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Plasma 3-hydroxyisobutyrate (3-HIB) and methylmalonic acid (MMA) are markers of hepatic mitochondrial fatty acid oxidation in male Wistar rats

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ABSTRACT

Objective: Discovery of specific markers that reflect altered hepatic fatty acid oxidation could help to detect an individual's risk of fatty liver, type 2 diabetes and cardiovascular disease at an early stage. Lipid and protein metabolism are intimately linked, but our understanding of this crosstalk remains limited.
Methods: In male Wistar rats, we used synthetic fatty acid analogues (3-thia fatty acids) as a tool to induce hepatic fatty acid oxidation and mitochondrial biogenesis, to gain new insight into the link between fatty acid oxidation, amino acid metabolism and TCA cycle-related intermediate metabolites in liver and plasma.
Results: Rats treated with 3-thia fatty acids had 3-fold higher hepatic, but not adipose and skeletal muscle, expression of the thioesterase 3-hydroxyisobutyryl-CoA hydrolase (*Hibch*), which controls the formation of 3-hydroxyisobutyrate (3-HIB) in the valine degradation pathway. Consequently, 3-thia fatty acid-stimulated hepatic fatty acid oxidation and ketogenesis was accompanied by decreased plasma 3-HIB and increased methylmalonic acid (MMA) concentrations further downstream in BCAA catabolism. The higher plasma MMA corresponded to higher MMA-CoA hydrolase activity and hepatic expression of GTP-specific succinyl-CoA synthase (*Suclg2*) and succinate dehydrogenase (*Sdhb*), and lower MMA-CoA mutase activity. Plasma 3-HIB correlated positively to plasma and hepatic concentrations of TAG, plasma total fatty acids, plasma NEFA and insulin/glucose ratio, while the reverse correlations were seen for MMA.
Conclusion: Our study provides new insight into TCA cycle-related metabolic changes associated with altered hepatic fatty acid flux, and identifies 3-HIB and MMA as novel circulating markers reflective of mitochondrial β-oxidation in male Wistar rats.

1. Introduction

Altered mitochondrial function in liver, adipose and muscle tissue has been suggested to underlie the development of insulin resistance and increased risk of developing several diseases [1–4]. In situations of excess hepatic lipid uptake, an adaptive increase in mitochondrial oxidative capacity can prevent fatty liver and related conditions including insulin resistance and type 2 diabetes [5]. A better understanding of the molecular mechanisms underlying reduced hepatic fatty acid oxidation could help identify individuals with developing insulin resistance and facilitate early prevention and treatment.

Metabolomic studies have revealed an elevation of fasting branched-chain amino acid (BCAA) concentrations in the circulation of people with obesity, insulin resistance and type 2 diabetes [6–9]. Circulating BCAA and metabolite concentrations depend on the net metabolism of BCAAs in several different tissues including liver, skeletal muscle, pancreas and adipose tissue [10]. Insulin resistance is associated with reduced BCAA catabolism in adipocytes [11,12] and a concomitant increase in BCAA catabolism in skeletal muscle [10]. BCAAs provide substrates for the TCA cycle. Impaired oxidation of BCAAs may result in

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excess accumulation of incompletely oxidized TCA intermediates and reduced fatty acid oxidation [13,14], and impaired flux of BCAAs into the TCA cycle has been linked to nonalcoholic fatty liver disease (NAFLD) [15]. We and others have recently reported altered levels of intermediary BCAA metabolites such as the valine-derived metabolite 3-hydroxyisobutyrate (3-HIB) in obesity, insulin resistance and type 2 diabetes [16–21]. We found increased 3-HIB levels according to degree of hyperglycemia and in established type 2 diabetes, that adipocytes release 3-HIB, and that 3-HIB modulates mitochondrial respiration, insulin-stimulated glucose uptake and fatty acid uptake with different effects in white and brown adipocytes [21]. Another study found that 3-HIB can promote insulin resistance in skeletal muscle by increasing endothelial fatty acid uptake [22]. The release of 3-HIB and other TCA-related dicarboxylic acids depends on the removal of Coenzyme A (CoA) groups via specific thioesterases, a type of hydrolases.

The 3-thia fatty acids, tetradecylthioacetic acid (TTA), 2-(tridec-12-yn-1-ylthio)acetic acid (1-triple TTA) and 1,10-biscarboxy-methylthiodecane (BCMTD) were previously shown to increase the rate of mitochondrial fatty acid β-oxidation in the liver, while they are not β-oxidizable themselves [23]. The 3-thia fatty acids have been reported to lower triacylglycerol (TAG) levels both in liver and plasma through activation of the peroxisome proliferator-activated receptors (PPARs) and mitochondria transcription factor A (TFAM) and thereby increase mitochondrial fatty acid oxidation capacity [24–27]. We have previously observed that the TTA-induced increase in hepatic mitochondrial fatty acid oxidation runs in parallel with increased ketogenesis [24,26] and with expression of genes indicative of higher TCA cycle activity [26] and reduced gluconeogenesis [28]. Moreover, reduced body weight after long term administration of TTA was accompanied with increased plasma levels of several amino acids, possibly due to reduced amino acid catabolism, thereby transferring energy from lipids to amino acids [29].

While indirect evidence links BCAA metabolism, TCA cycle activity and fatty acid oxidation, and that this crosstalk is altered in insulin resistance [13], direct measurement of altered TCA cycle metabolites in plasma could provide important insight into the mechanisms that govern mitochondrial fatty acid oxidation in the liver, and reveal novel biomarkers of changes in these processes. Therefore, we here investigated whether increased influx of fatty acids to the liver runs in parallel with changes in BCAAs catabolism reflected by changes in plasma 3-HIB and non-esterified fatty acids (NEFAs), liver and plasma triacylglycerols (TAGs), methylmalonic acid (MMA) and insulin/glucose ratio, as well as expression of genes and enzyme activities of regulatory proteins/enzymes.

2. Materials and methods

2.1. Compounds

In this study we used the synthetic fatty acid compounds 1-triple TTA (2-(tridec-12-yn-1-ylthio)acetic acid (CH ≡ C-$(CH_2)_{11}$-S-CH_2-COOH)), TTA (tetradecylthioacetic acid (CH_3-$(CH_2)_{13}$-S-CH_2-COOH) and BCMTD (3-dithiacarboxylic acid (HOOC-CH_2-S-$(CH_2)_{10}$-S-CH_2-COOH). In these fatty acids, the C3 in the acyl chain is substituted with a sulphur atom, preventing them to undergo β-oxidation [23]. However, TTA can be degraded at the ω-end. In contrast, 1-triple TTA has a triple bond at the ω-end, making it non-degradable at both ends. BCMTD has a carboxyl group and a sulphur atom at the other end in the acyl chain as well, hindering it to be degraded at both ends [30]. Chemical structures of the synthetic fatty acids can be found in Supplementary Fig. 1.

