results were obtained for tHcy, cystathionine, cysteine, sarcosine, glycine, serine and MMA. In the HF group, all urinary specimens were available for 9 animals, while 8 values were obtained for all analytes among TTA treated rats. Mean (±SD) metabolite concentrations and results from the ANOVA and the planned contrasts for all blood and urinary metabolites are presented in the enclosed manuscript Tables 1 and 2, respectively. The correlations and accompanying confidence intervals between blood and urinary concentration of metabolites are presented in the enclosed manuscript Table 3. In the following paragraphs, a short summary of the results from the planned contrasts will be presented, and Figures 6-12 provide bar plots that illustrate the differences between the intervention groups compared to the control group.

3.1 Plasma concentration of metabolites

Among plasma one-carbon metabolites, TTA treatment was associated with trends toward higher tHcy and lower methionine concentrations (Figure 7). Furthermore, significantly higher concentration of cystathionine was observed among the TTA treated animals, but plasma cysteine did not differ between the groups. The HF diet was not associated with any differences on these metabolites.

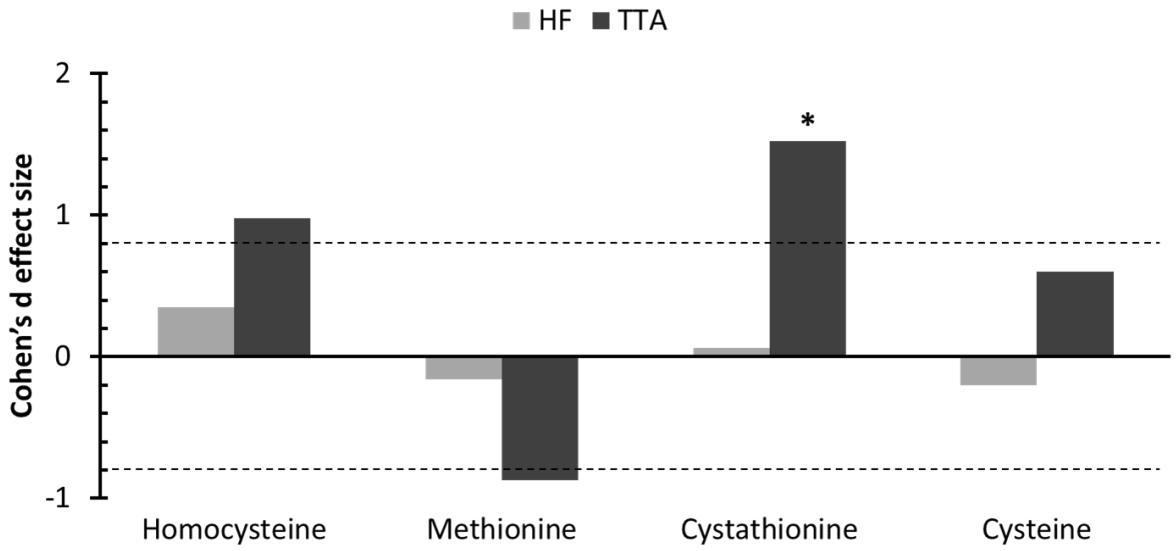

Figure 7 Results on plasma concentrations of one-carbon metabolites. The dashed lines represent Cohen's d = 0.8, and the asterix indicates statistical significance.

Along the choline oxidation pathway, higher plasma concentrations of DMG, glycine and serine were observed among TTA treated animals (Figure 8). A particular large difference was observed for plasma DMG. Again, the HF diet alone was not associated with any major between-group differences, apart from a trend towards lower concentration of plasma choline (p=0.057).

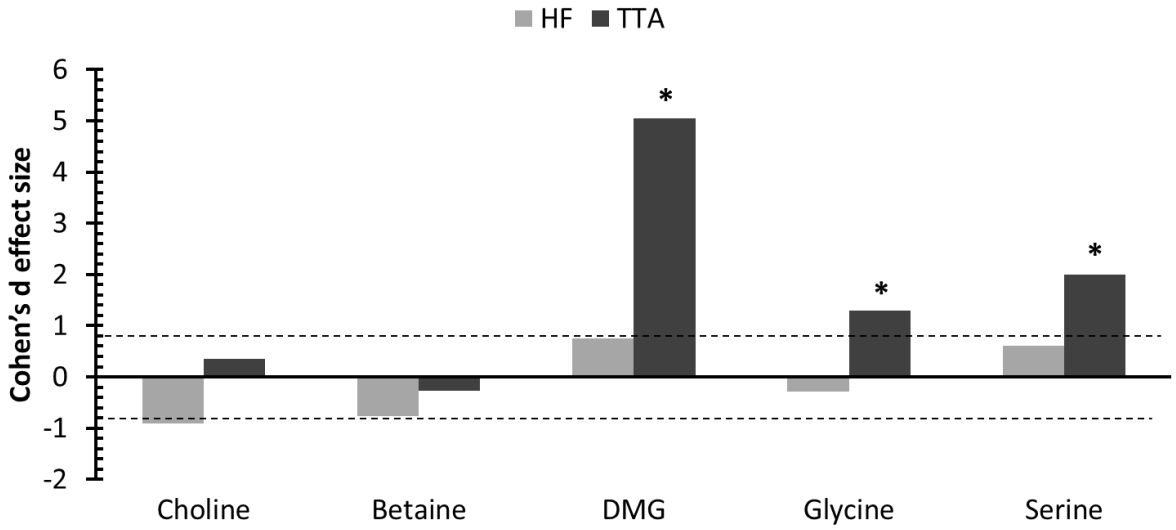

Figure 8 Results on plasma concentrations of choline and its oxidation products The dashed lines represent Cohen's d = 0.8, and the asterix indicates statistical significance. Abbreviation: DMG, dimethylglycine.

Several between-group differences were observed for plasma concentration of the different B2 and B3 metabolites, showed in Figure 9. TTA treatment was associated with lower riboflavin and FMN, whereas NAM and mNAM were strikingly higher compared to control. The HF diet was associated with trend toward lower riboflavin (p=0.011) and FMN (p=0.044), but no alterations in vitamin B3 status.

Figure 10 summarizes the results on plasma vitamin B6, folate and B12 status. The HF diet was associated with trend toward lower plasma concentration of PA (p=0.17), and higher concentrations of folate (p=0.023) and cobalamin (p=0.17). Interestingly, TTA treatment was associated with an opposite trend on plasma folate compared to what was seen with HF treatment, which was lower compared to control (p=0.024). Furthermore, pronounced higher concentrations of plasma PL and MMA were observed in the TTA group when compared to control.

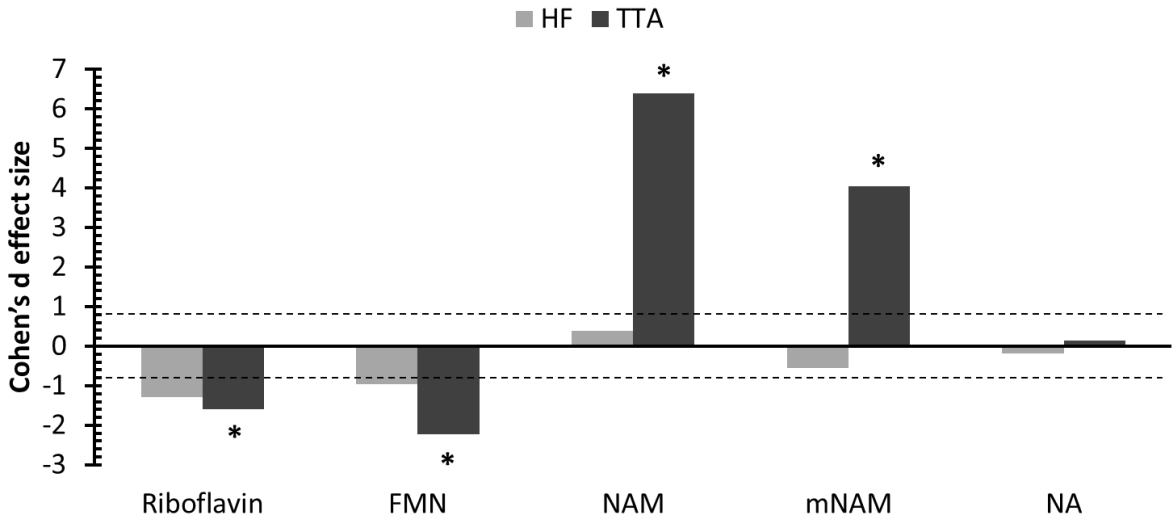

Figure 9 Results on plasma concentrations of B2 and B3. The dashed lines represent Cohen's d = 0.8, and the asterix indicates statistical significance. Abbreviations: FMN, flavin mononucleotide; mNAM, N^1-methylnicotinamide; NA, nicotinic acid; NAM, nicotinamide.

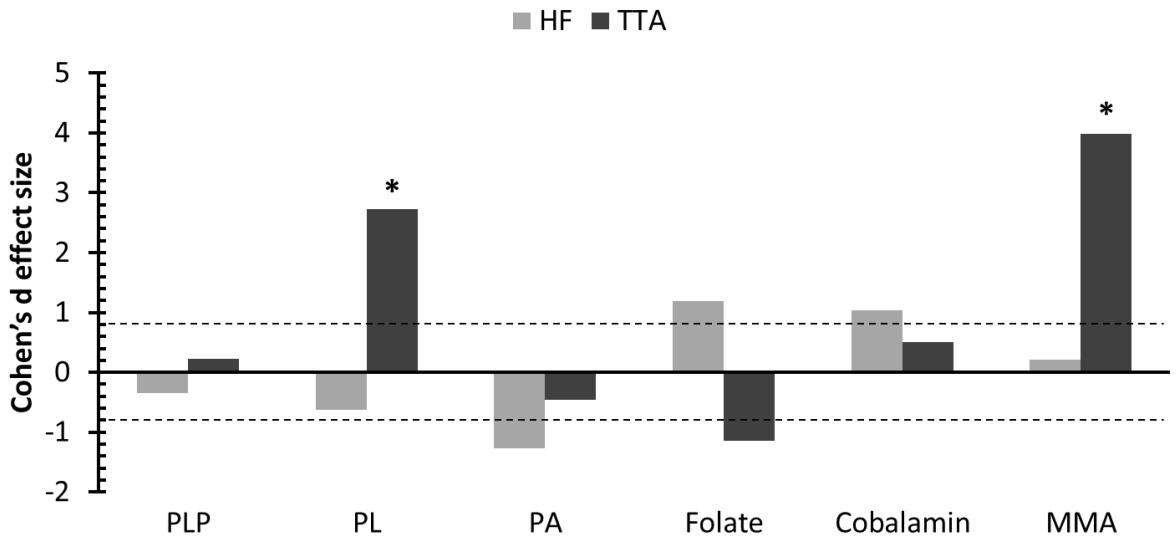

Figure 10 Results on plasma concentrations of B6, folate, cobalamin and MMA. The dashed lines represent Cohen's d = 0.8, and the asterix indicates statistical significance. Abbreviations: MMA, methylmalonic acid; PA, pyridoxic acid; PL, pyridoxal; PLP, pyridoxal-5-phopsphate.

