The valine-3-HIB pathway in obesity, insulin resistance and fatty liver

Mona Synnøve Bjune

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

The University of Bergen granted the PhD fellowship, and I was enrolled as a PhD candidate from May 2018 to March 2022 at the Department of Clinical Science, Faculty of Medicine, University of Bergen. I participated in the Postgraduate School of Clinical Medical Research at the Department of Clinical Science.

The work presented in this thesis was mainly performed at the Hormone Laboratory Research Group, Department of Medical Biochemistry and Pharmacology, Haukeland University Hospital (HUS) and Department of Clinical Science, University of Bergen, Bergen, Norway. My main supervisor was Associate Professor Simon E. Nitter Dankel at the Department of Clinical Science, and my co-supervisor was Professor dr. med. Gunnar Mellgren at the University of Bergen and HUS. The metabolite analyses were carried out by scientific staff at Bevital AS, and they were also collaborators of the first and third paper. Parts of the work were conducted in collaboration with Professor Rolf K. Berge and the Lipid Research Group, Department of Clinical Science, Faculty of Medicine, University of Bergen.

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Mona Synnøve Bjune

Mona Synnøve Bjune

Abbreviations

1-triple TTA	2-(tridec-12-yn-1-ylthio) acetic acid
3-HIB	3-hydroxyisobutyrate
AAs	Amino acids
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BCAAs	Branched-chain amino acids
BCAT	Branched-chain aminotransferase
BCKDH	Branched-chain alpha-keto acid dehydrogenase
BCMTD	1,10-biscarboxy-methylthiodecane
BMI	Body mass index
CoA	Coenzyme A
CRISPR	Clustered regularly interspaced short palindromic repeats
CVDs	Cardiovascular diseases
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
FAs	Fatty acids
FFAs	Free fatty acids
GC-MS/MS	Gas chromatography-tandem mass spectrometry
GLP-1	Glucagon-like peptide-1 receptor
GLUT4	Glucose transporter type 4
GO	Gene ontology
GSEA	Gene set enrichment analysis
GSH	Glutathione
GWAS	Genome wide association studies
HbA1c	Hemoglobin A1c
HDL	High-density lipoprotein
HIBCH	Hydroxyisobutyryl-CoA hydrolase
HOMA-IR	Homeostatic model assessment of insulin resistance

HUNT	Nord-Trøndelag health study
HUSK	Hordaland health studies
HUVECs	Human umbilical vein endothelial cells
IR	Insulin resistance
KD	Knockdown
KEGG	Kyoto encyclopedia of genes and genomes
LCFAs	Long chain fatty acids
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
MCTs	Monocarboxylate transporters
MMA	Methylmalonic acid
MMS	Methylmalonate semialdehyde
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem cells
MSUD	Maple syrup urine disease
mTORC1	Mammalian target of rapamycin complex 1
MVECs	Microvascular endothelial cells
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEFA	Non-esterified fatty acids
OCR	Oxygen consumption rate
PANTHER	Protein annotation through evolutionary relationship
PDC	Pyruvate dehydrogenase complex
PDH	Pyruvate dehydrogenase
PDK4	Pyruvate dehydrogenase kinase 4
PEPCK	Phosphoenolpyruvate carboxykinase
PPARγ	Peroxisome proliferator-activated receptor γ
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
SAT	Subcutaneous adipose tissue

SEM	Standard error of the mean
siRNA	Small interfering RNA
SNPs	Single-nucleotide polymorphisms
SVF	Stromal vascular fraction
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TAG	Triacylglycerol
TCA cycle	Tricarboxylic acid cycle
TTA	Tetradecylthioacetic acid
UCP1	Uncoupling protein 1
VAT	Visceral adipose tissue
VLDL	Very-low density lipoprotein
WAT	White adipose tissue
WHO	World health organization
WHR	Waist-hip ratio
WNOB	Western Norway obesity biobank

Abstract

The prevalence of obesity has increased greatly during the last decades, along with obesity-associated conditions and diseases including insulin resistance, type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD). Elevation in circulating branched-chain amino acid (BCAA) levels is associated with insulin resistance, NAFLD and risk of T2D. The valine-derived metabolite 3-hydroxyisobutyrate (3-HIB) is also elevated in insulin resistance and T2D. This points to a specific dysregulation in BCAA metabolism, but the underlying cellular mechanisms remain unclear, including which tissue(s) contribute. A greater understanding of the mechanisms may facilitate earlier detection and prevention of disease progression as well as provide a basis for the development new therapeutic treatment targets.

The aim of the present study was to gain new insight into the role of cellular BCAA metabolism in obesity and obesity-related diseases, combining data from *in vitro* cellular models (well-known experimental adipocyte and hepatocyte cell lines and primary and immortalized cell cultures from human and mouse), animal models (rats), and several human cohorts including people with insulin resistance, T2D, obesity and NAFLD.

In paper I, we identified the valine-derived metabolite 3-HIB as a marker of hyperglycemia and T2D, and as a regulator of white and brown adipocyte metabolism. Cellular consumption and catabolism of BCAAs as well as production of 3-HIB increased during adipogenesis in human and mouse adipocytes. Knockdown of the 3-HIB generating enzyme Hibch decreased 3-HIB release from adipocytes concomitant with reduced lipid storage, while cellular supplementation of 3-HIB showed opposite effects om mitochondrial respiration and ROS generation in white and brown adipocytes.

In paper II, rats were treated with synthetic fatty acids (FAs) that induce hepatic fatty acid oxidation and mitochondrial biogenesis, and we found marked changes in 3-HIB, methylmalonic acid (MMA) (a metabolite downstream of 3-HIB), TCA cycle

intermediates and other related intermediate metabolites. Gene expression and enzyme activity data for liver, adipose tissue and skeletal muscle support a particular role for altered Hibch activity in the liver, which controls the release of 3-HIB in the valine degradation pathway. Additionally, our data also link the MMA-CoA hydrolase activity to plasma levels of MMA, an established marker of vitamin B12 status, suggesting that circulating MMA does not only reflect activity of the MMA-CoA mutase.

In paper III, we found increased liver *HIBCH* expression in people with fatty liver and obesity, as well as positive correlations between both liver *HIBCH* mRNA expression and plasma 3-HIB concentrations with liver fat in human cohorts. To investigate mechanisms that may explain these correlations, we analyzed *HIBCH* mRNA and 3-HIB efflux in human Huh7 hepatocytes that were induced to increase their lipid storage by FA supplementation, and found that both *HIBCH* expression and 3-HIB efflux increased by FA treatment. By performing HIBCH overexpression and knockdown experiments, we established a causal positive relationship between *HIBCH* expression and 3-HIB efflux, suggesting that the liver may be a source of circulating 3-HIB. Additionally, the *HIBCH* perturbations as well as addition of 3-HIB to the Huh7 cells altered key processes in energy- and lipid metabolism, as did inhibition of PDK4. Taken together, the findings point to a regulatory relationship between HIBCH and PDK4 in the control of hepatocyte metabolism and metabolic flexibility, with possible implications for the development of fatty liver and associated diseases.

In conclusion, the 3-HIB generating enzyme HIBCH may serve as important regulator of cellular energy metabolism in adipose tissue and liver, and 3-HIB is a promising biomarker of metabolic changes in these tissues in different metabolic states, reflecting altered lipid storage in adipocytes and hepatocytes in association with reduced hepatic fatty acid oxidation.

List of Publications

Paper I

Nilsen* MS., *Jersin RÅ., Ulvik A., Madsen A., McCann A., Svensson P-A., Svensson MK., Nedrebø BG., Gudbrandsen OA., Tell GS., Kahn CR., Ueland PM., Mellgren G., Dankel SN. (2020): "3-Hydroxyisobutyrate, A Strong Marker of Insulin Resistance in Type 2 Diabetes and Obesity That Modulates White and Brown Adipocyte Metabolism", Diabetes, Vol. 69: 1903–1916*

** Name has changed to Bjune since the publication.*

Paper II

Bjune MS., *Lindquist C., Stafsnes MH., Bjørndal B., Bruheim P., Aloysius TA., Nygård O., Skorve J., Madsen L., Dankel SN., Berge RK. (2021) "Plasma 3-hydroxyisobutyrate (3-HIB) and methylmalonic acid (MMA) are markers of hepatic mitochondrial fatty acid oxidation in male Wistar rats", Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, Vol. 1866, Issue 4, 158887*

Paper III

Bjune MS., *Lawrence-Archer L., Laupsa-Borge J., Horn C., McCann A., Kern M., Blüher M., Mellgren G., Dankel SN. "The valine-3-hydroxyisobutyrate (3-HIB) pathway is associated with fatty liver disease and metabolic flexibility in human hepatocytes", Manuscript to be submitted.*

Related papers not included in the thesis

Jersin RÅ., Tallapragada D.S.P., Madsen A., Skartveit L., Fjære E., McCann A., Dyer L., Willems A., Bjune J-I., ***Bjune MS.****, Våge V., Nielsen HJ., Thorsen HL., Nedrebø BG., Busch C., Steen VM., Blüher M., Jacobson P., Svensson P-A., Fernø J., Rydén M., Arner P., Nygård O., Claussnitzer M., Ellingsen S., Madsen L., Sagen JV., Mellgren G., Dankel SN. (2021) "Role of the neutral amino acid transporter SLC7A10 in adipocyte lipid storage, obesity, and insulin resistance" Diabetes, Vol. 70: 680–695*

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1. Introduction

1.1 Obesity, type 2 diabetes and other obesity-related diseases

1.1.1 Obesity – definition and prevalence

Overweight and obesity are characterized by excessive accumulation of body fat that increases the risk of health complications. The body mass index (BMI, kg body weight / height in meters squared (kg/m^2)) estimates body fat based on the weight-to-height ratio and BMI categories (Table 1) are used to define whether a person is classified with overweight or obesity (WHO, 2021b).