2.2. Animal care and treatment

2.2.1. Animal study with 1-triple TTA

5-Weeks-old male Wistar rats (Taconic, Ejby, Denmark) were randomized on arrival by Research Randomizer [31] and housed in open cages in groups of four rats. In the 1-week acclimatization period and throughout the experiment, the rats were kept in standard 12 h light/12 h dark cycles at 22 ± 2 °C and with humidity conditions of 55 ± 5% and had ad libitum access to standard chow diet and water. At the beginning of the experiment, the rats were block randomized to their respective treatments [31] and paired-housed separated by cage walls, enabling sniffing interaction. 0.5 ml 0.5% methylcellulose (Hospital Pharmacy, Bergen, Norway) with or without 0.02 g 1-triple TTA (Synthetica AS, Oslo, Norway) was given orally by gavage to the rats in their respective groups daily over the 3-week period. The rats in the control group and 1-triple TTA group were weighed once a day and weekly feed consumption was registered. At the end of the experiment, the rats were euthanized by 5% isoflurane inhalation anaesthesia (Schering-Plough, Kent, UK) followed by cardiac puncture. The collected EDTA-blood was immediately chilled on ice and plasma samples obtained by centrifugation were stored at −80 °C. Liver, muscle and epididymal white adipose tissue (eWAT) were collected, weighed and snap-frozen in liquid nitrogen before storage at −80 °C. Parts of the fresh liver samples were immediately used for β-oxidation analysis (described later). Care was taken to improve the environment of the animals and to minimize suffering. The Norwegian State Board of Biological Experiments with Living Animals approved the protocol (Permit number 2015-7367).

2.2.2. Animal study with TTA and BCMTD

Male Wistar rats (Taconic, Ejby, Denmark), weighing 150–200 g, were paired-housed in wire cages. During the 1-week acclimatization period and throughout the experiment, the rats were kept in standard 12 h light/12 h dark cycles at 20 ± 3 °C and had unlimited access to standard chow diet and water. Palmitic acid (control), TTA and BCMTD were dissolved in 0.5% sodium carboxymethyl cellulose (CMC) as previously described [32] and were given at doses of 150 and 300 mg/kg body weight in a volume of 0.7–1 ml by gastric intubation to the rats in their respective groups each day for 1 week. 0.5% CMC was in some cases used as a second control. All rats were weighed daily and feed consumption was registered weekly. At the end of the experiment, the rats were euthanized by subcutaneous injection of Hynorm (Janssen, Pharmaceutica NV, Beerse, Belgium) and Dormicum© (F. Hoffman-La-Roche Ltd., Basel, Switzerland) followed by cardiac puncture. The collected liver tissue samples were immediately chilled on ice. The use of animals in the study was approved by the local ethical committee for animal experiments based on the Guidelines for the Care and Use of Experimental Animals, and in agreement with the Norwegian laws and regulations on laboratory animals in research.

2.3. Preparation of cellular fractions

Liver tissue from each rat was homogenized in ice-cold homogenization buffer containing 0.25 M sucrose, 10 mM Hepes (pH 7.4) and 2 mM EDTA. Subcellular fractions of the homogenate were prepared from each rat. The fractionation process was performed at 0–4 °C and consisted of several centrifugation steps with different time integrals to obtain light-mitochondrial (peroxisome-enriched), microsomal and cytosolic fractions as described earlier [33]. Parts of light-mitochondrial (peroxisome-enriched) fractions were used to prepare pure peroxisome fractions as described elsewhere [34] with some modifications. 2 ml light-mitochondrial fraction was overlaid with 15 ml of 25% Nycodenz in 0.25 M sucrose, 1 mM EDTA and 10 mM Hepes (pH 7.4). After centrifugation at 106,000 ×*g* for 90 min, the upper debris layer was removed and the remaining pure peroxisome pellet was dissolved in 1 ml 0.25 M sucrose, 1 mM EDTA and 10 mM Hepes (pH 7.4).

2.4. Hepatic gene expression analysis

Total RNA was isolated from frozen 20 mg frozen liver tissue by homogenization in RLT buffer (Qiagen) with 1% β-mercaptoethanol using TissueLyser II for 2 × 2 min at 25 Hz and by RNA purification using RNaesy Mini kit with DNase digestion (Qiagen), following the

manufacturer's protocols. 500 ng RNA input was included in the cDNA synthesis using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems). cDNA was mixed with 2× Taqman buffer (Applied Biosystems) and probes and primers (listed in Supplementary Table 1) and real-time quantitative PCR (RT-qPCR) was run on Sarstedt 384-well Multiply-PCR plates (Sarstedt Inc.) using the ABI Prism 7900HT Sequence detection system (Applied Biosystems). A standard curve was run for each probe by using a representative cDNA sample or cDNA from universal rat reference RNA (URRR, Agilent). mRNA levels were normalized to the reference gene *Rplp0* or *18S*.

2.5. Muscle and adipose tissue gene expression analysis

Total RNA was isolated from 100 mg frozen epididymal white adipose tissue (eWAT) and 30 mg frozen muscle tissue using RNA/DNA/Protein Purification Kit (Norgen Biotek Corp.) according to the manufacturer's instructions. 127 ng purified RNA was used as template for cDNA synthesis, using the High Capacity cDNA reverse Transcription Kit (Applied Biosystems) in accordance with the supplier's protocol. To analyze the cDNA, RT-qPCR was performed using LightCycler® 480 SYBR Green I Master mix (Roche) and primers for genes of interest (listed in Supplementary Table 2), following the manufacturer's protocol. mRNA levels were based on standard curves or the delta-delta Ct method and was normalized to the reference gene *Rplp0*.

2.6. Measurement of methylmalonyl-CoA mutase mRNA expression by hybridization analysis

Total RNA was purified by the guanidinium thiocyanate technique as previously described [35]. Gel electrophoresis on a 1% agarose mini-gel followed by ethidium bromide staining was carried out to evaluate the degree of RNA digestion. Previously described methods for RNA slot blotting onto nylon membranes [36] and hybridization to immobilized RNA [37] were followed. The relative mRNA expression of methylmalonyl-CoA was based on hybridization of radioactive probe to each RNA sample and was normalized to 28S RNA. The cDNA fragment of methylmalonyl-CoA was ^{32}P-labelled using the oligolabelling method [38].

