Biomarkers of one-carbon metabolism and B-vitamin status

Targeted metabolomics in rats and humans exploring the effects of PPARα-activation and dietary composition

Vegard Lysne

Thesis for the degree of Philosophiae Doctor (PhD)
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Scientific environment

The project culminating in this thesis was carried out from September 2015 to June 2019 at Centre for Nutrition, Department of Clinical Science, Faculty of Medicine, University of Bergen. My main supervisor was professor Ottar Kjell Nygård, and my co-supervisors were professor Jutta Dierkes, professor Per Magne Ueland and Dr. Elin Strand. The project was conducted in joint collaboration with the Department of Heart Disease, Haukeland University Hospital and Bevital AS.

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Vegard Lysne

Bergen, June 2019

Abbreviations

3Rs	the three R's (Reduction, Replacement, Refinement)
ACMS	2-amino-3-carboxymuconate semialdehyde
ACMSD	2-amino-3-carboxymuconate semialdehyde dehydrogenase
ALP	Alkaline phosphatase
AMI	Acute myocardial infarction
BHMT	Betaine-homocysteine methyltransferase
CAD	Coronary artery disease
CBS	Cystathionine β-synthase
CGL	Cystathionine γ-lyase
CVD	Cardiovascular disease
DAG	Directed Acyclic Graph
DMG	Dimethylglycine
DMGDH	Dimethylglycine dehydrogenase
FAD	Flavin adenine dinucleotide
FFQ	Food Frequency Questionnaire
FMN	Flavin mononucleotide
gMean	Geometric mean
GNMT	Glycine-N-methyltransferase
gSD	Geometric standard deviation

Hcy	Homocysteine
LOESS	Locally estimated scatterplot smoothing
MMA	Methylmalonic acid
mNAM	1-methylnicotinamide
MS	Methionine synthase
mTHF	5-methyltetrahydrofolate
MTHFR	Methylenetetrahydrofolate reductase
MUFA	Monounsaturated fatty acids
NAD	Nicotinamide dinucleotide
NAM	Nicotinamide
NNMT	Nicotinamide methyltransferase
PA	4-Pyridoxic acid
PAr	Pyridoxic acid index, PA/(PL+PLP)
PI	Prediction interval
PL	Pyridoxal
PLP	Pyridoxal-5-phosphate
PPAR	Peroxisome proliferator-activated receptor
PPRE	PPAR responsive elements
PUFA	Polyunsaturated fatty acids
QAPRT	Quinolinic acid phosphoribosyltransferase

RXR	Retinoid-X-Receptor
SAH	S-adenosyl-homocysteine
SAM	S-adenosyl-methionine
SAP	Stable angina pectoris
SARDH	Sarcosine dehydrogenase
SFA	Saturated fatty acids
SHMT	Serine hydroxymethyltransferase
SMD	Standardized mean difference
tHcy	Total Homocysteine
TTA	Tetradecylthioacetic acid
WENBIT	Western Norway B-vitamin Intervention Trial

Summary

BACKGROUND:

One-carbon metabolism is an overarching term describing central metabolic pathways involved in the transfer of one-carbon units. These include the methionine-homocysteine and folate cycles, as well as the choline oxidation and transsulfuration pathways, all of which depend on B-vitamins as cofactors. Circulating concentrations of several metabolites and intermediates of these metabolic pathways have been linked to chronic disease risk. Thus, extending our knowledge regarding the regulation of these pathways is warranted.

Targeted metabolomics offers the opportunity to study the concentration of several metabolites of these pathways simultaneously, and is thus required for the thorough investigation of the effects of diet and other factors on one-carbon metabolism. Diet provides both substrates, cofactors and one-carbon units, influencing the complex regulation of the different metabolic pathways. Furthermore, of particular interest is the activation of the nuclear receptor PPAR (peroxisome proliferator-activated receptor) α, a lipid sensor involved in the regulation of energy metabolism. The overall aim of this thesis was to utilize targeted metabolomics to explore the effect of PPARα-activation and dietary composition on of one-carbon metabolism and B-vitamin status. The effect of pharmacological PPARα-activation was addressed in *Paper I* and *II,* taking advantage of studies in laboratory animals. In *Paper III*, the effect of dietary macronutrient composition was investigated in a cross-sectional analysis of a human cohort.

METHODS:

Animal models: We utilized data from two animal experiments, where male Wistar rats were treated with PPAR-agonists. In the first experiment, the rats received a pan-PPAR-agonist for 50 weeks. In the second experiment, specific PPARα and γ-agonists were administered for 12 days. In both studies, plasma concentrations of metabolites were compared between treated and control animals.

Human study: This was a cross-sectional analysis in a cohort of 1928 patients with stable angina pectoris. Dietary data was derived from a food frequency questionnaire, and associations between dietary composition and plasma metabolite concentrations were assessed with multiple linear regression analyses.

In all studies, plasma one carbon metabolites and B-vitamin markers were quantified by applying mass spectrometric methods.

RESULTS:

Long-term (*Paper I*) and short-term (*Paper II*) pharmacological PPARα-activation influenced the one-carbon metabolome, with the strongest effects seen for increased plasma concentration of nicotinamide and methylnicotinamide (vitamin B3), pyridoxal (vitamin B6), methylmalonic acid (marker of vitamin B12 status), dimethylglycine and glycine (choline oxidation pathway metabolites), and reduced flavin mononucleotide (vitamin B2).

In humans, the observed effects of macronutrient intake were strongest for protein, where increased intake was associated with higher plasma concentrations of pyridoxal, pyridixal-5-phosphate and pyridoxic acid (vitamin B6), vitamin B12, riboflavin (vitamin B2) and methylnicotinamide. Further, we observed inverse associations between protein intake and plasma homocysteine and methylmalonic acid concentrations. When modeling the substitution of saturated with polyunsaturated fatty acids, we observed higher methylnicotinamide, pyridoxal, pyridixal-5-phosphate, cobalamin and dimethylglycine, as well as lower riboflavin concentrations.

CONCLUSION AND IMPLICATION:

PPARα-activation and dietary macronutrient composition altered the concentration of circulating biomarkers of one carbon metabolism. The effects of PPARα-activation were consistent across different conditions, and our results strongly suggest a central role for PPARα in the regulation of these metabolic pathways. Estimated effects of substituting saturated with polyunsaturated fatty acids yielded associations with the biomarkers similar to those observed with PPARα-activation in the animal studies.

This suggests that the effects of diet on one-carbon metabolism, especially related to dietary fatty acid composition, may be partly mediated through altered PPARα-activity.

This is the first metabolomic investigation targeting the majority of the metabolites of the one-carbon metabolism pathways simultaneously. Linking mechanistic studies in animals with observational data in humans provides novel information regarding metabolic regulations. The current investigations extend our understanding of how PPARα-activation and dietary composition influences the one-carbon metabolome. Application to the human situation will offer potential for more individualized dietary advice.

List of Publications

Paper I

Lysne V, Strand E, Svingen GF, Bjorndal B, Pedersen ER, Midttun O, Olsen T, Ueland PM, Berge RK, Nygård O. *Peroxisome Proliferator-Activated Receptor Activation is Associated with Altered Plasma One-Carbon Metabolites and B-Vitamin Status in Rats.* Nutrients. 2016;8(1).

Paper II

Lysne V, Bjørndal B, Grinna ML, Midttun Ø, Ueland PM, Berge RK, Dierkes J, Nygård, O and Strand E. *PPARα activation influences plasma one-carbon metabolites and B-vitamin status in rats.* Manuscript submitted to J Nutr 2019.

Paper III

Lysne V, Parys AV, Nygaard E, Olsen T, Strand E, Marienborg M, Laupsa-Borge J, Haugsgjerd T, McCann A, Ueland PM, Dierkes J and Nygård O. *Dietary macronutrient composition and plasma concentration of one-carbon metabolites and markers of B-vitamin status. A cross-sectional study.* Manuscript ready for submission.

Contents

1. Introduction

1.1 One-carbon metabolism

One-carbon metabolism is an overarching term collectively referring to the multitude of metabolic reactions involving the transfer of one-carbon units, molecules containing one carbon atom. These metabolic pathways are central in the regulation of many cellular processes, and is involved in the pathogenesis of several major lifestyle diseases. Thus, the regulation of these pathways is crucial to maintain health.

Transmethylation is a key reaction of one-carbon metabolism, where a methyl group is transferred from a methyl donor to a methyl acceptor. Transmethylation is involved in both synthesis and modifications of a large variety of molecules throughout the body. Metabolic pathways central to the one-carbon metabolism include the methionine-homocysteine cycle, the transsulfuration pathway, the folate cycle and the choline oxidation pathway. **Figure 1** provides an overview of the metabolic pathways discussed in this thesis, with key enzymes and their B-vitamin cofactors.

1.1.1 The methionine-homocysteine cycle and transsulfuration

Homocysteine (Hcy) is a sulfur-containing amino acid formed during transmethylation reactions from the essential amino acid methionine (1). The main methyl donor for these reactions is S-adenosyl-methionine (SAM), which is derived through adenosylation of methionine, by transferring an adenosyl group from adenosine triphosphate, in a reaction catalyzed by methionine adenosyltransferase (EC 2.5.1.6) (2). The various methyltransferases subsequently facilitate the transfer of the methyl group from SAM to the methyl accepting molecule, leaving S-adenosylhomocysteine (SAH) (3), which is further hydrolyzed into Hcy (4). Synthesis of creatine and phosphatidylcholine are believed to be the major sources for Hcy production (5). Excess Hcy in the cells may be exported out into the blood. In the circulation, most Hcy is found bound to protein, as a disulfide with other sulfur

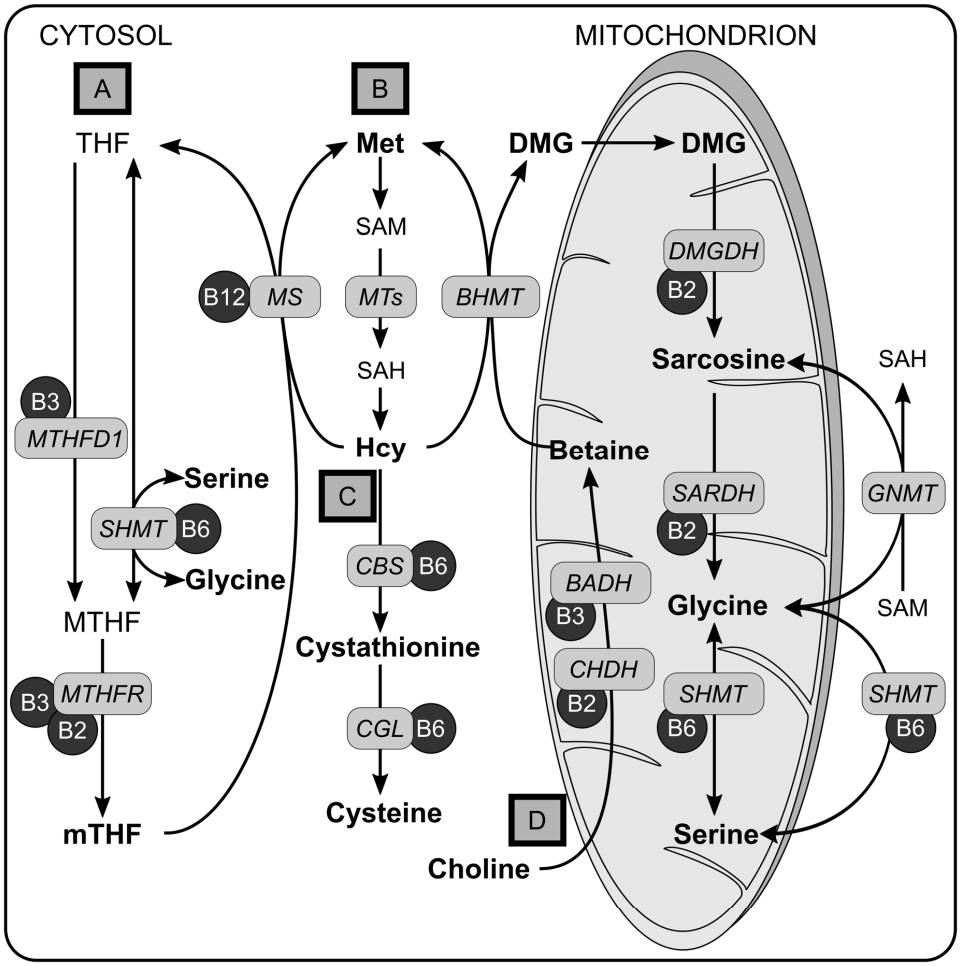

Figure 1. Overview of the metabolic pathways discussed: A) The folate cycle, B) the homocysteine-methionine cycle, C) the transsulfuration pathway and D) the choline oxidation pathway. The bold metabolites are measured in this project. Enzymes are shown in grey boxes and the B-vitamin cofactors in circles. In the folate cycle, a one-carbon unit is transferred to THF, forming mTHF via MTHF. mTHF donates the methyl group to Hcy, which is remethylated to Met in the homocysteine-methionine cycle, where Hcy is formed during transmethylation reactions. Hcy can be irreversibly catabolized to cysteine through the transsulfuration pathway. The choline oxidation pathway consists of the reactions metabolizing choline, and includes the transfer of a methyl group from betaine to Hcy, in a second remethylation pathway. This reaction also yields DMG, which is further metabolized in the mitochondrion. BADH, betaine aldehyde dehydrogenase; CBS, Cystathionine-β-synthase; CGL, cystathionine-γ-lyase; CHDH, Choline dehydrogenase; DMG, dimethylglycine; DMGDH, DMG dehydrogenase; GNMT, Glycine N-methyltransferase; Hcy, homocysteine; Met, methionine; MS, methionine synthase, mTHF, 5-methyltetrahydrofolate; MTHF; 5,10-methylenetetrahydrofolate MTHFD1, methylenetetrahydrofolate dehydrogenase complex 1; MTHFR, methylenetetrahydrofolate reductase; Methyltransferases; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SARDH, Sarcosine dehydrogenase; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate.

compounds, and minor amounts as free Hcy. When measuring circulating Hcy, it is mostly common to measure the total Hcy (tHcy) concentration, which includes all forms. Elevated tHcy is labeled hyperhomocysteinemia (6). When tHcy increases, two Hcy molecules can condensate to form homocystine, which can be subsequently excreted in the urine, referred to as homocystinuria (7).

Residing at a branch point of three metabolic pathways, Hcy has two main metabolic fates within the cells. It can be remethylated back to methionine, or irreversibly converted to cysteine through the transsulfuration pathway (1). Remethylation of Hcy to methionine occurs through either the cobalamin-dependent methionine synthase (MS; EC 2.1.1.13), using 5-methyltetrahydrofolate (mTHF) provided by the folate cycle as methyl donor (8–11), or through betaine-homocysteine methyltransferase (BHMT; EC 2.1.1.5), using betaine from the choline oxidation pathway as methyl donor (12–14). Of importance, MS-mediated remethylation is dependent on MS reductase (EC 1.16.1.8) for its initial activation and sustained activity (15,16). Equal contribution to Hcy remethylation of the two remethylation pathways has been demonstrated under normal conditions (17). While MS is ubiquitously found throughout the human body, BHMT is mainly expressed in liver and kidney (18). The transsulfuration pathway consists of two vitamin B6-dependent enzymes, mainly residing in the liver (18), converting Hcy to cysteine (19). First, cystathionine β-synthase (CBS; EC 4.2.1.22), catalyzes a condensation of Hcy and serine to form cystathionine (20–22). Second, cystathionine is further hydrolyzed to form cysteine by cystathionine γ-lyase (CGL; EC 4.4.1.1) (23,24). Thus, in most tissues Hcy must either undergo remethylation by MS, or be exported to be metabolized in other organs.

The metabolic fate of Hcy is determined according to the situation and requirement, and the coordination between these three pathways is regulated according to the requirement of methionine conservation (17). The SAM:SAH-ratio is frequently used as a measure of cellular methylation capacity, which is homeostatically maintained through the regulation of various methyltransferases (25). When SAM accumulates, transmethylation enzymes are stimulated. Through

scavenging of excess SAM, as well as by producing SAH, which inhibits methylation reactions (26), hepatic glycine-N-methyltransferase (GNMT; EC 2.1.1.20) is regarded central in the regulation of the methylation capacity (27). SAM concentrations in the liver are mainly regulated by phosphatidylcholine synthesis via phosphatidyletanolamine N-methyltransferase (EC 2.1.1.17). However, when SAM accumulates, other routes of SAM removal are also activated, such as the potential for histone methylation (28).

Catalytic activity of both remethylation enzymes is dependent on a zinc atom contained within the enzymes, which facilitates binding and activation of the Hcy molecule (29,30). CBS contains an iron-containing autoinhibitory domain which must be released for the enzymatic activity to occur (31,32). This leaves these three metabolic pathways susceptible to regulation by the redox potential of the cell. Cysteine has been demonstrated to be the limiting factor for the synthesis of our most abundant intracellular antioxidant glutathione. It has been demonstrated that cysteine derived from Hcy transsulfuration contribute to approximately half of the intracellular glutathione pool (33). Additionally, oxidation of the zinc-atoms of MS and BHMT inhibits their activity, limiting remethylation of Hcy in conditions of increased oxidative stress (34,35). SAM directly regulates several of the key Hcy-metabolizing enzymes (36). SAM accumulation inhibits the two remethylation pathways. First, MS-dependent remethylation is reduced by inhibiting methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20), the enzyme responsible for mTHF production (37–39). Second, SAM inhibits BHMT allosterically (40), although only at very high SAM concentrations (41). Further, SAM has been demonstrated to stimulate the removal of Hcy in the liver through the transsulfuration pathway by relieving the autoinhibitory domain of CBS (32,42–44).

Dietary intake of methyl groups influence the partitioning between remethylation and transsulfuration (45,46), and also seems to play a role in the distribution between the two remethylation pathways (47–50). Altogether, the intracellular concentration of Hcy is determined by the rates of production, remethylation and transsulfuration. Circulating tHcy concentration is further

dependent on cellular release into the blood, uptake by other tissues and renal excretion.

1.1.2 The folate cycle

The folate cycle constitutes the intracellular metabolism of folate, which exists in the cells as a group of interconvertible cofactors, sharing a common structure. Throughout the folate cycle the different folate cofactor forms are produced and interconverted, using the reduced folate form tetrahydrofolate as backbone (51).

Folate metabolism is uniquely linked to Hcy metabolism through MS, the only known enzyme able to accept the methyl group of mTHF (52). This reaction yields tetrahydrofolate, which can accept a one-carbon molecule from either formate via the trifunctional enzyme complex mehylenetetrahydrofolate dehydrogenase 1 (EC 6.3.4.3, 3.5.4.9 and 1.5.1.5) (53,54), or from serine in a reversible reaction catalyzed by serine hydroxymethyltransferase (SHMT; EC 2.1.2.1). Both reactions may form 5,10-methylenetetrahydrofolate, which is converted to mTHF by MTHFR. SAM binds to MTHFR and inhibits the activity, meaning mTHF production is enhanced in situations of low SAM and *vice versa*. This contributes to ensuring the requirement for methionine conservation by increasing the capacity for Hcy remethylation when SAM levels are low. Of importance, mTHF also allosterically inhibits GNMT activity (27). This provides a regulatory mechanism where increased SAM concentrations reduce mTHF levels, which subsequently releases the inhibition of GNMT. This facilitates scavenging of excess SAM. Indeed, it has been suggested that decreased Hcy synthesis through GNMT, and not increased remethylation, is the main explanation for the inverse association between folate status and tHcy (55).

1.1.3 The choline oxidation pathway

The choline oxidation pathway refers to the conversion of choline to glycine, through betaine, dimethylglycine (DMG) and sarcosine (56). Choline is a water-soluble quaternary ammonium compound mostly found incorporated into phospholipids as phosphatidylcholine. Choline may originate either from the diet or from endogenous synthesis by methylation of phosphatidyletanolamine to phosphatidylcholine (57).

This methylation occurs through phosphatidyletanolamine N-methyltransferase, and although ubiquitously expressed (18), its activity has been reported to primarily occur in the liver (56). Functions of choline are related to its effect on methylation capacity as a source of methyl groups as well as its involvement in hepatic lipid export (56). Further conversion of phosphatidylcholine to choline and phosphatic acid is catalyzed by phospholipase D (EC 3.1.4.4)(57).

The first step of the choline oxidation pathway is conversion of choline to betaine within the mitochondrion. This happens in two steps, catalyzed by choline dehydrogenase (EC 1.1.99.1) and betaine aldehyde dehydrogenase (EC 1.2.1.8) (58,59). Like choline, betaine may also be found in the diet in addition to being synthesized endogenously from choline. Betaine has two main functions, as an osmolyte involved in cell volume regulation, and as the methyl donor for BHMT-mediated remethylation of Hcy (60). The latter directly links the choline oxidation pathway to the methionine-Hcy cycle (**Figure 1**). In addition to methionine, the BHMT reaction also yields DMG (13). DMG diffuses back into the mitochondrion and is converted via sarcosine to glycine in two subsequent demethylation reactions, catalyzed by DMG dehydrogenase (DMGDH; EC 1.5.8.4) and sarcosine dehydrogenase (SARDH; EC 1.5.8.3), respectively (61). Both *DMGDH* and *SARDH* are mainly expressed in the liver and kidneys (18). Glycine may be reversibly interconverted with serine in a reaction catalyzed by SHMT, occurring both in the cytosolic (SHMT1) and mitochondrial (SHMT2) compartment (62,63). SHMT1 primarily promotes serine synthesis, while SHMT2 promotes glycine synthesis (64). Serine may additionally be synthesized from intermediates of glycolysis, providing a link between the choline oxidation pathway and glucose metabolism (65).