Table 1: Classification of obesity according to BMI

Category	**BMI (kg/m^2)**
Underweight	< 18.5
Normal weight	18.5-24.9
Overweight	≥ 25.0
Obesity	≥ 30.0
Obesity class I	30.0-34.9
Obesity class II	35.0-39.9
Obesity class III	≥ 40.0

[a] According to WHO guidelines (WHO, 2021b).

The worldwide prevalence of obesity has nearly tripled since 1975 (Bentham *et al.*, 2017), and the economic burden of obesity has become a major societal issue with significant healthcare costs as well as lower quality of life (Tremmel *et al.*, 2017). The World Health Organization (WHO) stated that in 2016 more than 1.9 billion adults (39%) were classified as being overweight (BMI ≥ 25 kg/m^2) and 650 million (13%) as having obesity (BMI ≥ 30 kg/m^2) (WHO, 2021b). According to the Nord-Trøndelag Health Study (the HUNT4 study: 2017-19), around 35-46% of the Norwegian population were classified with overweight and approximately 23% with obesity (Krokstad *et al.*, 2013; Åsvold *et al.*, 2021).

BMI provides a useful measure to define overweight and obesity on a population level but is less accurate on the individual level since the measure does not account for differences in muscle and fat mass. Additionally, BMI does not consider where the excess fat is located in the body (i.e., body fat distribution). Body fat distribution has a great impact on the risk of developing obesity-associated comorbidities, as storing fat in abdominal organs including visceral adipose tissue, the liver, intestines, kidneys and pancreas is associated with an greater health risk compared to storing excess fat primarily underneath the skin (subcutaneous adipose tissue) (Shuster *et al.*, 2012).

Waist-hip ratio (WHR) is found to be a better method to identify abdominal obesity and to predict obesity-related disease risk, estimating the relative accumulation of abdominal fat (both subcutaneous and intra-abdominal) (Björntorp, 1987). According to WHO, abdominal obesity is defined by a WHR above 0.85 for women and 0.90 for men (World Health Organization, 2008). Moreover, different imaging methods such as ultrasound, computed tomography (CT), magnetic resonance imaging (MRI) and Dual-Energy X-ray Absorptiometry (DEXA), as well as bioelectrical impedance analysis (BIA), have also been popular methods to estimate body fat percentage in research and clinical practice (Duren *et al.*, 2008; Cornier *et al.*, 2011), although these methods require more extensive equipment and resources.

1.1.2 Proposed causes of overweight and obesity

The primary cause of obesity is an imbalance between energy consumed and expended, linked to a higher intake of energy-dense food (typically high in both fat and sugar) combined with a sedentary lifestyle (WHO, 2021b).

Obesogenic environment

Over the past 20 years, the environment we live in has rapidly changed and the term “obesogenic environment” has been used to describe the sum of the surroundings, opportunities and other possible influences on life that together promote obesity (Swinburn, Egger and Raza, 1999). Urban design that encourages cars over walking and/or a high concentration of fast-food outlets nearby people’s home, school and

workplace has been linked to obesity (Lake and Townshend, 2006). Additionally, energy-dense and "ultra-processed" foods including sugar-sweetened beverages have been reported to disturb regulation of appetite, resulting in a further increase in energy intake (Hall *et al.*, 2019). At the same time, the overall energy expenditure has decreased since the time spent on physical activity after school and work has been replaced with time spent on sedentary activities such as television watching (Church *et al.*, 2011). Policies multilevel strategies that favor a health-promoting environment should therefore be made to take control of these key contribution to the obesity epidemic (Sallis and Glanz, 2009).

Other factors that are associated with obesity include socio-economic status (McLaren, 2007), malnutrition in the early-life period (Mameli, Mazzantini and Zuccotti, 2016), an altered gut microbiota (Muscogiuri *et al.*, 2019), mental health (Avila *et al.*, 2015), sleep deprivation (Knutson *et al.*, 2007) and frequent use of medications that have weight gain as a side effect (Apovian *et al.*, 2015). Increasing attention has also been given to food additives, hormone mimics and environmental toxins which are found in many everyday products (Kumar *et al.*, 2020). These endocrine disrupting chemicals can interfere with hormone receptors in the body and may give metabolic disturbances and increase the risk of obesity and related comorbidities (Schug *et al.*, 2011). Higher and more stable home temperatures have also been suggested to affect weight gain by lowering the contribution of heat-producing brown fat and thermogenesis to daily energy expenditure (Cypess and Kahn, 2010; Turner, Kumar and Koch, 2016).

Heritability

Genetic factors also play a role in obesity as multiple family, twin and adoption studies have demonstrated that around 40-80% of the inter-individual variation in BMI could be explained by genetics (Elks *et al.*, 2012). The genetic contribution to obesity is classified in two main categories; monogenic and polygenic obesity (Loos and Yeo, 2021). Monogenic obesity is typically rare, early-onset and severe and is caused by a single gene mutation, causing for instance a defect in one of the genes in the leptin–melanocortin hypothalamic pathway responsible for appetite regulation, which result

in leptin-deficiency and severe obesity (Clément *et al.*, 1998; Stagi *et al.*, 2017). Polygenic obesity is, on the other hand, a result of an interaction between the cumulative effect of several genetic variants (each of which have a modest effect) and the obesogenic environment (Loos and Yeo, 2021). More than 870 single-nucleotide polymorphisms (SNPs) have been identified to be strongly associated with BMI (Rohde *et al.*, 2019).

1.1.3 Health consequences of obesity

Having overweight and obesity affects several organs in the body and increases the risk of developing several obesity-related diseases, including cardiovascular diseases (CVD), heart disease, insulin resistance, type 2 diabetes (T2D), fatty liver disease and certain cancers (Bray, 2004; WHO, 2021b), and is associated with decreased life expectancy (Peeters *et al.*, 2003). The risk associated with obesity involves increased waistline along with high blood pressure, high fasting blood glucose levels, high triglyceride levels and low HDL cholesterol levels (Grundy *et al.*, 2004).

Type 2 diabetes and insulin resistance

The risk of developing T2D is strongly associated with higher body weight and a sedentary lifestyle. Other risk factors for T2D include increasing age and multiple genetic factors (Bellou *et al.*, 2018; Ingelsson and McCarthy, 2018). Diabetes is a disease of increased blood glucose, and its main diagnostic criterion is HbA1c above 48 mmol/L. HbA1c (glycated hemoglobin) is a reflection of the level of blood glucose over the past 8-12 weeks.

Worldwide, the prevalence of diabetes was approximately 463 million in 2019 and it is estimated that 700 million people will be diagnosed with diabetes by 2045 (Saeedi *et al.*, 2019). T2D is the most prevalent form of diabetes, which accounts for 90% of diabetes patients, whereas the remaining 10% have type 1 diabetes (T1D) or other rare forms of diabetes (Reed, Bain and Kanamarlapudi, 2021). According to estimates, half of the people living with diabetes globally are unaware of their condition (Saeedi *et al.*, 2019). Early diagnosis can be accomplished by measuring blood sugar levels (WHO,

2021a). When untreated, chronic elevated blood sugar levels in diabetes can give a variety of irreversible health complications over time, promoting, e.g., kidney disease vision and nerve damage in addition to cardiovascular/heart disease and poor blood circulation (Kopelman, 2000; Kahn, Cooper and Del Prato, 2014). According to WHO, diabetes was the direct cause of 1.5 million deaths in 2019 (WHO, 2021a).