2.7. Quantification of plasma and liver lipids

Lipids were extracted from frozen liver tissue as previously described [39]. Before the measurements, the lipid extract was evaporated with nitrogen and redissolved in isopropanol. Enzymatic measurements of lipids in liver and plasma samples were analyzed by the Hitachi 917 system (Roche Diagnostics) using the Triglyceride kit by GPO-PAP method (Roche Diagnostics) and NEFA FS kit (Diagnostic Systems).

2.8. Plasma glucose and insulin measurements

Glucose concentration in plasma was determined enzymatically by the Hitachi 917 system (Roche Diagnostics) using the GLUC2 kit (Roche Diagnostics). Insulin measurements in plasma were performed using the Rat/Mouse insulin ELISA kit (Merck).

2.9. Quantification of ketone body in plasma

Production of ketone bodies was determined in plasma samples using a β-hydroxybutyrate (ketone body) colorimetric assay kit (Cayman Chemical).

2.10. Hepatic mitochondrial fatty acid oxidation (β-oxidation analysis)

1 g fresh liver tissue was homogenized in 4 ml ice-cold buffer comprising 0.25 M sucrose, 10 mM Hepes and 1 mM Na_4EDTA (pH 7.4). The homogenates were centrifuged at 1030 RCF for 10 min at 4 °C, and the post-nuclear fraction was immediately used to measure palmitoyl-CoA oxidation as acid-soluble products, as described earlier [40]. The protein concentration was measured by the DC Protein Assay kit (Bio-Rad Laboratories).

2.11. Measurement of plasma amino acids and related metabolites

Metabolites and amino acids in plasma were measured by gas chromatography-tandem mass spectrometry (GC–MS/MS) at Bevital AS (Bergen, Norway; http://www.bevital.no) as earlier described [41].

2.12. Measurement of serum MMA and vitamin B_{12} levels

Methylmalonic acid (MMA) concentrations in serum samples were determined by capillary electrophoresis (CE) as previously described [42]. Vitamin B_{12} levels were measured using the competitive magnetic separation immunoassay by Technican Immuno 1® System (1995) (Bayer Corporation).

2.13. Measurements of free coenzyme A (CoASH), propionyl-CoA, methylmalonyl-CoA, and succinyl-CoA

The concentrations of short-chain acyl-CoA esters in liver tissue samples were quantified by reversed-phase high performance liquid chromatography (RP-HPLC) as described previously [43] with some modifications. Liver tissue was homogenized in ice-cold 5% sulfosalicylic acid containing 50 μM dithioerythritol to obtain 30% w/v homogenates. 500 μl homogenate was centrifuged at 600 ×*g* for 10 min and 50 μl supernatant was immediately transferred into a 100 × 4.6 mm HPLC column packed with 3-μm particle size of ODS Hypersil (C_{18}), equipped with a guard column filled with Pelliguard LC-18 packing. Absorbance was measured at 254 nm. Elution solvent A consisted of 100 mM sodium phosphate and 75 mM sodium acetate (pH 4.6) and solvent B contained 70% solvent A in methanol. The elution process was performed at room temperature and the flow rate was 1.5 ml/min. The following elution profile was obtained: 0 min, 90% A; 10 min, 60% A; 17.6 min, 10% A, and the baseline condition was achieved by washing the column with 90% A for 8 min.

2.14. Enzymatic assays in liver fractions

Propionyl-CoA synthetase activity was measured as previously described [44]. A linear relationship between time (0–30 min) and protein (0–1 mg/assay) was observed in the assay.

Propionyl-CoA carboxylase activity was determined as previously described [45] with some modifications. The assay mixture consisted of 1.30 mM propionyl-CoA, 68 mM Tris-HCl (pH 8), 3 mM $MgCl_2$, 100 mM KCl, 1.35 mM ATP, 1 μCi (^{14}C) 20 mM $NaHCO_3$, and 0.05–0.2 mg protein. Incubations were carried out in duplicates for 30 min at 37 °C. Control incubations contained no propionyl-CoA. 25 μl 5 M perchloric acid was used to stop the reactions, followed by centrifugation at 1700 ×*g* for 15 min. 25 μl supernatant was transferred onto Whatman 3MM paper and was dried overnight. Measurements of radioactivity were performed on the paper filters.

Methylmalonyl-CoA mutase activity was measured as the generation of (2-(3)-^{14}C)-succinyl-CoA from a racemic mixture of DL-(methyl-^{14}C)-methylmalonyl-CoA as described before [46]. Assays where the rats were fed palmitate alone (200 mg/kg body weight/day) had 20–100 μM palmitoyl-CoA and CoA-bound TTA, and identical concentrations of the free corresponding fatty acids (not CoA-bound).

Methylmalonyl-CoA hydrolase was based on the release of free CoASH from methylmalonyl-CoA and was measured spectrophotometrically with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) as described earlier [47]. The assay mixture contained 0.285 μM Methylmalonyl-CoA, 11.5 mM Hepes (pH 6.8), 0.24 mM DTNB, and 120 mM KCl. A linear relationship in the spectrophotometric assay was seen up to 250

μg of protein added.

Methylmalonyl-CoA, propionyl-CoA, and DTNB were obtained from Sigma Chemicals. All other chemicals used were purchased from common commercial sources and were of analytic grade.

2.15. Statistical analyses

Statistical analyses were performed using the software GraphPad Prism 8. To evaluate significant difference between two groups, two-tailed unpaired *t*-tests (assuming normal distribution) were performed. Ordinary one-way ANOVA (Sidak's post-hoc test) was performed to assess significant difference for more than two groups. Two-way ANOVA (Holm-Sidak's post-hoc test) was used to evaluate control and treatment groups for the different compartments in the subcellular liver fractions. Significant difference was defined as $p < 0.05$ and results are expressed as mean ± S·D. for 5–8 rats per group. Correlations were calculated by Spearman.