Glycine has several other fates. In the liver and pancreas (18), GNMT may convert glycine back to sarcosine in a SAM-dependent methylation reaction (66). As GNMT binds folate, it may also influence hepatic folate metabolism and folate-dependent reactions (67). A third fate of glycine is catabolism through the nicotinamide adenine dinucleotide (NAD)-dependent trifunctional enzyme complex glycine cleavage system (EC 1.4.4.2, 2.1.2.10 and 1.8.1.4), yielding CO_2, NH_3,

NADH and a one-carbon group which enters the folate cycle (68). Both SHMT (especially cytosolic SHMT1) and glycine cleavage is sensitive to vitamin B6 status, and the activity decreases during deficiency (69,70). In addition to being a building block in protein synthesis, functions of glycine include being a precursor for creatine, purines, glutathione and heme (71). Creatine synthesis includes transmethylation by guanidinoacetate-N-methyltransferase (EC 2.1.1.2), which together with GNMT provides a role for glycine in Hcy production and the regulation of methylation capacity (5).

1.1.4 Plasma one-carbon metabolites and disease risk

In humans, one-carbon metabolites are only available in their transport organ, the blood. Elevated plasma tHcy is a well-established marker of increased atherothrombotic cardiovascular disease (CVD) and coronary heart disease risk (72). Additionally, plasma tHcy has been linked to several other clinical outcomes, including cancer (73), pregnancy complications (74), neural tube defects (75,76), congenital defects of the heart (77), osteoporosis (78), Alzheimer's disease and dementia (79,80), depression (81), cognitive decline (82), hyperinsulinemia (83) and type 2 diabetes (84). However, although treatment with B-vitamins is an effective means of lowering plasma tHcy, secondary prevention of CVD through lowering tHcy with B-vitamins have largely failed to show clinical benefit (85). These observations have questioned the causal relationship, and suggest that elevated plasma tHcy perhaps should be regarded as a marker of underlying pathological mechanisms, rather than a risk factor *per se* (86). It also encourages the investigation into alternative explanations for the association between elevated plasma tHcy and adverse health outcomes, such as redox status. Indeed, the close relationship of methylation status and cellular redox signaling was recently referred to as *Methoxistasis*, and elevated tHcy was suggested to represent disturbances in this homeostasis (87). However, primary prevention with folic acid was shown to reduce the risk of ischemic stroke in a large, hypertensive Chinese cohort, without impacting mortality (88). They argued that the lack of benefit in prior trials could be due to their secondary preventive nature as well as being conducted in populations with high folate status contributing to a possible ceiling effect.

Plasma concentrations of metabolites along the choline oxidation and the transsulfuration pathways have been linked to risk of major life style diseases.

Elevated plasma choline concentrations has been associated with increased risk of cardiac events (89,90), acute myocardial infarction (91) and diabetes (92) among patients with established CVD. Both low and high plasma betaine concentrations have been linked to increased risk of cardiovascular events (93), while higher betaine concentration has been associated with a lower risk of colorectal cancer (94). In patients with established CVD, elevated plasma DMG has been associated with increased risk of acute myocardial infarction and mortality (95,96). Higher levels of sarcosine in the circulation has been associated with increased risk of prostate cancer (97), but the opposite has later been reported (98). In the transsulfuration pathway, plasma cystathionine has been associated with increased risk of acute myocardial infarction and mortality in patients with established CVD (99–101). Of interest, noncanonical reactions of both CBS and CGL yields the gaseous transmitter hydrogen sulphide (H_2S), which has been shown to modulate several physiological functions related to disease risk (102).

Glycine and serine metabolism have gained great attention for its role in cancer biology (65,103), and plasma glycine is inversely associated with acute myocardial infarction risk (104). Further, low plasma glycine concentrations have been observed in patients with obesity or diabetes type 2, and it also predicts diabetes type 2 incidence. Both weight loss and physical activity aimed at improving insulin resistance consistently lead to increased plasma glycine (105).

1.2 B-vitamins and markers of B-vitamin status

The B-vitamins are a heterogeneous group of essential water-soluble nutrients functioning as cofactors for enzymatic reactions in a myriad of physiological functions. Pathways in relation with one-carbon metabolism include enzymes requiring different B-vitamins as cofactors, as indicated in **Figure 1**.

1.2.1 Riboflavin

Riboflavin is a natural flavin belonging to the isoalloxazines. Higher organisms cannot synthesize these molecules, hence riboflavin is an essential nutrient. In the diet, riboflavin is obtained primarily from dairy products, eggs, meat (especially organ meat) and from some vegetables. Riboflavin was the second compound designated as one of the B-vitamins, and is thus known as vitamin B2 (106). In plasma vitamin B2 mainly exists as free riboflavin, but after cellular uptake most of it is found as its two cofactor forms; flavin mononucleotide (FMN) and flavin adenine dinucleotide, of which the latter comprises ~90% of the total flavin in the body. FMN is formed from riboflavin by the zink-dependent riboflavin kinase (EC 2.8.1.26) (107) and flavin adenine dinucleotide is formed from FMN (108). The two flavocoenzymes act as cofactors for flavoproteins which primarily catalyze redox reactions. The flavocoenzymes are also involved in energy metabolism as well as the metabolism of other vitamins such as folate, vitamin B6 and cobalamin. As plasma concentrations of flavin adenine dinucleotide are tightly regulated and does not respond to changes in riboflavin status, circulating concentrations of riboflavin or FMN have been suggested as better alternatives to assess vitamin B2 status in epidemiological settings (109).

1.2.2 Niacin

Niacin refers to nicotinamide (NAM) and nicotinic acid, and is known as vitamin B3. Nicotinic acid and NAM serve as precursor for the essential molecule NAD, which is involved as cofactor or substrate for a vast number of reactions, primarily redox-reactions. In energy metabolism, β-oxidation of fatty acids and substrate oxidation in the Krebs cycle is dependent on NAD^+ as an electron carrier. Some enzymes in the metabolic pathways discussed in this thesis also depend on NAD^+, such as MTHFR, betaine aldehyde dehydrogenase and MS reductase. Many NAD-dependent reactions yield NAM, which through the salvage pathway is recycled to NAD (110). Through a SAM-dependent methylation reaction catalyzed by nicotinamide-N-methyltransferase (NNMT, EC 2.1.1.1), NAM and nicotinic acid can be converted to N1-methylnicotinamide (mNAM). mNAM has been suggested to carry cardioprotective properties, and its production may be another mechanism for scavenging of excess

SAM (111). Ideally, niacin status is measured by quantification of tissue NAD. However, as the half-life of plasma NAD is short, the measurement of the other biomarkers are used more frequently (110).

Niacin is obtained from the diet as niacin, or through catabolism of the essential amino acid tryptophan via the kynurenine pathway. Food sources of niacin include dairy products, meat, fish, cereals, vegetables and peanuts. Total dietary niacin is measured in niacin equivalents, which also includes tryptophan as an NAD precursor (110). Most dietary tryptophan is catabolized through the kynurenine pathway (112,113). One of the end metabolites of this pathway is hydroxyanthranilic acid, which is further enzymatically converted to 2-amino-3-carboxymuconate semialdehyde (ACMS). In liver and kidney, ACMS may be catabolized to picolinic acid by the enzyme ACMS dehydrogenase (ACMSD; EC 4.1.1.45). When ACMS accumulates it may be non-enzymatically converted to quinolinic acid which through quinolinic acid phosphoribosyltransferase (QAPRT; EC 2.4.2.19) is a substrate for NAD synthesis. ACMSD and QAPRT are considered the rate-limiting enzymes for the tryptophan-to-NAD conversion, and inhibition of ACMSD has been shown to increase niacin synthesis (114,115).

1.2.3 Vitamin B6

Vitamin B6 collectively refers to six interconvertible compounds with a shared structure: pyridoxal (PL), pyridoxine, pyridoxamine and their phosphorylated forms. Several enzymes are involved in the regulation and interconversion between the different forms. The three primary B6-forms can be 5'- phosphorylated by PL kinase (EC 2.7.1.35) and the phosphorylated forms of pyridoxine and pyridoxamine is converted to PL-5-phosphate (PLP) by pyridoxine-5'-phosphate oxidase (EC 1.4.3.5) (70). Notably, this enzyme is a flavoprotein dependent on FMN as cofactor, which interconnects the metabolism of vitamin B2 and B6 (116). PLP is the active cofactor form of vitamin B6, serving as cofactor for more than 160 enzymatic reactions mostly involved in amino acid metabolism. Of relevance to this thesis, both enzymes of the transsulfuration pathway, as well as SHMT, depend on PLP as cofactor. In blood, vitamin B6 is mainly circulating as PLP (70-90%), and to a lesser degree as PL and

the catabolite 4-pyridoxic acid (PA). PLP is converted to PL by alkaline phosphatase (ALP; EC 3.1.3.1), which is necessary for uptake of B6 into the target cells, as the phosphorylated form cannot cross cell membranes.

Vitamin B6 is found in a wide variety of foods, mainly bound to protein. Among the main sources are meat, fish and whole-grains. Vitamin B6 status may be assessed directly, by measuring the concentration of B6 forms in blood or urine, or indirectly by evaluation of the activity of PLP-dependent enzymes or measurement of metabolites dependent on such reactions. The most commonly used method of direct assessment is plasma PLP concentration. However, factors other than vitamin B6 may have a strong influence on PLP concentrations, including inflammation and smoking (57), complicating the interpretation. Total circulating vitamin B6, as well as urinary excretion of PA, has been suggested as biomarkers (117), but the lack of evaluation criterions render these alternative measures less useful compared to plasma PLP. Several of the enzymes of the kynurenine pathway utilizes PLP as cofactor, and both the tryptophan loading test (117) and several ratios of kynurenine pathway metabolites have been suggested as functional markers (118).

Systemic vitamin B6 status has received attention for its inverse association with oxidative stress and inflammation, maybe reflecting an increased demand for PLP during inflammation (119–121). Low plasma PLP has been associated with several inflammatory conditions, with a tissue-specific reduction of liver PLP suggesting a mobilization of B6 to sites of inflammation (70,122). The ratio of PA/(PL + PLP), termed the PAr-index (PAr) is a suggested marker of the B6 catabolism during inflammation (123).

1.2.4 Folate

Folate is a generic term referring to a family of interconvertible coenzymes, as described in section 1.1.2. The term includes naturally occurring folates present in the reduced form, and synthetic folic acid found in supplements and fortified food products. Folic acid is reduced to tetrahydrofolate, and subsequently metabolized like the natural folates. Folate is found in a wide variety of foods, but among the main sources are green leafy vegetables, dark green vegetables and certain legumes such as

beans. However, in large parts of the world the main source is grain products due to mandatory fortification with folic acid (124). Folic acid is the oxidized and more stable form of the vitamin which is usually used for food fortification and in supplements. It also has higher bioavailability than natural folates. Women of reproductive age is recommended to supplement the diet with 400µg folic acid, as pre- and periconceptional supplementation has been shown to reduce the risk of neural tube defects (125).

The different folate coenzyme forms are involved in the synthesis and modifications of cellular components like nucleotides and amino acids (124). In the circulation, folate is primarily found as mTHF (126). However, when the capacity to reduce folic acid is exceeded, which is observed to happen at a single daily dose of > 200 µg (127), unmetabolized folic acid may appear in the circulation. This is frequently observed in populations consuming fortified foods (128,129), including pregnant women (130,131). However, although folic acid is not known to be a cofactor, there are currently no established associations between circulating unmetabolized folic acid and adverse events. Of particular relevance to the current thesis, is the role of mTHF as methyl donor for MS-mediated remethylation of Hcy. Folate supplementations effectively lowers circulating tHcy although this effect may be due to reduced Hcy production rather than increased remethylation (55). Further, expression of *GNMT* has been suggested to be a determinant of hepatic as well as plasma folate concentrations (67).

1.2.5 Cobalamin

Cobalamin (Vitamin B12) is a water-soluble micronutrient required as cofactor for two enzymatic reactions in higher animals such as humans. Methylcobalamin is cofactor for the cytosolic MS, involved in Hcy remethylation. Adenosylcobalamin is cofactor for the mitochondrial methylmalonyl-CoA mutase (EC 5.4.99.2) (132), catalyzing the catabolism of methylmalonyl-CoA, originating from the breakdown of branched-chained amino acids, odd-chained fatty acids and cholesterol, to succinyl-CoA. Succinyl-CoA is a precursor for heme biosynthesis, as well as a substrate for energy production through Krebs cycle. Cobalamin deficiency may be related to

inadequate intakes, or acquired or inherited defects leading to disrupted absorption, processing or transport. The former is considered a nutritional deficiency, while the latter would be referred to as a functional deficiency. The ultimate manifestation of cobalamin deficiency is inhibition of MS and methylmalonyl-CoA mutase, leading to an accumulation of Hcy and methylmalonic acid (MMA), a dicarboxylic acid formed non-enzymatically from methylmalonyl-CoA (133). In the diet, we primarily find cobalamin in foods of animal origin, such as meat, fish, dairy and eggs. Milk has been suggested to be the most bioavailable source of cobalamin (134). In food cobalamin is primarily found as hydroxycobalamin, and supplemental cobalamin is commonly in the cyanocobalamin form. It has been suggested that as all cobalamin forms follow the same metabolic route, which form we consume makes little difference (135). However, recent experiments in rats suggest a possible difference in tissue distribution, which may potentially be of clinical relevance (136).

Circulating status markers of vitamin B12 status include the static markers cobalamin and holo-transcobalamin, and the functional markers tHcy and MMA, which all have major weaknesses when utilized as standalone markers (132). Cobalamin is transported in the circulation bound to either transcobalamin or haptocorrin, whereof only the first is recognized by the transcobalamin receptors on the target cells. As most (~80%) of the circulating cobalamin is bound to haptocorrin, and thus not available for cellular uptake, total serum cobalamin is not necessarily reflective of status. Several studies have demonstrated discrepancies between circulating cobalamin and markers of cellular cobalamin status (137,138). Holo-transcobalamin is a direct marker of the active fraction of circulating cobalamin, and is regarded as a more accurate measure of status compared to serum cobalamin. However, limited knowledge on the metabolism and homeostasis of this marker limits its use as a first line test (132). Circulating and urinary concentrations of the functional biomarkers tHcy and MMA can be utilized as indicators of cobalamin function. However, as remethylation of Hcy depends on both folate and cobalamin, tHcy is of limited value as a standalone marker of cobalamin status. Compared to tHcy, MMA is a more specific marker of cobalamin function, but it has been shown that only ~16% of the variation in plasma MMA is accounted for by vitamin B12,

age, sex and kidney function (143), suggesting that cobalamin-independent factors are playing a part in the regulation of MMA concentrations. Of interest, a genome-wide investigation in healthy Irish adults identified two genetic variants which were strongly related to plasma MMA, the strongest being a single-nucleotide polymorphism in *HIBCH* which is involved in the catabolism of the branch-chained amino acid valine (144).

1.3 Diet and one-carbon metabolism

In addition to the direct link through intake of biomarkers, several published articles have suggested a role of dietary components in the regulation of one-carbon metabolism. Curtailing the intake of methyl groups through protein restriction was shown to increase the partitioning towards the two remethylation pathways (45,46). Short-term restriction of cysteine and methionine, while supplementing polyunsaturated fatty acids (PUFA), also altered the concentration of several metabolites, such as increased tHcy (145). Lower plasma tHcy were reported for increasing protein and PUFA intake, and at higher intakes of fish and eggs (146). In healthy volunteers, supplementation with krill oil rich in phosphatidylcholine and omega-3 PUFA reduced tHcy and increased the concentration of choline oxidation pathway metabolites (147). Further, in a metabolomic evaluation of a randomized controlled trial where ~6.5 E% from saturated fatty acids (SFA) was replaced by PUFA, several metabolites related to one-carbon metabolism differed between groups. Higher concentrations of glycine, serine, cystathionine and riboflavin, and lower concentrations of cysteine and cobalamin were observed in the intervention group (148). Total carbohydrate intake was positively associated with tHcy, while the opposite was seen with vegetables and whole-grain, indicating a role for carbohydrate quality (146). Indeed, whole-grain cereals are a main source of betaine (149), and higher intakes have been associated with higher plasma betaine (150).

In rodents, increased intake of betaine, when combined with methionine restriction, has been reported to induce *BHMT* mRNA (151). Further, dietary fat increased the genetic expression of *Choline dehydrogenase*, *BHMT* and *DMGDH*,

and downregulated *CBS* and *CGL*, possibly promoting flux through the choline oxidation pathway and limit transsulfuration flux (152). The intake of glycine has been shown to impact both the conversion of glycine to serine through SHMT, and glycine catabolism through the glycine cleavage system, both of which were increased in rats receiving a glycine-supplemented diet (153). The intake of B-vitamins also have an impact on one-carbon metabolism, as exemplified by the effect of folic acid and cobalamin on plasma tHcy (154). In mice, maternal protein restriction reduced the methylation of the PPARα-promoter, as well as increased expression of *PPARα* and several target genes in the offspring (155).

1.4 Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPAR) are ligand-activated nuclear hormone receptors, discovered on the basis of their activation by a class of rodent hepatocarcinogens causing proliferation of the peroxisomes (156). PPARs exist in three subclasses; PPARα, PPARγ and PPARβ/δ (157), which are all activated by a large variety of natural and synthetic ligands, including fatty acids and their derivatives (158,159). The PPARs are involved in the regulation of a myriad of target genes important for many physiological processes (157), and due to their important roles in lipid and glucose homeostasis they have been described as lipid and insulin sensors (158). PPARα has been described as a key nutritional sensor and central regulator of metabolic responses to dietary factors (160).

A common feature of the PPARs is the requirement of forming a heterodimeric complex with the retinoid-X-receptor (RXR) before binding to PPAR responsive elements (PPRE) on target genes (161,162). This heterodimer is considered permissive, meaning the complex may also to some degree be activated by RXR-ligands (163). The three subtypes share a high degree of homology, but their functions are subtype specific, with large differences in ligand specificity and tissue distribution. PPARα augments fatty acid catabolism and apolipoprotein synthesis, and is ubiquitously expressed in tissues with high rates of fat oxidation, such as liver, brown adipose tissue, heart, skeletal muscle and kidneys. PPARγ is mainly expressed

in adipose tissue where its activation stimulates adipocyte differentiation and lipid storage, but also in heart and liver (157). PPARα-agonists like fibrates have been used to improve dyslipidaemia, and PPARγ-agonists, like thiazolidinediones and glitazones, have been used to improve insulin resistance (158). PPARβ/δ is ubiquitously expressed, and mainly promotes fatty acid oxidation in the mitochondrion (162). Of the PPARs, PPARα is most relevant for this thesis, and is therefore discussed in more detail in the following section.

1.4.1 PPARα

PPARα is involved in the regulation of many important biological processes in the cells, and a vast amount of target genes have been identified (164). PPARα is a key regulator of energy metabolism (160), and has mainly been known for its role in upregulating genes responsible for microsomal, peroxisomal and mitochondrial β-oxidation (165,166). Additionally, PPARα plays a role in the regulation of carbohydrate and amino acid metabolism. PPARα upregulates gluconeogenetic and downregulate glycolytic genes (167), facilitating the continuous supply of glucose to glucose-dependent tissues like the brain during conditions of limited glucose availability. A possible mechanism underlying PPARα-mediated reduction in glucose utilization is inactivation of pyruvate dehydrogenase (EC 1.2.1.51) in numerous tissues (168). In amino acid metabolism, PPARα activation mainly suppresses amino acid degradation (169–171), with the exception of branch-chained amino acids where fibrate treatment was demonstrated to increase catabolism (172). In addition to being involved in the regulation of energy metabolism, PPARα-activation has been shown to reduce both inflammation and oxidative stress (173–178).

Regulation of metabolic homeostasis

Of particular interest, PPARα governs the shift in fuel utilization in response to the fasting state, by regulating genes necessary to increase hepatic oxidation of fatty acids, stimulate gluconeogenesis, glycogenolysis and ketogenesis, as well as minimizing amino acid breakdown (168). When fasted, PPARα-null mice suffer from metabolic abnormalities such as hypoglycemia, hypoketonemia as well as developing fatty liver due to diversion of lipids towards triglyceride synthesis (168). The hepatic

expression and activation of PPARα follows a diurnal rhythm inversely related to feeding status and is increased during fasting (179,180). The hepatic protein level correlates strongly with the genetic expression, but the increase in target genes is dependent on the presence of a PPARα ligand (180). It was previously suggested that fatty acids released from adipose tissue acted as ligands. However, it has been shown that only fatty acids provided by diet or produced in the liver, not those released from adipose tissue and transported as albumin bound free fatty acids, are able to activate hepatic PPARα (167,181).

Dietary activation of PPARα

Diet is directly linked to PPARα activation through supplying dietary fatty acids and its derivatives (182). Long-chained omega-3 polyunsaturated fatty acids (PUFA) and oxidized fatty acids are the most potent activators (158,168). Thus, it has been suggested that both amount and composition of dietary fatty acids may be of relevance when considering dietary influence on PPARα-activity (168). In mice, both high-fat feeding (152,183) and betaine supplementation (184) have been demonstrated to upregulate the expression of PPARα and its target genes, an effect suggested to possibly be mediated through increased BHMT flux (152,184).

Based on the observation that PPARα is induced in the fasting state, as well as dietary fats acting as activators, ketogenic diets are of particular interest. Ketogenic diets are very low in carbohydrates, and correspondingly high in fat, which metabolically mimics fasting in terms of fuel utilization. Higher proportion of PUFA in the diet could potentially lead to stronger PPARα-activation (168). Indeed, while low-carbohydrate diets with high proportions of saturated fat (SFA) typically increases circulating lipid levels compared to low-fat diets (185), this was not observed with a ketogenic diet low in saturated fatty acids (SFA) (186), potentially reflecting stronger PPARα-activation by the latter diet.

Pharmacological activation of PPARα

In addition to maintaining metabolic homeostasis by serving as a target for endogenous ligands, PPARα is a targeted by many synthetic ligands, including lipid lowering fibrate drugs, including fenofibrate, clofibrate and WY14,643.

Pharmacological activation of PPARα raises plasma high-density lipoprotein-cholesterol and reduces triglyceride levels. Hence PPARα agonists have been used to improve the circulating lipid profile and reduce CVD risk (178,187). Tetradecylthioacetic acid (TTA) is a modified saturated fatty acid with 16 carbon atoms and a sulfur atom incorporated into the third carbon, making it resistant to breakdown through β-oxidation. TTA is known as a pan-PPAR agonist with a particular high affinity towards PPARα (182,188), and administering TTA increases the hepatic expression of PPARα target genes. TTA treatment also elicits effects on lipid metabolism similar to what is observed with fibrate treatment (189–192), protects against weight gain (193), and carries anti-inflammatory and anti-oxidative properties (189,190). By being resistant to mitochondrial β-oxidation, TTA has been suggested to further contribute to PPARα-activation by facilitating the accumulation of an endogenous ligand (182).