Diabetes is a chronic, metabolic disorder where circulating glucose levels increase as a consequence of reduced cellular uptake and utilization of glucose. After a meal with carbohydrate-rich foods the circulating glucose levels increase. The β-cells in the pancreas release insulin, a hormone that is essential for keeping blood sugar levels in the normal range by promoting the entry of glucose into skeletal muscle and adipose cells and preventing the hepatic release of glucose. The amount of glucose and insulin in our bloodstream will therefore largely depend on the time, composition and size of the last meal. If cells in the body do not take up glucose normally in response to insulin (insulin resistance), the β-cells will release more insulin to compensate. This will keep the glucose level within the normal range for some time, but eventually the β-cells will fail to maintain a sufficient increase in insulin production, resulting in hyperglycemia (prediabetes) and T2D. While T2D is largely a consequence of a sedentary lifestyle and poor diet, which over time promotes overweight/obesity and affects insulin sensitivity, T1D is thought to primarily be triggered by an autoimmune reaction where the insulin-producing β-cells in the pancreas are destroyed/impaired leading to lack of insulin relative to the need to maintain normal glucose levels (Figure 1) (Kasuga, 2006; WHO, 2021a).

Systemic vs tissue-specific insulin resistance and crosstalk between tissues

Tissue-specific gene mutations in the insulin signaling pathway in mice have revealed that reduced insulin sensitivity can occur in specific tissues and give different metabolic effects, depending on the tissue that is manipulated or which gene that is knocked out (Kido *et al.*, 2000; Kasuga, 2006; Rask-Madsen and Kahn, 2012). For instance, liver-specific inactivation of insulin receptors resulted in systemic insulin resistance and glucose intolerance, likely due to a failure of insulin to inhibit glucose

production in the liver (Michael *et al.*, 2000). In contrast, knockdown of the insulin receptor specifically in adipose tissue of mice gave insulin resistance only locally in the adipose tissue shown by decreased ability of insulin to inhibit lipolysis, reduced insulin-stimulated uptake of glucose, and reduced lipogenesis in adipocytes (Blüher *et al.*, 2002). In other words, adipose-tissue specific knockout did not affect whole-body glucose metabolism and these mice were protected against obesity and obesity-related glucose intolerance (Blüher *et al.*, 2002). Moreover, mice that lack the insulin receptor in muscle display insulin resistance in muscle, hyperlipidemia and increased fat mass, but also normal levels of insulin and glucose in the blood as well as no significant change in body weight compared to control mice (Brüning *et al.*, 1998). These mice maintained whole-body glucose likely because glucose was shunted to other tissues, such as fat and liver for metabolism, making precursors for TAG storage in adipose tissue. Note that the insulin-stimulated uptake of glucose was still partly functional in muscle, possible because the muscle tissue also contained other cell types, including adipocytes that still had a functional insulin receptor (Brüning *et al.*, 1998).

So how does systemic insulin resistance develop, and which tissues contribute primarily to the systemic insulin resistance associated with obesity? Some studies have shown that insulin resistance in muscle and fat is not sufficient to develop T2D in the absence of β-cell and liver defects (Lauro *et al.*, 1998), while others have reported that for instance muscle-specific deletion of the gene that encodes the insulin-stimulated glucose transporter GLUT4 leads to severe insulin resistance and glucose intolerance (Zisman *et al.*, 2000). Additionally, studies have demonstrated that adipocyte-specific knockdown of GLUT4 in mice also resulted in insulin resistance in muscle and liver (Abel *et al.*, 2001), revealing the existence of organ crosstalk mediated not only by traditional hormones and the central nervous system, but also through bioactive signaling molecules with paracrine and/or endocrine actions (such as adipokines, myokines, and hepatokines) secreted by metabolically active organs (Herman and Kahn, 2006; Jensen-Cody and Potthoff, 2021; Romero and Eckel, 2021). However, more research is needed to understand this complex organ crosstalk and the signaling molecules and associated changes in metabolic processes.

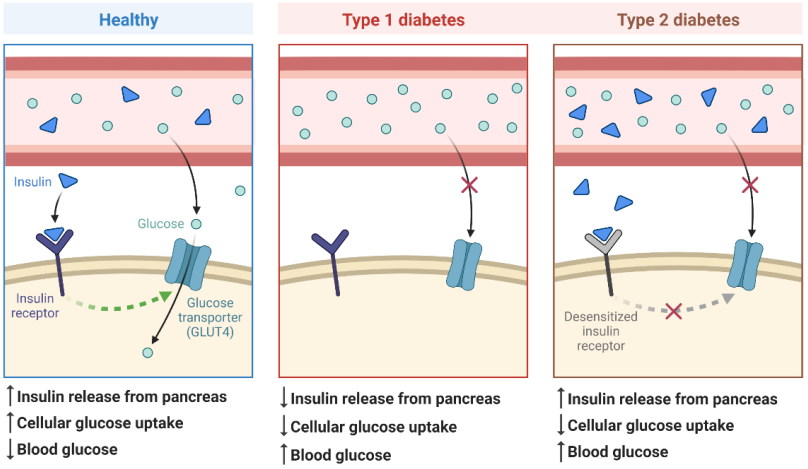

Figure 1. Regulation of glucose uptake after a meal and changes in diabetes. Created with BioRender.com

Fatty liver disease

The growing obesity rates worldwide contribute centrally to the dramatic increase in cases of non-alcoholic fatty liver disease (NAFLD), which is estimated to be about 24-30% of populations globally (Younossi *et al.*, 2016; Shabalala *et al.*, 2020). Approximately 90% of the patients with NAFLD have obesity and of these around 70% have insulin resistance or T2D (Shabalala *et al.*, 2020).

NAFLD is characterized by excessive accumulation of hepatic fat exceeding 5% of the liver's weight (Sherif, 2019). NAFLD can be divided into different stages based on the progression of the condition, ranging from simple steatosis, which is the earliest stage of NAFLD, to the more severe stages including non-alcoholic steatohepatitis (NASH) and liver cirrhosis, which both may develop further to hepatocellular carcinoma (HCC) (Figure 2) (Turchinovich *et al.*, 2018).

Both simple steatosis and NASH are reversible stages of NAFLD (Turchinovich *et al.*, 2018) and it is therefore important to diagnosis early. Unfortunately, NAFLD usually causes no symptoms and is often first discovered when testing for other health issues.

Additionally, there is stigma associated with liver disease since many people associate the disease with alcohol abuse due to stigma-associated terms in medical nomenclature (Karlsen *et al.*, 2022). According to Karlsen *et al*. liver disease is neglected both by the people affected, health professionals and society in general (Karlsen *et al.*, 2022). For instance, people with T2D are checked regularly for concomitant injuries to the heart, kidneys and eyes, but most guidelines fail to mention the risk of liver disease, which also affects this patient group. As a result, many people seek medical attention at a stage of the disease where it is no longer possible to reverse the damage. Since fatty liver disease is associated with metabolic dysfunction the term "MAFLD" (metabolic associated fatty liver disease) has recently been suggested as an appropriate term to describe these subtypes within this heterogeneous disease (Eslam *et al.*, 2020).

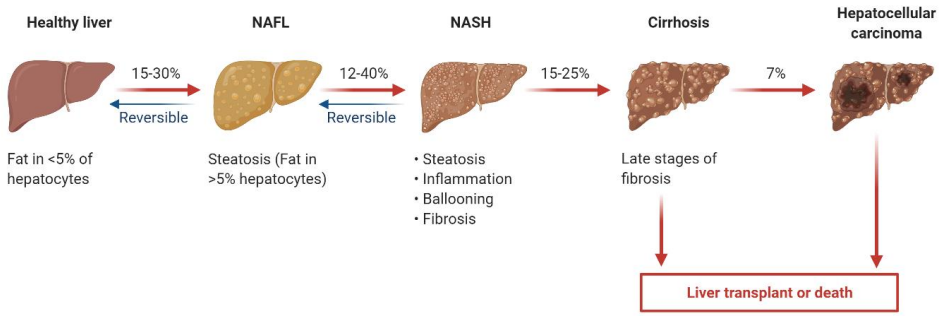

Figure 2. The progression and stages of NAFLD. Reprinted from "Non-Alcoholic Fatty Liver Disease (NAFLD) Spectrum", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates

1.1.4 Treatment of obesity and obesity-related diseases

Weight reductions of around 5-15% is recommended to improve health and reduce risk of obesity-associated comorbidities (Ryan and Yockey, 2017). Some of the most common approaches to treat obesity and obesity-related diseases such as T2D and NAFLD are discussed below.

Lifestyle intervention

Lifestyle modifications, focusing on change of diet (most common is calorie restrictive diets), exercise and cognitive behavioral therapy, are the main treatment for obesity, and many people have successful weight loss following an intensive lifestyle intervention, losing 5-10% of the original body weight that was maintained for 8 years (Wadden, 2014). According to a 20-year follow-up study, lifestyle interventions can also prevent or delay the incidence of T2D in the long term (Li *et al.*, 2008). Lifestyle changes to either obtain or maintain an healthy body weight is also the preferred method for prevention and treatment of NAFLD (Romero-Gómez, Zelber-Sagi and Trenell, 2017).