3. Results

3.1. Effects on fatty acid flux into liver and BCAA metabolites

We first investigated the impact of 3-thia fatty acid administration on hepatic fatty acid flux and plasma concentrations of BCAA metabolites. Three weeks of 1-triple TTA treatment led to increased hepatic gene expression of carnitine/acylcarnitine translocase (*Slc25a20*) (Fig. 1), together with increased expression of fatty acid transfer protein (*Cd36*), fatty acid binding protein 1 (*Fabp1*) and carnitine transporter (*Slc22a5*) as reported previously [26]. This was accompanied with increased expression of the gene encoding ELOVL fatty acid elongase 6 (*Elovl6*) (Fig. 1), which uses malonyl-CoA as a 2-carbon donor in the first and rate-limiting step of fatty acid elongation. This was further accompanied by decreased levels of plasma non-esterifies fatty acid (NEFA), decreased total fatty acids in plasma, increased levels of the plasma ketone bodies/total fatty acid ratio and decreased insulin/glucose ratio, but no difference in body weight gain or feed efficiency as reported previously [26,28].

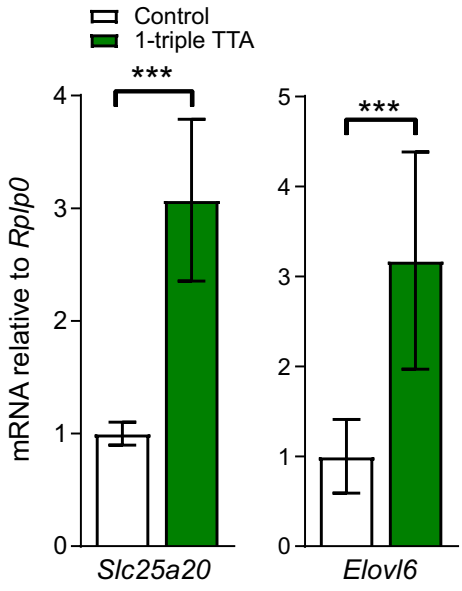

Fig. 1. Effects of 1-triple-TTA on genes regulating fatty acid flux into the liver in vivo.
Relative hepatic mRNA expression of *Slc25a20* and *Elovl6*, relative to *Rplp0* in control (n = 7) and 1-triple TTA-treated Male Wistar rats (n = 7). The results are presented as mean ± SD.
*p < 0.05, **p < 0.01, ***p < 0.001.

Metabolites from BCAA degradation, including 3-HIB and MMA, may enter the TCA cycle as acetyl-CoA or succinyl-CoA (Fig. 2A). 1-triple TTA treatment did not change the circulating levels of valine, leucine and isoleucine, but the plasma levels of 3-HIB and MMA were decreased and increased respectively (Fig. 2B), reflected in a strong inverse correlation between 3-HIB and MMA (Fig. 2C).

3.2. Effects on regulatory enzymes in BCAA catabolism

In 1-triple TTA-treated rats the gene expressions of *Bcat2*, *Bckdha* and *Hibadh* in liver, muscle and adipose tissue remained unchanged by 1-triple TTA administration (Fig. 2D). Interestingly, hepatic expression of *Hibch*, encoding a mitochondrially located enzyme which hydrolyzes 3-hydroxyisobutyryl-CoA to 3-HIB, was increased 3-fold (Fig. 2D). The effect of 1-triple TTA on tissue 3-HIB formation may be specific for liver, since no changes in *Hibch* expression were seen in samples from muscle and epididymal fat (Fig. 2D).

3.3. Correlations of 3-HIB with changes in mitochondrial functions and parameters linked to lipid and glucose metabolism

The mitochondrial fatty acid oxidation increased after treatment with 1-triple TTA [26], and both plasma levels of 3-HIB and 3-HIB/MMA-ratio were negatively correlated with mitochondrial fatty acid oxidation across the control and treatment groups (Fig. 2E, Supplementary Table 3). On the other hand, plasma level of 3-HIB correlated positively to both plasma and liver TAG, and plasma total fatty acids and NEFA (Fig. 3A). Additionally, both plasma levels of 3-HIB and 3-HIB/MMA-ratio were correlated inversely to hepatic total fatty acid levels and plasma ketone/NEFA ratio, an indicator of fatty acid oxidation and ketogenesis in the liver (Fig. 3A, Supplementary Table 3). In the 1-triple TTA-treated rats, the plasma ratio of insulin/glucose was lower than in controls [28]. Notably, the insulin/glucose ratio was positively correlated to both 3-HIB, TAG and NEFA (Fig. 3B). Similarly, across the groups we also found significant correlations between MMA and/or 3-HIB/MMA ratio with the parameters of lipid and glucose metabolism (Supplementary Table 3).

3.4. Degradation of hepatic 3-HIB

The strong correlations between circulating 3-HIB and several parameters in lipid and glucose metabolism prompted us to further investigate the enzymatic processes that regulate 3-HIB metabolism and degradation in the liver. 3-HIB can be converted to propionyl-CoA and CoASH by HIBADH and ALDH6A1 via the intermediate metabolite MMS (Fig. 2A). Interestingly, the hepatic concentrations of both free CoASH and propionyl-CoA were increased by TTA and BCMTD administration (Fig. 4A). We also observed higher hepatic concentrations of MMA-CoA in TTA-treated rats than in controls (Fig. 4A).

Propionyl-CoA is formed by degradation of valine and leucine, as well as of odd-chain fatty acids, but can also be converted from propionate and CoASH by propionyl-CoA synthetase (Fig. 2A). Propionyl-CoA can further be converted to MMA-CoA by propionyl-CoA carboxylase. In post-nuclear fractions of rats treated with BCMTD, we found increased activity of propionyl-CoA synthetase, but no change in propionyl-CoA carboxylase activity (Fig. 4B). Moreover, MMA-CoA can be converted to MMA by MMA-CoA hydrolase or to succinyl-CoA by MMA-CoA mutase (Fig. 2A). We observed a decrease in MMA-CoA mutase activity and an increase in MMA-CoA hydrolase activity by BCMTD treatment (Fig. 4B). In subcellular fractions from the liver, we observed that most of the activity of MMA-CoA mutase was located to the mitochondrial and cytosolic fractions, showing decreased mutase activity by BCMTD (Fig. 4C). Low mutase enzyme activities were found in the pure peroxisomal fraction, the peroxisome-enriched fractions containing peroxisomes and lysosomes, and in the microsomal fractions (Fig. 4C). Most of the MMA-CoA hydrolase activity was found in peroxisomes and in the