3.2 Urine concentration of metabolites

All urinary results are depicted in Figure 11 and 12. No between-group differences were found according to the urinary concentration of one-carbon metabolites. A pronounced higher concentration of MMA was observed with TTA treatment as compared to control. The urinary concentration DMG and sarcosine was higher among the animals receiving TTA.

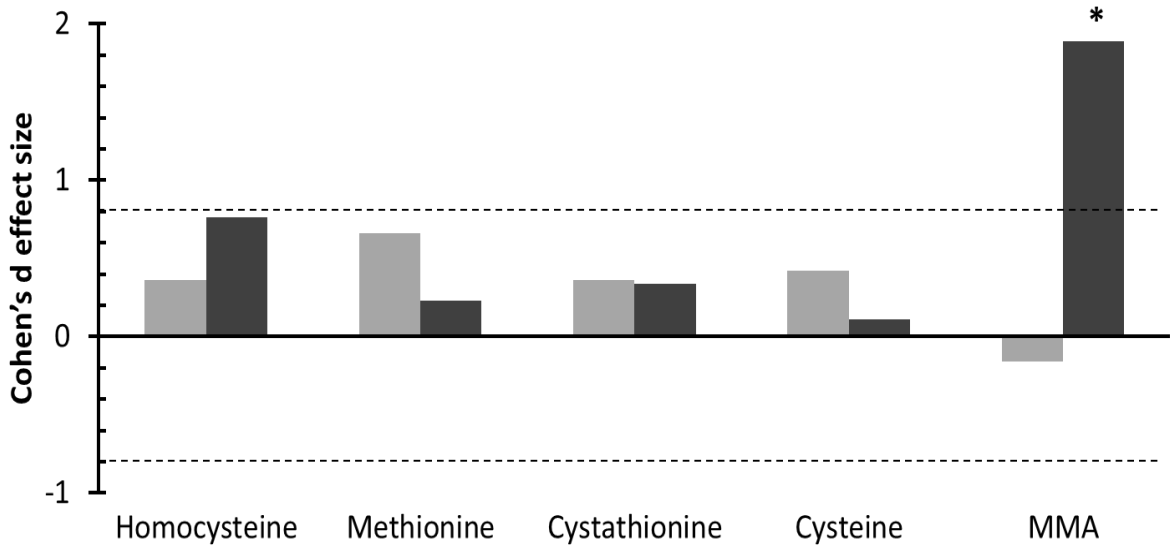

Figure 11 Results on urinary concentrations of one-carbon metabolites and MMA. The dashed lines represent Cohen's d = 0.8, and the asterix indicates statistical significance. Abbreviation: MMA, methylmalonic acid.

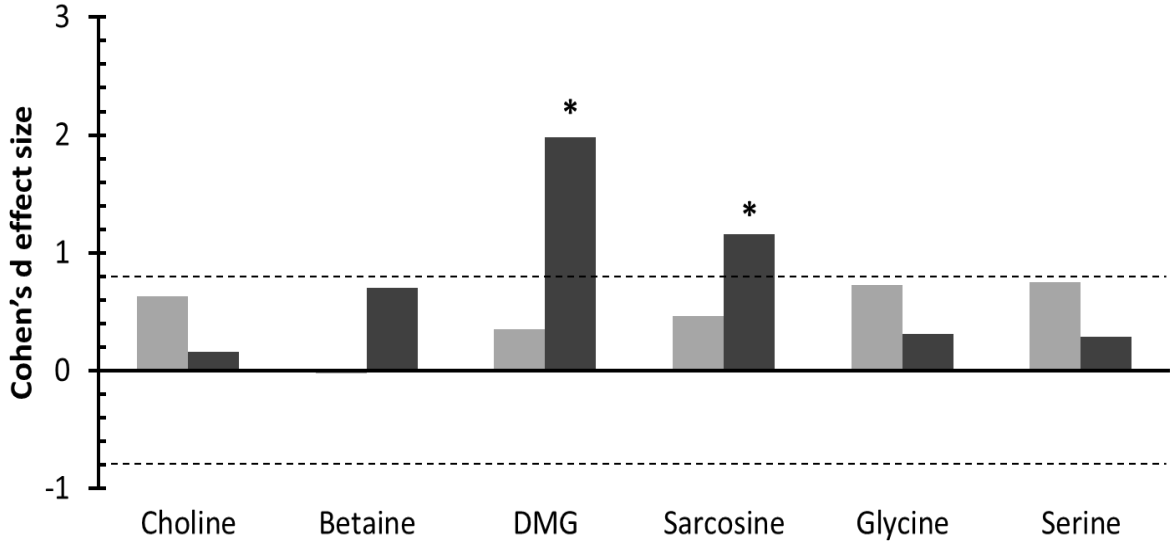

Figure 12 Results on urinary concentrations of choline and its oxidation products. The dashed lines represent Cohen's d = 0.8, and the asterix indicates statistical significance. Abbreviation: DMG, indicates dimethylglycine.

3.3 Correlations between plasma and urine concentrations

Strong correlations between the plasma concentrations of betaine, DMG and MMA was seen with their respective urinary concentrations (r=.59, .82 and .85). No correlations were observed for any of the other metabolites analyzed in both plasma and urine. All correlation coefficients are provided in the enclosed manuscript Table 3.

3.4 Post hoc analyses

The post hoc tests yielded more or less the same results as the planned contrasts, with the exceptions of the higher concentrations of urinary DMG (p=0.016) and sarcosine (p=0.019) which were only borderline statistically significant. The results of the post hoc tests are provided in Table 3 for plasma metabolites and in Table 4 for urinary metabolites.

Table 3 Results from the post hoc tests, plasma metabolites. Dunnet's test was performed when variance was equal, and Dunnet's T3 was chosen whenever heteroscedasticity was observed.

	HF vs. control	TTA vs. control
	p	p
Methionine	0.91	0.14
Homocysteine	0.68	0.09
Cystathionine	0.99	0.018
Cysteine	0.88	0.25
Choline	0.16	0.81
Betaine	0.42	0.72
DMG	0.34	<0.0001
Glycine	0.90	0.005
Serine	0.60	<0.001
Riboflavin	0.02	0.001
FMN	0.12	0.001
NAM	0.77	<0.001
mNAM	0.90	<0.001
NA	0.91	0.92
PLP	0.81	0.82
PL	0.45	<0.001
PA	0.29	0.37
Folate	0.06	0.07
Cobalamin	0.30	0.52
MMA	0.95	<0.001

Abbreviations: DMG, dimethylglycine; FMN, flavin mononucleotide; HF, high fat; mNAM, N^1-methylnicotinamide; NA, nicotinic acid; NAM, nicotinamide; PA, pyridoxic acid; PL, pyridoxal; PLP, pyridoxal-5-phosphate; MMA, methylmalonic acid; TTA, tetradecylthioacetic acid.

Table 4 Results from the post hoc tests, urinary metabolites. Dunnet's test was performed when variance was equal, and Dunnet's T3 was chosen whenever heteroscedasticity was observed.

	HF vs. control	TTA vs. control
	p	p
Methionine	0.44	0.95
Homocysteine	0.71	0.24
Cystathionine	0.78	0.67
Cysteine	0.56	0.97
Choline	0.28	0.97
Betaine	0.99	0.21
DMG	0.84	0.016
Sarcosine	0.91	0.019
Glycine	0.35	0.89
Serine	0.22	0.85
MMA	0.97	<0.001

Abbreviations: DMG, dimethylglycine; HF, high fat; MMA, methylmalonic acid; TTA, tetradecylthioacetic acid.

4 Discussion

4.1 Methodological strengths and limitations

4.1.1 Experimental procedures

In this study among male Wistar rats, we studied possible effects of treatment with the pan-PPAR agonist TTA. Although TTA is known to activate all subtypes of PPAR, it is demonstrated to preferentially activate PPARα [134]. TTA was thus previously suggested to activate PPARα in a dual manner, both direct and by promoting the synthesis of endogenous ligands [134]. Regardless, it is not straightforward to argue that the observations specifically result from PPARα activation. However, a proteomic study among the same animals investigated in this thesis showed consistent results compared to studies using more specific PPARα agonists like WY 14.643 [158-160], which strongly suggests the current findings to primarily be a result of PPARα activation.

A well-known effect of short term TTA-treatment among rodents fed a HF diet is reduced weight gain despite higher feed intake [153], which was also observed during long-term TTA treatment of the rats currently investigated [155]. All animals underwent a surgical procedure affecting the gastrointestinal tract, which may potentially affect nutritional status. To deal with these issues, a separate experiment was conducted, comparing animals that underwent the surgical procedure to animals that did not. It was concluded that the surgery did not influence body weight gain or nutritional status [156].

The long-term dietary intervention is a major strength of the current study, as this provides the opportunity to investigate the longitudinal, and not only the acute, effects of PPAR activation. However, when trying to attribute the observations to PPAR activation, one cannot exclude that PPAR-independent actions of TTA may also have affected the findings [150]. Another advantage of our study is the possibility to examine the effect of dietary fat content alone, as dietary fatty acids and their derivatives are also known to bind and activate PPARs. It has been suggested that HF feeding enhances the genetic expression of PPARα [147], and it was previously demonstrated that increased amount of PPARα was a potent enhancer of the induction of target genes following treatment with WY14.643 [144]. This may indicate that the effects observed with TTA combined with HF diet may be an overestimation of a suggested PPARα effect. However, the PPARα mRNA in liver did not differ between the HF and the control group, whereas it was about doubled in the TTA group

(data not shown). Nevertheless, also including a group combining the low fat control diet with TTA treatment would have been advantageous for the current investigation.