However, the weight loss is in many cases not sustainable, and many people struggle with weight regain later (Hall and Kahan, 2018). Typically, people who have lost weight following lifestyle modifications regain about 30-35% of their lost weight one year after the intervention and around 50% of them have returned to their original weight within five years (Blomain *et al.*, 2013). There are different theories why this happens. A lower body weight will require less energy than a higher body weight and it is expected that the energy intake and expenditure must be adapted to the new body weight (Hill, Wyatt and Peters, 2012). To maintain the new body weight will in other words require permanent behavior change which most people will struggle to do long-term. Several compensatory processes/mechanisms, that in theory are responses that shall protect humans against adverse effect of starvation, also make it harder to keep the weight off long term. For instance, there is evidence that the body make physiological adaptions after weight loss, which favors weight regain by affecting hunger and satiety for example by increasing circulating levels of appetite-stimulating hormones such as ghrelin and decreasing levels of leptin, as well as by giving greater reductions in resting energy expenditure than expected based on the change of body weight and composition (Greenway, 2015). If long-term sustained weight loss is hard to obtain, other approaches may be needed, or offered as complementary treatment to facilitate continued weight loss and/or maintenance.

Pharmacological treatment

If lifestyle intervention is insufficient on its own, pharmacological therapy is recommended for individuals having a BMI above 30 or above 27 with obesity-related co-morbidities (Apovian *et al.*, 2015). Most of the anti-obesity drugs work by affecting hunger and satiety and are approved for long-term use to treat obesity combined with a calorie-restrictive diet and increased physical activity (Tchang, Kumar and Aronne, 2021). The most used obesity drugs currently include Liraglutide, Orlistat and Bupropion-Naltrexone. Liraglutide (which is also used to treat T2D) and Semaglutide (a recently approved anti-obesity drug) are GLP-1 receptor agonists that work by decreasing appetite and increasing fullness/satiety (Pi-Sunyer *et al.*, 2015; Wilding *et al.*, 2021) Orlistat (pancreatic lipase inhibitor) promotes weight loss by decreasing dietary fat absorption from the gastrointestinal tract by around 30% (Tchang, Kumar and Aronne, 2021). A combination of Bupropion and Naltrexone decreases appetite by affecting the reward pathway in different parts of the brain (Apovian *et al.*, 2013). Of note, many of these drugs can give side effects. Choice of anti-obesity drugs should therefore be carefully considered based on the patient's disease history and associated comorbidities before use (Son and Kim, 2020).

Metformin is the most common drug prescribed for T2D and is thought to mainly work by reducing glucose production in the liver (suppressing gluconeogenesis through several molecular mechanisms) and by improving glucose uptake and insulin sensitivity (Rena, Hardie and Pearson, 2017). However, there is a demand for better drugs that improve insulin resistance, and there are currently no approved pharmacological agents for NAFLD or NASH. Some ongoing clinical trials of NAFLD and NASH evaluate antidiabetic and anti-obesity medication as major treatment options (Zhang and Yang, 2021). More knowledge of the underlying molecular mechanism of the pathogenesis of NAFLD and insulin resistance is needed to offer new and better treatment options.

Bariatric surgery

Bariatric surgery is considered the most effective treatment to reduce body weight of people with morbid obesity and is documented to give sustained weight reduction and an overall long-term reduction in mortality (Sjöström *et al.*, 2007) as well as improved quality of life (Nielsen *et al.*, 2022). Additionally, improvement in insulin resistance and/or remission of T2D is often seen within days to weeks after surgery, at least partly independent of weight loss (Kashyap *et al.*, 2010; Gudbrandsen *et al.*, 2019). Bariatric surgery also seems to improve NAFLD in patients with obesity or even reverse the pathological changes in the liver after surgery in these patients (Laursen *et al.*, 2019). Despite these positive effects, surgery is not an optimal way to overcome the obesity "epidemic" and its related diseases for many reasons. Bariatric surgery is an invasive procedure, involving either food-restrictive procedures such as adjustable gastric banding or by removing a large portion of the stomach to about 15% of its original size (sleeve gastrectomy) and combined restrictive and malabsorptive procedures that also modify the anatomy of the gastrointestinal tract (Roux-en-Y gastric bypass and biliopancreatic diversion), as well as other various forms of surgical techniques that changes the absorption of nutrients (Elder and Wolfe, 2007). Short- and long-term negative physical side effect and/or complications can occur, for instance abdominal pain, dumping syndrome, fatigue and nutrient/vitamin deficiencies (Ma and Madura, 2015). Additionally, a negative psychological impact is reported, including alcohol abuse, depression and anxiety (Kubik *et al.*, 2013; Plecka *et al.*, 2013). Therefore, other options such as lifestyle and pharmacological interventions should be considered before bariatric surgery is recommended. Of note, bariatric surgery is often (depending on the country) only available for people fulfilling the inclusion criteria of having a BMI above 40 or above 35 with obesity related co-morbidities, such as T2D, hypertension and NAFLD ('Gastrointestinal surgery for severe obesity: National Institutes of Health Consensus Development Conference Statement', 1992).

1.2 Adipose tissue

Adipose tissue is a highly dynamic organ and takes up between 4% to above 40% of the total body weight in adult humans (Ikeda, Maretich and Kajimura, 2018). In the following section the distribution, characteristics, composition, and general function of adipose tissue will be presented. Furthermore, specific functions of different adipocytes and the role of adipose tissue related to disease risk will be discussed.

1.2.1 Distribution, characteristics and composition of adipose tissue

Subcutaneous and visceral adipose tissue

For many years, the most common way to classify fat depots has been by their general location in the body, namely subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT). Additionally, these adipose tissue compartments can be subdivided further based more specifically on where they are localized and the organism in question. SAT is located below the skin of the upper and lower body depots and accounts for around 80-90% of the adipose tissue in lean humans. SAT is therefore the depot where most excess energy is stored as well as being the main supplier of circulating FFAs to the liver and other tissues. VAT surrounds internal organs in the abdominal cavity (Kwok, Lam and Xu, 2016; Reddy *et al.*, 2019).

SAT and VAT distribution in humans depends on sex, age and ethnicity. Women typically have about 10% higher body fat when matched to men with same BMI (Karastergiou *et al.*, 2012). Women typically have more SAT, especially stored in the lower part of the body (hips, thighs and buttocks), creating a “pear-shaped” distribution, while men typically have more fat distributed to VAT around the abdominal organs creating an “apple-shaped” body phenotype (Karastergiou *et al.*, 2012). VAT makes up approximately 10-20% of the total body mass in men and 5-10% in women (Lee, Wu and Fried, 2013) and as a consequence of these sex differences in adiposity men have in general higher risk of developing obesity-related complications (Chang, Varghese and Singer, 2018). SAT is typically higher in African American women, whereas VAT tend to be higher in Caucasians, and women of Asian

origin have higher VAT content than Caucasian women (Karastergiou *et al.*, 2012). Additionally, aging increases adiposity in both sexes and a redistribution of fat storage in the subcutaneous to the abdominal area (especially visceral abdominal fat storage), and post-menopausal women often have a body fat distribution more similar to that in men with more VAT (Kuk *et al.*, 2009).

White, brown and beige adipocytes

Adipose tissue is also classified as white (WAT), brown (BAT) and beige (or "brite", brown-in-white) (Kahn, Wang and Lee, 2019) (Figure 3). White and beige adipocytes appear to arise predominantly from a myogenic factor 5 negative (Myf5−) mesenchymal lineage, whereas brown adipocytes derive from Myf5+ precursor cells, which also give rise to skeletal muscle cells (Seale *et al.*, 2008; Sanchez-Gurmaches *et al.*, 2012)

White adipose tissue (WAT) is widely distributed in humans and makes up approximately 90% of the body fat mass. In humans, WAT is located in subcutaneous as well as in visceral regions of the upper body, and for SAT in lower body. White adipocytes are large cells defined by a large, unilocular lipid droplet occupying more than 95% of the cell volume. Because of the cell's high lipid storage capacity, the main function for WAT is fat storage and utilization (Rosen and Spiegelman, 2014; Kwok, Lam and Xu, 2016).