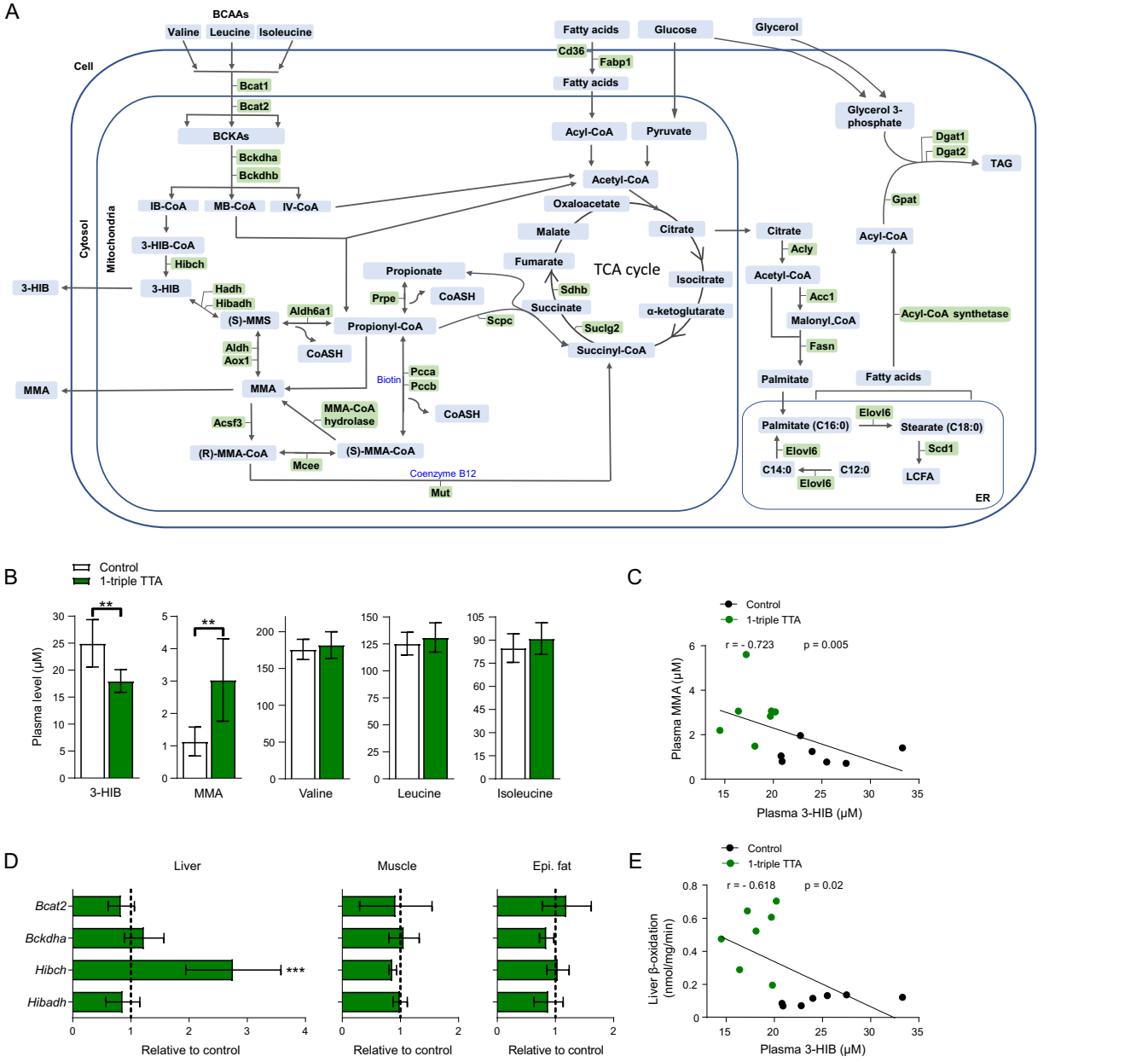

Fig. 2. Comparison of liver, muscle and fat in BCAA metabolism and the effect of 1-triple TTA on fatty acid oxidation in the liver in vivo.
(A) Overview of cellular BCAA catabolism and TCA cycle. Amino acids, metabolites and intermediates are marked in blue and relevant enzymes in green. (B) Plasma levels of 3-HIB, MMA and the BCAAs (valine, leucine and isoleucine) in control (n = 7) and 1-triple TTA-treated Male Wistar rats (n = 7). (C) Spearman correlations for plasma 3-HIB and plasma MMA concentrations in control (n = 7) and 1-triple TTA-treated Male Wistar rats (n = 7). Spearman r and p-value stated in the figures. (D) Relative mRNA expression of genes encoding enzymes in BCAA catabolism (relative to *Rplp0*) in liver, muscle and epididymal white adipose tissue dissected from 1-triple TTA-treated Male Wistar rats (n = 7) relative to control (n = 7). The results are presented as mean ± SD. (E) Spearman correlations of plasma 3-HIB concentration and liver β-oxidation in control (n = 7) and 1-triple TTA-treated Male Wistar rats (n = 7). Spearman r and p-value stated in the figures.
*p < 0.05, **p < 0.01, ***p < 0.001.

mitochondrial and cytosol fractions, and the hydrolase activity was increased in these fractions after BCMTD administration (Fig. 4C). The microsomal fraction had only diminutive levels of hydrolase activity (Fig. 4C). Although the mutase activities both in mitochondria- and cytosol-enriched fractions were decreased (Fig. 4C), the hepatic mRNA levels of MMA-CoA mutase were elevated 1.5–2-fold by TTA and BCMTD treatment (Fig. 4D). We also observed higher circulating concentrations of MMA in TTA-treated rats although a tendency of lower MMA concentrations in BCMTD-treated rats, while serum vitamin B12 levels were lower in both TTA- and BCMTD-treated rats (Fig. 4E).