4.1.2 Differences between rodents and humans

Lipid metabolism in rodents differs from that among humans, including a markedly higher responsiveness to hormonal control among the former. Thus, how data obtained from rodent models apply to human physiology may not be adequately addressed [171]. In humans, the majority of cholesterol is transported in low-density lipoprotein particles, while in rodents cholesterol is transported almost exclusively in high-density lipoprotein particles [172]. Rats also differ from humans according to PPAR expression and function. Humans express much lower levels of PPARα as compared to rodents [173], and while the liver peroxisomes in rats respond to PPARα activation by increasing in number and size, resulting in hepatomegaly and liver cancer, this response is not seen in humans [174, 175]. Between-species differences in PPAR activity may also be influenced by different availability of several coactivators or corepressors [174]. Interspecies differences in PPARα ligand affinity [176] or differences in the promoter region of PPARα target genes has also been suggested [177]. Two strains of humanized PPARα mice have been generated. Although human PPARα seemed to yield the same effects as mouse PPARα on the regulation of lipid metabolism and peroxisome proliferation, the human variant did not resemble the distinct hepatomegaly seen in rodent models [178, 179]. This was also seen after long-term treatment [180]. As humans possess lower expression of PPARs, combined with species differences in lipid metabolism and in the response to PPAR activation, extrapolation of results derived from rodent experiments to humans is not straightforward, and should mainly be hypothesis generating according to human health.

4.1.3 Statistical methods

When testing a scientific hypothesis, we calculate the probability of gaining the observed results given that no effect really existed, and this is referred to as null-hypothesis testing [170]. Our hypothesis was that the intervention groups would differ from control animals, thus the corresponding null-hypothesis was that the groups did not differ. The p-value represents the probability of obtaining the current results assuming the null-hypothesis is true. The level of statistical significance is a preset p-value, usually set at $p=0.05$, and if the obtained p-value is under this threshold of statistical significance, we conclude that the result is statistically significant. This provides confidence in rejecting the null-hypothesis, thus

strengthening the confidence in the current hypothesis. However, statistical significance is no guarantee of the observation representing a genuine effect. If no effect exist, one still could expect one p-value to be <0.05 for every 20 test just by chance, constituting a false positive finding (Type 1 error) [170]. The p-value is calculated based on the variance, which is majorly affected by sample size. The standard error is calculated by dividing the SD with the square root of the sample size, and thus smaller samples will present with larger standard errors. Small sample sizes provide poor statistical power, thus increasing the probability of not discovering a real effect, also referred to as a false negative (Type 2 error) [170]. In general, a smaller sample size requires a larger difference to reach statistical significance and vice versa.

Even though small sample sizes are frequently regarded a major limitation in human studies, a small number of samples are rather common in animal studies as the animals may be assumed to be more similar to each other as opposed to humans. However, smaller sample sizes could be a challenge because the data will seldom be normally distributed. Indeed, although most metabolites in the current investigation proved to be normally distributed as evaluated with the Kolmogorov-Smirnov test (data not shown), there were some exceptions. However, as the three groups were compared with one-way ANOVA, which is regarded to be fairly robust to deviations from normal distribution as long as the group sizes are equal, these deviations from normality were not regarded a limitation [170]. A statistically significant F-statistic from the ANOVA does not specifically address which groups differ from each other, and such information may be obtained from using planned comparisons or one of several post hoc analyses. As we wanted to investigate the associations related to HF feeding or TTA treatment compared to low fat feeding, we chose to perform planned comparisons. Ideally, a group should only be singled out in one of the comparisons, meaning that the comparisons were orthogonal. However, as we compared both intervention groups against the control group, the planned contrasts were non-orthogonal. This prompts for careful interpretation of the results, as the p-values for the various between-group comparisons will be somewhat related to each other. Hence, it is appropriate to select a more strict cutoff for statistical significance [170]. The statistical analyses were not adjusted for multiple comparisons, which increases the probability of obtaining false positive results. Such adjustment could have been done by Bonferroni correction, dividing the level of statistical significance by the number of analyses performed, thus controlling the rate of Type 1 errors. However, such adjustment

tends to be too conservative when performing a large number of analyses [170]. Hence, we chose to set the cutoff for statistical significance at $p<0.01$.

Notably, a recently published paper called for care to be taken when interpreting p-values, as these are demonstrated to underestimate the probability of discovering false positives and are majorly affected by sample size [181]. The article emphasized the importance of estimating effect sizes, as these are not affected by sample size and provide valuable information about the magnitude and relative importance of an effect [181]. Even if an analysis does not reach statistical significance, one cannot exclude the existence of an effect. It simply suggests that the potential effect is not large enough to be found in the current study [170]. Actually, it has been underlined by the American Psychology Association that one always should present some estimates of the effect size alongside the p-values [182]. According to this, we mainly focus on the Cohen's d effect sizes when evaluating the findings in this study, giving information on the extent of the differences observed. Also, information on effect sizes is of importance when executing power calculations of future studies [182].

4.2 The targeted metabolomic approach

Research fields like genomics and transcriptomics provide information about the genome and the expression of genes, while proteomics deal with measurement of protein levels in biological tissues. Downstream these fields we find metabolomics, which refer to the analysis of molecules present in biologic mediums such as blood, urine or tissue – the metabolome. [183]. The metabolomic approach can be used to distinguish between groups based on the metabolic profile, which may be useful both in treatment and prevention of disease [184]**.** A metabolomic approach with an initial hypothesis and focus on certain metabolites, like the current study, is categorized as targeted metabolomics [183].

Metabolomics has been proposed as an essential tool to understand the function of PPARα, not least to investigate the clinical effects of different PPARα-agonists [185]. An untargeted urinary metabolomic study was carried out in 2007, where both wild-type and PPARα-null mice were treated with WY14.643 to identify specific urinary biomarkers reflecting PPARα activity [186]. Two years later, another metabolomics investigation was performed on rats treated with fenofibrate [187]. Among other markers, an increase in the urinary excretion of several metabolites in the tryptophan-niacin pathway was discovered after PPARα activation, suggesting these to be potential biomarkers of PPARα activity.

4.3 Discussion of results

In this study, long-term supplementation with TTA was associated with pronounced alterations in plasma concentrations of metabolites along the choline oxidation pathway and markers of B-vitamin status in rats when compared to a low-fat control diet. The most pronounced differences were observed for plasma concentrations of DMG, NAM, mNAM and MMA, which were all higher, and FMN and PL which were lower in the TTA group. In urine, higher concentrations of DMG and sarcosine, as well as MMA, were observed in the TTA group. No statistically significant between-group differences were observed according to the amount of dietary fat, neither in plasma nor urine.

4.3.1 Effect of dietary fat content

Compared to the low-fat control group, no statistically significant differences were observed in either systemic or urinary metabolite concentrations for the HF group. However, the small sample size may have impeded statistical significance, as some of the observed differences reached the threshold of what was considered a large effect. No between-group differences were observed in the concentration of one-carbon metabolites (Figure 7). In the choline oxidation pathway, the HF diet was associated with trends towards lower choline and betaine while DMG was slightly higher compared to control diet (Figure 8). HF treatment was also associated with trends toward lower vitamin B2 (Figure 9) as well as higher circulating folate and cobalamin (Figure 10) concentrations. It has been suggested that dietary fat promotes flux through BHMT in mice, with observed increases in both mRNA and protein levels of BHMT. These findings were accompanied by decreased hepatic concentrations of betaine, and the HF treatment also led to increased expression of *PPARα* mRNA as well as some selected target genes [147]. As previously mentioned, betaine supplementation is shown to increase the genetic expression of PPARα [146], and the amount of PPARα protein is closely related to *PPARα* mRNA expression [144]. These observations point towards an elevated *PPARα* expression following HF feeding, and that increased flux through BHMT is involved. Hence, there was reason to believe that the HF group could have had a higher expression of *PPARα* as compared to the control group in the current study. This was further supported by the trends toward lower plasma betaine and higher DMG concentrations among TTA treated rats, being in accordance with what has been previously observed in mice receiving a HF diet [147]. However, no differences in *PPARα* mRNA according to dietary fat were observed in the liver (data not shown) in the current study, rendering this explanation unlikely. Lack of statistical significant differences according to dietary fat thus implies that the availability of

PPARα agonists is not sufficient to lead to any substantial changes, as that seen with the TTA treatment in this study. This suggests that the availability of such ligands may predominantly be determined by metabolic or genetic factors and not by diet alone. Indeed, *PPARα* expression alone does not affect PPARα target gene expression in the absence of a PPARα ligand [144]. Other possible explanations could be 1) species-differences between mice and rats, 2) that the amount of fat given to the HF group in the current study was not high enough to replicate previous findings, or 3) that the dietary intervention in our study lasted long enough for the rats to adapt to the HF diet. The mice in the previous investigation were given 34% (w/w) fat for twelve weeks [147], whereas the HF treated rats in our study received 25% (w/w) fat for 50 weeks.

4.3.2 TTA treatment and homocysteine metabolism

Plasma cystathionine was higher in the TTA group, which may indicate reduced flux through the transsulfuration pathway, a previously suggested feature of PPARα activation [158] and TTA treatment [160]. Additionally, trends toward higher levels of plasma tHcy and lower Met were observed among the TTA-treated rats, both reaching the threshold of what was considered a large effect (Figure 7). Elevated tHcy concentrations is also a known effect from fibrate treatment [157], most probably due to enhanced PPARα activation. Elevated tHcy may be due to increased production. It is generally believed that the synthesis of creatine and choline are the major metabolic sources of Hcy [26], but also the endogenous synthesis of carnitine may be of importance, as each molecule of carnitine produced consumes three molecules of SAM [188]. Notably, carnitine synthesis is demonstrated to be upregulated by PPARα activation [189, 190], and has also been shown after TTA treatment [155]. Elevated tHcy may also be caused by reduced remethylation. Reduced Hcy remethylation through MS may be due to lower availability of cobalamin, as indicated by the higher concentrations of MMA observed in the TTA treated rats. Also, lower concentrations of vitamin B2 may have affected this remethylation pathway as it 1) is involved in the production of mTHF [89] and 2) is important for the function of MSR [114], which maintains MS function.

As elevated plasma tHcy may be caused by disturbances in either the remethylation or the transsulfuration pathways, it is of interest to note that these metabolic pathways are known to be regulated by redox status, where increased oxidative stress inhibits the former [29, 30] and activates the latter [19]. Disturbances in redox balance is thus suggested to be a possible mechanism causing hyperhomocysteinemia [191]. This represents another aspect of one-

carbon metabolism possibly influenced by PPARα activity as both PPARα activation [139-143] and TTA treatment [149, 154] are shown to have anti-inflammatory and anti-oxidative effects.