Brown adipose tissue (BAT) was for a long time thought to only exist in human infants, accounting for about 5% of their total body mass and critical to maintain their body temperature through non-shivering thermogenesis (Enerbäck, 2010; Urisarri *et al.*, 2021). However, since the identification of functional BAT in adult human in 2009 (Cypess *et al.*, 2009), numerous studies have been conducted to investigate whether activation and/or expansion of BAT could be a new strategy to treat obesity and metabolic diseases (McNeill, Suchacki and Stimson, 2021). BAT has a more limited distribution in the body than WAT, representing about 1-2% of the body fat mass in adult humans (Kahn, Wang and Lee, 2019). BAT is primarily located in the lower neck

area between the shoulder blades and along the spinal cord of adult humans, but can also be found in other part of the body, for instance interspersed within the adipose tissue that surrounds the kidneys (Cheong and Xu, 2021). Brown adipocytes are smaller in size relative to white adipocytes and are characterized by a multilocular lipid droplet morphology, high density of mitochondrion and expression of brown fat-specific genes, including *UCP1* and *PRDM16* (Ikeda, Maretich and Kajimura, 2018).

The prevalence and activity of BAT depends on sex, age, BMI and temperature, among other factors. For instance it has been found that the prevalence of BAT is higher in women than men and in lean individuals compared to people with obesity, as well as in younger compared to older people (Cypess *et al.*, 2009; Lee *et al.*, 2010). The BAT activity also shows seasonal variation being higher in the winter than the summer months (Wang *et al.*, 2015).

Beige adipocytes have no defined anatomical locations but are interspersed within WAT, primarily in subcutaneous depots (Wu *et al.*, 2012). Although the beige adipocytes share many of the functions and characteristics of brown adipocytes, they are thought to develop from different embryonic precursor populations (Seale *et al.*, 2008). Beige adipocytes arise when WAT is exposed to low temperature or other stimuli such as exercise or treatment with β3-adrenergic or PPARγ agonists (Cannon and Nedergaard, 2004; Stanford, Middelbeek and Goodyear, 2015; Merlin *et al.*, 2018).

Cellular heterogeneity of adipose tissue depots

Several distinct functional differences between adipose tissue depots have been reported (Lee, Wu and Fried, 2013; Cheong and Xu, 2021). While BAT is a relatively homogenous tissue, primarily composed of brown (mature) adipocytes, WAT has been found to be highly heterogeneous both in terms of cellular composition and function (Kwok, Lam and Xu, 2016). Studies show that mature adipocytes only represent about 20-40% of the cell population present in the adipose tissue (Rosen and Spiegelman, 2014). The remaining part of the adipose tissue consists of other cell types, including mesenchymal stem cells, preadipocytes, endothelial cells, various immune cells and

inflammatory macrophages (Kershaw and Flier, 2004). This cellular heterogeneity of adipose tissue depots gives rise to distinct, functional characteristics including the degree of beiging capacity, storage and utilization of lipids, glucose uptake and inflammation and release of adipokines, which in turn can affect disease risk (Schoettl, Fischer and Ussar, 2018; Cheong and Xu, 2021; Sakers *et al.*, 2022)

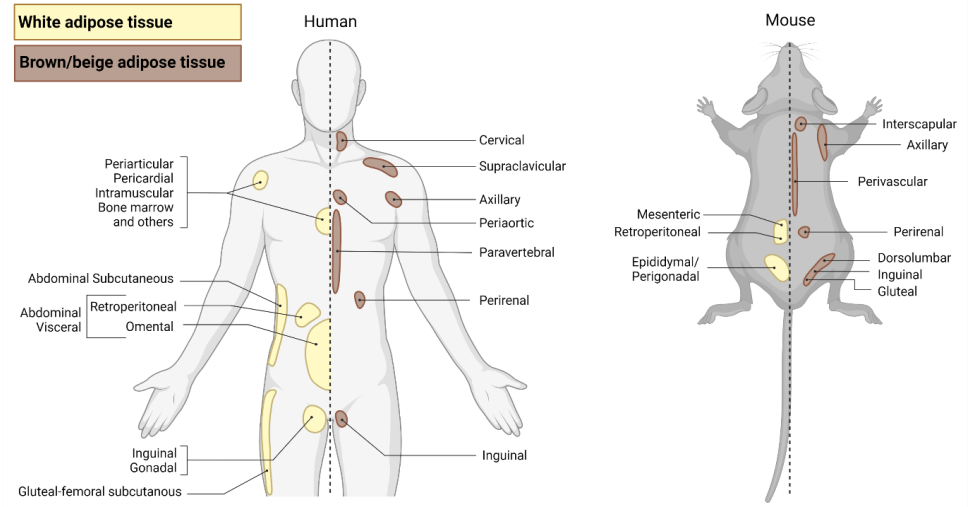

Figure 3. Distribution of adipose tissue depots in human and mouse. Adipose tissue depots including white subcutaneous, white visceral and brown adipose tissue in humans and mice are shown. Created with BioRender.com.

1.2.2 General function of adipose tissue

Adipose tissue expansion

A basic function of adipose tissue is to isolate against cold and protect against dermal infection and external mechanical damage on the body (Kwok, Lam and Xu, 2016). Moreover, key roles of adipose tissue are to store excess energy as lipids by lipogenesis and to mobilize this stored fuel through lipolysis in adipocytes to provide nutrients to other tissues when less energy is available. Excess energy increases the concentration of free fatty acids (FFAs) in the circulation. To maintain energy balance and whole-body homeostasis, the FFAs are converted into triacylglycerols (TAGs), and the TAGs

are stored in the adipocytes as lipid droplets. However, a prolonged positive energy balance makes the adipose tissue expand to increase lipid storage capacity, protecting ectopic tissues (including the visceral adipose depots) from excess lipid storage which can result in lipotoxicity. Adipose tissue expansion occurs by a combination of an increase in the size of the existing adipocytes (hypertrophy) and an increase in the number of adipocytes through *de novo* adipogenesis (hyperplasia) (Choe *et al.*, 2016; Longo *et al.*, 2019).

Adipogenesis, the process of creating mature adipocytes from mesenchymal stem cells (MSCs), involves a complex interaction of transcriptional regulators and other factors in several signaling pathways, in two distinct phases. In the first phase, known as the commitment phase, the fate of MSCs is restricted to the adipocyte lineage, which involves loss of ability to develop into other cell types, such as myocytes, osteoblasts and chondrocytes. In the second phase, called terminal differentiation, the newly formed preadipocytes with their fibroblast-like shape are changed to obtain the functional and morphological characteristics of mature adipocytes, including lipid droplets and a transcriptional program that allows the metabolic functions and hormonal responses unique to adipocytes (Rosen and MacDougald, 2006; Tang and Lane, 2012; Rosen and Spiegelman, 2014). For example, the committed adipocytes respond to the peptide hormone insulin by decreasing lipolysis and increasing adipogenesis, glucose uptake and lipogenesis (Cignarelli *et al.*, 2019).

Even though the stored TAG in the lipid droplets of the adipocytes are constantly hydrolyzed into glycerol and FFAs (lipolysis) and resynthesized to TAG (TAG synthesis), depending on the energy demand, the adipocyte number in our body is largely determined during childhood and adolescence, and normally remains more or less constant throughout adulthood, independent of weight gain or loss (Spalding *et al.*, 2008). In adult humans with a stable body weight, the adipocyte turnover rate is high and around 10% of adipocytes are renewed within a year, by adipogenesis and adipocyte death (Spalding *et al.*, 2008; Choe *et al.*, 2016). However, during development of obesity the number of adipocytes can also increase (Arner *et al.*, 2010).

Endocrine activity

Adipose tissue displays other important functions than storing excess energy as TAG in lipid droplets. As a highly metabolically active endocrine organ, adipose tissue plays a central role in maintaining whole-body energy metabolism and balance, participating in the regulation of glucose and lipid metabolism, thermogenesis, insulin sensitivity, inflammatory response and vascular endothelial function (Kershaw and Flier, 2004; Kwok, Lam and Xu, 2016; Sakers *et al.*, 2022).

Adipose tissue produces and secretes a variety of bioactive peptides, known as adipokines, which can act both as local (paracrine/autocrine) and systemic (endocrine) signals. For instance, adiponectin secreted from adipocytes regulates the metabolism of both glucose and lipids in the liver by increasing glycolysis and fatty acid oxidation (breakdown of glucose and FAs) and decreasing gluconeogenesis (reducing the production of glucose from non-carbohydrate precursors such as amino acids (AAs), lactate and glycerol) (Gamberi *et al.*, 2018; Zhang *et al.*, 2019). Another example is leptin, which when circulating at low levels (reflective of a low adipose tissue mass) acts in the hypothalamus to increase feeding and lower energy expenditure (Ghantous *et al.*, 2015). Furthermore, adipose tissue expresses various receptors, allowing interaction with other tissues/organs through the endocrine system and the central nervous system (Kershaw and Flier, 2004). The endocrine signaling also differs between adipose tissue depots. For instance, SAT has a greater secretion of leptin and adiponectin, whereas VAT secretes more of the pro-inflammatory cytokine interleukin 6 (Kershaw and Flier, 2004)

Adipose Tissue as a thermal regulator

As previously mentioned, BAT has a crucial function to regulate body temperature, converting the energy we obtain from food into heat by non-shivering thermogenesis (Cannon and Nedergaard, 2004). Brown adipocytes have a great density of mitochondria with high basal expression of uncoupling protein-1 (UCP1), while beige adipocytes must be stimulated to express UCP1 by cold or β-adrenergic signaling to

obtain thermogenic activity (typically with much lower total UCP1 activity and thermogenic capacity) (Nedergaard and Cannon, 2013). UCP1 increases the cell's oxygen consumption by facilitating leakage of protons from the inner to the outer mitochondrial membrane, leading to heat generation instead of ATP (adenosine triphosphate) production (Carpentier *et al.*, 2018; Ikeda, Maretich and Kajimura, 2018). Activation of human BAT and "beiging"/"browning" of WAT by using specific drugs that activate β-adrenergic signaling, thereby stimulating lipolysis and thermogenesis, has been considered as potential treatment of obesity and metabolic disorders (Cero *et al.*, 2021).