1.4.2 Between-species differences in response to PPARα-activation

Although PPARα is ubiquitously expressed across mammalian species, some major quantitative and qualitative between-species differences must be addressed. First, PPARα-activation in rodents increases the number and size of liver peroxisomes as well as elevated expression of proinflammatory cytokines, ultimately resulting in hepatomegaly and liver carcinogenesis. Humans are responsive to the lipid-lowering effects of PPARα-activation, but not to the detrimental effects on the liver (194,195). Accordingly, rodents belong to the so-called *proliferating species*, while humans do not. There are several suggested mechanisms for the between-species differences in response to PPARα-agonists. One potential explanation is quantitative differences in expression level. The expression level of hepatic PPARα was previously thought to be lower in humans compared to rodents, but recent data suggests that expression levels are similar. However, humans partly produce a truncated and dysfunctional protein, which may explain some functional differences (166). However, in human hepatocytes overexpressing PPARα to the levels found in rodents, it was demonstrated that although the expression of other PPARα target genes were increased, markers of peroxisome proliferation were not affected (196). This suggests

that lack of detrimental effects on the liver is due to factors other than PPARα expression level. PPARα-null mice expressing human PPARα respond similarly to humans when exposed to PPARα-agonists, and do not develop liver cancer (197–199). This indicates that human and rodent PPARα differ in their gene regulating profiles (200). A transcriptomic analysis of chimeric mice carrying human liver cells receiving treatment with a PPARα-agonist verified that PPARα play an important role in the regulation of lipid metabolism in the human liver. It also provided evidence that compared to mice, gene expression in human hepatocytes are in general less sensitive to PPARα-activation (201). Another potential source of differences in PPARα-activation in rodents compared to humans may be related to lipid transport, providing endogenous agonists.

1.4.3 PPARα and one-carbon metabolism

Several links have been demonstrated between PPARα activation and one-carbon metabolism. Treatment with fibrates has been consistently associated with increased tHcy in humans (202,203), and although the mechanism has not been fully elucidated, it has been suggested to be related to decreased kidney function (204). Indeed, fibrate treatment leads to an increase in some kidney function markers such as creatinine and cystatin C, but not others such as symmetric dimethylarginine (204,205). However, the increase in tHcy is preventable by co-administration of tHcy-lowering B-vitamins (206,207), suggesting that kidney function does not fully explain the observations. Based on animal and cell experiments, inhibition of Hcy catabolism through the transsulfuration pathway could be involved (170,208). Further, increased urinary excretion of choline, betaine and DMG has been observed both in humans (209–212) and animals (138,170), which may reflect upstream accumulation of metabolites due to PPARα-dependent downregulation of *DMGDH* and *SARDH* (170,213). Related to this, it has been suggested that the association between elevated plasma DMG and CVD risk may partly be related to enhanced endogenous PPARα-activity (95,96).

Higher plasma concentrations of glycine and serine are other consistent findings when treating animals with PPARα-agonists (138,170,215,216). Based on metabolic

tracer experiments, increased plasma glycine was demonstrated to primarily be due to increased rate of appearance, rather than decreased removal, suggesting a role for PPARα in de novo synthesis of these amino acids (215). Reduced intracellular conversion to sarcosine through GNMT may also contribute to glycine accumulation, as PPAR α activation has been reported to inhibit hepatic GNMT on the gene and protein level (170,214). The previously published effects of PPARα-activation on the enzymes of the choline oxidation pathway are indicated in **Paper II: Figure 1**.

When considering B-vitamin status, some consistent links have been demonstrated after PPARα-activation. Increased urinary excretion of NAM and mNAM has been reported repeatedly in animal experiments (138,170,216–218). Mechanistically, this has been shown to be caused by increased tryptophan-to-NAD conversion in the liver (220). Treatment of rodents with PPARα-agonists have been shown to reduce gene expession and enzymatic activity of ACMSD, as well as enhance the activity of QAPRT (170,208,217,219,221,222). This would contribute to increased partitioning of tryptophan catabolism towards NAD synthesis. PPARα-activation has been demonstrated to upregulate *ALP*, the circulating enzyme responsible for the dephosphorylation of PLP to PL, providing a role for PPARα in the regulation of vitamin B6 status markers (217,219,223,224). Potential roles of PPARα in cobalamin metabolism include increased catabolism of odd-chained fatty acids and branch-chained amino acids, which both are yielding Succinyl-CoA, which is metabolized through methylmalonyl-Coenzyme A mutase.

1.5 Knowledge gaps and rationale for this thesis

As metabolites of the one-carbon metabolism are linked to the risk of major lifestyle diseases, unraveling their regulation is of interest both when considering treatment and prevention. Involvement of PPARα in the regulation of one-carbon metabolism has been demonstrated both on the gene and protein level. However, the regulatory role of PPARα has not been fully elucidated. Although some effects on the one-carbon metabolome have been reported in the literature, a targeted analysis including the majority of the biomarkers has not been implemented. Many of the biomarkers of

interest in this thesis are provided by diet, and dietary composition has been linked to altered concentration of some one-carbon metabolites. Health effects of diet may partly be explained through effects on one-carbon metabolism, e.g. as suggested for the intake of whole grains (150). However, a targeted metabolomic approach to evaluate the associations between dietary composition and the one-carbon metabolome is lacking.

Combining mechanistic data from animal studies and observational data in humans may fill knowledge gaps regarding the role of dietary composition in the regulation of one-carbon metabolism. This may in the future contribute to a framework for metabolic phenotyping when considering personalized medicine or precision nutrition.

2. Objectives

This thesis concerns the intersection between three interrelated domains: One-carbon metabolism, PPARα-activation and diet. The overall aim was to utilize targeted metabolomics to explore the regulation of one-carbon metabolism and B-vitamin status. We hypothesized that pharmacological PPARα-activation, as well as changes in dietary composition would influence the activity in these metabolic pathways, and that this would be reflected by the one-carbon metabolome. We investigated these hypotheses using targeted metabolomics, first in animal studies and second in a cohort of patients with CVD.

Specific aims

- **Paper I**: Investigate the effect of a pan-PPAR agonist, primarily activating PPARα, on circulating and urinary concentration of one-carbon metabolites and systemic markers of B-vitamin status in male Wistar rats. (**Published 2016**)

- **Paper II**: Investigate the effect of specific activation of PPARα and PPARγ on circulating concentrations of one-carbon metabolites and related markers of B-vitamin status in male Wistar rats. (**Submitted 2019**)

- **Paper III**: Explore the associations between dietary intake of carbohydrate, protein and fat on circulating one-carbon metabolites and markers of B-vitamin status in a cohort of patients with stable angina pectoris. (**Manuscript ready for submission**)

3. Materials and methods

3.1 Quantification of outcome metabolites

The outcome metabolites of interest were the same across the three included papers, namely metabolites related to Hcy metabolism, the choline oxidation pathway and markers of B-vitamin status. Study-specific analyses were carried out by the laboratory at Bevital AS (www.bevital.no), using automated methods based on mass-spectrometry (225). The plasma metabolites were analyzed using gas- or liquid chromatography coupled with tandem mass spectrometry (226–228) or microbiological assay (229). **Table 1** provides an overview of the outcome metabolites, the analytical quantification method used and which metabolites were included in each paper. The intraclass correlation coefficient is a descriptive statistic of within-person reproducibility, i.e. how representative a single measure is of long-term average exposure. By expert agreement, an intraclass correlation coefficient < 0.40 is considered to represent poor reproducibility, while values > 0.75 is considered excellent.

Table 1. Overview of outcome metabolites and analytical method across papers

			Paper		
Plasma metabolite	**Analytical method**	**ICC**	**I**	**II**	**III**
Homocysteine metabolism					
Methionine	GC-MS/MS	0.33	X	X	X
Total homocysteine	GC-MS/MS	0.72	X	X	X
Cystathionine	GC-MS/MS	0.63	X	X	X
Cysteine	GC-MS/MS	0.56	X	X	X
Choline oxidation					
Choline	LC-MS/MS	0.36	X	X	X
Betaine	LC-MS/MS	0.65	X	X	X
Dimethylglycine	LC-MS/MS	0.64	X	X	X
Sarcosine	GC-MS/MS	0.68			X
Glycine	GC-MS/MS	0.81	X	X	X
Serine	GC-MS/MS	0.71	X	X	X
B-vitamin status markers					
Riboflavin	LC-MS/MS	0.79	X	X	X
Flavin mononucleotide	LC-MS/MS	0.69	X	X	
Nicotinamide	LC-MS/MS	N/A	X	X	X
1-methylnicotinamide	LC-MS/MS	N/A	X	X	X
Nicotinic acid	LC-MS/MS	N/A		X	
Pyridoxal	LC-MS/MS	0.62	X	X	X
Pyridoxal-5-phosphate	LC-MS/MS	0.70	X	X	X
Pyridoxic acid	LC-MS/MS	0.58	X	X	X
PA-ratio	Calculated	N/A		X	X
Folate	Microbiological assay	0.56	X		X
5-methyltetrahydrofolate	LC-MS/MS	N/A		X	
Cobalamin	Microbiological assay	0.82	X	X	X
Methylmalonic acid	GC-MS/MS	0.81	X	X	X

Source: www.bevital.no, data published in references 225-229.

3.2 Study design and statistical analyses

To meet the aims of the thesis, three studies were performed. **Table 2** summarize key characteristics of the included papers.

Table 2. Key characteristics of the included papers

	Paper I	**Paper II**	**Paper III**
Species	Rats	Rats	Humans (SAP patients)
Study design	Intervention	Intervention	Cross-sectional
Sample size	20	20	1928
Duration	50 weeks	12 days	-
Background diet	Low-fat	High-fat	-
Exposure	Pan-PPAR activation with TTA	Specific PPARα and PPARγ activation	Self-reported macronutrient intakes by FFQ
Outcome	Plasma and urine concentration of biomarkers at end of study	Plasma concentration of biomarkers at end of study	Plasma concentration of biomarkers at baseline

FFQ, food frequency questionnaire; PPAR, peroxisome proliferator-activated receptor; SAP, stable angina pectoris; TTA, tetradecylthioacetic acid

3.2.1 Paper I

Study design

Paper I was based on materials from a previous animal experiment (231), originally set out to investigate the effect of the pan-PPAR agonist TTA and/or fish oil on the incidence of ventricular cancer. A total of 210 male Wistar rats, aged 8 weeks on arrival, were initially obtained. Related to the primary aim of the study, the rats went through an initial surgical gastroenterostomy procedure, whereof 25 rats died. The remaining 185 rats were randomized into five experimental groups; 1) Low-fat diet, 2) high-fat diet, 3) high-fat diet supplemented with TTA, 4) high-fat diet supplemented with fish oil and 5) high fat diet supplemented with fish oil and TTA. The rats were housed 5 per cage, and had free access to water and experimental diet for the study duration of 50 weeks. The animals were sacrificed under non-fasting conditions, after being anaesthetized by Isoflurane inhalation, and blood, urine and tissue were collected. After the publication of the original study, plasma and urine one-carbon metabolites and B-vitamins have been quantified in 10 animals per group.

For this substudy, only rats from the high-fat control (n = 10) and high-fat with TTA (n = 10) groups were included. TTA was the exposure of interest, as it is known to be a pan-PPAR agonist with a particular high affinity towards the PPARα subtype (182,188). The experimental timeline is shown in **Figure 2**.

The experiments complied with the Guidelines for the Care and Use of Experimental Animal use and the study protocols were approved by the Norwegian State Board for Biological Experiments with Living animals (“Forsøksdyrutvalget”, permit number 2005140).

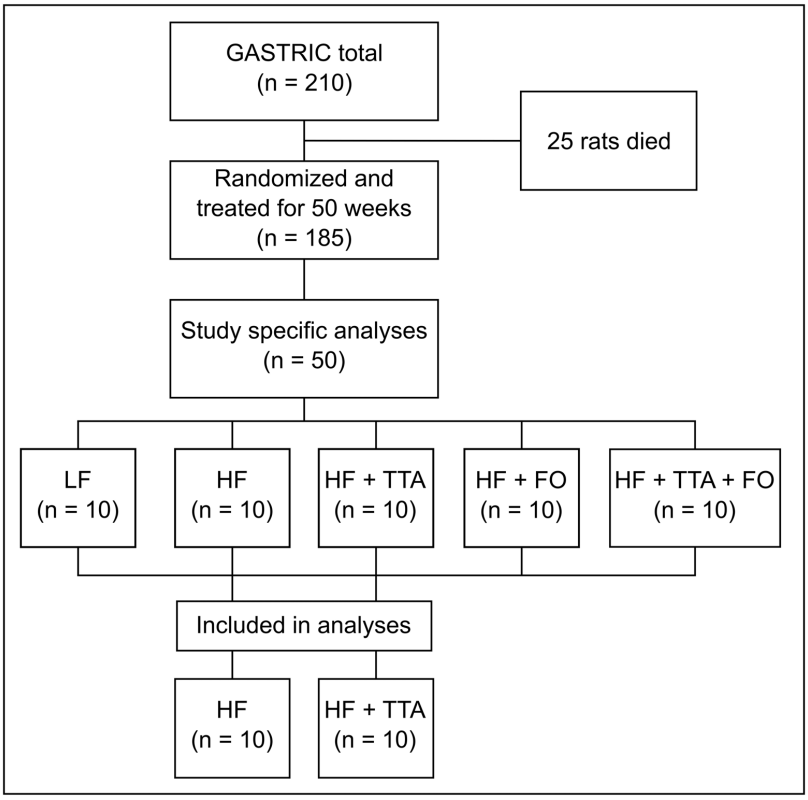

Figure 2. Flowchart illustrating the study design of the animal experiment in Paper I. Only rats from the HF and the HF + TTA groups were included in the statistical analyses. FO indicate fish oil; HF, High-fat; LF, Low-fat; TTA, tetradecylthioacetic acid.

Statistical analyses and presentation of results

Plasma and urinary metabolite concentrations were presented as arithmetic means with their standard deviations (SD). The urinary metabolites were standardized to urinary creatinine to correct for dilution. Correlations between plasma and urinary metabolites were assessed with Pearson's correlations. Between-group differences were evaluated with independent samples t-tests, and effects were given as standardized mean differences (SMD)(95% confidence interval [CI]) which indicate differences in pooled SD units. The individual rat was considered the experimental unit of analysis. As we performed many tests, we used the procedure of Benjamini and Hochberg to calculate a cutoff for statistical significance at $p<0.01$ (232).

3.2.2 Paper II

Study design

Paper II was based on another previous animal experiment, the PPAR2014 study (233), designed to investigate the effects of short-term activation of PPARα and PPARγ on plasma lipids, fatty acid composition and gene regulation in liver and adipose tissue. In total, 20 male Wistar rats were block randomized to receive PPARα-agonist (WY-14,643) PPARγ-agonist (Roziglitazone) or placebo for a study period of 12 days. The animals received a standard low-fat chow diet, and a daily supplement of 300 µl muffin dough with or without the PPAR-agonist. The animals were housed 2-3 per cage, and all animals within the same cage belonged to the same intervention arm. However, the animals were taken out of the cages to receive the treatment, so for statistical analyses the individual rat was considered the experimental unit. The animals were weighed at day 0, 6, and 12, and food intake was estimated by weighing the food provided to the cages and subtracting residual feed after the experiment. Randomization was applied to all study phases; cage placement, all study procedures and termination. After receiving anesthesia with isoflurane, the animals were sacrificed under fasting conditions by cardiac puncture and exsanguination. Blood was collected in EDTA-tubes and centrifuged, and tissues harvested, before being snap-frozen and stored at -80°C until analyses.

The experiment was conducted according to the Norwegian legislation and regulations governing experiments using live animals, and the protocol was approved by the Norwegian Committee for Experiments on Animals (FOTS ID: 2014/6187).

Statistical analyses and presentation of results

Plasma metabolite concentrations are given as geometric means (gMean) with their geometric SD (gSD), which has been suggested to better represent log-normally distributions, typical of biological measurements (234). The metabolite concentrations were log-transformed before analysis, and the between-group differences were assessed by one-way analysis of variance with planned contrasts performed for the two PPAR agonist groups versus the control group. Effect estimates were given as SMD (95% CI), and shown visually. The proportion of the

between-group variance explained by the models were evaluated by calculating the η^2, and we adopted Cohen's cutoff to define a large proportion of the variance explained at $\eta^2 > 0.35$ (235). As the animals were taken out of the cages to receive treatment, we regarded the individual animal the experimental unit.

3.2.3 Paper III

Study design

Paper III is a cross-sectional analysis based on the Western Norway B-vitamin Intervention Trial (WENBIT), consisting of 3090 participants initially randomized to receive tHcy-lowering B-vitamins. The source population for the trial was patients referred to coronary angiography for suspected coronary artery disease (CAD). Only patients diagnosed with stable angina pectoris (SAP) was included (n = 2573). The patients answered a food frequency questionnaire (FFQ) at baseline (**Appendix I)**. Patients not completing the FFQ (n = 485) or leaving more than one page empty (n = 80), as well as those reporting very high (>15,000 kJ/day for women and >17,500 kJ/day for men) or low (<3000 kJ/day for women and <3300 kJ/day for men) total energy intake (n = 27) were excluded. Additionally, we excluded 52 patients who reported > 10 % of the total energy intake from alcohol, and one participant missed data on all outcome metabolites. After exclusions, the final study population consisted of 1928 participants, and **Figure 3** provides a flow chart illustrating the selection process.

The study protocol was in accordance with principles of the Declaration of Helsinki, and the study was approved by the Regional Committee for Medical Research Ethics, the Norwegian Medicines Agency, and the Data Inspectorate. The trial is registered at www.clinicaltrials.gov, with the identifier NCT00354081.

Statistical analyses and presentation of results

Baseline characteristics and plasma concentration of metabolites were represented by gMean (95% prediction interval [PI]) for continuous variables, and counts (%) for categorical variables, respectively. We chose to indicate the distribution by 95% PI instead of gSD for interpretability reasons. The 95% PI provides the limits of the

interval defined by ($gMean/gSD^2$, $gMean \times gSD^2$). Dietary variables were adjusted using the density method (236) and expressed as energy percentage (E%) for nutrients contributing to total energy intake or as g/1000 kcal for food groups.

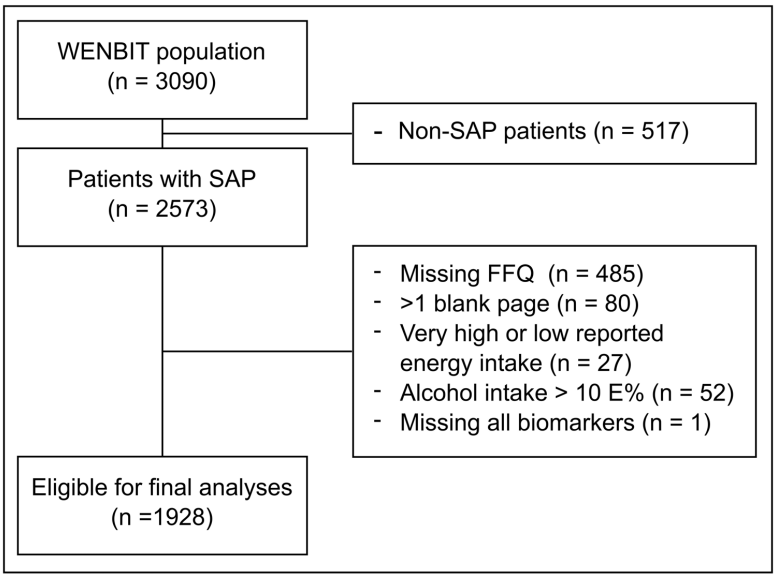

Figure 3. Flowchart illustrating the selection of patients from the WENBIT source population for inclusion in paper III. FFQ indicate food frequency questionnaire; SAP, stable angina pectoris; WENBIT, Western Norway B-vitamin Intervention Trial

Partial correlation was used to assess associations between dietary composition of macronutrients and intake of food groups, and linear regression was used to assess associations between intake of macronutrients and plasma concentrations of outcome biomarkers. We assessed these associations in a simple model adjusted for reported energy intake (Model 1), and in an additionoal model further adjusted for age, sex, BMI, smoking and alcohol intake (Model 2). Metabolite concentrations were log-transformed before analysis, and by back-transforming the β-estimates the associations were expressed as % change in the outcome metabolite for an isoenergetic increment of 1 E% in the exposure nutrient (237). To evaluate the associations on the continuous scale, we fitted locally estimated scatterplot smoothing (LOESS)-curves adjusted for model 2 covariates. To visualize the uncertainty, we

generated hypothetical outcome plots where we plotted LOESS-curves based on 25 bootstrapped samples of the data, methods provided by the R-package *ungeviz* (238). These plots represent hypothetical associations compatible with the data, and hence demonstrate the uncertainty in the observed associations.

When the relative intake of one macronutrient increases, the relative intake of another must necessarily decrease. Both are of interest, as the net effect of increasing the intake of one nutrient could differ depending on what is replaced. Substitution analyses are increasingly utilized to mimic feeding studies of such dietary substitutions, especially when studying the substitution between energy-yielding macronutrients (239). In **Paper III** we mimicked all the specific dietary substitutions between the macronutrients by carrying out substitution analyses where all macronutrients except the one being replaced were included as covariates. Accordingly, the estimates indicated % change in the outcome metabolite per 1 E% increment in the exposure nutrient replacing the nutrient not included in the model. For example, we modeled the effect of protein replacing carbohydrates in a model where protein was the independent variable, and fat, alcohol and total energy intake were included as covariates. Theoretically, when fat, alcohol and total energy intake are fixed, any increase in protein intake must be accompanied by an isoenergetic decrease in carbohydrate intake. To evaluate the role of fat composition, we performed substitution analyses for the isoenergetic substitution between SFA, MUFA and PUFA.