4.3.3 TTA treatment and the choline oxidation pathway

Several metabolites along the choline oxidation pathway strongly differed between the rats receiving TTA and the control rats (Figure 8). A particularly pronounced difference was observed for DMG, the plasma and urine concentrations of which were higher in TTA-treated animals. Higher urinary DMG concentrations probably indicate a spill-over effect from higher plasma concentrations, as suggested by the strong correlation between blood and urinary concentrations. DMG is solely produced from betaine in the BHMT reaction [68], and is either excreted in the urine or catabolized to sarcosine and further to glycine inside the mitochondrion [57]. Higher concentrations of plasma DMG may thus be a result of increased production, decreased catabolism or urinary excretion, or a combination thereof. As PPARα activation is demonstrated to reduce the genetic transcription [158] and protein level [159] of the DMG catabolizing enzymes, the latter of which also was shown with TTA treatment [160], decreased catabolism is probably a major contributor to elevated plasma DMG in the current study. It was not possible to determine plasma sarcosine due to analytical interference from the EDTA in tubes used for blood sampling. However, we can assume that higher urinary sarcosine concentrations among TTA treated rats reflects higher circulating levels, as the urinary concentration of both betaine and DMG correlated strongly with their respective plasma concentrations. In addition to the apparent direct negative effect of PPARα activation on the expression of these enzymes, both DMGDH and SARDH are dependent on vitamin B2. A TTA induced reduction in vitamin B2 levels may thus contribute to decreased DMG catabolism. Vitamin B2 is also essential in the MTHFR related making of mTHF [89], the donor for MS-mediated Hcy remethylation. If folate-dependent remethylation of Hcy is reduced, this may lead to a compensatory increase in BHMT flux, hence also DMG production, to fulfill the requirements for Hcy remethylation [51]. Hence, the higher concentration of plasma and urinary DMG is probably influenced both by reduced catabolism and increased production.

Apart from DMG catabolism, sarcosine may also be formed in the cell cytosol from methylation of glycine by GNMT. However, as PPARα activation is demonstrated to reduce *GNMT* mRNA [158], the increased urinary concentration of sarcosine is most probably not a

result of increased cytosolic sarcosine production. In fact, a reduced GNMT flux due to PPARα activation may have contributed to the higher plasma concentrations of glycine and serine observed among TTA-treated rats, by decreasing glycine catabolism to sarcosine. PPARα activation is generally assumed to reduce amino acid catabolism [138], and higher plasma concentrations of both glycine and serine have previously been reported with fenofibrate treatment [187]. Correspondingly, higher concentrations of most amino acids were observed in plasma after TTA treatment [155]. Metabolic sources of glycine are depicted in Figure 13. In addition to the SARDH reaction, which most probably is inhibited by PPARα-dependent downregulation of *SARDH* mRNA, glycine may be formed reversibly from serine by SHMT [87], and results from *in vitro* studies have suggested that this is primarily carried out by the mitochondrial SHMT2 [192]. Serine is an end product of glycolysis, but as PPARα is known to inhibit glycolytic activity [133, 136] this may not be a substantial source of either serine or glycine in the current study. A third option for glycine synthesis is by threonine catabolism initiated by NAD-dependent threonine dehydrogenase (EC 1.1.1.103), which is known to be the major pathway of threonine catabolism in rats [161]. The circulating concentrations of threonine in the TTA treated animals currently investigated was higher as compared to the HF group [155], making this a possible metabolic source of glycine. A fourth source of glycine is carnitine biosynthesis [188], and it has previously been demonstrated that carnitine synthesis is upregulated by PPARα activation [189, 190]. Notably, a previous study of the same animals investigated in this thesis found a TTA-induced increase of most genes in carnitine synthesis [155]. Thus, it is plausible that the major source of higher glycine concentration is through upregulated carnitine synthesis combined with reduced catabolism through GNMT and a possible contribution from threonine degradation. Interestingly, the cytosolic isoform of SHMT is suggested to be involved in carnitine synthesis as it seems to be identical to 3-hydroxytrimethyllysine aldolase (EC 4.1.2.X), one of the key enzymes in the carnitine synthesis pathway which, like SHMT, is shown to be dependent on vitamin B6 [193, 194]. This further underlines the interrelationship of these metabolic pathways, including B-vitamin status. Serine is a substrate for the first enzyme of the transsulfuration pathway, where it condenses with Hcy to form cystathionine [14]. As already mentioned, PPARα is suggested to reduce transsulfuration flux [158], which suggests the higher serine concentrations to at least in part be related to decreased catabolism through transsulfuration.

No difference between the TTA group and control were observed for neither choline nor betaine concentration in plasma (Figure 12). As discussed above, the HF treatment was

associated with a trend towards lower concentrations of both these metabolites. This may be explained by a saturation of BHMT flux among TTA treated rats caused by the considerably higher plasma concentrations of DMG, as it is demonstrated that DMG is a potent inhibitor of BHMT activity [4, 195]. Also, an increased flux through BHMT following HF feeding is thought to increase the genetic expression of PPARα. Thus, one might suggest that increased PPARα activity following TTA treatment would abolish this mechanism.

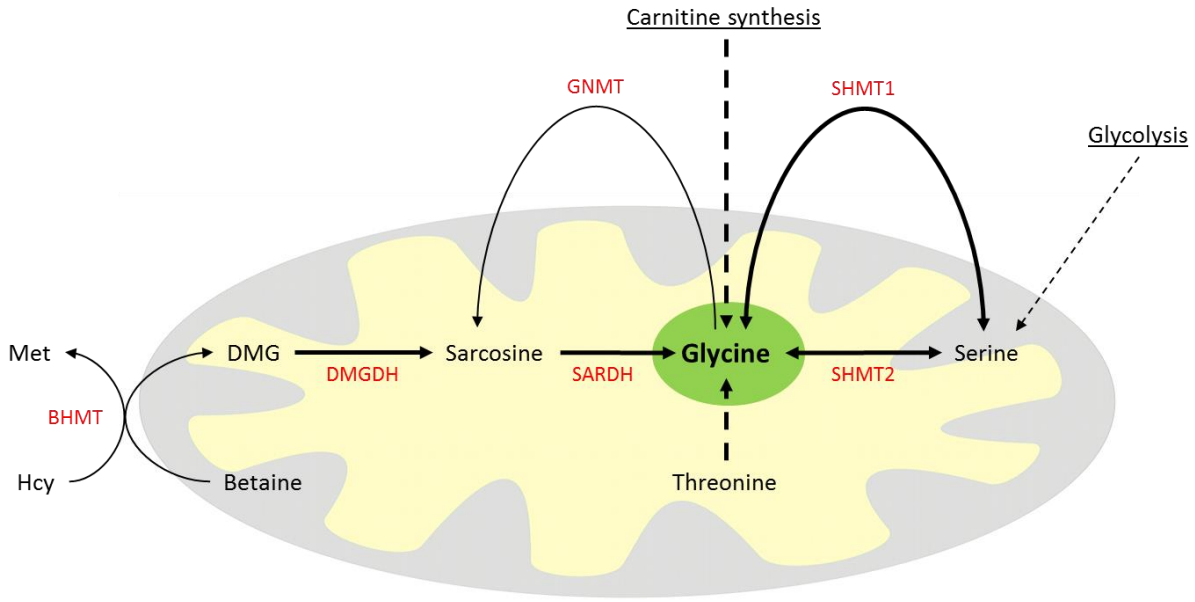

Figure 13 Major metabolic sources of glycine. Glycine may be derived from the choline oxidation pathway, from the glycolytic pathway, from threonine degradation or as a product of carnitine biosynthesis. BHMT indicates betaine-homocysteine methyltransferase; DMG, dimethylglycine; DMGDH, dimethylglycine dehydrogenase, GNMT, glycine N-methyltransferase; Hcy, homocysteine; Met, methionine; SARDH, sarcosine dehydrogenase; SHMT, serine hydroxymethyltransferase

4.3.4 TTA and plasma vitamin B2 status

Among TTA treated rats, lower concentrations of both riboflavin and FMN were observed (Figure 9). Also, rats receiving the HF diet alone had a trend towards lower concentrations of these metabolites compared to the control group. As several enzymes in the metabolic pathways discussed are dependent on vitamin B2 as a cofactor, reduced availability of this vitamin is likely of importance for the activity of these enzymes, including MTHFR, MSR, CHDH, DMGDH and SARDH. In a previous metabolomic study on fenofibrate treatment, higher concentration of riboflavin was reported both in plasma and urine at day 14 [187]. However, no information was given on the composition of the diet, and in the current study the B2 vitamers were lower also among the HF treated rats as compared to control. This suggests that the lower plasma concentration of riboflavin and FMN observed with TTA

treatment in the current investigation may be prone to interference from dietary fat intake, and possibly also a PPAR-independent effect by TTA.

4.3.5 TTA and plasma vitamin B3

A marked increase was observed for the concentration of the vitamin B3 metabolite NAM and the breakdown product mNAM in the TTA treatment group (Figure 9). As NAM is produced during catabolism of tryptophan, an effect on this metabolic pathway may be a potential causal mechanism explaining the observation. Previously, an increased conversion of tryptophan to niacin was observed after PPARα activation in rats [196], and increased hepatic concentrations of NAD was shown with clofibrate treatment [197]. Treatment with PPARα agonists, such as clofibrate and WY14.643, has been reported to increase the activity of quinolinate phosphoribosyltransferase (EC 2.4.2.19) [198] as well as reducing the genetic transcription and activity of 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase (EC 4.1.1.45), which are key enzymes in the tryptophan catabolic pathway in both rats [158, 198-200] and mice [201]. These alterations in gene expression favor the production of NAM, and both NAM and mNAM concentrations have been observed to be higher in urine after PPARα activation in rodents [158, 186, 187, 199, 200]

NAD(P) is also an important electron carrier in the β-oxidation of fatty acids, which is well known to be stimulated by PPARα activation. As fibrate treatment has been shown to enhance the hepatic concentrations of NAD [197], altered concentrations of vitamin B3 metabolites may be related to increased requirements caused by a PPARα-mediated stimulation of fatty acid catabolism. Indeed, it has been suggested that upregulation of the tryptophan-niacin pathway is a response to the increased requirements of NAD for β-oxidation [199].