1.2.3 Adipose tissue dysfunction and disease risk

Evidence in humans supports that risk of developing the obesity-related disease is a consequence of impaired function of storing lipids in SAT, highlighting the importance of maintaining normal development and expansion of SAT (Arner *et al.*, 2010; Gustafson, Nerstedt and Smith, 2019). Excess energy is mainly stored in SAT. Once the adipocytes in SAT reach their limit of lipid storage capacity, *de novo* adipogenesis is initiated to increase further fat storage (Figure 4). The recruitment and differentiation of adipose precursor cells, rather than expanding the cells above their upper limit, is the preferred response and would be protective against the obesity-associated metabolic complications, preventing excess adipocyte hypertrophy. In metabolically unhealthy obesity, the capacity to store fat in SAT may however be limited, either due to the lack of ability to make enough new adipocytes (limited hyperplasia) or to expand the existing adipocytes further (limited hypertrophy). Further caloric overload over time will result in excess accumulation of fat in VAT and ectopic tissues (e.g., liver, skeletal muscle, pancreas, and heart), which is associated with increased health complications (Chait and den Hartigh, 2020). Loss of hyperplastic potential and/or hypertrophic expansion of the already large lipid-filled adipocytes, especially in VAT, is strongly associated with risk of developing of metabolic diseases (Kim *et al.*, 2014; Longo *et al.*, 2019). Overall, changes in both the number and size of the adipocytes affect the microenvironment of the expanded fat tissue as well as excessive ectopic lipid storage, which may produce various consequences such as local hypoxia, reduced

oxygen consumption, cell stress (reactive oxygen species), inflammation, autophagy and insulin resistance, and associated shifts in energy fuel and metabolite fluxes with the potential to affect the whole-body metabolism (Choe *et al.*, 2016; Longo *et al.*, 2019).

Another aspect to consider regarding VAT compared to SAT and disease risk is the portal theory (Bjorntorp, 1990), which suggests that the negative outcome of having too much visceral fat is because VAT is drained by the hepatic portal vein with direct flow into the liver, whereas SAT is drained systemically (by inferior or superior venae cava) and is therefore diluted in the circulation before affecting different tissues/organs. In other word, people with abnormal VAT amounts may expose their liver to higher levels of FFAs and pro-inflammatory cytokines, which may help explain their particular risk of developing hepatic insulin resistance and liver steatosis (Item and Konrad, 2012; Kwok, Lam and Xu, 2016).

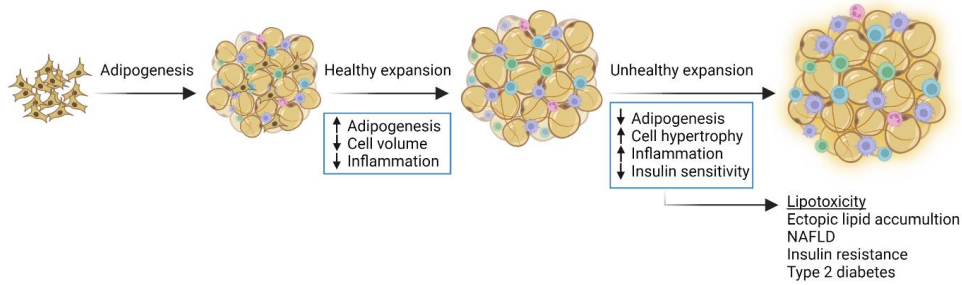

Figure 4. Adipose tissue expansion and associated changes in cellular and physiological processes. Created with BioRender.com

There is furthermore evidence for the importance of an altered lipid turnover in the development of obesity and related metabolic complications, with a decreased lipid removal rate seen in obesity and with aging (Arner *et al.*, 2011, 2019). Interestingly, recent discoveries have also implicated altered cell cycle regulation in mature adipocytes in obesity and hyperinsulinemia in the mechanisms of adipose tissue inflammation and disease risk (Li *et al.*, 2021).

1.3 The liver

The liver is the largest solid internal organ in humans, weighing approximately 1-1.5 kg (in adult humans) (Frayn, Keith N. Evans, 2019). In the following section the characteristics, composition, and general function of the liver will be presented. Additionally, the role of liver in obesity-associated conditions such as insulin resistance and NAFLD will be discussed.

1.3.1 Location, composition and functional characteristics of the liver

Unlike other organs in the body, the liver is supplied with blood through two main vessels: the hepatic artery and the portal vein system (Figure 5). Around 75-80% of hepatic blood supply is delivered to the liver through the portal vein, giving the liver a unique role in metabolism (Frayn, Keith N. Evans, 2019). Importantly, the pancreatic veins also join the portal vein, carrying blood that contains the hormones insulin and glucagon from pancreas. These hormones will therefore first exert their effect on the liver before leaving the liver through several hepatic veins which enter the main blood vessel in the body (the systemic circulation) (Carneiro *et al.*, 2019). Other important vessels connected with the liver include those that transport bile from the liver to the gallbladder (Frayn, Keith N. Evans, 2019).

The liver is mostly composed of hepatocytes, which account for about 80% of the liver volume, but other cell types are also present, including endothelial cells and Kupffer cells (macrophages) (Si-Tayeb, Lemaigre and Duncan, 2010). The arrangement of the hepatocytes in the liver gives the hepatocytes specific functions, depending on their location in this structure. The periportal hepatocytes (hepatocytes located outside of each lobule) are immediately exposed to incoming blood from the portal vein and artery, while perivenous hepatocytes (hepatocytes located closer to center of each lobule) are less exposed. It has been found that gluconeogenesis mainly takes place in periportal hepatocytes while glycolysis and ketogenesis occur more frequently in perivenous hepatocytes (Braeuning *et al.*, 2006). However, it appears that each individual hepatocyte can perform all different functions according to the current biological condition (Frayn, Keith N. Evans, 2019).

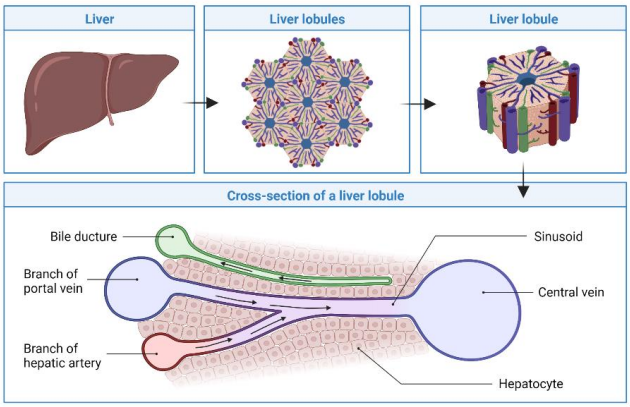

Figure 5. Anatomy of the liver. Hepatocytes are organized as smaller structures called lobules, which are hexagonally shaped, and at the corners of the lobule (hexagon) is a trad of three vessels: branches of the portal vein, the bile duct and the hepatic artery, which are located in between the hepatocytes. Blood flows from the triads towards the central vein, which is positioned in the middle of each lobule. During transportation of the blood, the blood from the hepatic artery and the portal vein is mixed together in a type of capillaries called sinusoids, before entering the central vein. The sinusoids are equivalent of the capillaries found in other tissues in the body and the transported blood in the sinusoids is in direct contact with the hepatocytes. Bile produced by hepatocytes is transported in the opposite direction to the blood flow in the bile duct (Frayn, Keith N. Evans, 2019). Created with BioRender.com

1.3.2 General function of the liver

The liver has an essential role in metabolism. Hepatocytes show a great flexibility in metabolic fuel selection, and respond to and mediate both nutritional and hormonal signals. An overview of some of the specific metabolic processes will be presented in this section.