We further investigated the metabolite flux from BCAA degradation towards the TCA cycle in the liver, by measuring the gene expression of key enzymes involved. *Aldh6a1*, which is responsible for conversion between (*S*)-MMS and and propionyl-CoA, showed no difference in expression between treated and untreated rats (Fig. 4F). Of interest, the hepatic gene expression of methylmalonyl-CoA synthetase (*Acsf3*), which converts MMA to (*R*)-MMA-CoA, was upregulated by 1-triple TTA (Fig. 4F). The expression of the genes *Pcca* and *Pccb*, which together encode the enzyme propionyl-CoA carboxylase, was also increased in the liver by 1-triple TTA (Fig. 4F). We additionally observed increased expression of methylmalonyl-CoA emirase (*Mcee*), which is responsible for converting (*R*)-MMA-CoA to (*S*)-MMA-CoA, in 1-triple TTA-treated

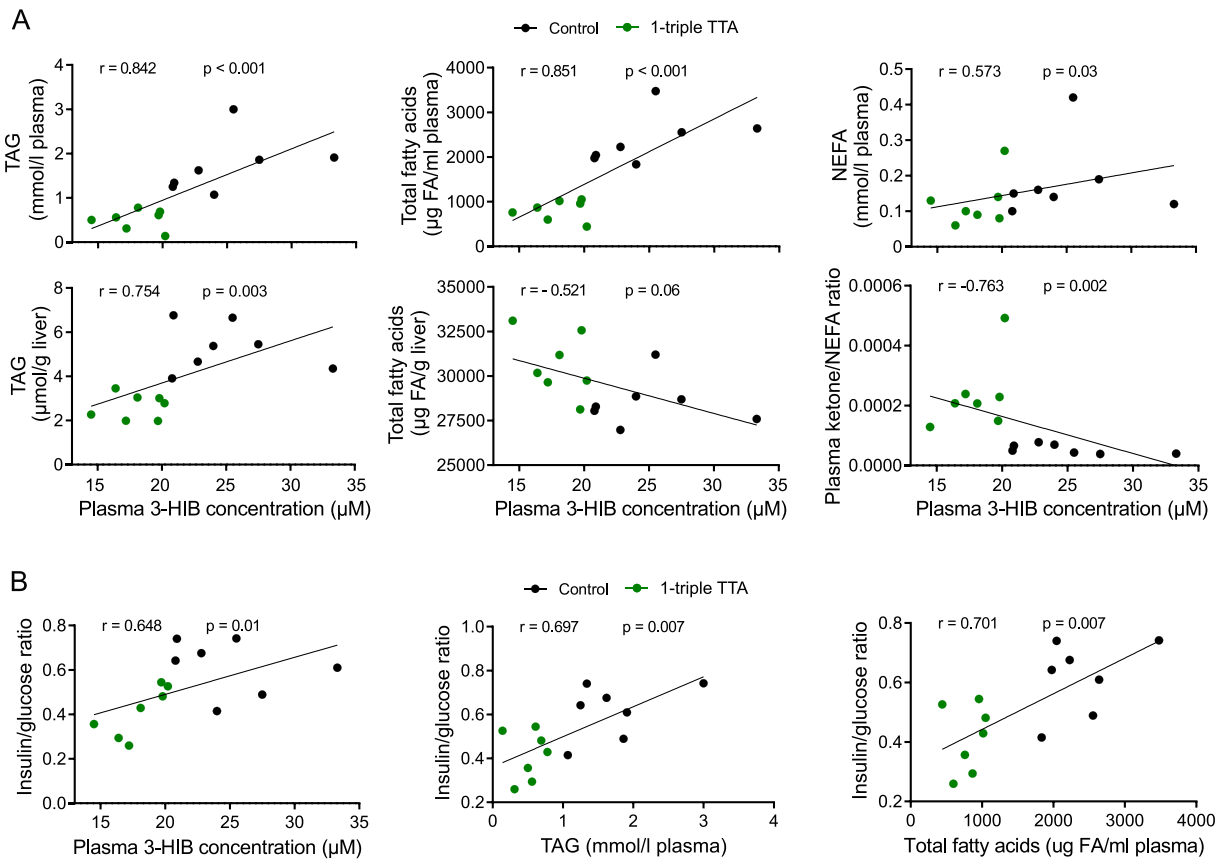

Fig. 3. Plasma 3-HIB levels correlates with changes in mitochondrial functions and parameters linked to lipid and glucose metabolism.
(A–B) Spearman correlations of plasma 3-HIB concentrations and parameters related to lipid and glucose metabolism in control (n = 7) and 1-triple TTA-treated Male Wistar rats (n = 7).
TAG; triacylglycerol, NEFA; non-esterified fatty acids.
Spearman r and p-values are stated in the figures.

rats (Fig. 4F). An increase in hepatic *Mut* expression was also seen in 1-triple TTA-treated rats (Fig. 4F).

The liver concentrations of succinyl-CoA were not significantly changed by TTA or BCMTD treatment (Fig. 4A). However, an increased level of plasma succinate in 1-triple TTA-treated rats was accompanied with increased expression of genes encoding both GTP-specific succinyl-CoA synthetase (*Suclg2*), succinate dehydrogenase (*Sdha*) and the iron-sulphur subunit of succinate dehydrogenase (*Sdhb*), the latter involved in complex II of the mitochondria electron transport chain and responsible for transferring electrons from succinate to ubiquinone (Fig. 4F). Furthermore, plasma succinate levels were inversely correlated to circulating 3-HIB levels (Fig. 4G).

4. Discussion

In this study, we used mitochondria-targeted fatty acid analogues as tools to investigate BCAA catabolism and cellular mechanisms related to TCA cycle activity, mitochondrial β-oxidation and lipid metabolism relevant in progression of metabolic diseases. We observed that treatment of rats with these liver-targeting compounds reduced plasma levels of 3-HIB and increased plasma levels of MMA. Previous studies have reported that 3-HIB is released from different tissues such as muscle, heart and adipocytes and acts as a signaling molecule [21,22,48,49]. However, as the fatty acid analogues used in this study primarily target the liver mitochondria, our results indicate that the circulating levels of 3-HIB and MMA may be controlled, at least in part, by the liver, which also has high BCAA catabolic activity [10]. In line with this, 3-thia fatty acid treatment altered gene expression and activity of BCAA catabolizing enzymes in the liver, while no such changes were found in skeletal muscle and epididymal white adipose tissue.

The reduced plasma levels of 3-HIB in 3-thia fatty acid-treated rats might have resulted from increased 3-HIB catabolism towards the TCA cycle. This is supported by the higher liver content of free CoASH, propionyl-CoA and succinyl-CoA, higher MMA-CoA hydrolase activity, and higher gene expression of MMA-CoA mutase, succinyl-CoA synthase and succinate dehydrogenase compared to control. Of note, even though treatment with 3-thia fatty acids increased expression of the gene encoding MMA-CoA mutase, thereby promoting formation of the TCA-cycle intermediate succinyl-CoA, we found a decrease in MMA-CoA mutase activity. Still, the tendency towards increased hepatic succinyl-CoA levels was accompanied with increased plasma levels of succinate, indicating an increased degradation of BCAA intermediates that can be used as fuel in the TCA cycle.