4.3.6 TTA, vitamin B6 and inflammation

Pronouncedly higher concentrations of PL were observed among the TTA treated as compared to the control rats (Figure 10). However, no between-group differences were found for either PLP or PA. Even though PLP was comparable between the groups, TTA treatment seems to result in a marked increase in systemic vitamin B6 concentrations. As the production of PLP is shown to be dependent on vitamin B2 as a cofactor [100], lower availability of B2 in the TTA treatment group is a possible explanation of these observations. As PLP is not able to cross cell membranes, it has to be converted to PL. This conversion is catalyzed by alkaline phosphatase, and notably, alkaline phosphatase activity was previously shown to be

upregulated after PPARα activation in rats [202]. Thus, this may partly explain why we observe higher concentrations of PL after TTA treatment.

B6 deficiency has previously been reported to be associated with increased oxidative stress and inflammation [101-103]. Thus, our observations may be regarded markers of attenuated oxidative stress and inflammation, features previously demonstrated by PPARα activation [139-143] and TTA treatment [149, 154]. Another way of presenting the B6 status is by calculating the ratio of PA to the total PL and PLP. This PA-ratio was recently demonstrated to be a more sensitive and reliable marker of inflammation than the other markers of vitamin B6 status [203]. Increasing PA-ratio may represent either enhanced catabolism of B6 as seen during inflammation, or redistribution of PL and PLP between the intracellular, extracellular and the blood compartment [203]. The higher concentration of PL among the TTA treated rats lower the PAR, further supporting the argument of the TTA treated rats having a reduced level of inflammation.

4.3.7 TTA treatment and folate metabolism

Plasma folate was lower in the TTA compared to control group (Figure 10). Interestingly, the HF treatment seemed to have the opposite effect. None of these differences reached statistical significance, but the effect sizes were nevertheless large. As previously mentioned, limited availability of vitamin B2 as seen in the TTA treated rats may have reduced the production of mTHF. The precursor of mTHF, MTHF, may be synthesized by two different enzymes, namely MTHFD1 and SHMT1. It is previously demonstrated that SHMT1-derived MTHF is primarily partitioned towards *de novo* synthesis of thymidylate and not remethylation of Hcy [91, 92]. As SHMT1 is a PLP-dependent enzyme, higher availability of this cofactor may also enhance MTHF synthesis through this pathway. The production of MTHF through MTHFD1 is dependent on sufficient supply of formate, such as the formate derived from the mitochondrial demethylation reactions of the choline oxidation pathway, conversion of serine to glycine and from glycine catabolism through the glycine cleavage system (Figure 2). As PPARα activation is inhibiting the transcription of several enzymes involved in these reactions, the formate production may be decreased. Thus, more MTHF is possibly originating from the SHMT1 reaction, supporting the hypothesis that mTHF synthesis is reduced due to MTHF being increasingly partitioned towards thymidylate synthesis, in addition to the decreased availability of vitamin B2 as cofactor for MTHFR.

4.3.8 TTA treatment and vitamin B12

Another striking between-group difference was observed for MMA, with much higher plasma and urinary concentrations observed in the TTA treated rats as compared to the rats receiving the control diet (Figure 10). However, no difference was found between the groups regarding plasma cobalamin, suggesting a TTA-induced functional, albeit not overt, cobalamin deficiency. MMACHC, the chaperone responsible for making cobalamin available for MS and mut, is dependent on vitamin B2 and glutathione for its function [113]. Additionally, MSR, an enzyme crucial for maintaining MS activity and function, is also dependent on vitamin B2 [114]. Reduced availability of vitamin B2 may thus be of major importance for the observed increase in MMA. Lower grade of inflammation as discussed earlier, in addition to the suggested PPARα-mediated inhibition of the transsulfuration pathway [158], could cause a reduction in glutathione synthesis [19], further contributing to the proposed reduction in MMACHC function. If the TTA treatment is contributing to such a metabolic cobalamin deficiency through PPARα activation, this also sheds further light on the higher concentration of DMG by reducing MS function as previously discussed.

5 Conclusion

In this study, we have demonstrated that PPAR activation with the pan-PPAR agonist TTA is associated with altered blood and urinary concentrations of one-carbon metabolites and markers of B-vitamin status. A particular large effect was observed for DMG, NAM, mNAM and MMA, which were all higher among TTA-treated rats. Based on current and previous results, these effects are probably mainly mediated through PPARα, but future investigations need to address the issue of relating these effects to a specific PPAR subtype using more subtype specific agonists as well as evaluating the additional effects of dietary fat.

6 Future perspectives

Today, dietary advice is generally based on mean effects found in large epidemiological studies. Such studies will not be able to take individual variability into account, and subgroups of participants who may respond in opposite directions will thus not be revealed. When evaluating whole populations this may wrongly be interpreted as a null-effect, hence increasing the risk of making Type 2 errors. Extremely large sample sizes and the exploration of several relationships simultaneously will increase the experimental error rate by yielding many statistically significant associations having small effect sizes [204], hence increases the probability of making Type 1 errors. Personalized nutrition has been used to treat rare, inborn errors of metabolism like phenylketonuria. Under such circumstances, metabolic factors lay the foundation for very specific dietary advice. However, recent research is addressing the implementation of a similar approach aimed at the general population, targeting personalized nutrition [205].

As PPARs play a central role as master regulators of metabolism, biomarkers of PPAR activity, among which several are proposed in the current study, could help identifying subgroups which could benefit of certain nutritional advice. Hence, more research using the targeted metabolomics approach to explore PPAR function is warranted. The results of the current study suggested elevated plasma DMG, MMA, and the B3 metabolites NAM and mNAM as candidate biomarkers of PPARα activity, but as TTA is a pan-PPAR agonist, these metabolites should also be evaluated in animal models using more subtype-specific PPAR agonists. Such a study has recently been conducted in our research group, where both specific agonists and antagonists of PPARα and PPARγ, respectively, were used. The results from this study may shed further light on the relationship between PPARs and the one-carbon metabolism related metabolome and B-vitamin status in blood and urine. In this study, gene

expression of already established PPAR targeted genes and also genes involved in one-carbon metabolism will be measured, exploring the metabolome in light of PPAR regulation.

The applicability of these putative biomarkers of PPARα activity to humans is another aspect which should be targeted in future studies. First, associations between diet and clinical outcome should be addressed in subgroup analyses of existing cohorts, preferably randomized controlled trials, where the participants are stratified according to their metabolic profile [206]. Such analyses are already being conducted in our research group, based on cohort studies of patients with stabile angina [207] and acute myocardial infarction [208]. However, it should be noted that subgroup analyses increases the number of statistical tests performed, thus increasing the likelihood of Type 1 errors [209]. It should also be kept in mind that in a large data set, lots of spurious subgroup differences will exist [209]. Also, as the number of participants and clinical events in the various subgroups is reduced as compared to the total population, the statistical power decreases, increasing the risk of Type 2 errors [206]. These limitations should thus be kept in mind when performing such analyses, and subgroup effects arising from post hoc hypotheses should always be validated in a different data set [209].

Findings from the screening studies should in the end lead to randomized dietary intervention trials where participants receive dietary advice according to their metabolic profile, and the ultimate aim will be to discover biomarkers which could be used as basis for individualization of nutritional advice.

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Peroxisome proliferator-activated receptor activation is associated with altered plasma one-carbon metabolites and B-vitamin status in rats

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Abstract

Introduction: Increased systemic concentrations of metabolites along the choline oxidation pathway have previously been linked to adverse cardiovascular risk, and altered genetic transcription of key enzymes mediated by the peroxisome proliferator-activated receptors (PPARs) may be involved. We investigated the effect of PPAR activation on blood and urine concentrations of one-carbon metabolites and markers of B-vitamin status in rats.

Methods: During 50 weeks, Male Wistar rats received either a low fat control diet, a high fat (HF) diet or a HF diet supplemented with tetradecylthioacetic acid (TTA), a pan-PPAR agonist with pronounced affinity towards PPARα. Blood and urine samples were analyzed, and results were compared by one-way ANOVA. Planned comparisons versus control were made for both intervention groups, and we report the respective Cohen's d effect sizes.

Results: Essentially no between-group differences were observed in blood or urine according to dietary fat intake. However, compared to control, TTA-treated animals had higher plasma dimethylglycine (d=5.05), glycine (d=1.3), serine (d=1.99) and cystathionine (d=1.52), nicotinamide (d=6.4), methylnicotinamide (d=4.05), methylmalonic acid (d=3.98) and pyridoxal (d= 2.73), whereas plasma riboflavin (d= -1.6) and flavin mononucleotide (d= -2.22) were lower. Urinary concentrations of dimethylglycine (d=1.98), sarcosine (d=1.16) and methylmalonic acid (d=1.89) were higher among TTA treated rats ($p < 0.01$ for all comparisons).

Conclusion: Long-term TTA treatment was associated with pronounced alterations in blood and urinary concentrations of one-carbon and choline metabolites, as well as markers of B-vitamin status in rats. Our findings add to the evidence of the one-carbon metabolism being regulated by PPARs.

Introduction

Elevated plasma total homocysteine (tHcy) is related to increased risk of cardiovascular disease (CVD) [1], but lowering tHcy with B-vitamins has not improved prognosis among CVD patients [2], questioning a causal relationship. This encourages investigation into novel mechanisms associated with elevated plasma tHcy [3]. Of interest, circulating and urinary concentrations of various metabolites along the choline oxidation pathway have been related to major lifestyle diseases, including CVD and diabetes [4-8]. We have recently shown that higher plasma dimethylglycine (DMG) concentration is associated with increased risk of acute myocardial infarction as well as total and cardiovascular mortality, independent of traditional risk markers such as elevated plasma tHcy [6, 7].

Homocysteine (Hcy) reside at a branch point of three metabolic pathways. Remethylation of Hcy back to methionine is catalyzed either by the cobalamin-dependent methionine synthase (MS) or betaine-homocysteine methyltransferase (BHMT), using 5-methyltetrahydrofolate (mTHF) or betaine as the methyl donor, respectively. Hcy catabolism to form cysteine is carried out by the vitamin B6 dependent transsulfuration pathway [9] (Fig 1). The choline oxidation pathway is linked to Hcy metabolism through BHMT. This reaction produces DMG [10], and flux through BHMT has been shown to be associated with demethylation of the promoter region of the Peroxisome proliferator-activated receptor (PPAR) α gene in mice, resulting in increased expression of *PPARα* and its target genes [11]. DMG is further oxidized to sarcosine and glycine by the two mitochondrial flavoproteins DMG dehydrogenase (DMGDH) and sarcosine dehydrogenase (SARDH) [12]. In rats, activation of PPARα was demonstrated to reduce the transcription of both DMGDH and SARDH [13]. PPARα activation also affects the genetic expression of both enzymes of the transsulfuration pathway in rats [13], further supporting a relationship between PPARs and the one-carbon and choline metabolism. Hence, we previously suggested that the association between elevated DMG concentrations and CVD risk may partly be explained by enhanced PPARα activity [6, 7].