GLUCOSE METABOLISM

Glycolysis and glycogenesis

After a meal, glucose is absorbed from the intestine into the portal vein, and the hepatocytes (especially the periportal cells) are exposed to high glucose concentrations (Figure 6). While insulin released from the pancreatic β-cells is required to induce the incorporation of glucose into extrahepatic tissues via the insulin-responsive glucose transporter GLUT4, the liver can take up glucose without insulin (Bryant, Govers and

James, 2002; Chadt and Al-Hasani, 2020). The glucose from the blood enters hepatocytes predominantly via GLUT2, which is independent of insulin and acts quickly when glucose concentrations are high (but only when concentration of incoming glucose in the portal vein surpasses the systemic glucose concentration)(Rui, 2014; Chadt and Al-Hasani, 2020). Inside the hepatocytes, glucose is phosphorylated to form glucose 6-phosphate, which can be further catabolized to pyruvate (aerobic conditions) or lactate (anaerobic conditions). This process is called glycolysis and is the main process in the fed state when glucose is abundant. The glycolytic products are used further to synthesize lipids, AA and other important molecules as well as being completely oxidized to generate ATP. Glucose 6-phosphate may also enter the pentose phosphate pathway (to produce NADPH) or glycogenesis (conversion of excess glucose into glycogen for storage in the liver for use later when energy is required). Of note, most of the energy the liver requires for various metabolic functions comes from oxidation of FAs and AAs rather than glucose. The main use of glucose in the liver is to be converted to acetyl-CoA via pyruvate for lipogenesis and ultimately to be stored as energy in the form of lipids, which are transported to the circulation for storage in adipose tissue (Rui, 2014; Frayn, Keith N. Evans, 2019).

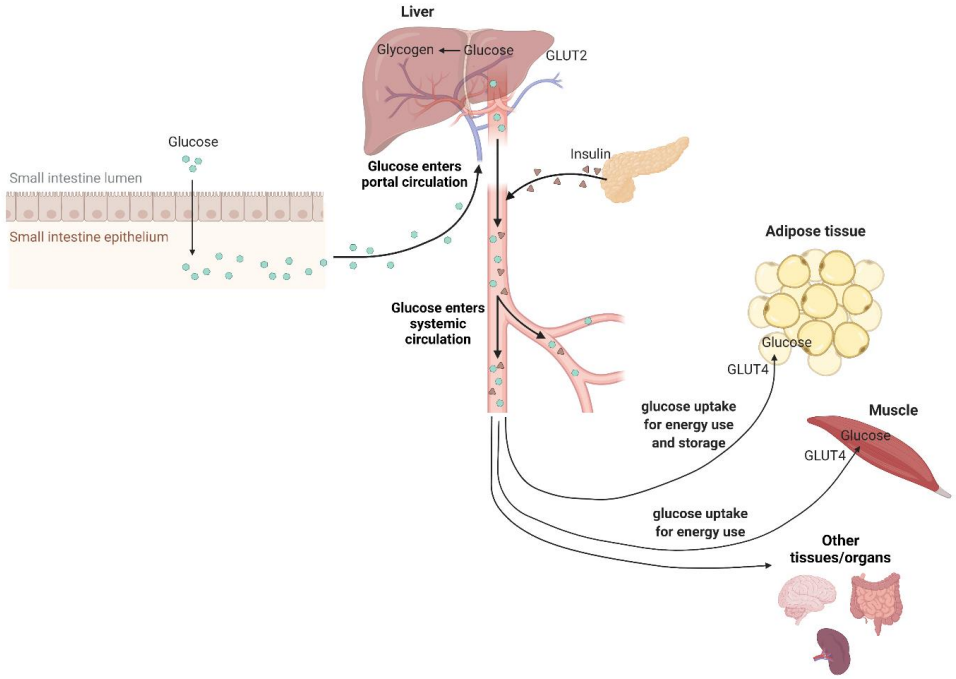

Figure 6. Routes of dietary glucose to the liver, muscle and adipose tissue. Created with BioRender.com.

Glycogenolysis

During short-term fasting periods and exercise, the glucose levels in the blood are reduced. To prevent too low blood glucose levels, glucagon is produced and secreted from pancreatic α-cells to promote glycogenolysis, the process of converting the stored glycogen in the liver to glucose. Thus, glucagon has the opposite effect of insulin, promoting increased glucose concentrations in the bloodstream. Glycogenolysis provides the main source of glucose during the first hours of fasting and mainly occurs in the liver. Once the liver has generated glucose, it is released into the systemic circulation and is accessible to both the liver and extrahepatic tissues (Quesada *et al.*, 2008; Chourpiliadis and Mohiuddin, 2021).

Gluconeogenesis

Glucose does not have to come from a carbohydrate-rich diet or from the glycogen stores in the liver, but can also be produced from non-carbohydrate sources such

gluconeogenic substrates including glycerol, pyruvate, lactate and glucogenic AAs (e.g., alanine, methionine, histidine, valine) as well as glucogenic and ketogenic AAs (e.g., phenylalanine, isoleucine, threonine, tryptophan) (Figure 7) (Chourpiliadis and Mohiuddin, 2021). This process is called gluconeogenesis (also known as *de novo* glucose synthesis).

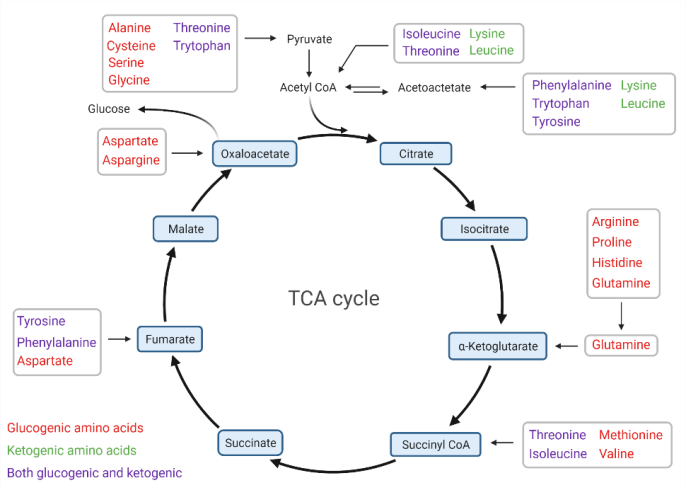

Figure 7. Amino acid catabolism. Glucogenic and ketogenic AAs are used as substates to generate glucose and ketone bodies in the liver. Created with BioRender.com

Glucagon secreted from pancreatic α-cells promotes not only glycogenolysis, but also stimulates the uptake of AAs for gluconeogenesis in the liver. Hepatic gluconeogenesis mainly occurs during prolonged fasting periods or exercise when the glycogen stores are more or less depleted and is suppressed by insulin (Rui, 2014; Chourpiliadis and Mohiuddin, 2021)

The rate of gluconeogenesis is controlled by both the availability of gluconeogenic substrates as well as the expression or activation of specific enzymes related to steps in gluconeogenesis. The gluconeogenic substrates can either be produced in the liver or be delivered to the liver from extrahepatic tissues (i.e., adipose tissue, muscle etc.) through the systemic circulation (Rui, 2014). For instance, glycerol is released from adipose tissue through lipolysis and skeletal muscle produces pyruvate through

glycogenolysis and glycolysis, making both glycerol and pyruvate available for the liver as gluconeogenic substrates. Additionally, prolonged fasting and exercise will also lead to protein degradation and production of lactate in skeletal muscle. AAs and lactate are released into the circulation and are also important gluconeogenic substrates for the liver as a source of energy (Rui, 2014; Zhang *et al.*, 2019).

FATTY ACID METABOLISM

Dietary lipid uptake and transport

After a meal, dietary fat is mainly digested and absorbed in enterocytes (epithelial cells) of the small intestine (Figure 8). Since the lipids are not water-soluble, they need to be packed into transport vehicles called lipoproteins, which are composed of four components: cholesterol, phospholipids, TAG and proteins. The enterocytes are responsible for packaging TAGs into chylomicrons (the largest of the lipoproteins). The chylomicrons (containing the TAGs) are transported through the lymphatic system into the bloodstream, where they release TAGs. The enzyme lipoprotein lipase (LPL), localized at the surface of the endothelial cells of the blood vessels outside the extrahepatic tissues, breaks down TAGs into FAs and glycerol. The FAs can be used as an energy source (by oxidation, which generates ATP) right away or the FAs and glycerol are formed back to TAGs to be stored mainly in the adipose tissue as lipid droplets for later use. After chylomicrons have delivered the FAs to target tissues, chylomicron remnants are taken up by the liver, where they are disassembled and incorporated into very-low density lipoprotein (VLDL). The VLDL particles can be used later by the liver for delivering self-produced TAGs to extrahepatic tissues via the bloodstream. FAs (e.g., in phospholipids) are also used as building blocks of the cell membranes (Rui, 2014; Frayn, Keith N. Evans, 2019).