Numerous studies have shown increased circulating BCAA concentrations in obesity and insulin resistance [6–8]. Of note, the 3-thia fatty acid-induced increase in circulating levels of BCAA metabolites was not accompanied with reduced BCAA levels. However, the present study indicates that downstream intermediates in BCAA metabolism, here most notably 3-HIB and MMA, are direct and specific reflectors of altered cellular functions. This is supported by our recent study showing specific changes in circulating 3-HIB and not valine within 1 week after bariatric surgery [21], and another previous study showing a correlation of 3-HIB, but not of the BCAAs, and improvements in glucose metabolism following dietary intervention [19]. Notably, a recent study showed that whole-body knockout of the mitochondrial BCAA catabolic enzyme BCKDH kinase in mice increased circulating 3-HIB

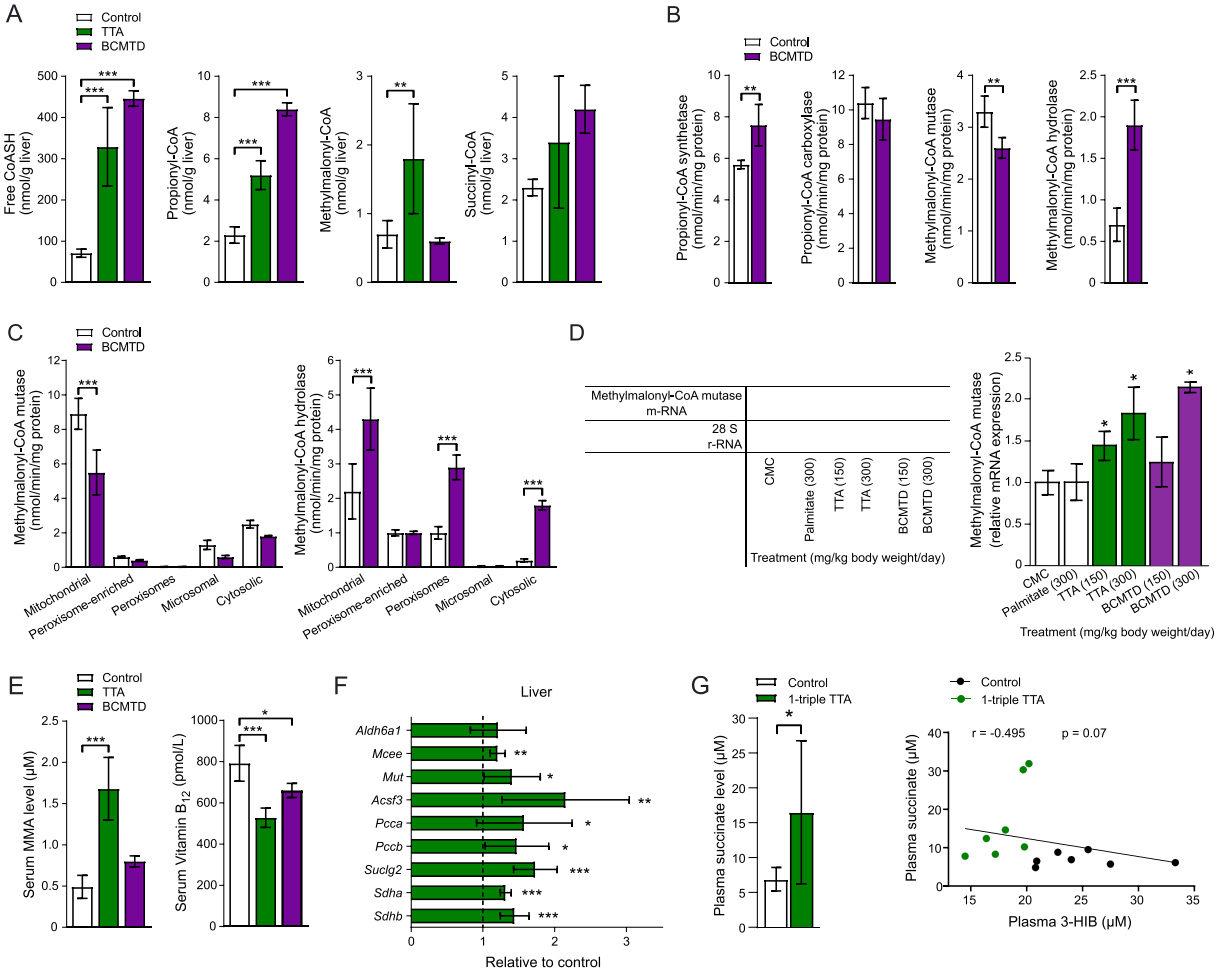

Fig. 4. 3-Thia fatty acids affect pathways of hepatic BCAA catabolism and 3-HIB degradation.
(A) Levels of various short chain acyl-CoA derivatives in liver homogenates of rats treated with TTA (n = 5) or BCMTD (n = 5) for 7 days. The results are presented as mean ± S.D. (One-way ANOVA, Sidak's). (B) Effect of BCMTD on the activity of main enzymes in propionyl-CoA metabolism linked to the BCAA pathway, measured in post nuclear liver fractions of rats treated for 7 days. The results are presented as mean ± S.D. of 5 animals per group. Treatment doses for palmitic acid and BCMTD were 300 ng/kg body weight/day. (C) Specific activities of methylmalonyl-CoA mutase and methylmalonyl-CoA hydrolase in each of the subcellular liver fractions from rats treated with or without BCMTD for 7 days. Treatment doses for palmitic acid and BCMTD were 300 ng/kg body weight/day. The results are presented as mean ± S.D. of 5 animals per group (Two-way ANOVA, Holm-Sidak's). (D) Effect of TTA on the mRNA expression of methylmalonyl-CoA mutase in rat liver. Animals were treated with 2 doses of TTA. One representative slot blot of a total of four is shown. Cumulative results of slot blots are shown in the graph. Results represent means ± S.D. of four animals. (E) Levels of MMA and vitamin B12 in serum of rats treated with 300 mg/kg/day of palmitic acid (control), TTA or BCMTD for 7 days. The results are presented as mean ± S.D. of 5 animals per group (One-way ANOVA, Sidak's). (F) Relative hepatic gene expression of key enzymes of the BCAA pathway in the direction to the TCA cycle, relative to *18S* in control (n = 7) and 1-triple TTA-treated Male Wistar rats (n = 7). The results are presented as mean ± SD. (G) Plasma levels of succinate, and Spearman correlation for plasma 3-HIB and plasma succinate concentrations in control (n = 7) and 1-triple TTA-treated Male Wistar rats (n = 7). Spearman r and p-values are stated in the figures.
*p < 0.05, **p < 0.01, ***p < 0.001.

concentrations while decreasing circulating valine [10]. Additionally, the authors reported that mice with obesity and insulin resistance show a redistribution in whole-body BCAA metabolism, with a shift in BCAA oxidation from fat and liver towards skeletal muscle, concomitant with elevated circulating BCAA levels [10]. Together, these studies reveal a complex inter-organ regulation of BCAA metabolism, and together with our data support that specific BCAA-related metabolites may capture altered cellular and physiological states, such as fatty acid oxidation, more directly than the BCAAs.