PPARα is a key regulator of energy metabolism [14], with a vast number of identified target genes [15]. This nuclear receptor is shown to be activated by dietary or endogenous fatty acids and their derivatives [16]. Tetradecylthioacetic acid (TTA) is a sulfur-containing fatty acid analogue and pan-PPAR agonist with a pronounced affinity towards PPARα [17], and recently, long-term TTA treatment was associated with lower protein levels of DMGDH

and SARDH in rats [18]. Whether TTA treatment affects the related metabolites has not been previously investigated. While the status of folate, B6 and B12 is known to influence tHcy concentration in blood [19], the relation between B-vitamins and other one-carbon metabolites are not fully elucidated. Most reactions in the metabolic pathways discussed depends on B-vitamins as cofactors, and accordingly, their availability is likely important. PPARα regulate key enzymes in the synthesis of vitamin B3 from tryptophan [20], but whether PPARα activation influences the status of other B-vitamins are uncertain.

Thus, in the current substudy, we aimed to investigate the association between PPAR activation, by either a high fat (HF) diet alone or additional TTA supplementation, and blood and urinary concentrations of components of the choline oxidation pathway and one-carbon metabolites, as well as systemic markers of B-vitamin status.

Methods

Animals and diets

The animals (n=30) were randomly allocated to receive one out of three diets for 50 weeks: 1) A low fat control diet (Control) with 7% fat (5% lard, 2% soybean oil, w/w); 2) a HF diet with 25% fat (23% lard, 2% soybean oil, w/w); 3) a HF diet supplemented with TTA treatment (22.6% lard, 2% soybean oil, 0.4% TTA, w/w). All diets were isoenergetic and isonitrogenous (20% protein, w/w), had the same amounts of micronutrients, and the rats had free access to water and feed. Casein (Tine BA, Oslo, Norway) was used as the protein source. Fat sources were lard (Ten Kate Vetten BV, Musselkanaal, The Netherlands) and soybean oil (Dyets Inc., Bethlehem, Pa, USA). TTA was provided by the Lipid Research Group (Department of Clinical Science, University of Bergen, Bergen, Norway). Tert-butyl-hydroquinon was obtained from Sigma-Aldrich (St. Louis, MO, USA), and the rest of the ingredients (cornstarch, sucrose, fiber, AIN-93 G mineral mix, AIN-93 vitamin mix, L-cysteine and choline bitartrate) were obtained from Dyets Inc (Bethlehem, Pa, USA). The pellets were made by Nofima Ingredients (Bergen, Norway). The animals investigated were part of a larger study, and more detailed description of this experiment has previously been reported [21].

After 50 weeks, 4-6 h into the light cycle, the animals were sacrificed under non-fasting conditions, anaesthetized by Isofluorane (Forane, Abbott Laboratories, Abbott Park, IL, USA) inhalation. Blood was drawn by cardiac puncture and collected in BD Vacutainer

tubes containing EDTA (Becton-Dickinson, Plymouth, UK). Urine was collected directly from the urinary bladder.

The animal experiments were designed to comply with the Guidelines for the Care and Use of Experimental Animals and the study protocol was approved by the Norwegian State Board for Biological Experiments with Living animals.

Biochemical analyses

Except plasma cobalamin, which was measured in seven rats per group due to limited amounts of plasma, blood metabolites were analyzed in 10 rats per group. Urinary specimens were not available for all rats. In the control group, 9 urine samples were available for methionine, choline, betaine and DMG, while 8 samples were available for tHcy, cystathionine, cysteine, sarcosine, glycine, serine and MMA. In the HF group, all urinary analytes were available for 9 animals, while 8 values were obtained for all analytes among TTA treated rats. All analyzes were performed at Bevital A/S (Bergen, Norway, http://www.bevital.no). In plasma, methylmalonic acid (MMA), tHcy, serine and glycine were analyzed by gas chromatography- tandem mass spectrometry (GC-MS/MS) [22]. Plasma choline, betaine, DMG, methionine and cysteine [23], as well as all vitamin B2, B3, and B6 metabolites, cystathionine and the B3 catabolite N^1-methylnicotinamide [24] were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Plasma folate and cobalamin were measured by microbiological assays [25, 26]. In urine, cysteine, cystathionine, sarcosine, glycine, serine and MMA were measured by GC-MS/MS [22], and methionine, choline, betaine and DMG by LC-MS/MS [23].

Statistical analyses and presentation of data

The results are presented as means (standard deviation [SD]), and between-group differences were tested by one-way analysis of variance (ANOVA). We carried out planned contrasts to compare both intervention groups to the control group. Cohen's d effect sizes were calculated by pooling the SDs and are provided for all comparisons.

The concentrations of urinary metabolites were corrected for urine creatinine concentrations, to adjust for dilution. We evaluated the relationship between plasma and urinary concentrations of metabolites by calculating Pearson's correlation coefficients. Statistics were performed by using IBM SPSS Statistics for Windows, version 21 (SPSSIBM.,

NY, USA). Because the results were not adjusted for multiple comparisons, p-values <0.01 were considered statistically significant. A d >0.8 were considered a large effect [27].

Results

Mean (± SD) concentrations for all plasma and urinary metabolites and results from the comparisons are presented in Tables 1 and 2, respectively. HF treated rats did not significantly differ from the control group on any of the analyzed metabolites. Compared to rats receiving the control diet, rats treated with TTA differed in most metabolites and markers of B-vitamin status. For components of the transsulfuration pathway, higher concentration of plasma cystathionine was observed (d=1.52, p=.006). Along the choline oxidation pathway, rats in the TTA intervention group had higher concentrations of plasma DMG (d=5.05, p<.001), glycine (d=1.3, p=.003) and serine (d=1.99, p<.001). In urine, higher concentrations were observed for DMG (d=1.98, p=.006) and sarcosine (d=1.16, p=0.01).

Among the B-vitamers and metabolites, we observed lower concentrations of plasma riboflavin (d=-1.6, p<.001) and FMN (d=-2.22, p<.001) whereas plasma nicotinamide (NAM) (d=6.4, p<.001), N^1-methylnicotinamide (mNAM) (d=4.05, p<.001) and pyridoxal (PL) (d=2.73, p<.001) were higher in the TTA treated rats as compared to control. Plasma folate tended to be lower (d=-1.15, p=0.024) whereas no difference was observed for plasma cobalamin, albeit the plasma concentrations of the functional marker of cobalamin deficiency, MMA, was higher (d=3.98, p<.001) in the TTA treated group. Accordingly, urine concentration of MMA was also significantly higher in TTA treated rats when compared to those receiving the control diet (d=1.89, p<0.001). The largest effect sizes observed in plasma are shown in figure 2.

As shown in Table 3, there were positive correlations between plasma and urinary betaine (r=.59), DMG (r=.82) and MMA (r=.85) concentrations.

Discussion

Main Findings

This long-term, 50 week, animal study indicates that TTA treatment was associated with pronounced effects on systemic concentrations of metabolites along the choline oxidation pathway and one-carbon metabolism, as well as markers of B-vitamin status. The largest effect sizes were observed for plasma concentrations of DMG, NAM, mNAM and MMA, which were all higher, and FMN and PL which were lower in the TTA group.

Previous studies

The PPARs have been thoroughly explored according to their role in lipid and glucose metabolism, but the relationship between PPARs and other metabolic pathways has only recently gained some attention. Involvement of PPARα in amino acid metabolism has been demonstrated [28], and fibrates, which are specific PPARα ligands have consistently been associated with elevated plasma tHcy [29]. For components of the choline oxidation pathway, treatment with the specific PPARα agonist WY14,643 has demonstrated a reduction in *DMGDH* and *SARDH* mRNA in rats [13] and lower protein level of SARDH in mice [30].These results are in accordance with the previously reported proteomic findings among the animals currently investigated [18]. Moreover, as TTA is known to preferentially activate PPARα over the other PPAR subtypes [17], our observations are most likely mediated mainly through PPARα. The present study thus extends previous findings by demonstrating that PPARs also likely affect the flux through the choline oxidation pathway and one-carbon metabolism pathways, as well as the status of closely related B-vitamins.

Possible mechanisms

TTA treatment and the choline oxidation pathway

The particularly high concentration of DMG associated with TTA treatment could be explained by TTA induced alterations in DMG production, catabolism, urinary excretion or a combination thereof. At least part of the association is probably explained by decreased catabolism of both DMG and sarcosine, as supported by the lower protein levels of DMGDH and SARDH among TTA-treated animals in the current investigation [18]. Data on plasma sarcosine could have shed further light on a potential accumulation of metabolites downstream of DMG, but unfortunately we were not able to determine sarcosine in plasma due to analytical interference from the EDTA in tubes used for blood sampling. However, urinary concentrations of both DMG and sarcosine were higher in the TTA treated rats, indicating a spill-over effect from higher plasma concentrations. This was further supported by the strong correlations between plasma and urinary concentrations of DMG and betaine. Sarcosine can also be produced from glycine in the cell cytosol, via glycine N-methyltransferase (GNMT). As PPARα is suggested to inhibit flux through GNMT [13, 30], decreased cellular sarcosine production may contribute to the higher plasma concentrations of glycine and serine among the TTA treated rats. Moreover, glycine may be formed from serine which can be derived through glycolysis [31], which is known to be inhibited by PPARα [15].

Thus, higher plasma concentrations are probably not due to increased flux through the glycolytic pathway. Another possibility of glycine synthesis is from threonine catabolism [31], and as the plasma threonine concentration was markedly higher in the TTA treated rats [32], threonine degradation is a possibly contributing to increased glycine concentrations. Notably, increased glycine concentration could also be related to enhanced carnitine synthesis [33], and as both PPARα activation [34] and TTA treatment [32] is associated with increased activity of this pathway, it is reasonable to suspect this pathway being a main contributor to the elevated glycine concentration.