3.3 Statistical software

The statistical analyses in **Paper I** was performed with IBM SPSS Statistics for Windows, version 21 (240). In **Paper II** and **III**, statistical analysis were performed with R (241), and the following packages within the *tidyverse* (242); *broom* (243), *tidyr* (244), *dplyr* (245), *forcats* (246), *ggplot2* (247), *haven* (248), *magrittr* (249), *purrr* (250), *rlang* (251) and *stringr* (252), as well as *effsize* (253), *forestplot* (254), *naniar* (255), *visdat* (256), *ungeviz* (238), *flextable* (257), *officer* (258) and *rmarkdown* (259).

4. Summary of main results

4.1 Paper I

In **Paper I**, we wanted to explore the effect of pan-PPAR-agonist TTA on plasma and urine concentration of one-carbon metabolites.

The most pronounced between-group differences observed (SMD [95% CI]) were higher plasma concentrations of NAM (6.1 [3.9, 8.2]), mNAM (4.3 [2.2, 6.5]), DMG (4.0 [2.4, 5.5]), MMA (3.8 [2.0, 5.5]) and PL (3.4 [2.0, 4.8]) in TTA-treated rats compared to the control group. Smaller differences were observed regarding urinary excretion of metabolites, but the large increase in plasma DMG and MMA was followed by their increased urinary excretion (SMD 1.7 [0.6, 2.8] and 2.1 [0.8, 3.2], respectively).

4.2 Paper II

In **Paper II**, the main goal was to pursue and extend the observations from **Paper I**, by investigating the effects of specific activation of PPARα or γ. Further, we wanted to see whether the same observations would appear under different conditions. **Figure 4** shows the gene expression of the PPARs and the two PPARα target genes *LPL* and *ACOX1* in the intervention groups relative to control. PPARα expression was stronger in the liver in both intervention groups. The hepatic expression of *LPL* and *ACOX1* was markedly increased after treatment with PPARα-agonist. Altogether, these findings confirmed activation of PPARα.

Many metabolites differed between the groups, and in general the model explained a high proportion of the variance. PPARα-activation was associated with a number of metabolites, as well as larger differences from the control group compared to PPARγ-activation. The strongest differences observed between the PPARα group and control were increased NAM (SMD [95% CI] 6.6 [3.6, 9.5], η^2 = 91 %), mNAM (5.9 [3.2, 8.6], η^2 = 90 %), glycine (4.3 [2.2, 6.5], η^2 = 81 %), DMG (3.4 [1.1, 5.3], η^2 = 68 %), PL (3.9 [1.9, 5.9], η^2 = 74 %) and MMA (3.0 [1.3, 4.7], η^2 = 73 %).

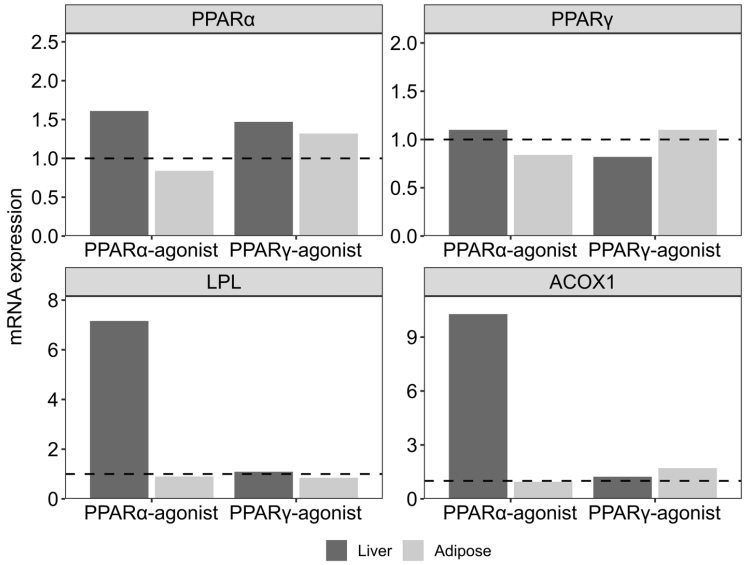

Figure 4. Liver and adipose tissue gene expression of PPARα, PPARγ, and the PPARα target genes LPL and ACOX1 in the PPAR-agonist groups relative to the control group represented by the dotted line. ACOX1, Acyl-CoA Oxidase 1; LPL, lipoprotein lipase.

4.3 Paper III

In **Paper III**, we set out to explore the associations between dietary composition and the one-carbon metabolome. As diet is known to influence PPARα-activity, we also wanted to compare the observations towards the two animal studies.

Key baseline characteristics of the patient cohort are provided in **Table 3**. gMean (95% PI) age was 61 (44, 85) years, body mass index 26 (20, 34) kg/m^2, and 1532 (79.5 %) received lipid lowering statin treatment.

When considering carbohydrate, protein and fat individually, the strongest associations towards the plasma one-carbon metabolites and markers of B-vitamin status were seen for increasing protein intake. For an isoenergetic increment of 1 E% from protein, we observed higher PLP (% change [95% CI], 3.1 [2.1, 4.1]), cobalamin (2.9 [2.1, 3.7]), riboflavin (2.4 [1.1, 3.7]), PA (2.2 [1.3, 3.2]) and mNAM (2.1 [1.1, 3.1]), and lower tHcy (-1.4 [-1.9, -0.9]) and MMA (-1.4 [-2.0, -0.8]). The substitution analyses indicated that it did not make a big difference whether protein

replaced carbohydrate or fat, suggesting that increased protein intake was the main determining factor. Increasing carbohydrate or fat intake was not strongly associated with plasma concentration of any outcome metabolite. The substitution analyses modeling changes in dietary fatty acid composition demonstrated that replacing SFA with MUFA or PUFA was associated with higher circulating mNAM, PL, PLP and folate, and lower plasma riboflavin, MMA and tHcy.

Table 3. Baseline characteristics of patients included in paper III.

	Total population	Female	Male
n	1928	390	1538
Male	1538 (79.8 %)		
Age, y	61 (43.9, 84.8)	63.2 (45.2, 88.4)	60.4 (43.7, 83.7)
Waist Circumference, cm	96 (76, 121)	88 (67, 117)	98 (80, 119)
BMI (kg/m^2)	26.1 (19.8, 34.4)	25.8 (18, 37)	26.2 (20.3, 33.7)
Smoking[1]	559 (29.0 %)	109 (27.9 %)	450 (29.3 %)
HbA1c	5.82 (3.76, 9.02)	5.86 (3.8, 9.04)	5.82 (3.75, 9.01)
Diabetes[2]	592 (30.7 %)	117 (30.0 %)	475 (30.9 %)
Hypertension	911 (47.3 %)	200 (51.3 %)	711 (46.2 %)
Previous AMI	835 (43.3 %)	145 (37.2 %)	690 (44.9 %)
Statin use	1532 (79.5 %)	310 (79.5 %)	1222 (79.5 %)

Variables are given as geometric mean (95% prediction interval) or as counts (%). AMI, acute myocardial infarction; BMI, body mass index; HbA1c, glycated haemoglobin.
[1]Smoking is calculated from self-report, or plasma cotinine > 85 nmol/L
[2]Diabetes is defined according to preexisting diagnosis, HbA1c > 6.5% or a blood glucose measurement > 7 mmol/L (fasting) or > 11.1 mmol/L (nonfasting)

5. Methodological aspects

Preface

This thesis includes data from studies carried out in rats and humans, where different exposures and conditions were investigated in relation to the same outcome variables. Methodological considerations pertaining to the different model systems are discussed separately in *section 5.1* and *5.2*, before joint considerations regarding outcome metabolites and statistical analyses are discussed in *section 5.3* and *5.4*.

5.1 Animal studies

Paper I and **II** were based on data derived from two studies in male Wistar rats, an outbred strain of *Rattus norvegicus*, which is an animal model frequently used in research (260). Some methodological considerations regarding these studies are merited, relating to the ethical aspects of animal research and the study design.

5.1.1 The 3Rs and ethical considerations

First developed in the 1950's, the concept of the three R's (3Rs; replacement, reduction, refinement) has become a fundamental part of the guidelines and legislation concerning the use of animals in research (261). Acknowledging that we will depend on the use of animal experiments in the foreseeable future, the 3Rs provide a basis for public acceptance of animal research by placing emphasis on causing a minimum amount of suffering to a minimum number of animals (262,263).

The first R is *replacement*, and refers to all measures taken to avoid the use of animals. The replacement can be full, where the use of animals is avoided completely, or partial, where animals are replaced with other animals considered less capable or incapable of suffering, or with primary cells or tissues. The second R is *reduction*, and refers to all actions taken to reduce the number of animals used per experiment. This includes designing a study which maximize the amount of information collected from each animal, as it allows achieving the goal using less animals. The final R is *refinement*, which is related to all methods used to minimize pain and suffering to the animals. Factors like pain and stress are known to influence

the physiology of the animal. Hence, *refinement* contributes to better data quality, which again increases the potential for *reduction*.

The principle of reduction is of relevance to this thesis, as both **Paper I** and **II** are based on available data from experiments originally investigating other outcomes. The data from **Paper I** was initially used to study the effect of TTA on gastric cancer (231), and the data from **Paper II** initially set out to explore short-term effects of PPAR-activation on lipid metabolism (233). By utilizing these data sets to estimate the effects of PPAR-activation on plasma concentration of one-carbon metabolites and B-vitamin status, we contribute to the literature suggesting a role of PPARα in the regulation of one-carbon metabolism, without sacrificing more animals. Data from animal experiments proves valuable also outside the scope of the initial outcomes of interest, and especially when exploring metabolic effects of specific interventions. Secondary use of animal data for targeted or untargeted metabolomics complies with the 3Rs and should be encouraged whenever possible, in order to colllect maximum information from each animal sacrificed.

5.1.2 Cage effects and identification of the experimental unit

The animals included in **Paper I** and **II** were not single-housed, meaning cage effects could be a potential source of bias. Cage effects refer to the situation where external factors influences one cage different from the other, introducing systematic differences between animals belonging to different cages. An example of such factors are vertical and horizontal cage placement in the racks. This may potentially cause different conditions with regard to light, temperature, noise etc. Another potential source of cage effects is when study procedures are performed cage by cage, on different days or time of day, or even by different personnel. To limit the influence of cage effects, randomization should apply to all aspects of the experiment, including cage placement and sequence of every experimental procedure and sacrifice. Single housing can eliminate cage effects, but is in general not recommended, as rats are social animals. The burden of being single housed could be a bigger concern than cage effects.

When analyzing data it is also important to identify the experimental unit of analysis: the smallest unit that can be allocated to different exposures. When all animals in a cage belong to the same experimental group, the cage would usually be considered the experimental unit. Treating each animal as independent observations may not be justified considering the potential for cage effects rendering the animals not truly independent. However, in some circumstances, using the individual animal as the experimental unit can be justified. This would be the case if the study treatment is given independent of the cages, and in a way in which it overrides the cage effect. In **Paper II**, animals of the same experimental group were housed in the same cages. However, on the basis that the animals were taken out of the cages to receive the study treatment, which was believed to affect the animals more profoundly compared to any potential cage effects, we decided to treat the individual animal as the experimental unit. We also performed sensitivity analyses where the cages were considered the experimental unit, which yielded similar results.

Paper I was based on a subsample of a larger experiment, and at the time of analysis information regarding which animals belonged to the same cages, were not available. Hence, it was not possible to evaluate the potential for cage effects. We did, however, consider the individual rat as the experimental unit, although the potential for cage effects were high given that the treatment was given in the diet which was distributed per cage. Data on cage distribution has later been obtained from the original data, and **Figure 5** illustrates a sensitivity analysis towards cage effects similar to what we did in **Paper II**. This suggests that although we treated the individual rat as the experimental unit, cage effects were not a big problem in **Paper I**.

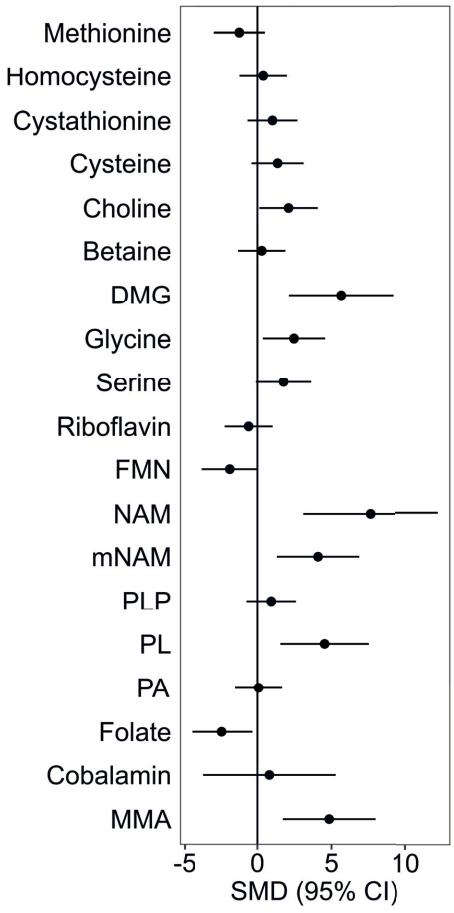

Figure 5. Standardized mean differences (SMD) in plasma concentration of metabolites for between cages of rats receiving tetradecylthioacetic acid (TTA) treatment versus control. DMG, dimethylglycine; FMN, flavin mononucleotide; mNAM, methylnicotinamide; MMA, methylmalonic acid; PA, pyridoxic acid; PL, pyridoxal; PLP, pyridoxal-5-phosphate.

5.1.3 Generalizing from rodents to humans

The ultimate goal of health research is to provide useful information regarding human health, and ideally such knowledge should be generated in humans. However, for several reasons we are often not able to study a phenomenon in humans. Animals are used to unravel biochemical mechanisms, toxicity, as well as initial testing of drugs and novel therapies, which would be unethical or impractical to study in humans. Hence, we are still dependent on the use of animals, which has offered tremendous impact on human health and welfare (264,265). Single biological mechanisms may be explored in vitro, but although the 3Rs emphasize the need to replace animal research with cell culture experiments where possible, in vivo experiments are necessary to take into account the complex interactions between different organs going on within living organisms. Compared to human trials, the possibility of harvesting tissues in animal experiments facilitate a deeper investigation into physiological and mechanistic impact of diet and drugs. However, although humans and animals are astonishingly similar with regard to anatomy and physiology, some key between-species differences exists which complicates cross-species generalization. Hence, results from in-vitro and animal experiments should ultimately be tested and confirmed in human trials. Although

inbred animals are often more useful when studying specific metabolic mechanisms due to their genetic similarities, outbred strains better resembles the variance observed in humans, and the results may be of more relevance to the general human population. When comparing rodents and humans, one main difference of relevance to this thesis should be emphasized: rodents are a *proliferating species*, with more and stronger responses to PPARα-activation, as demonstrated in humanized mice models of PPARα-activation (197–201). This means that effects of PPARα-activation seen in rodents are not necessarily relevant for human physiology. However, some similarities have been demonstrated regarding the role of PPARα in the regulation of energy metabolism, especially in lipid metabolism (166), meaning some generalization is possible.

5.2 Human study

Paper III was a cross-sectional study linking self-reported dietary composition and baseline plasma concentration of outcome metabolites related to one-carbon metabolism and B-vitamin status. The observational nature of the study prevents causal conclusions, but as dietary exposure was recorded retrospectively, we may assume a temporal association between exposure and outcome. Accordingly, this study should be regarded as exploratory, and the results should be used to generate hypotheses that may be tested in other cohorts or experimental studies. Some main methodological limitations pertaining to **paper III** merits further consideration, primarily the potential sources of confounding and bias, the inherent limitations in self-reported dietary exposure data, and model building strategies.

5.2.1 Confounding and bias

Observed associations in epidemiological studies may or may not reflect a true exposure-outcome association. Considering the possibility of other explanations, such as confounding or bias, is paramount when attempting to untangle potential causal relationships. Confounding distorts the association, and bias refer to all factors leading to a systematic deviation between the observed and the truth. In observational research, many different sources of bias have been identified, broadly categorized

into information bias and selection bias. Confounding and bias threatens the *internal validity*, the extent to which a study measures what it is supposed to measure, within the context of data collection.

Confounding

A *confounder* is a factor associated with both the exposure and the outcome, but is not on the causal path between them. In randomized controlled trials, the purpose of randomization is equal distribution of factors other than the exposure of interest. Hence, differences between groups can be attributed to the exposure. Due to lack of randomization, observational research is prone to confounding, and indeed there are many examples of experiments failing to reproduce observational findings (266). Confounding cannot be mitigated by increasing the sample size, and must be handled otherwise, e.g. through stratification or adjustment. In **Paper III**, potential confounders would be factors associated with both dietary choices and the outcome metabolites, and the selection of covariates is discussed in more detail below.

Two important concepts when handling confounding are especially relevant, namely *residual confounding* and *unmeasured confounding*, both of which are present in epidemiological studies (267). *Residual confounding* is due to nondifferential measurement error in a confounder, attenuating the confounder-outcome relation. The effect on the exposure-outcome association is unpredictable, and depends on the direction of bias (267,268). Statistical adjustment is not able to fully remove the confounding when confounders are measured imprecisely (269), and one should be cautious in claiming independence based on persisting association after statistical adjustment (269,270). However, adjusting may still be better than not adjusting, as some confounding is removed. *Unmeasured confounding* refers to all the potential confounders not included in the model, of which the analyses cannot be conditioned upon. In the recent years an assumptionless measure of sensitivity toward unmeasured confounding has emerged (271,272), but unfortunately no straightforward way exists for its application in linear regression analyses.

Considering dietary exposures, confounding due to *healthy/unhealthy consumer bias* is of special relevance, as intake of food is closely related to other

health-related variables, such as physical activity and smoking (266). Socioeconomic status is also closely related to diet quality (273). Self-selection of dietary and other health related exposures yield a high chance of bias due to confounding. A limitation in **Paper III** is our inability to account for *healthy-consumer bias* and *socioeconomic status*, which may be related to the inclination to follow official dietary guidelines and thus dietary composition.

Selection bias

Selection bias is related to the inclusion of participants, and is concerning the representativeness towards the source population. All studies are by definition conditioned on the hidden factor of being included or not, and systematic differences between the two may bias the estimates and limit external validity. The source population for **Paper III** was patients in Western-Norway, referred to coronary angiography for suspected CAD and diagnosed with SAP. Of the referred patients, 3090 were originally randomized in the WENBIT trial. Characteristics of this cohort were comparable to other cohorts of patients with verified CAD (274). As SAP is not necessarily verified by angiography, it can be argued that compared to the general SAP population, our source population has more severe disease. On the other hand, willingness to participate in a trial may reflect personal traits such as health-consciousness. This is a potential source of selection/nonresponse bias known as the *healthy volunteer effect* (275).

There may be systematic differences between those willing to complete an FFQ and those who are not, which may be a concern for the study population in **Paper III**. This may relate to the FFQ being cognitively challenging and exhausting, potentially excluding the most severely ill patients. This may be further exacerbated by the *healthy volunteer effect* where health-conscious individuals are more likely to comply with the study protocol. Out of the 2573 eligible patients, 2088 (81 %) completed the FFQ. This was comparable to the FFQ completion rate in the full WENBIT cohort (80 %) (276), indicating that SAP-patients did not differ from non-SAP-patients in their willingness to complete the FFQ. Further, we decided to exclude participants who left at least one blank page, or reported very high or low

intakes, and those reporting a high alcohol intake. The topic of excluding implausible reporters has been given some attention, and many methods exist (277–279). Using simple cutoffs, such as the ones used in **Paper3**, was shown to yield similar results as more sophisticated methods based on estimating energy requirements (278).

Table 4 provides a numerical comparison of some key characteristics between participants included and those not included from the 2573 eligible patients. Compared to excluded individuals, participants were less likely to smoke, being diagnosed with diabetes or having experienced a previous AMI, and their CAD was somewhat less severe. Taken together, *healthy volunteer effects* and the inclusion process in **Paper III** may have introduced some *selection bias*.

Table 4. Comparison of health-related characteristics between included and excluded participants in Paper III

	Included	Not included
Age, y	61 (43.9, 84.8)	61.1 (43, 86.8)
Male	1538 (79.8 %)	512 (79.4 %)
Fasting	671 (34.8 %)	264 (40.9 %)
BMI, kg/m^2	26.1 (19.8, 34.4)	26.3 (19.7, 35.2)
Waist Circumference, cm	95.7 (75.9, 120.8)	97 (77.2, 122)
Smoking[1]	559 (29.0 %)	264 (40.9 %)
Diabetes[2]	592 (30.7 %)	237 (36.7 %)
Hypertension	911 (47.3 %)	304 (47.1 %)
eGFR, ml/min/1.73m^2	88.1 (59, 131.5)	88.8 (57.9, 136.3)
Previous AMI	835 (43.3 %)	308 (47.8 %)
Statin use	1532 (79.5 %)	497 (77.1 %)
No significant stenosis	218 (11.3 %)	64 (9.9 %)
1 vessel disease	543 (28.2 %)	187 (29.0 %)
2 vessel disease	532 (27.6 %)	155 (24.0 %)
3 vessel disease	635 (32.9 %)	239 (37.1 %)

Variables are given as geometric mean (95% prediction interval) or as counts (%). AMI, acute myocardial infarction; BMI, body mass index; HbA1c, glycated haemoglobin.
[1]Smoking is calculated from self-report, or plasma cotinine > 85 nmol/L
[2]Diabetes is defined according to preexisting diagnosis, HbA1c > 6.5% or a blood glucose measurement > 7 mmol/L (fasting) or > 11.1 mmol/L (nonfasting)

Information bias

Information bias refers to all factors influencing data collection, leading to misclassification of participants. Proper measurement of outcomes and covariates are important to maximize *internal validity*. However, most biological and physiological variables vary over time, following different patterns. Thus, measurement at a single time point does not necessarily reflect long-term exposure. Extreme values are typically followed by less extreme values on the next occasion, a statistical concept known as *regression to the mean* (275). This variation is a source of random measurement error, yielding *regression dilution bias*. Depending on the variable affected, this may cause attenuation (bias towards the null) or *residual confounding*, due to reduced influence of the variable in the model (268). This is a widely recognized effect of nondifferential measurement error, which most often holds in large samples (280), and we may thus assume that this is the case in **Paper III**.