The present study implicates 3-HIB as a reflector of, and potential player in, fatty acid oxidation, ketogenesis and glucose production and storage, metabolic processes in which the liver plays an essential role [50]. The plasma levels of 3-HIB showed significant positive correlations to both plasma and liver TAG as well as to plasma total fatty acids and NEFA, and inverse associations with hepatic β-oxidation and to plasma ketone/NEFA ratio. Mitochondrial fatty acid oxidation has previously been implicated in the regulation of both liver and plasma TAG [24–27]. The altered expression of the 3-HIB-forming enzyme HIBCH in the liver but not adipose and muscle tissue suggests that 3-HIB may serve as a marker of altered hepatic fatty acid metabolism. Measurement of 3-HIB might therefore have clinical value with respect to monitoring patients at risk of developing fatty liver and related insulin resistance due to impaired hepatic fatty acid oxidation and altered BCAA catabolism. Although we here analyzed changes in 3-HIB in rats with pharmacologically activated fatty acid oxidation and ketogenesis, we and others have previously demonstrated a marked increase in circulating 3-HIB levels in the context of obesity and insulin resistance [16–21]. Our study therefore suggests that high circulating 3-HIB levels in these conditions reflect reduced hepatic fatty acid oxidation, and that therapies that lower 3-HIB levels might act at least in part by increasing fatty acid oxidation. These possibilities however need to be tested in future studies. Conversely, it is of interest to test if 3-HIB blocks fatty acid

oxidation and ketogenesis in the liver.

Our study points to a mechanism of increased circulating MMA concentrations in the context of increased hepatic fatty acid oxidation that is at least in part independent of vitamin B12 activity, as circulating vitamin B12 levels were decreased by 3-thia fatty acid treatment. MMA-CoA mutase, which is responsible for converting MMA-CoA to succinyl-CoA, is a vitamin B12-dependent protein, and high MMA levels are used as an indicator of vitamin B12 deficiency [51]. Long-term use of metformin, a first-line pharmacologic treatment of type 2 diabetes which is thought to improve mitochondrial function [52,53], has been found to promote vitamin B12 deficiency accompanied by increased plasma MMA levels [54,55]. Elevated MMA levels are also observed in patients with the genetic disorder methylmalonic aciduria, which can be caused by mutations in the gene that encodes the MMA-CoA mutase and result in disturbed amino acid metabolism [56]. Circulating MMA levels are therefore though to largely depend on the activity of the B12-dependent MMA-CoA mutase. Additionally, impairment of MMA-CoA mutase has been found to result in mitochondrial dysfunction and altered lipid metabolism in skeletal muscle [14]. However, recent studies have also demonstrated that circulating MMA levels can be affected via the valine degradation pathway by heritability of specific HIBCH variants, independently of the level of coenzyme B12 [57,58], and that this mechanism also involves impaired mitochondrial function [58]. In the present study, gene expression of *Hibch*, encoding 3-hydroxyisobutyate-CoA hydrolase which can support MMA formation by generating free 3-HIB, increased 3-fold in liver after 1-triple TTA administration. Our study further supports the importance of the B12-independent enzyme MMA-CoA hydrolase in this context. MMA-CoA hydrolase is responsible for splitting off CoA from MMA-CoA, and thereby controls the production of MMA [59]. Indeed, we observed a marked increase in the enzyme activity of MMA-CoA hydrolase by 3-thia administration in rats, while MMA-CoA mutase activity surprisingly decreased. Taken together, these data support that the altered circulating MMA levels could primarily be due to the increased hepatic *Hibch* expression as well as increased MMA-CoA hydrolase activity observed upon 3-thia fatty acid treatment in the present study, independent of vitamin B12.

Our study has some limitations. First, our observations were made in male rats, and we cannot necessarily extrapolate the findings to female rats, or to humans. Additionally, the experiments were performed in lean insulin sensitive and not also in obese/insulin resistant rats. Moreover, the data represent the net whole-body metabolic effect of the 3-thia fatty acids, and although these compounds primarily target fatty acid oxidation in the liver [26], we did not trace intracellular or whole-body fluxes of BCAA and TCA cycle intermediates. The relationship between BCAAs, the TCA cycle and fatty acid metabolism is complex. BCAAs and fatty acids function in a cooperative manner with effects on mitochondrial biogenesis, glycolysis, and insulin signal transduction, but the primary mechanisms and complex cross-tissue regulation underlying altered insulin sensitivity and mitochondrial dysfunction remain unclear [13,60].

In conclusion, the present study shows that enhanced mitochondrial β-oxidation, induced by 3-thia fatty acids that target the liver, is accompanied by lower plasma levels of 3-HIB and higher plasma levels of MMA and succinate. These changes correspond to altered hepatic expression and activity of 3-HIB-CoA and MMA-CoA-targeting thioesterases which remove the CoA moieties from intermediary metabolites linked to BCAA catabolism and the TCA cycle. Our data support 3-HIB and MMA as novel circulating markers of altered hepatic mitochondrial fatty acid oxidation, at least in male Wistar rats.

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CRediT authorship contribution statement

Mona Synnøve Bjune: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Carine Lindquist:** Investigation. **Marit Hallvardsdotter Stafsnes:** Resources. **Bodil Bjørndal:** Conceptualization, Investigation. **Per Bruheim:** Investigation. **Thomas A. Aloysius:** Investigation. **Ottar Nygård:** Resources. **Jon Skorve:** Resources. **Lise Madsen:** Conceptualization, Resources, Writing – original draft. **Simon N. Dankel:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Rolf Kristian Berge:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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