Lower circulating B_2 vitamers were observed in the TTA treated rats compared to those in the control group. Both DMGDH and SARDH are flavoproteins [35], and lower availability of the B2-vitamers may thus directly reduce DMG catabolism. Moreover, folate-dependent remethylation of Hcy utilizes mTHF, which is produced from methylenetetrahydrofolate by methylenetetrahydrofolate reductase (MTHFR). The activity of MS is also dependent on another enzyme, MS reductase (MSR), and notably, both MTHFR and MSR are flavoproteins [36, 37]. Thus, reduced MS flux due to lower availability of vitamin B2, also suggested by a trend towards lower plasma folate in the TTA treated rats, may reflect a compensatory increase in BHMT-mediated remethylation, enhancing DMG production.

The current findings therefore suggest that higher DMG concentration may be explained by a combination of increased production and decreased catabolism, and lower availability of B2-vitamers may be involved in both mechanisms. The elevated concentrations of glycine and serine may be due to PPARα-dependent increased synthesis of carnitine in addition to decreased cellular sarcosine production, with a possible contribution from threonine degradation which should be evaluated in future studies.

TTA treatment and vitamin B3

B3 vitamers are cofactors for a vast number of enzymatic redox reactions, thereof in the β-oxidation of fatty acids and in substrate oxidation in Krebs cycle [38]. In one-carbon metabolism related pathways, NADP is used as a reducing agent for both MTHFR and MSR [36, 37], as well as in the production of betaine from betaine aldehyde [39]. The primary cofactor form, nicotinamide adenine dinucleotide (phosphate) (NAD(P)), is formed from the precursors NAM and NA, and in the current study TTA treatment was associated with significant higher concentrations of NAM and also its breakdown metabolite mNAM. PPARα

activation by WY14.643 was previously suggested to increase the production of NAM originating from the catabolism of tryptophan [13, 20, 40], and accordingly, such treatment has consistently been associated with elevated urinary concentrations of both NAM and mNAM [13, 41, 42]. Higher concentrations of NAM and mNAM observed in the TTA group may thus be due to PPARα-induced increased production, and may also be related to increased requirements for vitamin B3 due to enhanced β-oxidation, a well-known PPARα effect [43].

TTA treatment and vitamin B6

The transsulfuration pathway is activated by oxidative stress [44], and systemic vitamin B6 deficiency has previously been associated with both increased oxidative stress [45] and inflammation [46]. Of the B6 vitamers, only PL differed significantly between groups, with higher concentration being observed in the TTA group. Although the most commonly used marker of vitamin B6 status is PLP, the total amount of circulating vitamin B6 is regarded a more precise measurement of B6 status [47]. Higher total plasma vitamin B6, as indicated by the much higher PL among TTA treated rats, may therefore suggest treatment induced lower inflammation and oxidative stress, consistent with the anti-inflammatory and anti-oxidative effects seen by PPARα activation [48, 49] and TTA treatment [50, 51].

TTA treatment and vitamin B12 status

The only known biological function of cobalamin is to serve as a cofactor for the enzymes MS and methylmalonyl-Coenzyme A mutase (Mut). Mut catalyzes the catabolism of methylmalonyl-Coenzyme A in the mitochondria. During cobalamin deficiency, MMA is formed from methylmalonyl-CoA (Fig 1), hence plasma MMA is utilized as a clinical marker of cobalamin deficiency [52]. The intracellular processing of cobalamin is complex and involves several chaperones. Of particular interest is the methylmalonic aciduria combined with homocystinuria type C (MMACHC) protein, a flavoprotein responsible for making free cobalamin available for production of the two cofactor forms [53]. Dealkylation of cobalamin by MMACHC is dependent on glutathione transferase activity [54], linking cobalamin metabolism to the transsulfuration pathway, as the latter is a substantial source of cysteine, the limiting amino acid for glutathione synthesis [55]. In our study, higher concentrations of MMA were observed in both plasma and urine among the TTA treated rats. Plasma cobalamin concentration, however, were unaffected, indicating a metabolic cobalamin deficiency not associated with low circulating cobalamin. Because MMACHC is dependent on vitamin B2,

lower availability of B2-vitamers may have contributed to the higher MMA concentrations. Lower flux through the transsulfuration pathway, as previously suggested with PPARα activation [13], and supported by the higher plasma cystathionine concentrations in the TTA group, may also have contributed due to the role of glutathione in MMACHC function. As cobalamin is a crucial cofactor for MS, a functional cobalamin deficiency may also contribute to increased DMG production caused by a compensatory enhanced BHMT-flux, as discussed above.

Taken together, the current as well as previous studies suggest that various pathways of the one-carbon and choline oxidation pathways may interconnect with and be regulated by PPARα related mechanisms, probably both through a direct effect on important enzymes and by influencing the availability of B-vitamin cofactors.

Strengths and limitations

The main strength of this study is its controlled, long-term dietary intervention design. Although we cannot establish a certain time-dependent effect by TTA, the large and highly statistically significant between-group differences in terms of concentrations of several metabolites are suggestive of an effect by the TTA intervention per se. Extrapolation of the results to humans may, however, not be straightforward, as PPARα affects rodents differently and to a larger extent than humans [56, 57]. It is also important to keep in mind that blood concentrations of the various metabolites do not necessarily reflect their extravascular or intracellular concentrations, prompting carefulness in the interpretation in terms of metabolic flux [58].

Clinical application

The involvement of PPARs in all aspects of nutrient metabolism is well established. Thus, information on the activity of PPARs, and PPARα in particular, may be of future interest when considering tailored treatment or nutritional advice to the individual person. Metabolomics has been proposed as an important tool to understand PPARα function [42], and targeted metabolic profiling focusing on one-carbon metabolites may prove to supply valuable information regarding PPAR activity.

Conclusions

We have demonstrated that long-term treatment with the pan-PPAR agonist TTA is associated with altered plasma and urinary concentration of several one-carbon and choline metabolites,

as well as markers of B-vitamin status in rats, with the largest effect sizes observed for plasma DMG, NAM, mNAM and MMA. Our findings should motivate further investigation into the relationship between PPARs and these metabolic pathways.

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Table 1. Plasma concentration of one-carbon metabolites, choline oxidation products and markers of B-vitamin status, according to dietary intervention group.

	Dietary treatment group			ANOVA	HF vs. Control		TTA vs. Control	
	Control	HF	TTA	p†	d	p‡	d	p‡
One-carbon metabolites, µmol/L								
Methionine	73.3 ± 9.72	71.7 ± 10.8	65.3 ± 8.7	0.18	-0.16	0.716	-0.87	0.080
tHcy	7.2 ± 1.8	7.9 ± 2.5	9.3 ± 2.5	0.13	0.35	0.467	0.98	0.047
Cystathionine	0.64 ± 0.11	0.65 ± 0.13	1.02 ± 0.34	0.002	0.06	0.905	1.52	0.006
Cysteine	171.4 ± 30.9	166.2 ± 20.8	189.3 ± 28.3	0.15	-0.20	0.670	0.60	0.150
Choline metabolites, µmol/L								
Choline	9.2 ± 1.4	7.8 ± 1.6	10.0 ± 2.9	0.09	-0.91	0.057	0.36	0.439
Betaine	78.9 ± 15.5	68.0 ± 12.7	72.4 ± 31.2	0.52	-0.77	0.262	-0.27	0.498
DMG	4.4 ± 1.2	5.6 ± 2.0	15.5 ± 2.9	<0.001	0.75	0.207	5.05	0.000
Glycine	283.1 ± 45.5	268.6 ± 54.2	408.6 ± 129.0	0.002	-0.29	0.706	1.30	0.003
Serine	368.9 ± 45.	408.0 ± 78.7	586.9 ± 148.4	<0.001	0.61	0.392	1.99	<0.001
Markers of B-vitamin status								
B2 vitamers, nmol/L								
Riboflavin	27.1 ± 7.7	19.0 ± 4.4	15.1 ± 7.3	0.001	-1.29	0.011	-1.6	<0.001
FMN	24.5 ± 11.0	14.3 ± 10.1	6.3 ± 3.6	0.001	-0.97	0.044	-2.22	<0.001
B3 vitamers, nmol/L								
NAM	1658 ± 432	1832 ± 467	6138 ±890	<0.001	0.39	0.544	6.4	<0.001
mNAM	88.2 ± 93.1	50.7 ± 22.6	1154.0 ± 360.2	<0.001	-0.55	0.244	4.05	<0.001
NA	83.2 ± 20.2	79.8 ± 18.9	86.7 ± 26.1	0.78	-0.18	0.695	0.15	0.728
B6 vitamers, nmol/L								
PLP	260.7 ± 40.7	243.8 ± 54.2	277.3 ± 99.5	0.57	-0.35	0.591	0.22	0.596
PL	2271 ± 34.7	207.6 ± 26.5	350.3 ± 53.5	<0.001	-0.63	0.283	2.73	0.000
PA	40.5 ± 9.7	30.9 ± 4.4	32.0 ± 24.5	0.33	-1.27	0.175	-0.46	0.226
Folate, nmol/L	66.9 ± 6.4	86.3 ± 22.1	53.1 ± 15.7	0.001	1.19	0.023	-1.15	0.024
Cobalamin, pmol/L	613 ± 42	671 ± 68	654 ± 107	0.37	1.03	0.173	0.5	0.333
MMA, µmol/L	0.35 ± 0.08	0.37 ± 0.09	1.04 ± 0.23	<0.001	0.21	0.646	3.98	<0.001

DMG indicates dimethylglycine; FMN, flavin mononucleotide; HF, high fat; LF, low fat; MMA, methylmalonic acid; mNAM, N^1-methylnicotinamide; NA, nicotinic acid; NAM, nicotinamide; PA, pyridoxic acid; PL, pyridoxal; PLP, pyridoxal-5-phosphate; tHcy, total homocysteine, TTA, tetradecylthioacetic acid

†One-way ANOVA

‡Planned contrasts

Table 2. Urinary concentrations of one-carbon metabolites, choline oxidation products and methylmalonic acid, according to dietary intervention group. The values are adjusted according to urinary creatinine concentration.