The main concern regarding information bias in **Paper III** pertains to the collection of self-reported dietary intake data. In nutritional epidemiology, we are mainly concerned with the long-term dietary exposure. As this is not directly observable, we depend on estimates derived from self-reported data. Dietary assessment in **Paper III** was done using an FFQ designed to capture the habitual Norwegian diet (281,282). As the FFQ presents the participants with the complex cognitive task of averaging their own intake, this is a common source of information bias, including systematic misreporting due to *social desirability* or *recall bias*. The inherent imprecision in dietary data has been the basis of a heated debate regarding the validity and usefulness of such data. Some go as far as claiming self-reported dietary data holds almost no value (283–286), while others emphasize that the data is valuable when handled correctly (287,288). The next section offers some perspectives on the perceived limitations of self-reported dietary data.

5.2.2 Perspectives on dietary data

Error structure in dietary instruments

Several methods exist for dietary assessment, with different strengths and limitations. Common instruments include food records, 24h recalls and FFQs, which are

variously affected by measurement error. Food records and 24h recalls are heavily influenced by day-to-day and seasonal variation, which are considered sources of random measurement error when estimating long-run intake (290). In comparison, FFQs are more influenced by systematic errors related to misreporting, (291). However, although day-to-day variation have less influence, random and person-specific errors still prevail (290,291). While random error can be mitigated by repeated measures, systematic errors will cause a persistent shift in the intake distribution. Intake-related measurement error such as under- or overreporting will also cause minor changes in the shape of the distribution. Random error mainly increases the variation (292). Based on simulated data, **Figure 6** illustrates the expected effects of measurement error on the intake distribution as well as the association with an outcome metabolite, assuming a normally distributed true intake.

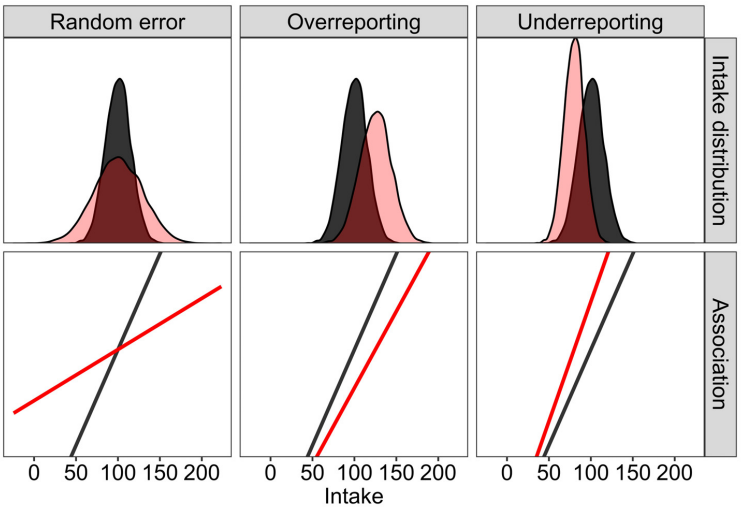

Figure 6. Simulated data, demonstrating the expected effect of measurement error on the observed intake distribution and the association between dietary intake and an outcome. Grey color represents true intake distribution and association between intake and the outcome, red color represents the observed intake distribution and association with the outcome when different types of error are added to the intake variable.

The different types of measurement error never appear in isolation, but in combination, meaning the data is still prone to bias (290,291,293). Based on the error structure, and the expected effect of the different measurement errors, it is clear that FFQs are not a suitable method to describe intake distributions in a population.

However, its ability to rank individuals according to their intake level make it suitable to assess diet-outcome associations. Hence, FFQ-derived dietary data may be considered suitable for the estimation of associations between dietary composition and biomarker concentrations in **paper III**.

Dealing with measurement error in dietary data

It is important to note that measurement error in cohort studies is most likely nondifferential, meaning it does not differ systematically according to the outcome. Accordingly, the main concerns regarding self-reported dietary data has traditionally been related to attenuation of associations and loss of statistical power, both related to *regression dilution bias* (294). Through validation studies, the amount of expected attenuation can be quantified, by evaluating how well the reported intake resembles true intake. Ideally, validation should be performed toward an unbiased estimate of the true intake, which unfortunately exists only for a few nutrients. The alternative is relative validation towards an instrument with less bias, such as repeated 24h recalls or food records. A potential pitfall when relying on relative validation is that the agreement between the methods are inflated due to correlated errors, overestimating the validity of the instrument.

Some mitigation strategies exist to combat the measurement error in self-reported dietary intake data. Energy adjustment takes advantage of the fact that the reporting errors in different foods, and nutrients, are highly correlated with reported total energy intake. There are several reasons to adjust reported intakes for total energy. First, expressing dietary intake relative to reported energy intake gives better estimates of the true intake (290,291,293,294). Second, total energy intake is closely related to factors such as body weight and physical activity level, and hence it is associated with several health outcomes. If total energy is associated with the outcome, so will all the nutrients making up the total intake, making total energy intake a *confounder* (236). Third, energy adjustment reduces the attenuation and increases precision in estimated associations (290,291,293–295). Fourth, by including energy intake, we estimate the effect of increasing the intake of one nutrient while simultaneously decreasing the intake of another. Such substitution effects are

ubiquitous in nutrition, also reflected in dietary guidelines. With some exceptions, dietary guidelines are based on substitutions, such as replacing some SFA with PUFA (296). Hence, when estimating diet-outcome associations, the adjusted risk estimates may often be more relevant, as they refer to changes in dietary composition.

In **Paper III** we decided to use the density method, and express macronutrient intakes as E% and foods as g/1000 kcal. Using E% has the added benefit of being a well-established concept which is easy to relate to. By pooling the results from five validation studies of FFQ and 24h recalls, it was demonstrated that associations based on absolute protein intake was heavily attenuated, but less so when using protein density (expressing protein as E%) (291). The FFQ used in **Paper III** has previously been validated for energy intake and some macronutrients, demonstrating fairly good ability to rank participants according to macronutrient intakes when expressed as E% (297,298). Unfortunately, it was not possible to perform an internal validation in our cohort, which should be regarded a limitation. However, our decision to convert dietary intake to densities was justified in the literature and by previous validations studies.

5.2.3 Model building strategies

Selection of covariates for inclusion in the statistical model is an everlasting challenge, and no strict guidelines exists (299). One strategy is purposeful selection, where potential covariates are kept in the model if they satisfy a pre-specified criterion, which may be related to its influence on the exposure-outcome estimate (*epidemiological criterion*), or whether the variable is statistically significant in the model (*statistical criterion*). However, as this approach is data driven, and runs the risk of overfitting, it is regarded to be more appropriate in settings where prediction - and not causal inference - is the primary objective (299). Others have noted that this approach also runs the risk of ignoring true confounders (267,300).

Another strategy has grown as part of the *"new causality movement"* in epidemiology (301). Here we take advantage of prior knowledge on the causal relationships between variables to identify parameters which are likely confounding the exposure-outcome association. This may be graphically represented by drawing

directed acyclic graphs (DAG) (300). Within this framework, one should carefully select covariates based on whether they are thought to confound the association you want to estimate, and avoid unnecessary adjustments potentially causing *overadjustment bias* (302). In **Paper III**, this approach was used for model building, and a simplified DAG is shown in **Figure 7**, where the variables in red was identified as relevant confounding variables. When modeling the associations to many different outcomes, a variable may be a confounder for one outcome, and not for another, which is a limitation. However, for simplicity and comparability reasons, we decided to apply the same model to all outcome metabolites.

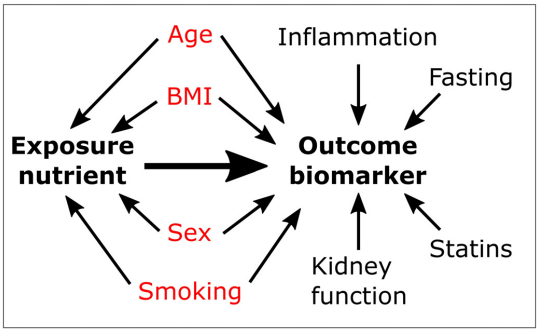

Figure 7. Simplified directed Acyclic Graph (DAG) for the model building process in Paper III. The red variables were identified as confounders for the association between the exposure nutrient and the outcome biomarker, and included in the model. BMI, body mass index.

Two clear predictors of many plasma biomarkers are kidney function and fasting status. The case could be made to include these variables in the linear regression models for the purpose of increasing precision (302). However, these parameters were assumed not to be related to the exposure, and thus not considered confounders. In the name of parsimony, we therefore opted to not include them in the final model. The same decision was made for inflammation and medication use such as statins. As recommended, we additionally adjusted the model for reported energy intake (287). For interpretation reasons we also decided to include reported alcohol intake in the model, as we wanted to estimate the effect of increasing the intake of one macronutrient at the expense of another, not at the expense of alcohol.

Another issue is related to categorization of continuous variables, typically leading to misclassification (303), and adjustment for categorical variables removes less confounding (299). Although inherently continuous, smoking exposure was

dichotomized into smokers and non-smokers, based on self-report and plasma cotinine. Taking advantage of cotinine, a biomarker of nicotine exposure, reduces the risk of smoker misclassification. However, lack of data on smoking intensity is a potential source of residual confounding (303).

5.2.4 Generalizability

The first prerequisite for generalizability is *internal validity*. Good internal validity does, however, not guarantee *external validity*. The source population in **Paper III** has been reported to be fairly representative for the general CAD population, and although some *selection bias* is likely, the results should be at least partly generalizable. However, the cohort mostly consisted of middle-aged to elderly Caucasian men, of which most were treated with lipid-lowering medications. Hence, it can be argued that extrapolation of our findings to healthy populations with other age, gender or ethnical compositions should be done with caution.

5.3 Collection of outcome metabolites and covariates

The outcome metabolites for all three papers included in this thesis were quantified at a single time point from blood samples, and some methodological considerations need addressing. First, nutritional biomarkers are often influenced by fasting status. In **Paper I**, the animals were sacrificed in the non-fasting state, while the animals in **Paper II** were fasted. Given the focus on PPARα, which is induced in the fasting state (179,180), this could be a source of variation between the studies. However, the results of **Paper I and II** are largely consistent, suggesting that the pharmacological PPARα-activation overrides the potential effect of fasting. Imprecise measurements of the outcome metabolites may stem from analytical variation, and all the outcome variables in the current thesis were measured with some degree of uncertainty as indicated by the intraclass correlation coefficients reported in **Table 1**. However, when used as outcome variables, these measurement errors are not contributing to the *regression dilution bias*, as the regression analyses estimate the average response which is not influenced by random error. Hence, analytical sources of variation is not considered a major limitation in this project.

5.4 Statistical modeling and the garden of forking paths

The three papers included in this thesis represents a journey through *the garden of forking paths*, a concept first described by statisticians Andrew Gelman and Eric Loken, related to the phenomenon of multiple comparisons. By forking paths, they refer to all the choices we make when building statistical models, also referred to as *researcher degrees of freedom* (304). They argue that when decisions are made contingent on the data, different data would lead to different choices; hence we would be testing a different model.

5.4.1 Presentation of data

Descriptive statistics provide a numerical summary of the data, most often by providing information on the central tendency and the variability/spread. Comparing descriptive statistics directly provide an informal way of between-group comparisons, and assess representativeness between populations such as the WENBIT population (274). Distributions are commonly characterized by a central tendency, typically the mean, and a measure of variance, typically the SD. The interval within ± 1 SD from the mean is expected to cover ~68% of the distribution, while ± 2 SD covers ~95%. These properties do not hold true for log-normal distributions, which are commonly encountered in biological measurements (234). As the data are skewed, the interval will commonly span too far below the central estimate, and not far enough above. As most observations lie below the mean, these intervals will contain a larger proportion of the values, and frequently also contain negative values. The corresponding intervals for log-normal distributions are (gMean/gSD, gMean×gSD) and ($gMean/gSD^2$, $gMean \times gSD^2$), yielding an asymmetric interval surrounding the gMean containing ~68% and ~95% of such distributions.

Figure 8 illustrates a log-normal distribution with geometric mean 100 and gSD 2. The red area represents the interval within the mean (128.2) ± one (28.8, 227.6) or two (-70.7, 327.1) SD, containing 84.4 and 95.5 % of the distribution, respectively. However, this interval also contain negative values, which are biologically impossible. The grey area illustrates the geometric mean (100) and the

intervals contained within one (50, 200) or two (25, 400) gSD, containing 68.0 and 95.4 % of the distribution.

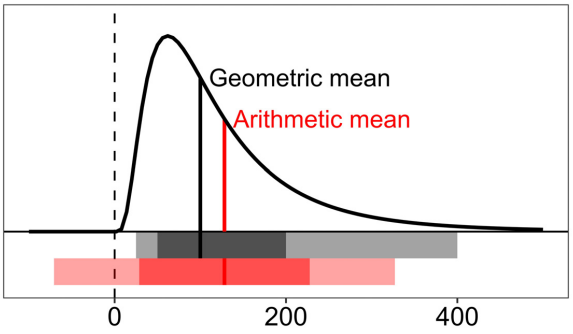

Figure 8. Illustration of a log-normal distribution and its descriptive statistics measured on different scales. *The red area shows the interval contained within one or two standard deviations from the arithmetic mean, and the grey area shows the intervals covered within one or two geometric standard deviations from the geometric mean.*

In **Paper I**, we reported arithmetic means (SD), which in hindsight was a suboptimal choice, as exampled by some of the intervals within ± 2SD contained negative values for several metabolites. In **Paper II and III**, we followed the recommended approach by reporting the gMean (gSD). However, as gSD is a multiplicative measure of variation, it is not intuitively interpreted. Accordingly, we opted to calculate the 95% PI, corresponding to the grey area in **Figure 8**, when presenting data in **Paper III**. Although different choices were made regarding data transformation in **Paper I** and **II**, the standardized effect size facilitated between-study comparability, and in this case also easier comparison between the different metabolites present in very different concentrations. A later reanalysis (not shown) of **Paper I**, when metabolites were log-transformed before analysis, yielded very similar results.

Categorization of continuous variables is common practice, and the main advantage is related to interpretability, as it facilitates comparison between categories. However, categorization invariably leads to loss of power due to reduced influence of the data range, Also, the (often inappropriate) assumption of homogeneity within groups almost never holds. A third disadvantage is that groups will be defined differently across studies, making cross-study comparison difficult (305). Analyses on the continuous scale are more powerful, but interpretation is harder. In **Paper III**, we kept the dietary exposure variables continuous, and reported

the point estimates per isoenergetic increment of 1 E%. However, in presence of non-linearity, this approach by itself is not sufficient. Hence, the main results were shown graphically by plotting the continuous association between the exposure nutrient and the outcome metabolite concentration predicted by the model. This approach demonstrates potential non-linear associations without the drawbacks of categorization. The graphical approach also encourages a focus on the direction of effects as well as potential thresholds where an effect occurs, changes or ceases, which is highly relevant in terms of making broader, clinically relevant inferences.

5.4.2 Multiple comparisons

Multiple comparisons continue to be a hot topic for debate, and is of relevance for all papers included in this thesis. In **Paper I**, we adjusted the analyses for multiple comparisons by using the false discovery rate method of Benjamini and Hochberg (232). After careful consideration we opted not to perform any correction for multiplicity in **Paper II** and **III**, a decision made on the basis of arguments in the literature (306). Traditionally, the main concern when testing many associations is related to the inflated risk of falsely claiming an association. Methods of multiple comparison correction reduces the false positive rate, although on the expense of decreasing statistical power and inflating the risk of false negatives. Accordingly, it has been argued that corrections only make truly sense if studying random numbers, where all associations per definition are false positives (306). Further, Gelman and Loken stress the fact that multiple comparisons not only concern all the different comparisons that were made, but all the potential comparisons that could have been made, which in theory is endless (304). They emphasize that a single association may be analysed in a multitude of ways, with many *researcher degrees of freedom*. **Paper I**, **II** and **III** were all based on data where many comparisons have already been published, and many more comparisons were made within the current papers. For **Paper III**, we also have the issue of covariate selection, inflating the number of potential comparisons drastically. The final decision of not correcting for multiple comparisons in **Paper II** and **III** was made on the basis of the mainly explorative nature of these papers, and in hindsight it can be argued that the correction applied to **Paper I** was unnecessary.

5.4.3 Statistical significance and replication

The cornerstone in statistical inference has for long been *null-hypothesis significance testing*, where an observation is deemed *statistically significant* or not based on its p-value. This culture has received harsh criticism for contributing to publication bias, overestimation of effects and ultimately the current replication crisis (307). Formally, the p-value is a continuous measure of the compatibility between the observed data and a specified statistical model, commonly the null-hypothesis of no difference, given that all assumptions of the test holds (308). Due to many widespread misconceptions regarding p-values, the American Statistical Association issued a statement on statistical significance and p-values, emphasizing the many drawbacks of relying on p-values when making conclusions (309). One major pitfall of relying on declaration of statistical significance is that two studies may be largely consistent, yet be wrongly interpreted as being in conflict, when the decision is made based on the presence or absence of statistical significance (310–312). Arguably, least as important as not claiming something to be true or important when $p < 0.05$, is to avoid claiming "no effect" when $p > 0.05$, frequently referred to as *"absence of evidence is not evidence of absence"* (313).

In 2019, another statement was issued (314), effectively calling to abandon the dichotomous practice of declaring a result statistically significant or not. This has been supported by others (300). Accordingly, in **Paper II** and **III** we did not determine a cutoff for statistical significance, and reported all results without dichotomization. This decision was based on the explorative nature of these studies, and the notion that formal statistical inferences is not valid for this purpose (315).

6 Discussion of results and future considerations

The main aim of this thesis was to study the effects of PPARα-activation and dietary composition on the circulating one-carbon metabolome. Consistent findings from the animal studies included increased concentrations of NAM, mNAM, DMG, PL and MMA. In the human study, the strongest observed associations were related to protein intake, which was associated with increased PLP, PL, cobalamin, riboflavin and mNAM, as well as lower tHcy and MMA. Interestingly, estimated effects of changing fat composition suggested that replacing SFA with PUFA elicited similar alterations in metabolite concentrations as was observed with PPARα-activation in the animal studies, including higher mNAM and PL, and lower riboflavin. The main overall strength of the included studies in this thesis is the targeted metabolomic approach to characterize the effects on the one-carbon metabolome, using both animal and human data.

6.1 Potential mechanisms

6.1.1 The effect of pharmacological PPARα-activation

In **Paper I** and **II**, we observed consistent patterns of higher plasma concentration of several outcome metabolites after PPARα-activation. An important finding is that these observations were consistent across the very different study durations, the context of different background diets as well as the fasting vs non-fasting blood samples. Collectively, this demonstrates that PPARα-activation has a sustained and more pronounced effects on the one-carbon metabolome compared to these other factors.

The increased plasma concentrations of NAM and mNAM is likely due to increased tryptophan-to-NAD conversion in the liver, as PPARα-activation inhibits *ACMSD* and stimulates *QAPRT* (170,217,219,221,222). PPARα stimulates β-oxidation of fatty acids, a process that depends on NAD^+, and it is possible that a concomitant increase in NAD availability is an adaptation related to increased requirements (217). PPARα-activation is known to reduce inflammation, and

increased methylation of NAM to mNAM has been suggested to be an adaptive response to inflammation (111). Indeed, NNMT is not normally saturated, and increased NAM levels will subsequently lead to increased mNAM (316). This could also contribute to the higher tHcy levels seen with fibrate treatment (202). The regulation of *NNMT* has not been extensively studied (316), and whether it is a PPARα target gene is not known. However, *NNMT* expression is associated with effects on the circulating lipid profile similar to what is observed with PPARα-activation (316). Interestingly, although not normally considered a major consumer of hepatic SAM, increased NAM methylation may reduce the flux through GNMT, which usually acts as a regulator of cellular methylation capacity. This may have contributed to the increased glycine concentrations observed.

We observed a marked increase in PL in the treatment groups. However, PLP is normally the main circulating B6 form. ALP is the enzyme responsible for converting PLP to PL, and previous studies have reported increased expression of *ALP* after PPARα-activation (217,219,223,224). Vitamin B6 is known to be inversely associated with inflammation (70). Hence, consistent with the anti-inflammatory properties of PPARα, higher concentrations of vitamin B6 biomarkers may be related to lower inflammation. A potential partitioning towards PL may be due to increased conversion through ALP. Further, the conversion of the phosphorylated forms of pyridoxine and pyridoxamine to PLP depend on FMN, which was lower after PPARα-activation, potentially limiting PLP synthesis.

Increased MMA was consistently observed after PPARα-activation, and may be related to increased production or reduced metabolism of methylmalonyl-CoA. Precursors of methylmalonyl-CoA includes odd-chain fatty acids and branch-chained amino acids, both of which encounter increased catabolism during PPARα-activation. However, increased MMA may also be related to PPARα-induced interference with cobalamin function. Indeed, a search in the *PPARgene* database (317) identified two key enzymes in the intracellular processing of cobalamin, *MMACHC* and *MMADHC*, as predicted PPARα target genes. Further, *MMACHC* is dependent on riboflavin and glutathione for its function (318,319). We observed lower riboflavin and FMN, and

could thus expect glutathione to be reduced due to inhibition of the transsulfuration pathway as well as the anti-oxidative properties of PPARα.

In the choline oxidation pathway, the most pronounced effect was seen for increased DMG. The most evident explanation for this observation is related to reduced catabolism, as PPARα-activation downregulates both *DMGDH* and *SARDH* (170), resulting in lower protein levels (213,214). Reduced oxidative stress following PPARα-activation may facilitate BHMT flux (35), consistent with the observed increase in DMG. Further, PPARα downregulates both enzymes of the transsulfuration pathway (170,208), and the resulting increase in intracellular Hcy increases substrate availability for the Hcy remethylation enzymes. As serine condenses with Hcy to form cystathionine, reduced transsulfuration flux may also contribute to the increased concentrations of serine and glycine.

6.1.2 The effect of dietary composition

In **Paper III**, we observed several associations between dietary composition of macronutrients and the one-carbon metabolome, mainly with increasing protein intake. Of particular interest for this thesis, the modeled effects of substituting SFA with PUFA, which has been suggested to elicit stronger activation of PPARα, were similar to the effects seen with PPARα-activation in **Paper I** and **II**.

The most obvious explanation for the associations seen with increasing protein intake is related to the food profile. Higher protein intake in this cohort was characterized by increased intakes of fish, meat, dairy and vegetables, and lower intakes of fruit, berries, grains and potatoes. Protein-rich food of animal origin are rich sources of vitamin B6, niacin, folate and cobalamin, which is consistent with the higher concentrations of these vitamins. Further, dairy is the main dietary source of riboflavin, which is in accordance with higher plasma riboflavin. An inverse association between protein intake and plasma tHcy is consistent with what has previously been reported (320). Based on the inverse association between tHcy and folate, vitamin B6 and cobalamin, lower tHcy would be expected with higher intake of these nutrients. Further, increased intake of methionine provides the precursor for SAM synthesis, which stimulates Hcy catabolism through the transsulfuration

pathway. Increased intracellular SAM may activate mechanisms to scavenge the excess methyl groups, such as GNMT. This may partially explain the inverse association between protein intake and plasma glycine. Additionally, increased methylation of NAM is another potential methyl sink (316), and is consistent with the higher concentrations of mNAM observed. The higher plasma concentrations of methionine and sarcosine, as well as lower tHcy and glycine, may thus be related to dietary influences on methylation status. The lower plasma MMA is consistent with increasing cobalamin intake following higher intakes of animal food. As the associations with increasing protein intake were consistent regardless of whether protein replaced carbohydrate or fat, this suggests that the associations are mainly driven by protein.When modeling the substitution between different fatty acid classes, replacing SFA with PUFA was related to higher mNAM, PLP, PL, PA, folate, as well as lower riboflavin, tHcy and MMA. Based on unsaturated fatty acids being more potent PPARα-activators, such change has previously been hypothesized to yield stronger PPARα-activation (168). There are similarities between some of these observations and what was observed with PPARα-activation in **Paper I** and **II**. Hence, we may speculate that diet-induced alterations in PPARα-activity may be involved. As we did not measure PPARα expression or activity in the current study, this must be explored in future studies.

A direct comparison to the findings in **Paper I** and **II** is hampered by the much more complex situation faced in **Paper III**. Altering dietary composition changes many variables simultaneously, and other factors including physical activity, smoking, body weight and composition and nutritional status such as over- or undernutrition will unavoidably influence the metabolic phenotype. Further, although substitution analyses attempt to simulate feeding studies, they do not directly assess an actual dietary substitution. Thus, we speculate that dietary macronutrient composition, and fatty acid composition, may influence one-carbon metabolism partly through altering PPARα-activity. However, this is not necessarily directly mediated through binding of a PPARα-ligand. Activity of the PPARα/RXR-complex may also be influenced by binding of RXR-ligands (163). Further, RXR also heterodimerize with other nuclear receptors such as retinoic acid receptor, vitamin D

receptor, liver X receptor and thyroid hormone receptor. In total, it is estimated that RXR dimerize with a third of all human nuclear receptors, facilitating their function (163). Thus, altered PPARα-activity may depend on RXR availability, which depend on the requirement to dimerize with other nuclear receptors. Hence, dietary influence on PPARα-activity may be both direct by providing PPARα-agonists, and indirect through interfering with other RXR-dependent receptors.

6.2 Clinical application and the way forward

There are no direct clinical application of the findings in this project at the current time. However, the findings lays the foundation for future research into the regulation of one-carbon metabolism, with potential implications for treatment or personalized medicine and nutrition. The following sections provides a discussion on how the findings presented herein could form the basis for future research, aiming to further increase our knowledge of the intersection between one.carbon metabolism, PPARα and diet.

6.2.1 Association, causation and the issue of adaptive mechanisms

When the concentration of a biomarker is associated with the risk of a clinical endpoint, the biomarker may be considered a risk marker. However, this does not necessarily mean that the biomarker is causally linked to the disease. There is always a possibility that the metabolite concentration is changed as a response to other factors associated with the disease, and may even be part of an adaptive or protective mechanism. Hence, treatment aimed at "correcting" the levels of a biomarker for disease risk is not necessarily beneficial. Accumulation of firefighters in the proximity of a fire is a helpful analogy, where the firefighters can be seen as a marker associated with fire risk. Although high concentrations of the risk marker is associated with increased risk of the outcome, removing the risk marker when first present will obviously be a bad idea. This is of course a clear-cut example of *reverse causality*, but the same logic may be applied to biological biomarkers of disease risk. One example is inflammation, which is part of the physiological response to harmful stimuli.

When a metabolite is associated with the risk of an outcome, it is always important to consider the possibility that the metabolite is altered as a response to disease mechanisms. This is crucial when making a decision of whether to correct the metabolite level or not. If the metabolite concentration is altered as part of a protective mechanism, "correcting" the concentration may do more harm than good. Conversely, if the metabolite is mediating the effect of the primary cause on the outcome, or has negative health effects *per se*, treatment targeting the metabolite level may be appropriate. The challenge is identifying which is which, and when interpretation of adaptation vs causality is difficult, we should also be conservative in our decision to treat (321). Related to one-carbon metabolism, tHcy is a good example of a metabolite associated with increased disease risk, but where lowering concentrations have mostly failed to improve prognosis (85), suggesting that elevated tHcy is an adaptive marker of underlying pathology (86,87). This means that it is the cause of elevated tHcy, and not the tHcy *per se* which is problematic.

6.2.2 A note on the future of nutritional epidemiology

As argued by Beaton and others (287,322), it is absolute key to continue increasing our ability to handle the inevitable measurement errors in dietary self-reports. Thus, when planning future epidemiological studies, it is important to include mitigation strategies for measurement error, to improve estimation (323). Methods exist to correct this error, but may require additional data to be collected. The most widespread method of correcting for measurement error is regression calibration, a form of exposure modeling where predicted intakes replace reported intakes in the model (294). Predicted intakes are obtained from a calibration equation from reported intakes and relevant covariates. A prerequisite for this approach is the availability of more accurate data in a subset, to perform an internal validation study. As recovery biomarkers are sparse, the best alternative is to plan for the collection of repeated 24h recall interviews in addition to the FFQ. Collecting repeated 24h recalls in a subgroup allows for regression calibration (294), but if collected for the whole cohort, combining the instruments have proven superior (324). Another way of improving dietary intake data is combining self-reported data with biomarkers (325,326). To

facilitate valid inferences on diet-outcome associations in future studies, properly planning the collection of dietary data is of utmost importance.

6.2.3 A note on extending the use of metabolomics

Metabolomics refer to the comprehensive and quantitative analysis of all metabolites. A less global approach is *targeted metabolomics*, where the focus is on a smaller, more specific set of metabolites. All papers in the current thesis belongs to the field of *targeted metabolomics* (327). Modern laboratories, such as *Bevital A/S*, have developed methodology allowing simultaneous quantification of many metabolites from low volumes of biological fluids. Metabolomic analyses of epidemiological studies are a great starting point for exploring the metabolic profile in relation to different exposures, such as we did with dietary composition in **Paper III**. Metabolomic analyses of animal experiments may contribute to the unraveling of the complex regulation of different metabolic pathways, which was our focus in **Paper I** and **II**. However, the real strength lies in the use of metabolomics in clinical intervention studies, e.g. as we did using data from a randomized trial of changed dietary fat composition (148). The observed associations from the current thesis could be further explored by including the quantification of one-carbon metabolites in other observational and experimental studies of dietary exposure.

6.2.4 A note on precision nutrition and the importance of subgroup analyses

In studies, whether observational or experimental, we are mostly concerned with effects on the group level. This is fundamentally different from determining the best approach on the individual level (328). Many scenarios may lead to the same average effect in a population, including but not limited to large effects in small subgroups, and untangling them could potentially be of paramount importance to clinical practice. The existence of heterogeneity of individual responses is widely acknowledged, and the concept of precision nutrition has become a hot topic (329).

To improve clinical decision making, the main crux is to identify those who will benefit from, or potentially be harmed by, a given recommendation or treatment. The first step in this process is the screening for putative indicators, and an accessible tool

is subgroup analyses to explore potentially diverging patterns within different subgroups. As subgroup analyses run a high risk of bias, their results have often been looked at with disdain (330). However, the importance of exploratory subgroup analyses have been emphasized (315), although results should always be regarded hypothesis generating and must be externally confirmed (331).

Another possible tool is genetic analyses, successfully implemented in the treatment of inborn errors of metabolism. Genetic screenings for the purpose of personalized nutritional advice are already readily available commercially, but its validity has been questioned (332). Related to one-carbon metabolism, a genetic variant in *MTHFR* has been demonstrated to increase the risk of hypertension, which may be treated with riboflavin supplementation (333).

Metabolic phenotyping represents another approach to personalized nutrition. In **Paper I** and **II**, we suggest several potential biomarkers of PPARα, including plasma concentration of mNAM, NAM, DMG, PL and MMA, which individually or combined may provide information on PPARα-activity. Given the crucial role of PPARα in the regulation of energy metabolism, information on its endogenous activation may provide clues when considering tailored nutritional advice. Indeed, some interactions between genetic PPARα-variants and diets have been reported (160), indicating that endogeneous PPARα activity may mediate inter-individual variations in response to dietary exposures. To extend the findings from **Paper I** and **II** to humans, targeted metabolomics should be applied to (existing) human trials of fibrate treatment. However, given the central question of causation vs adaptation, it remains to be unraveled if different dietary advice should be given to persons with high or low endoogenous PPARα-activity. A natural next step is therefore to explore the effect of different dietary exposures in cohort and intervention studies, using targeted metabolomic profiling to identify subgroups of interest. If consistent patterns emerge, the ultimate test would be to carry out a randomized trial of such advice with block randomization according to metabolic phenotype.

7. Conclusions

The main finding of this thesis is that the one-carbon metabolome is influenced by both PPARα-activation and dietary composition. The effects of pharmacological PPARα-activation were consistent across different conditions, suggesting a profound role of PPARα in the regulation of one-carbon metabolism. The most striking findings were higher mNAM, NAM, PL, DMG, glycine and MMA, suggesting these metabolites as potential biomarkers of PPARα-activity. This also suggests links between lipid and one-carbon metabolism, which have not been investigated thoroughly so far.

In humans, dietary composition was associated with the one-carbon metabolome, with the strongest associations observed for protein intake. Higher protein intake was associated with higher concentration of PLP, cobalamin, riboflavin and mNAM. The modeled effect of substituting SFA with PUFA in humans showed similarities with the metabolic profiles observed after PPARα-activation in the animal studies, especially regarding the concentrations of riboflavin, mNAM, PL, PLP and DMG. This suggests that dietary fatty acid composition influences one-carbon metabolism, potentially through interfering with PPARα-activity.

Intervention studies in humans would be the next step to further our understanding regarding the role of PPARα and dietary composition in one-carbon metabolism. Similar to the studies conducted in this thesis, it would be beneficial to apply targeted metabolomics to existing studies with stored biological samples and well-characterized diets, instead of conducting new trials. These analyses may also provide information on individual variation in response to dietary exposure, and may thus aid the identification of subgroups for personalized dietary 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: Plasma concentrations of metabolites along the choline oxidation pathway have been linked to increased risk of major lifestyle diseases, and peroxisome proliferator-activated receptors (PPARs) have been suggested to be involved in the regulation of key enzymes along this pathway. In this study, we investigated the effect of PPAR activation on circulating and urinary one-carbon metabolites as well as markers of B-vitamin status. Male Wistar rats (n = 20) received for 50 weeks either a high-fat control diet or a high-fat diet with tetradecylthioacetic acid (TTA), a modified fatty acid and pan-PPAR agonist with high affinity towards PPARα. Hepatic gene expression of PPARα, PPARβ/δ and the enzymes involved in the choline oxidation pathway were analyzed and concentrations of metabolites were analyzed in plasma and urine. TTA treatment altered most biomarkers, and the largest effect sizes were observed for plasma concentrations of dimethylglycine, nicotinamide, methylnicotinamide, methylmalonic acid and pyridoxal, which were all higher in the TTA group (all $p < 0.01$). Hepatic *Pparα* mRNA was increased after TTA treatment, but genes of the choline oxidation pathway were not affected. Long-term TTA treatment was associated with pronounced alterations on the plasma and urinary concentrations of metabolites related to one-carbon metabolism and B-vitamin status in rats.

Keywords: dimethylglycine; methylmalonic acid; one-carbon metabolism; peroxisome proliferator-activated receptors; tetradecylthioacetic acid

1. Introduction

Elevated plasma total homocysteine (tHcy) is related to increased risk of atherothrombotic cardiovascular disease (CVD) [1]. However, lowering of tHcy with B-vitamins has not improved prognosis among CVD patients [2], which is questioning a causal relationship and encourages investigation into novel mechanisms associated with elevated plasma tHcy [3]. Circulating and urinary concentrations of various metabolites along the choline oxidation pathway, which is linked to remethylation of Hcy, have been related to major lifestyle diseases including CVD and diabetes [4–8]. We have recently shown that higher plasma dimethylglycine (DMG) concentrations are associated with

increased risk of acute myocardial infarction as well as total and cardiovascular mortality, independent of traditional risk markers including elevated plasma tHcy [6,7].

Homocysteine (Hcy) resides at a branch point of three metabolic pathways. Remethylation of Hcy back to methionine is catalyzed either by the cobalamin-dependent methionine synthase (MS, EC 2.1.1.13) or betaine-homocysteine methyltransferase (BHMT, EC 2.1.1.5), 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] (Figure 1). Hcy metabolism is linked to the choline oxidation pathway by BHMT, which demethylates betaine to form DMG [10]. DMG is further oxidized to sarcosine and glycine by two mitochondrial flavoenzymes, *i.e.*, DMG dehydrogenase (DMGDH, EC 1.5.8.4) and sarcosine dehydrogenase (SARDH, EC 1.5.8.3) [11]. Interestingly, increased flux through BHMT has also been associated with decreased DNA methylation of the promoter region of the peroxisome proliferator-activated receptor (PPAR) α gene in mice, resulting in increased gene expression of PPARα and its target genes [12]. In rats, activation of PPARα has been demonstrated to reduce the genetic transcription of DMGDH, SARDH and glycine N-methyltransferase (GNMT, EC 2.1.1.20), as well as both enzymes of the transsulfuration pathway [13]. This indicates a relationship between PPARα and these pathways, and hence, we previously suggested that the association between elevated plasma DMG and CVD risk may partly be related to enhanced endogenous PPARα activity [6,7].

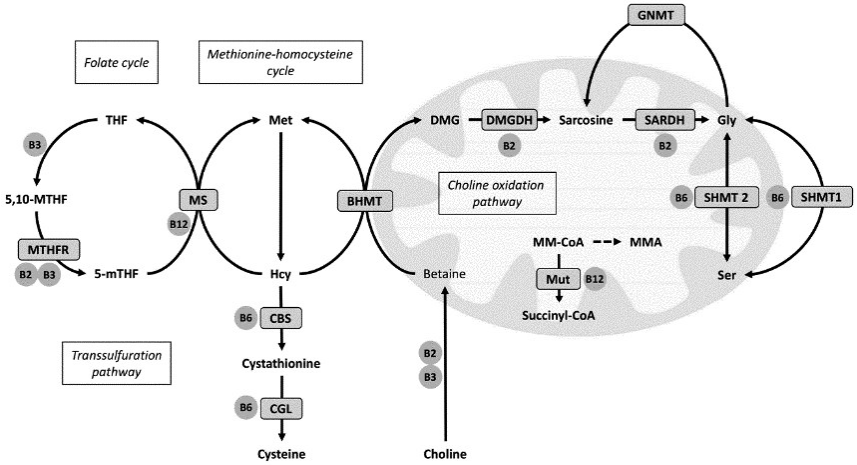

Figure 1. Overview of one-carbon metabolism related pathways. 5-mTHF indicates methyltetrahydrofolate; 5,10-MTHF, methylenetetrahydrofolate; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; CGL, cystathionine-γ-lyase; DMG, dimethylglycine; DMGDH, dimethylglycine dehydrogenase; Gly, glycine; GNMT, glycine N-methyltransferase; Hcy, homocysteine; Met, methionine; MMA, methylmalonic acid; MM-CoA, methylmalonyl CoA; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; Mut, methylmalonyl-CoA mutase; Sarc, sarcosine; SARDH, sarcosine dehydrogenase; Ser, serine; SHMT, serine-hydroxy-methyltransferase; THF, tetrahydrofolate.

PPARα is a key regulator of energy metabolism [14], with a large number of identified target genes [15]. PPARα is activated by dietary or endogenous fatty acids and their derivatives [16]. Tetradecylthioacetic acid (TTA) is a sulfur-containing fatty acid analogue with a high affinity towards PPARα [17], and we have previously demonstrated significant increases in PPARα target genes in the liver after TTA treatment, also accompanied by reduced plasma and hepatic lipid levels [18]. Although PPARα has been thoroughly explored according to its role in lipid and glucose metabolism,

the relationship between PPARα and other metabolic pathways has only recently gained attention. Involvement in amino acid metabolism has been demonstrated [19,20], and fibrates, which are specific PPARα ligands have consistently been associated with elevated plasma tHcy [21], as well as being associated with elevated urinary output of choline, betaine and DMG [22,23], linking PPARα to one-carbon metabolism. In terms of the choline oxidation pathway, PPARα activation has in rodent models been associated with a reduction in *Dmgdh* and *Sardh* mRNA [13] and lower protein level of SARDH [24], and recently, long-term TTA treatment was associated with lower protein expression of BHMT, DMGDH and SARDH [25]. However, whether TTA treatment affects the related metabolites has yet to be explored. Also, activation of PPARα has been shown to increase the synthesis of vitamin B3 from tryptophan by regulating key enzymes in this pathway [26], but whether PPAR activation influences the status of other B-vitamins is uncertain.

The aim of the current study was to investigate how PPAR activation by TTA supplementation affected blood and urinary concentrations of components of the choline oxidation pathway and one-carbon metabolites, as well as systemic markers of B-vitamin status.

2. Materials and Methods

2.1. Animals and Diets

Male Wistar rats (n = 20), 8–10 weeks old on arrival and weight 260–270 g (Taconic Europe A/S, Lille Skensved, Denmark), were randomly allocated to receive either a high fat control diet (Control) with 25% fat (23% lard, 2% soybean oil, weight/weight) or a high fat diet supplemented with TTA (TTA) (22.6% lard, 2% soybean oil, 0.4% TTA, weight/weight). The diets had the same amounts of micronutrients, and the rats had free access to water and feed during the study period. The animals investigated were part of a larger study, and more detailed descriptions of this experiment and the composition of the diets have previously been published [27]. Feed intake was comparable between groups, but the TTA treated rats gained less weight as compared to Controls [28].

After 50 weeks, the animals were sacrificed under non-fasting conditions by anaesthetization with 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.

2.2. Ethics Statement

The animal experiments complied with the Guidelines for the Care and Use of Experimental Animal use and the study protocols were approved by the Norwegian State Board for Biological Experiments with Living animals ("Forsøksdyrutvalget", permit number 2005140).

2.3. Biochemical Analyses

With the exception of plasma cobalamin, which was measured in only seven rats per group due to limited amounts of plasma, all blood metabolites were analyzed in 10 rats per group. Urinary metabolites were analyzed in 9 control animals and in 8 TTA treated rats. All analyses were performed at Bevital A/S (Bevital, Bergen, Norway). In plasma, methylmalonic acid (MMA), tHcy, cystathionine, serine and glycine were analyzed by gas chromatography coupled with tandem mass spectrometry (GC-MS/MS) [29]. Plasma choline, betaine, DMG, methionine and cysteine, as well as all vitamin B2, B3, and B6 forms and metabolites were analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) [30,31]. Plasma folate [32] and cobalamin [33] were measured by microbiological assays. In urine, cysteine, cystathionine, sarcosine, glycine, serine and MMA were measured by GC-MS/MS [29], and methionine, choline, betaine and DMG by LC-MS/MS [30].

2.4. Gene Expression Analysis

RNA was purified from frozen liver samples and cDNA was produced as previously described [34]. Using probes and primers from Applied Biosystems (Foster City, CA, USA), real-time PCR was performed with Sarstedt 384 well multiply-PCR Plates (Sarstedt Inc., Newton, NC, USA) on *Ppara* (Rn00566193), *Ppard* (Rn 00565707), *Bhmt* (Rn00578255_m1), *Dmgdh* (Rn00594751), *Sardh* (Rn00454657_m1) and *Gnmt* (Rn00567215_m1). Three reference genes were included: 18s (Kit-FAM-TAMRA (Reference RT-CKFT-18s)) from Eurogentec (Seraing, Belgium), glyceraldehyde-3-phosphate dehydrogenase (Gapdh, Mm99999915_g1) from Applied Biosystems, and ribosomal protein, large, P0 (Rplp0, Gene ID 11837) from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The absolute quantification was normalized according to the reference genes as previously described [28], and the result is presented normalized to 18s, which was selected by the NormFinder algorithm which ranks the candidate reference genes according to their expression stability [35].

2.5. Statistical Analyses and Presentation of Data

The plasma concentrations of metabolites are presented as means (SD). Normality was assessed by the Kolmogorov-Smirnov test, and the groups were compared with independent samples *t*-tests. Standardized mean differences (SMD) (95% confidence interval) were calculated.

The concentrations of urinary metabolites were given as μmol of metabolite per mmol creatinine (SD) to correct for dilution. We evaluated the relationship between plasma and urinary concentrations of metabolites by calculating Pearson's correlation coefficients.

Statistics were performed using IBM SPSS Statistics for Windows, version 21 (SPSS IBM., Chicago, IL, USA), Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) and Microsoft Excel 2010. p-values < 0.01 were considered statistical significant, according to the Benjamini and Hochberg method of controlling the false discovery rate [36].