	Dietary treatment group			ANOVA	HF vs Control		TTA vs Control	
	Control	HF	TTA	p†	d	p‡	d	p‡
One-carbon metabolites, µmol/L								
Methionine	81.5 ± 65.2	150.4 ± 132.0	100.3 ± 98.3	0.36	0.66	0.19	0.23	0.65
tHcy	0.94 ± 0.45	1.1 ± 0.48	1.31 ± 0.54	0.32	0.36	0.49	0.76	0.14
Cystathionine	7.6 ± 9.18	10.6 ± 7.5	11.6 ± 14.2	0.73	0.36	0.56	0.34	0.45
Cysteine	41.4 ± 34.9	56.9 ± 37.9	45.0 ± 29.6	0.63	0.42	0.37	0.11	0.82
Choline metabolites, µmol/L								
Choline	333 ± 369	842 ±1088	414 ± 617	0.33	0.63	0.17	0.16	0.83
Betaine	23.2 ± 10.5	23.0 ± 8.96	32.8 ± 16.5	0.20	-0.02	0.96	0.70	0.19
DMG	7.4 ± 4.5	10.4 ± 11.3	45.2 ± 27.5	0.003	0.35	0.48	1.98	0.006
Sarcosine	0.67 ± 0.44	0.97 ± 0.79	3.11 ± 2.94	0.02	0.46	0.73	1.16	0.01
Glycine	162 ± 115	295 ± 226	202 ± 134	0.25	0.73	0.15	0.31	0.54
Serine	349 ± 296	685 ± 544	453 ± 412	0.26	0.75	0.13	0.29	0.64
Markers of B-vitamin status								
MMA, µmol/L	3.26 ± 1.49	3.04 ± 1.40	8.21 ± 3.39	<0.001	-0.16	0.84	1.89	<0.001

DMG indicates dimethylglycine; HF, high fat; MMA, methylmalonic acid; tHcy, total homocysteine; TTA, tetradecylthioacetic acid

† One-way ANOVA

‡Planned contrasts

Table 3. Correlations between blood and urinary metabolites in all rats

	r (CI)[1]	P†
Methionine	0.02 (-0.34, 0.43)	0.91
tHcy	0.17 (-0.01, 0.23)	0.43
Cystathionine	0.21 (-0.09, 0.56)	0.31
Cysteine	0.01 (-0.40, 0.46)	0.96
Choline	-0.06 (-0.51, 0.29)	0.78
Betaine	0.59 (0.05, 0.85)	0.002
DMG	0.82 (0.65, 0.94)	<0.001
Glycine	0.00 (-0.3, 0.38)	0.99
Serine	-0.02 (-0.38, 0.35)	0.93
MMA	0.85 (0.77, 0.93)	<0.001

DMG indicates dimethylglycine; MMA, methylmalonic acid; tHcy, total homocysteine.

[1]Pearsons correlation coefficient with bootstrapped confidence intervals

†p-value for correlation

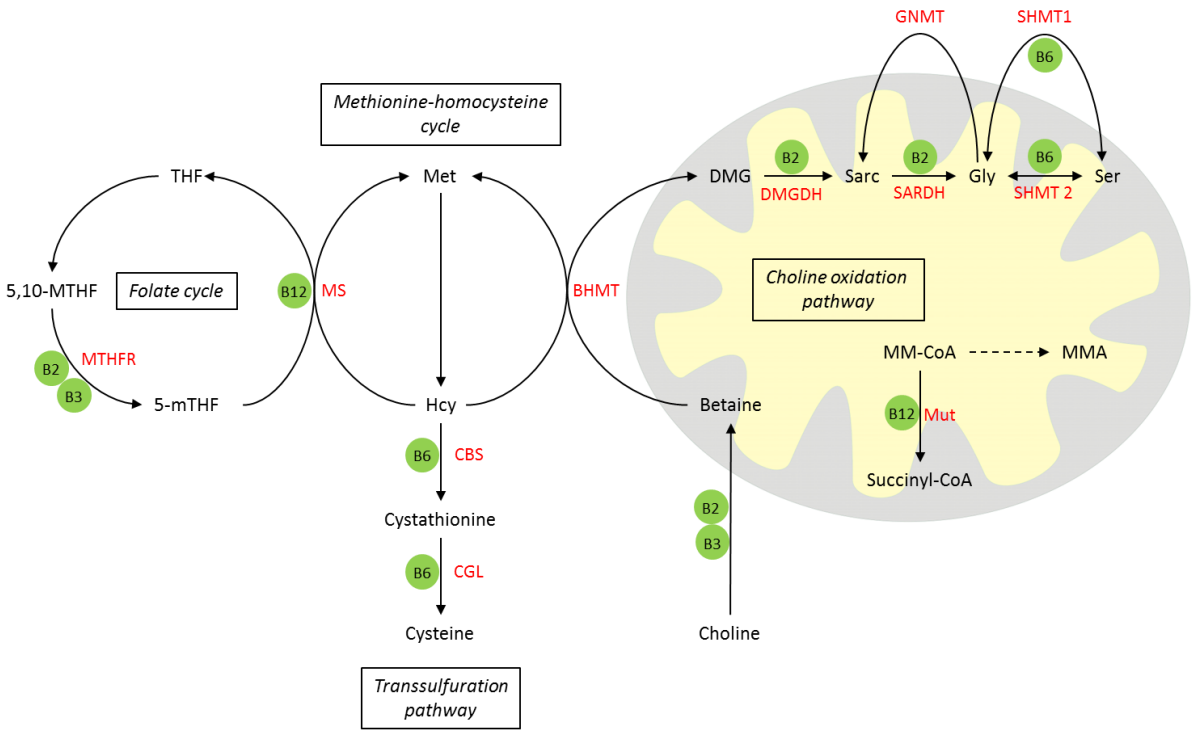
Figure legends

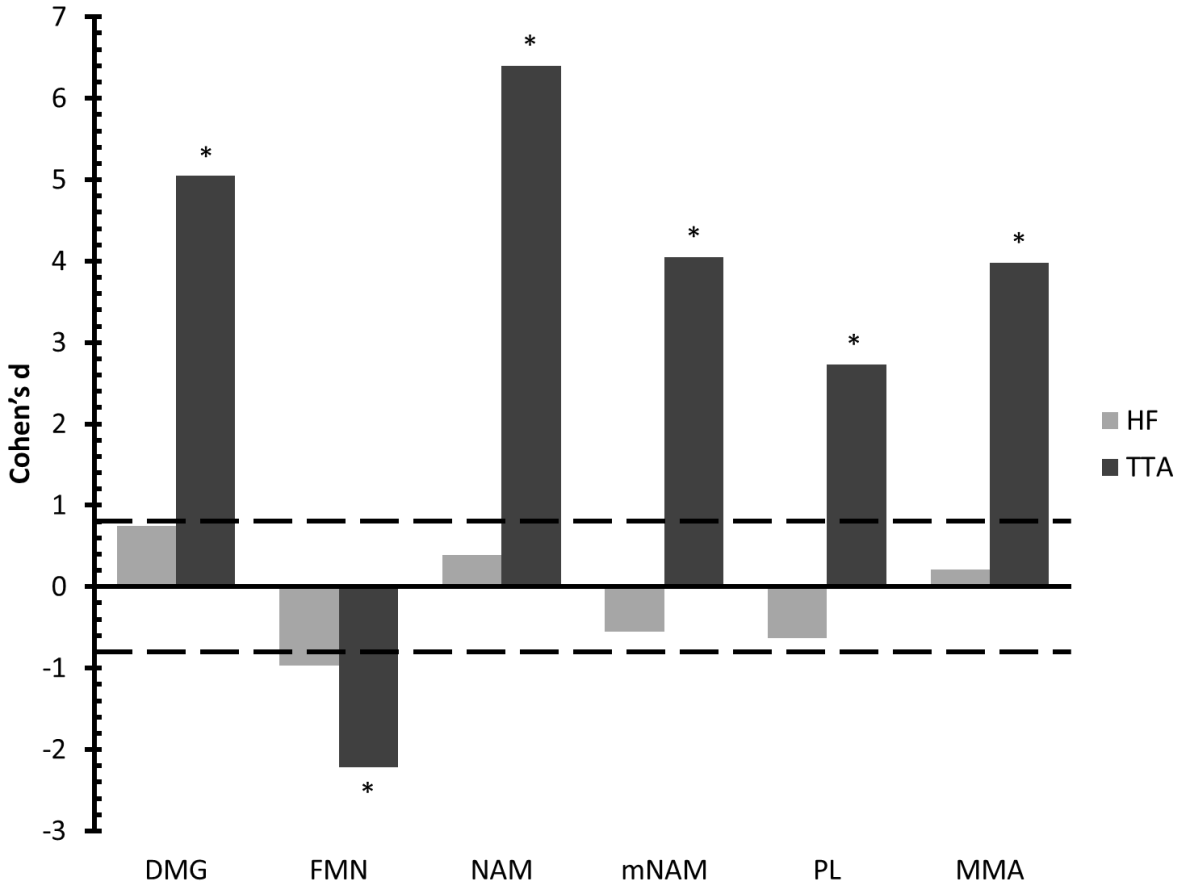
Fig 1.

Overview of relevant metabolic pathways. 5-mTHF indicates methyltetrahydrofolate; 5,10-MTHF, methylenetetrahydrofolate; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; CGL, cystathionine-γ-lyase; DMG, dimethylglycine; DMGDH, dimethylglycine dehydrogenase; Gly, glycine; GNMT, glycine N-methyltransferase; Hcy, homocysteine; Met, methionine; MMA, methylmalonic acid; MM-CoA, methylmalonyl coenzyme A; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; Mut, methylmalonyl-CoA mutase; Sarc, sarcosine; SARDH, sarcosine dehydrogenase; Ser, serine; SHMT, serine-hydroxymethyltransferase; THF, tetrahydrofolate;

Fig 2.

Cohen's d effect sizes for the most pronounced differences in plasma metabolite concentrations between the high fat diet alone and TTA treatment as compared to the control diet. The dashed lines represent Cohen's=0.8 and the asterix indicates $p<0.001$. DMG indicates dimethylglycine; FMN, flavin mononucleotide; MMA, methylmalonic acid; NAM, nicotinamide; PL, pyridoxal.


Methionine-homocysteine cycle
THF
Met
DMG
B2
DMGDH
Sarc
B2
SARDH
Gly
B6
SHMT 2
Ser
GNMT
SHMT1
B6
5,10-MTHF
Folate cycle
B12
MS
BHMT
Choline oxidation pathway
MM-CoA
MMA
B2
B3
MTHFR
5-mTHF
Hcy
Betaine
B12
Mut
Succinyl-CoA
B6
CBS
Cystathionine
B6
CGL
B2
B3
Cysteine
Choline
Transsulfuration pathway


Cohen's d
7
6
5
4
3
2
1
0
-1
-2
-3
*
*
*
*
*
*
DMG
FMN
NAM
mNAM
PL
MMA
HF
TTA