For the gene expression analyses, normality was analyzed by the D'Agostino and Pearson omnibus normality test. The expression of *Ppara*, *Bhmt*, *Dmgdh*, *Sardh* and *Gnmt* mRNA was compared by *t*-test while the expression of *Ppard* mRNA was compared by Mann-Whitney U test.

3. Results

Mean (SD) concentrations for all plasma and urinary metabolites and the SMD between groups are presented in Figures 2 and 3. Compared to rats receiving the control diet, rats treated with TTA differed in most metabolites and markers of B-vitamin status.

In terms of the transsulfuration pathway, higher concentration of plasma cystathionine was observed (SMD = 1.56 [0.45–2.44], $p = 0.004$). Regarding components of the choline oxidation pathway, rats in the TTA intervention group had higher concentrations of plasma DMG (SMD = 3.96 [2.39–5.49], $p < 0.001$), glycine (SMD = 1.42 [0.41–2.39], $p = 0.005$) and serine (SMD = 1.51 [0.48–2.49], $p = 0.003$). In urine, the TTA intervention group had higher concentrations of DMG (SMD = 1.69 [0.55–2.80], $p = 0.003$).

Among B-vitamers and their respective metabolites, we observed higher concentrations of plasma nicotinamide (NAM) (SMD = 6.06 [3.89–8.19], $p < 0.001$), N1-methylnicotinamide (mNAM) (SMD = 4.32 [2.16–6.45], $p < 0.001$) and pyridoxal (PL) (SMD = 3.38 [1.96–4.77], $p < 0.001$) in the TTA treated rats as compared to controls, whereas plasma folate was lower (SMD = −1.73 [−2.76–−0.68], $p = 0.001$). Riboflavin (SMD = −0.65, [−1.55–0.26], $p = 0.13$) and FMN (SMD = −1.05 [−2.0–0.05], $p = 0.039$) tended to be lower after TTA treatment. No difference was observed for plasma cobalamin, albeit both plasma and urine concentrations of MMA were higher among TTA treated rats vs controls (SMD = 3.77 [2.0–5.51] and 2.05 [0.83–3.22], respectively, both $p < 0.001$).

As shown in Table 1, there were strong, positive correlations between plasma and urinary concentrations for betaine ($r = 0.62$), DMG ($r = 0.79$) and MMA ($r = 0.73$), all $p < 0.01$.

Table 1. Correlations between blood and urinary concentrations of metabolites. Male Wistar rats were treated with either a low-fat control diet or a high-fat diet with or without additional TTA treatment, n = 17. Pearson correlation coefficients were calculated between the plasma and urinary concentration of metabolites.

	r (95% CI)	*P*
Methionine	−0.02	0.93
tHcy	−0.10	0.71
Cystathionine	0.25	0.33
Cysteine	0.09	0.73
Choline	0.08	0.93
Betaine	0.62	0.008
DMG	0.79	<0.001
Glycine	−0.13	0.63
Serine	−0.17	0.51
MMA	0.73	<0.001

DMG indicates dimethylglycine; MMA, methylmalnic acid; tHcy, ttal hmcysteine.

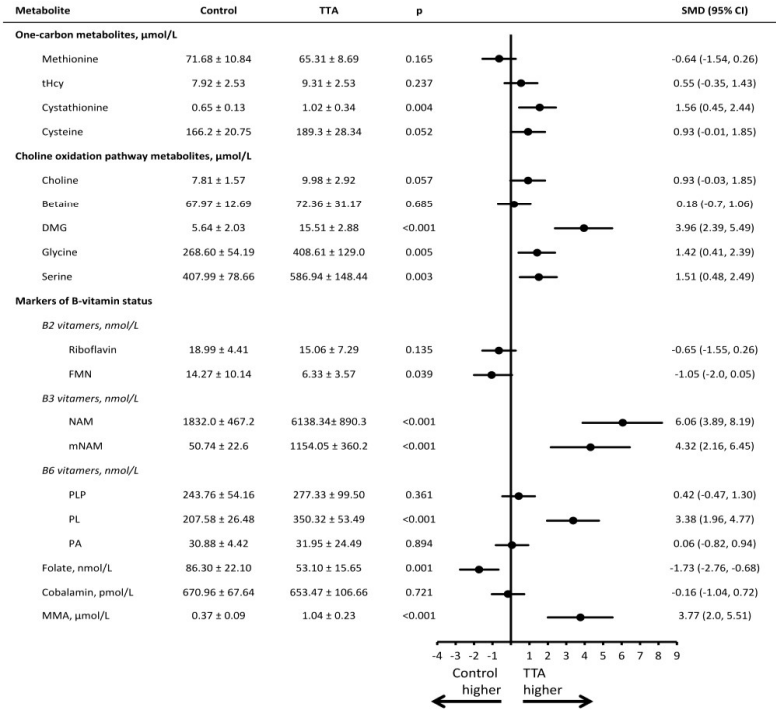

Figure 2. Plasma concentrations of metabolites in rats receiving a high fat control diet or a high fat diet with additional TTA treatment. Male Wistar rats were treated with a high-fat diet with or without additional TTA treatment, n = 10 in each group. Mean (SD) plasma concentration of the metabolites, as well as the standardized mean difference (95% CI) between the control and the TTA treated animals. DMG indicates dimethylglycine; FMN, flavin mononucleotide; MMA, methylmalonic acid; mNAM, N1-methylnicotinamide; NAM, nicotinamide; PA, pyridoxic acid; PL, pyridoxal; PLP, pyridoxal-5′-phosphate; SMD, standardized mean difference; tHcy, total homocysteine; TTA, tetradecylthioacetic acid.

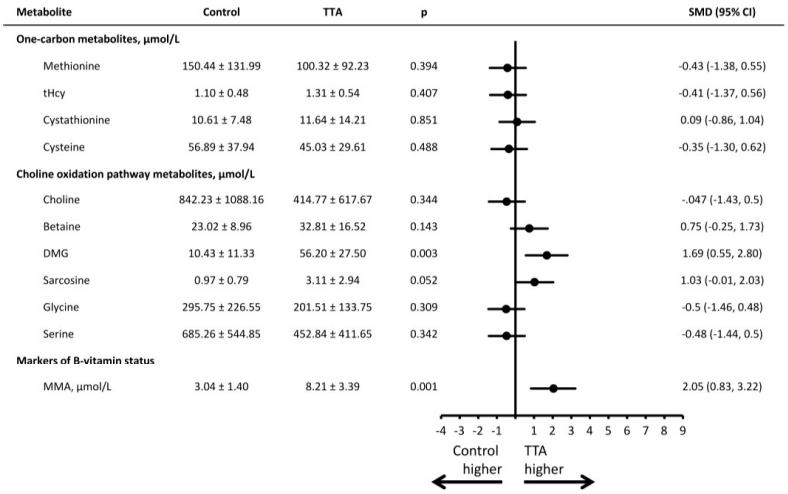

Figure 3. Urinary concentrations of metabolites in rats receiving a high fat control diet or a high fat diet with additional TTA treatment. Male Wistar rats were treated with a high-fat diet with or without additional TTA treatment, $n = 10$ in each group. Mean (SD) urinary concentration of the metabolites, as well as the standardized mean difference (95% CI) between the control and the TTA treated animals. DMG indicates dimethylglycine; MMA, methylmalonic acid; SMD, standardized mean difference; tHcy, total homocysteine; TTA, tetradecylthioacetic acid.

The hepatic gene expression analyses showed no difference in the expression of *Bhmt* (Figure 4A), *Dmgdh* (Figure 4B), *Sardh* (Figure 4C) or *Gnmt* (Figure 4D). However, *Ppara* mRNA was increased 2.1-fold ($p < 0.001$) (Figure 4E) and *Ppard* mRNA was increased 1.5-fold ($p < 0.001$) (Figure 4F) in the TTA group.

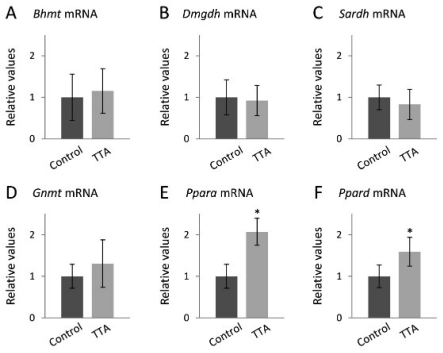

Figure 4. Gene expression in liver of rats receiving a high fat control diet or a high fat diet with additional TTA treatment. Male Wistar rats were treated with a high-fat diet with or without additional TTA treatment, $n = 10$ in each group. RNA was purified from frozen liver samples, and gene expression was quantified relative to a reference gene. The bars represent the relative gene expression of *Bhmt* (**A**); *Dmgdh* (**B**); *Sardh* (**C**); *Gnmt* (**D**); *Pparα* (**E**); and *Ppard* (**F**) compared to the Control group. Group differences are evaluated with *t*-test and Mann-Whitney U test, and * indicates $p < 0.001$. Bhmt indicates betaine-homocysteine methyltransferase; Dmgdh, dimethylglycine dehydrogenase; Gnmt, glycine N-methyltransferase; Ppar, peroxisome proliferator-activated receptor; Sardh, sarcosine dehydrogenase; TTA, tetradecylthioacetic acid.

4. Discussion

4.1. Principal Findings

This long-term, 50 weeks, animal study indicated that TTA treatment was associated with pronounced effects on the hepatic gene expression of PPARα and PPARβ/δ and on circulating 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, MMA and PL, which were all higher in the TTA group. Our results extend on previous findings by demonstrating that PPARs may also affect the plasma concentrations of metabolites in the choline oxidation and one-carbon metabolism pathways, as well as circulating levels of closely related B-vitamins.

4.2. Possible Mechanisms

4.2.1. TTA Treatment and the Choline Oxidation Pathway

The particularly high concentration of DMG associated with TTA treatment, both in plasma and in urine, 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 previously reported in TTA-treated animals [25]. However, as the hepatic gene expression of *Dmgdh* and *Sardh* was not different between groups, this could be related to post-transcriptional regulation or other mechanisms such as limited availability of cofactors. Both DMGDH and SARDH are flavoproteins [37], and lower circulating concentrations of vitamin B2 as observed in this study may thus reduce DMG catabolism. Moreover, folate-dependent remethylation of Hcy by MS utilizes mTHF, which is produced from methylenetetrahydrofolate by methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20), and the reaction depends on MS reductase (MSR, EC 1.16.1.8). Notably, both MTHFR and MSR are flavoproteins [38,39]. Accordingly, we observed markedly lower levels of folate in the TTA-treated rats, and as mTHF makes up the majority of circulating folate [40], this suggests reduced MTHFR flux. Hence, reduced MS flux due to lower concentrations of mTHF, may lead to a compensatory increase in BHMT-mediated remethylation, enhancing DMG production.

Data on systemic sarcosine may have shed further light on potential changes of metabolites downstream of DMG. 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 sarcosine tended to be higher in the TTA treated rats, indicating an increased glomerular filtration or decreased fractional tubular reabsorption secondary to a probable higher plasma concentration. This was further supported by the strong correlations between plasma and urinary levels of the closely related metabolites DMG and betaine. Sarcosine can be produced from glycine in the cell cytosol, via GNMT. As PPARα activation is suggested to inhibit flux through GNMT [13,24], decreased cellular sarcosine production from glycine may have contributed to the higher plasma concentrations of glycine and serine observed among the TTA treated rats. In line with this, a recent study found increased concentrations of glycine and serine after PPARα activation, and a metabolic tracer experiment revealed that increased rate of appearance into plasma, not decreased clearance or catabolism, was the main mechanism responsible for this observation [41].

There are several possible routes for glycine synthesis that may be affected by TTA. Increased production from sarcosine via SARDH is unlikely, due to the known inhibitory effect of PPARα activation on SARDH [13,25]. Moreover, glycine may be formed from serine, which can be derived through glycolysis [42], but as the glycolytic pathway is known to be inhibited by PPARα activity [15] this is also not a likely source. Another possible route of glycine synthesis is from threonine catabolism [42], which should be further explored as the plasma threonine concentration has previously been reported to be markedly higher among the TTA-treated rats [28]. Notably, both

PPARα activation [43] and TTA treatment [28] are associated with increased synthesis of carnitine, which plays an essential role in fatty acid metabolism [44]. Because the production of each molecule of carnitine also yields one molecule of glycine [44], it is reasonable to suspect increased carnitine synthesis being a contributor to the elevated glycine concentrations.

4.2.2. TTA Treatment and Vitamin B3

B3 vitamers are cofactors for a vast number of enzymatic redox reactions, such as the β-oxidation of fatty acids and substrate oxidation in Krebs cycle [45], as well as in the synthesis of carnitine [46]. In one-carbon metabolism, vitamin B3 is used as a reducing agent for both MTHFR and MSR [38,39], as well as in the conversion of choline to betaine [47]. The primary cofactor form of vitamin B3, nicotinamide adenine dinucleotide (phosphate) (NAD[P]), is formed from NAM and NA, and in the current study TTA treatment was associated with significantly higher concentration 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 [13,26,48], and accordingly, such treatment has consistently been associated with elevated urinary concentrations of both NAM and mNAM [13,49,50]. Higher plasma NAM and mNAM observed in the TTA group may thus be due to PPARα-induced increased production, and may be related to increased requirements for vitamin B3 due to enhanced β-oxidation, a well-known PPARα effect [51].

4.2.3. TTA Treatment and Vitamin B6

The transsulfuration pathway is activated by oxidative stress [52], and systemic vitamin B6 deficiency has previously been associated with both increased oxidative stress [53] and inflammation [54]. Of the B6 vitamers, only PL differed significantly between groups, with higher concentrations being observed in the TTA group. Although the most commonly used marker of vitamin B6 status is pyridoxal-5′-phosphate (PLP), total plasma B6-aldehyde (PL+PLP) is suggested as a direct measure of B6 status [55]. Higher B6-aldehyde, as indicated by the higher levels of PL, may thus represent improved B6 status, which is associated with lower inflammation and oxidative stress [53,54]. This is consistent with the anti-inflammatory and anti-oxidative effects previously seen by PPARα activation [14,15] and TTA treatment [18,56]. Notably, it has been demonstrated in cell studies that the gene expression of alkaline phosphatase (EC 3.1.3.1), the enzyme responsible for conversion of PLP to PL in plasma, is upregulated after PPARα activation [57]. This may explain why PL, and not PLP, was higher after TTA treatment. However, the production of PLP from pyridoxine and pyridoxamine is catalyzed by the flavoprotein pyridoxamine-pyridoxine 5-phosphate oxidase, and lower availability of vitamin B2 may limit PLP production via this route, possibly adding to the explanation why PLP is not increased [58].

4.2.4. TTA Treatment and Vitamin B12 Status

In this study, higher concentrations of MMA were observed in both plasma and urine among the TTA treated rats. Plasma cobalamin, however, was unaffected, indicating a metabolic cobalamin deficiency not reflected by low circulating cobalamin levels. This is in accordance with the observation that serum cobalamin is often poorly correlated with clinical signs and the functional markers of B12 deficiency [59]. The intracellular processing of cobalamin is complex and involves several enzymes which, to our knowledge, have not been evaluated as candidate PPARα targets. The protein expression of methylmalonyl-CoA mutase (MUT, EC 5.4.99.2), which catalyzes the catabolism of methylmalonyl-CoA in the mitochondria, was reported to be elevated after TTA treatment [25]. This is not in agreement with the elevated plasma and urinary MMA observed in the present study, and may be a compensatory up regulation due to other TTA-induced metabolic alterations upstream of the MUT reaction. One potential mechanism could be inhibition of the methylmalonic aciduria combined with homocystinuria type C (MMACHC) protein. MMACHC is a flavoprotein responsible for making free cobalamin available for cofactor synthesis [60], and lower availability of vitamin B2 may thus reduce

MMACHC function. Also, MMACHC is dependent on glutathione transferase activity [61], linking cobalamin metabolism to the transsulfuration pathway, which is a substantial source of cysteine for glutathione synthesis [62]. PPARα activation is known to inhibit the transcription of both enzymes in the transsulfuration pathway [13,63]. Elevated plasma cystathionine, observed after TTA treatment, has previously been associated with impaired transsulfuration due to B6-restriction [64]. However, B6 depletion was not followed by decreased glutathione synthesis, indicating that glutathione synthesis might primarily be regulated by other mechanisms [52]. The anti-inflammatory and anti-oxidative properties of TTA may result in decreased glutathione synthesis, which then might hamper MMACHC function. Together, lower availability of B2 and glutathione might reduce MMACHC function, leading to a functional cobalamin deficiency, a known effect of MMACHC defects [65]. In terms of MMA synthesis, the precursor for methylmalonyl-CoA is propionyl-CoA, derived from the catabolism of both odd-chained fatty acids and branched-chained amino acids. Increased oxidation of fatty acids is a well-known PPARα-effect, and it has been suggested that the catabolism of branched-chained amino acids is stimulated by PPARα activity [66], possibly facilitating increased MMA production.

4.3. Strengths and Limitations

The main strength of this study is its randomized and controlled long-term dietary intervention design. Extrapolation of the results to humans may, however, not be straightforward, as PPARα activation affects rodents differently and to a larger extent than humans [67,68]. We can also not exclude the possibility of PPAR-independent effects of TTA treatment influencing the results, thus limiting the interpretation of the observations simply being due to PPARα activation. The animals were sacrificed under non-fasting condition, which can be regarded a limitation of the assessment of metabolites. In humans, we have shown that DMG concentrations are higher among non-fasting individuals as compared to those with fasting samples [6]. Furthermore, blood and urinary concentrations of the various metabolites do not necessarily reflect their tissue concentrations, prompting careful interpretation in terms of metabolic flux [69]. It should also be acknowledged that the different B-vitamin cofactors are involved in a myriad of metabolic pathways not discussed herein, which could be of clinical interest related to PPAR activity. In terms of genes, it should be regarded a limitation that we only have analyzed the hepatic expression, and not the expression in other tissues which might be different.

4.4. Clinical Application

The involvement of PPARs in 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 [50], and targeted metabolic profiling focusing on one-carbon metabolites may provide valuable information regarding PPARα activity. DMG, MMA and vitamin B3 metabolites could be promising targets for such metabolic profiling in humans.

5. Conclusions

We have demonstrated that long-term treatment with TTA is associated with altered plasma and urinary concentrations of metabolites related to one-carbon metabolism and B-vitamin status in rats. We did not observe any differences in the hepatic gene expression of genes related to the choline oxidation pathway, but the expression of PPARα was increased. Our findings should motivate further investigation into how these metabolic pathways are regulated, specifically by looking into the suggested role of PPARα and explore whether these metabolites may reflect hepatic PPARα activity.

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Appendix 1

HVA SPISER DU?

I dette skjemaet spør vi om dine spisevaner slik de **vanligvis** er. Vi er klar over at kostholdet varierer fra dag til dag. Prøv derfor så godt du kan å gi et **"gjennomsnitt"** av dine spisevaner. Ha det siste året i tankene når du fyller ut skjemaet. Der du er usikker, anslå svaret.

Skjemaet skal leses av en maskin, og derfor er det viktig at du setter et tydelig kryss i avmerket rute.

Riktig markering er slik: ☒

Bruk helst bløt blyant. Feil kan da rettes med viskelær.
Kulepenn og svart tusjpenn kan også brukes.

Av hensyn til den maskinelle lesingen pass på at arkene ikke blir brettet.

Alle svar vil bli behandlet strengt fortrolig.

EKSEMPEL PÅ UTFYLLING AV SPØRSMÅL 1.

Kari Nordmann spiser daglig 5 skiver brød og ett knekkebrød. Hun spiser vanligvis kneippbrød, men i helgene blir det en del loff. I tillegg spiser hun ett knekkebrød hver dag. Hun fyller ut første spørsmål slik:

1. HVOR MYE BRØD PLEIER DU Å SPISE?

Legg sammen det du bruker til alle måltider i løpet av en dag.

(1/2 rundstykke = 1 skive, 1 baguett = 5 skiver, 1 ciabatta = 4 skiver)

Antall skiver pr. dag

	0	1/2	1	2	3	4	5	6	7	8	9	10	11	12+
Fint brød (loff, baguetter, fine rundstykker o.l.)	☐	☐	☒	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Mellomgrovt brød (lys helkorn, lys kneipp, lyst hj.bakt o.l.)	☐	☐	☐	☐	☐	☒	☐	☐	☐	☐	☐	☐	☐	☐
Grovt brød (fiberkneipp, mørk kneipp, mørkt hj.bakt o.l.)	☒	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Knekkebrød (kavring, grov skonrok o.l.)	☐	☐	☒	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Sum skiver pr. dag = 6

Antall skiver pr. uke: 6 x 7 = 42 Tallet brukes i spørsmål 5.

1. HVOR MYE BRØD PLEIER DU Å SPISE?

Legg sammen det du bruker til alle måltider i løpet av en dag.

(1/2 rundstykke = 1 skive, 1 baguett = 5 skiver, 1 ciabatta = 4 skiver)

	Antall skiver pr. dag													
	0	1/2	1	2	3	4	5	6	7	8	9	10	11	12+
Fint brød (loff, baguetter, fine rundstykker o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Mellomgrovt brød (lys helkorn, lys kneipp, lyst hj.bakt o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Grovt brød (fiberkneipp, mørk kneipp, mørkt hj.bakt o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Knekkebrød (kavring, grov skonrok o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Sum skiver pr. dag = ____

Antall skiver pr. uke: ____ x 7 = ____. Tallet brukes i spørsmål 5.

2. HVA PLEIER DU Å SMØRE PÅ BRØDET?

Merk av både for hverdag og helg, selv om du bruker det samme.

Hverdager		Lørdager, søndager
☐	Bruker ikke	☐
☐	Smør (meierismør)	☐
☐	Bremykt, Smøregod	☐
☐	Brelett	☐
☐	Soft, soyamargarin (pakke, beger)	☐
☐	Solsikke	☐
☐	Oliven	☐
☐	Vita	☐
☐	Olivero	☐
☐	Omega	☐
☐	Soft light	☐
☐	Vita lett	☐
☐	Annen margarin	☐

3. OM DU BRUKER FETT PÅ BRØD, HVOR MYE BRUKER DU?

En porsjonspakning på 12 g rekker til antall skiver

1 ☐
2 ☐
3 ☐
4 ☐
5 ☐

4. MELK SOM DRIKK

(1 glass = 1,5 dl)

	Drikker sjelden/ ikke	Antall glass pr. dag								
		1/2	1	2	3	4	5	6	7	8+
Helmelk, søt, sur	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Lettmelk, søt, sur	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Lettmelk, ekstra lett	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Skummet melk, søt, sur	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

5. PÅLEGGSSORTER

Bruk sum skiver pr. uke fra spørsmål 1.

	Til antall skiver pr. uke										
	0	1/2	1	2-3	4-5	6-7	8-14	15-21	22-28	29-35	36+
Brun ost, prim	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Hvit ost, helfet, 27% fett (Jarlsberg, Norvegia o.l., smøreost; eske, tube)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Hvit ost, halvfet, 16% fett (Jarlsberg, Norvegia o.l., smøreost; eske, tube)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Ost med mer enn 27% fett (kremoster, Normanna, Ridderost)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

	0	1/2	1	2-3	4-5	6-7	8-14	15-21	22-28	29-35	36+
Leverpostei, vanlig	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Leverpostei, mager	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Servelat, vanlig	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Lett servelat, kalverull, kokt skinke, okserull o.l.	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Salt pølse, spekepølse (fårepølse, salami o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

	0	1/2	1	2-3	4-5	6-7	8-14	15-21	22-28	29-35	36+
Kaviar	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Makrell i tomat, røkt makrell	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sardiner, sursild, ansjos o.l.	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Laks, ørret	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Reker, krabbe	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

	0	1/2	1	2-3	4-5	6-7	8-14	15-21	22-28	29-35	36+
Syltetøy, marmelade, frysetøy	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Honning, sirup, sjokolade-, nøttepålegg	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

	0	1/2	1	2-3	4-5	6-7	8-14	15-21	22-28	29-35	36+
Grønnsaker som pålegg (agurk, tomat o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Frukt som pålegg (banan, eple o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Salater med majones	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Majones på smørbrød	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

6. EGG

	Antall pr. uke							
	0	Mindre enn 1	1	2	3-4	5-6	7	8+
(kokt, stekt, eggerøre, omelett)	☐	☐	☐	☐	☐	☐	☐	☐

7. FROKOSTGRYN, GRØT OG YOGHURT

Svar enten pr. måned **eller** pr. uke. <1 betyr sjeldnere enn 1 gang.

	Gang pr. måned					Gang pr. uke						Mengde pr. gang				
	0	<1	1	2	3	1	2-3	4-5	6-7	8+		1	1 1/2	2	3+
Havregryn, kornblandinger (4-korn, usøtet müsli o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	☐	☐	☐	☐
Cornflakes, puffet ris, havrenøtter o.l.	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	☐	☐	☐	☐
Havregrøt	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1-2 ☐	3-4 ☐	5-6 ☐	7+ ☐
Sukker til frokostgryn, grøt	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(ts)	1 ☐	2 ☐	3-4 ☐	5+ ☐
Yoghurt, naturell, frukt	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(beger)	1/2 ☐	1 ☐	1 1/2 ☐	2+ ☐
Lettyoghurt	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(beger)	1/2 ☐	1 ☐	1 1/2 ☐	2+ ☐
Go´morgen yoghurt inkl. müsli	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(beger)	1/2 ☐	1 ☐	1 1/2 ☐	2+ ☐
Melk søt, sur på gryn, grøt og dessert	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	3/4 ☐	1 ☐	2 ☐	3+ ☐

8. KAFFE OG TE

(1 kopp kaffe = 1,2 dl 1 kopp te = 2 dl)

	Drikker ikke/ikke daglig	Antall kopper pr. dag: 1/2	1	2	3-4	5-6	7-8	9-10	11+
Kaffe, kokt	☐	☐	☐	☐	☐	☐	☐	☐	☐
Kaffe, traktet, filter	☐	☐	☐	☐	☐	☐	☐	☐	☐
Kaffe, pulver (instant)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Kaffe, koffeinfri	☐	☐	☐	☐	☐	☐	☐	☐	☐
Te	☐	☐	☐	☐	☐	☐	☐	☐	☐
Nypete, urtete	☐	☐	☐	☐	☐	☐	☐	☐	☐

	Antall teskjeer eller biter pr. kopp: 0	1/2	1	2	3	4+
Sukker til kaffe	☐	☐	☐	☐	☐	☐
Sukker til te	☐	☐	☐	☐	☐	☐
Kunstig søtstoff til kaffe eller te	☐	☐	☐	☐	☐	☐
Fløte til kaffe	☐	☐	☐	☐	☐	☐

9. ANDRE DRIKKER?

Svar enten pr. måned **eller** pr. uke. < 1 betyr sjeldnere enn 1 gang.
Merk at porsjonsenhetene er forskjellige. 1/3 liter tilsvarer en halvflaske øl og 2/3 liter tilsvarer en helflaske.

	Gang pr. måned					Gang pr. uke						Mengde pr. gang					
	0	<1	1	2	3	1	2-3	4-5	6-7	8+		1/2	1	2	3	4	5+
Vann	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(glass)	☐	☐	☐	☐	☐	☐
Appelsinjuice	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(glass)	☐	☐	☐	☐	☐	☐
Annen juice, most, nektar	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(glass)	☐	☐	☐	☐	☐	☐
Saft, solbærsirup m. sukker	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(glass)	☐	☐	☐	☐	☐	☐
Saft, kunstig søtet	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(glass)	☐	☐	☐	☐	☐	☐
Brus, Cola, Solo o.l., med sukker	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(liter)	☐	☐	☐	☐	☐	☐
Brus, Cola, Solo o.l., kunstig søtet	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(liter)	☐	☐	☐	☐	☐	☐
Farris, Selters, Soda o.l.	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(liter)	☐	☐	☐	☐	☐	☐
Alkoholfritt øl, vørterøl, lettøl	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(liter)	☐	☐	☐	☐	☐	☐
Pilsnerøl	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(liter)	☐	☐	☐	☐	☐	☐
Vin	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(glass)	☐	☐	☐	☐	☐	☐
Brennevin, likør	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(1 dram = 4 cl)	☐	☐	☐	☐	☐	☐

	1/4	1/3	1/2	2/3	1	11/2+
Brus, Cola, Solo o.l., med sukker	☐	☐	☐	☐	☐	☐
Brus, Cola, Solo o.l., kunstig søtet	☐	☐	☐	☐	☐	☐
Farris, Selters, Soda o.l.	☐	☐	☐	☐	☐	☐
Alkoholfritt øl, vørterøl, lettøl	☐	☐	☐	☐	☐	☐
Pilsnerøl	☐	☐	☐	☐	☐	☐

	1	2	3	4	5	6+
Vin	☐	☐	☐	☐	☐	☐
Brennevin, likør	☐	☐	☐	☐	☐	☐

10. MIDDAGSRETTER

Vi spør både om middagsmåltidene og det du spiser til andre måltider. Tell til slutt sammen antall retter du har merket for og se om summen virker sannsynlig.
En "dl" tilsvarer omtrent mengden i en suppeøse. Med "ss" menes en spiseskje.

	Gang pr. måned										Mengde pr. gang					
	0	<1	1	2	3	4	5-6	7-8	9+		1/2	2/3	1	11/2	2+	
Kjøttpølse, medisterpølse	☐	☐	☐	☐	☐	☐	☐	☐	☐	(kjøttpølse)	☐	☐	☐	☐	☐	
Hamburger, karbonader o.l.	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐	
Grill- og wienerpølse	☐	☐	☐	☐	☐	☐	☐	☐	☐	(pølse)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐	
Hamburger-, pølsebrød, lomper	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐	
Kjøttkaker, medisterkaker, kjøttpudding	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐	
Kjøttdeigretter (saus eller gryte med kjøttdeig, lasagne o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐	
Taco (med kjøtt og salat)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐	
Pastaretter	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐	

	Gang pr. måned										Mengde pr. gang					
	0	<1	1	2	3	4	5-6	7-8	9+		1/8	1/4	1/2	3/4	1+	
Pizza (500-600 g)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(pizza)	☐	☐	☐	☐	☐	
Biff (alle typer kjøtt)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1/2	1	1 1/2	2	2 1/2+	
Koteletter (lam, okse, svin)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1/2	1	1 1/2	2	2 1/2+	
Stek (lam, okse, svin)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(skive)	1-2	3-4	5-6	7-8	9+	
Stek (elg, hjort, reinsdyr o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(skive)	1-2	3-4	5-6	7-8	9+	
Gryterett med helt kjøtt, frikassé, fårikål o.l.	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1-2	3-4	5-6	7-8	9+	
Lapskaus, suppelapskaus, betasuppe	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1-2	3-4	5-6	7-8	9+	
Bacon, stekt flesk	☐	☐	☐	☐	☐	☐	☐	☐	☐	(skive)	1-2	3-4	5-6	7-8	9+	
Kylling, høne	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1/4	1/3	1/2	3/4	1+	
Leverretter	☐	☐	☐	☐	☐	☐	☐	☐	☐	(skive)	1-2	3-4	5-6	7-8	9+	
	0	<1	1	2	3	4	5-6	7-8	9+		1	2	3	4	5+	
Fiskekaker, fiskepudding, fiskeboller	☐	☐	☐	☐	☐	☐	☐	☐	☐	(kake)	☐	☐	☐	☐	☐	
Fiskepinner	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1-2	3-4	5-6	7-9	10+	
Torsk, sei, hyse (kokt)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1	2	3	4	5+	
Torsk, sei, hyse (stekt, panert)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1	2	3	4	5+	
Sild (fersk, speket, røkt)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(filet)	1	2	3	4	5+	
Makrell (fersk, røkt)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(filet)	1/2	1	1 1/2	2	3+	
Laks, ørret (sjø, oppdrett)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(skive)	1	2	3	4	5+	
Fiskegryte, -grateng, suppe med fisk	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1-2	3-4	5-6	7-8	9+	
Reker, krabbe	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl, renset)	1	2	3	4	5+	
	0	<1	1	2	3	4	5-6	7-8	9+							
Risgrøt, annen melkegrøt	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1-2	3-4	5-6	7-8	9+	
Pannekaker	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1-2	3-4	5-6	7-8	9+	
Suppe (tomat, blomkål, ertesuppe o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1-2	3-4	5-6	7-8	9+	
Vegetarrett, vegetarpizza grønnsakgrateng, -pai	☐	☐	☐	☐	☐	☐	☐	☐	☐	(bit/dl)	1-2	3-4	5-6	7-8	9+	
	0	<1	1	2	3	4	5-6	7-8	9+		1/2	1	1 1/2	2	2 1/2+	
Brun/hvit saus	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	☐	☐	☐	☐	☐	
Smeltet margarin, smør til fisk	☐	☐	☐	☐	☐	☐	☐	☐	☐	(ss)	1-2	3-4	5-6	7-8	9+	
Bearnaisesaus o.l.	☐	☐	☐	☐	☐	☐	☐	☐	☐	(ss)	1	2	3	4	5+	
Majones, remulade	☐	☐	☐	☐	☐	☐	☐	☐	☐	(ss)	1	2	3	4	5+	
Ketchup	☐	☐	☐	☐	☐	☐	☐	☐	☐	(ss)	1	2	3	4	5+	

11. POTETER, RIS, SPAGHETTI, GRØNNSAKER

Svar enten pr. måned **eller** pr. uke. <1 betyr sjeldnere enn 1 gang.
Disse spørsmålene dreier seg først og fremst om tilbehør til middagsretter, men spiser du for eksempel en rå gulrot eller salat til lunsj, skal det tas med her.

	Gang pr. måned					Gang pr. uke						Mengde pr. gang				
	0	<1	1	2	3	1	2-3	4-5	6-7	8+		1	2	3	4	5+
Poteter, kokte	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	☐	☐	☐	☐	☐
Pommes frites, stekte poteter	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Potetmos, -stuing, gratinerte poteter	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Ris	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1-2 ☐	3-4 ☐	5-6 ☐	7-8 ☐	9+ ☐
Spaghetti, makaroni, pasta	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1-2 ☐	3-4 ☐	5-6 ☐	7-8 ☐	9+ ☐
Gulrot	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1/2 ☐	1 ☐	1 1/2 ☐	2 ☐	3+ ☐
Hodekål	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(skalk)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Kålrot	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(skive)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Blomkål	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(bukett)	1-2 ☐	3-4 ☐	5-6 ☐	7-8 ☐	9+ ☐
Brokkoli	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(bukett)	1-2 ☐	3-4 ☐	5-6 ☐	7-8 ☐	9+ ☐
Rosenkål	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1-2 ☐	3-4 ☐	5-6 ☐	7-8 ☐	9+ ☐
Grønnkål	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Løk	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(ss)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Spinat, andre bladgrønns.	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Sopp	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1-2 ☐	3-4 ☐	5-6 ☐	7-8 ☐	9+ ☐
Avocado	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1/4 ☐	1/2 ☐	3/4 ☐	1 ☐	1 1/4 + ☐
Paprika	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(strimmel)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Tomat	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1/2 ☐	1 ☐	1 1/2 ☐	2 ☐	3+ ☐
Tomatbønner, bønner/linser	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Mais	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(ss)	1-2 ☐	3-4 ☐	5-6 ☐	7-8 ☐	9+ ☐
Erter, frosne grønnsak-blandinger	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Salatblandinger	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Dressing	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(ss)	1/2 ☐	1 ☐	2 ☐	3 ☐	4+ ☐
Rømme	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(ss)	1/2 ☐	1 ☐	2 ☐	3 ☐	4+ ☐

Hvor mange ganger om dagen spiser du vanligvis grønnsaker utenom grønnsakene du spiser til middag?

0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5+ ☐

12. TYPE FETT TIL MATLAGING

Smør/margarin	Oljer
☐ Smør (meierismør)	☐ Olivenolje
☐ Bremykt	☐ Soyaolje
☐ Melange, Per	☐ Maisolje
☐ Soft-, soyamargarin (pakke, beger)	☐ Solsikkeolje
☐ Solsikke	☐ Valnøttolje
☐ Oliven	☐ Andre oljer
☐ Annen margarin	

13. FRUKT

Svar enten pr. måned **eller** pr. uke. < 1 betyr sjeldnere enn 1 gang.

	Gang pr. måned					Gang pr. uke						Mengde pr. gang			
	0	<1	1	2	3	1	2-3	4-5	6-7	8+		1/2	1	2	3+
Eple	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	☐	☐	☐	☐
Appelsin, mandarin, grapefrukt	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	☐	☐	☐	☐
Banan	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	☐	☐	☐	☐
Druer	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(klase)	☐	☐	☐	☐
Eksotisk frukt (kiwi, mango)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	☐	☐	☐	☐
Annen frukt (fersken, pære m.v.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	☐	☐	☐	☐
Jordbær, bringebær (friske, frosne)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	☐	☐	☐	☐
Blåbær	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	☐	☐	☐	☐
Multer	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	☐	☐	☐	☐

Hvor mange frukter spiser du vanligvis pr. dag?

0	1	2	3	4	5	6	7	8	9+
☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

14. DESSERT, KAKER, GODTERI

Svar enten pr. måned **eller** pr. uke. < 1 betyr sjeldnere enn 1 gang.

	Gang pr. måned					Gang pr. uke						Mengde pr. gang			
	0	<1	1	2	3	1	2-3	4-5	6-7	8+					
Hermetisk frukt, fruktgrøt	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1/2 ☐	1 ☐	2 ☐	3+ ☐
Puddinger (sjokolade, karamell o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4+ ☐
Is (1 dl = 1 pinne = 1 kremmerhus)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4+ ☐
Boller, julekake, kringle	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1 ☐	2 ☐	3 ☐	4+ ☐
Skolebrød, skillingsbolle	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1 ☐	2 ☐	3 ☐	4+ ☐
Wienerbrød, -kringle o.l.	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1 ☐	2 ☐	3 ☐	4+ ☐
Smultring, formkake	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1 ☐	2 ☐	3 ☐	4+ ☐
Vafler	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(plate)	1/2 ☐	1 ☐	2 ☐	3+ ☐
Sjokoladekake, bløtkake, annen fylt kake	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1/2 ☐	1 ☐	2 ☐	3+ ☐
Søt kjeks, kakekjeks (Cookies, Bixit, Hob Nobs)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1-2 ☐	3-4 ☐	5-6 ☐	7+ ☐
Sjokolade (60 g)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(plate)	1/2 ☐	1 ☐	2 ☐	3+ ☐
Drops, lakris, seigmenn o.l.	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1-2 ☐	3-4 ☐	5-6 ☐	7+ ☐
Smågodt (1 hg = 100g)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(hg)	1/2 ☐	3/4 ☐	1 ☐	1 1/2+ ☐
Potetgull (1 pose 100g = 7 dl)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1-2 ☐	3-4 ☐	5-6 ☐	7+ ☐
Annen snacks (skruer, crisp, saltstenger, lettsnacks o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1-2 ☐	3-4 ☐	5-6 ☐	7+ ☐
Peanøtter, andre nøtter (1 pose 100g = 4 never)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(neve)	1 ☐	2 ☐	3 ☐	4+ ☐

15. KOSTTILSKUDD (bs = barneskje, ts = teskje)

	Hele året	Bare vinter-halvåret	Gang pr. uke: 0	<1	1	2-3	4-5	6-7		Mengde pr. gang
Tran	☐	☐	☐	☐	☐	☐	☐	☐		1 ts ☐ 1 bs ☐ 1 ss ☐
Trankapsler	☐	☐	☐	☐	☐	☐	☐	☐	kapsler	1 ☐ 2+ ☐
Fiskeoljekapsler	☐	☐	☐	☐	☐	☐	☐	☐	kapsler	1-2 ☐ 3-4 ☐ 5-6 ☐ 7+ ☐
Multipreparater										
Sanasol	☐	☐	☐	☐	☐	☐	☐	☐	bs	1 ☐ 2 ☐ 3 ☐ 4+ ☐
Biovit	☐	☐	☐	☐	☐	☐	☐	☐	bs	1 ☐ 2 ☐ 3 ☐ 4+ ☐
Vitaplex	☐	☐	☐	☐	☐	☐	☐	☐	tablett	1 ☐ 2 ☐ 3 ☐ 4+ ☐
Kostpluss	☐	☐	☐	☐	☐	☐	☐	☐	tablett	1 ☐ 2 ☐ 3 ☐ 4+ ☐
Vitamineral	☐	☐	☐	☐	☐	☐	☐	☐	tablett	1 ☐ 2 ☐ 3 ☐ 4+ ☐
Annet	☐	☐	☐	☐	☐	☐	☐	☐	tablett	1 ☐ 2 ☐ 3 ☐ 4+ ☐

Hvis annet, hvilket? .....

Jernpreparater										
Ferro C	☐	☐	☐	☐	☐	☐	☐	☐	tablett	1 ☐ 2 ☐ 3 ☐ 4+ ☐
Hemofer	☐	☐	☐	☐	☐	☐	☐	☐	tablett	1 ☐ 2 ☐ 3 ☐ 4+ ☐
Duroferon Duretter	☐	☐	☐	☐	☐	☐	☐	☐	tablett	1 ☐ 2 ☐ 3 ☐ 4+ ☐
Annet	☐	☐	☐	☐	☐	☐	☐	☐	tablett	1 ☐ 2 ☐ 3 ☐ 4+ ☐

Hvis annet, hvilket? .....

B-vitaminer	☐	☐	☐	☐	☐	☐	☐	☐	tablett	1 ☐ 2 ☐ 3 ☐ 4+ ☐
C-vitamin	☐	☐	☐	☐	☐	☐	☐	☐	tablett	1 ☐ 2 ☐ 3 ☐ 4+ ☐
D-vitamin	☐	☐	☐	☐	☐	☐	☐	☐	tablett	1 ☐ 2 ☐ 3 ☐ 4+ ☐
E-vitamin	☐	☐	☐	☐	☐	☐	☐	☐	tablett	1 ☐ 2 ☐ 3 ☐ 4+ ☐
Folat (folsyre)	☐	☐	☐	☐	☐	☐	☐	☐	tablett	1 ☐ 2 ☐ 3 ☐ 4+ ☐
Kalktabletter	☐	☐	☐	☐	☐	☐	☐	☐	tablett	1 ☐ 2 ☐ 3 ☐ 4+ ☐
Fluortabletter	☐	☐	☐	☐	☐	☐	☐	☐	tablett	1 ☐ 2 ☐ 3 ☐ 4+ ☐
Annet	☐	☐	☐	☐	☐	☐	☐	☐	tablett	1 ☐ 2 ☐ 3 ☐ 4+ ☐

Hvis annet, hvilket? .....

16. NÅR SPISER DU PÅ HVERDAGER?

HOVEDMÅLTIDER som frokost, formiddagsmat, middag, kvelds.

Omtrent klokken

6	8	10	12	14	16	18	20	22	24	2	4
☐ ☐	☐ ☐	☐ ☐	☐ ☐	☐ ☐	☐ ☐	☐ ☐	☐ ☐	☐ ☐	☐ ☐	☐ ☐	☐ ☐

MELLOMMÅLTIDER som kaffe, frukt, godteri, snacks m.v.

Omtrent klokken

6	8	10	12	14	16	18	20	22	24	2	4
☐ ☐	☐ ☐	☐ ☐	☐ ☐	☐ ☐	☐ ☐	☐ ☐	☐ ☐	☐ ☐	☐ ☐	☐ ☐	☐ ☐

17. MENER DU SVARENE I SPØRRESKJEMAET GIR ET BRUKBART BILDE AV KOSTHOLDET DITT?

Ja	Nei
☐	☐

Er det matvarer/produkter du regelmessig bruker, og som ikke er nevnt i skjemaet?

.....

.....

18. ER DU FORNØYD MED KROPPSVEKTEN DIN SLIK DEN ER NÅ?

- ☐ Ja
- ☐ Nei, jeg ønsker å slanke meg
- ☐ Nei, jeg ønsker å legge på meg

19. KJØNN

Mann	Kvinne
☐	☐

Vennligst se etter at du har svart på alle spørsmål.

Takk for innsatsen!

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