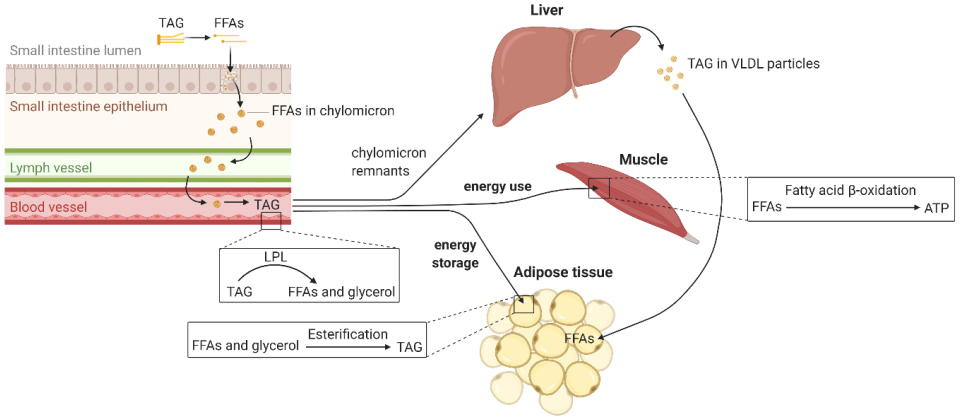

Figure 8. Routes of dietary fat to the liver, muscle and adipose tissue. Created with BioRender.com.

Lipogenesis

During excess glucose availability, the liver not only consumes glucose as the main metabolic fuel, but can also convert carbohydrates into lipids for later use. To do so, the liver initiates *de novo* lipogenesis, which includes (1) fatty acid synthesis and (2) TAG synthesis. The fatty acid synthesis takes place in the cytoplasm of the hepatocytes. Carbon precursors of acetyl-CoA (derived from excess carbohydrate and/or protein consumption) are converted to malonyl-CoA, and both malonyl-CoA and NADPH are used as precursors to form palmitic acid (the main product of lipogenesis) and other FAs. FAs can furthermore be elongated and desaturated by specific enzymes. The production of FAs is followed by TAG synthesis. This process occurs in the endoplasmic reticulum and involves the esterification of three FAs to a glycerol, which generates TAG. TAG synthesis also involves the use of FAs obtained from the diet. The produced TAGs are packed into VLDL particles, which are transported through the bloodstream to extrahepatic tissues (primarily adipose tissue). These TAGs can later provide energy through fatty acid β-oxidation (Ameer *et al.*, 2014; Duwaerts and Maher, 2019). Although lipogenesis primarily occurs in the liver, the process also takes place in adipose tissue as well as other tissues such as gut and kidney (Kume *et al.*, 2007; Song, Xiaoli and Yang, 2018; Hoffman, Alvares and Adeli, 2019).

Lipogenesis is regulated by the availability of lipogenic substrates and enzymes. Pyruvate, the main product of glycolysis, is used as a carbon source for lipogenesis and connects glycolysis to lipogenesis. Insulin promotes lipogenesis, and NADPH generated from malate and/or the pentose phosphate pathway, provides the reducing power for lipogenesis (Rui, 2014).

Lipolysis, fatty acid β-oxidation and ketogenesis

As previously mentioned, the liver will oxidize AAs and use them as gluconeogenic substrates such as during prolonged fasting. Additionally, by TAG hydrolysis, the liver will obtain energy from fat by breaking down each TAG molecule into one glycerol and three FAs. This process is called lipolysis and occurs in the cytoplasm of the cell. The liver will oxidize the resulting FAs into acetyl-CoA in the mitochondria by fatty acid β-oxidation. Further, acetyl-CoA is either completely oxidized via the TCA cycle and oxidative phosphorylation in the electron transport chain to yield ATP or converted into water-soluble ketone bodies (acetoacetate and β-hydroxybutyrate as well as acetone) through ketogenesis (Nguyen *et al.*, 2008). Fatty acid β-oxidation occur in many cells of the body, including adipocytes. For instance, BAT perform β-oxidation to fuel thermogenesis (Carpentier *et al.*, 2018). Liver, on the other hand, is the primary producer of ketone bodies (with some contribution also by the kidneys). Ketone bodies are exported from the liver to the systemic circulation to be used as an energy source for extrahepatic tissues. Notably, ketone bodies can also be generated using acetyl-CoA derived from other substrates than FAs, for instance AAs (Rui, 2014; Frayn, Keith N. Evans, 2019).

Metabolic processes shared between liver and adipose tissue

Most of the metabolic processes described above (except for gluconeogenesis and ketogenesis) occur in the adipose tissue as well. Glycogenesis and glycogenolysis also occur in adipose tissue as very small glycogen stores are found in the adipose tissue. However, the function of glycogen metabolism in adipose tissue remains unclear (Ceperuelo-Mallafré *et al.*, 2016). A partly parallel process to gluconeogenesis also occurs in adipose tissue, called glyceroneogenesis. These two processes share some of

the same key enzymes, including PEPCK (encoded by the gene *PCK1*) (Hanson and Reshef, 2003). However, in adipose tissue the purpose of this process is to make glycerol for FA storage in TAG, as opposed to glucose for release from the liver.

Other important functions of the liver

The liver has other essential roles in the body. Since most of the the blood that leaves the stomach and intestines passes through the liver via the portal vein, the liver has an important role processing all the products of digestion, which includes handling nutrients and eliminating toxins (for instance ethanol after alcohol intake). Additionally, the liver is also responsible for metabolizing drugs into forms that are easier to use for the rest of the body or that are nontoxic (Remmer, 1970). Moreover, amino acid (AA) catabolism in the liver is also a safe way to remove excess accumulation of toxic ammonia (nitrogen) by producing the less toxic substance urea via the urea cycle, involving deamination of glutamine (Watford, 2006).

The liver also has an essential role in digestion of food, producing bile (a greenish yellow fluid that consists of waste products, bile salts and cholesterol), which is needed to help digest and absorb fats and fat-soluble vitamins from the intestine. The produced bile is transported from the liver to the gallbladder for storage in between meals and emptied during digestion through the common bile duct into the small intestine (Chiang and Ferrell, 2018). Moreover, the liver produces critical blood proteins such as albumin, which helps keep fluid in the blood as well as facilitating the transport of hormones, FAs, and drugs throughout the body (Spinella, Sawhney and Jalan, 2016). Further, the liver is responsible for making various substances that help the blood clot after injury (Amitrano *et al.*, 2002).

1.3.3 Development of NAFLD and hepatic insulin resistance

The most common cause of NAFLD is increased calorie intake combined with reduced energy expenditure (i.e., a positive energy balance over time), although genetic predisposition to altered metabolism may also contribute (Birkenfeld and Shulman, 2014; Trépo and Valenti, 2020). The mechanisms of NAFLD pathogenesis are

incompletely understood, but FFAs play a central role. For several years a "two-hit" model have been used to describe the pathogenesis of NAFLD (Day and James, 1998). The "first hit" involves the intrahepatic accumulation of FAs, which is closely linked with insulin resistance. FFAs originating from lipolysis of TAGs in adipose tissue are delivered to the liver through the circulation. In addition to increased hepatic uptake of FAs from the circulation, *de novo* lipogenesis (producing TAGs from carbohydrates and protein) is stimulated in the liver, contributing to even more available FFAs. This accumulation of potentially hepatotoxic FFAs in the liver makes the liver more vulnerable to the "second hit", involving production of toxic lipid metabolites from FFAs that act as reactive oxygen species (ROS), promoting oxidative stress, mitochondrial dysfunction and production of inflammatory cytokines/adipokines, which eventually leads to fibrosis. Moreover, liver β-oxidation becomes overwhelmed as a result of increased FFA load in the context of NAFLD, which increases ROS production in the liver (Day and James, 1998; Dowman, Tomlinson and Newsome, 2009). There is, however, also evidence that hepatic TAG accumulation may be a protective mechanism by preventing the toxic effects of unesterified FFAs as they can directly trigger toxicity by activating inflammatory pathways and increasing oxidative stress (Yamaguchi *et al.*, 2007).

In recent years the multiple-hit model of NAFLD has been presented, which considers several factors acting together such as insulin resistance, adipokines, nutritional factors and gut microbiota as well as genetic and epigenetic factors (Buzzetti, Pinzani and Tsochatzis, 2016). Insulin resistance is one of the key factors that contributes to the development of NAFLD. In systemic insulin resistance, blood glucose and/or insulin concentrations are increased. As a result of insulin resistance, adipocytes release increasing amounts of FFAs into the circulation, which are taken up by the liver. Increased flux of FFAs into the liver promotes hepatic lipid storage. The liver further attempts to limit potential damage caused by excess FAs by directing the FAs into TAGs for transportation in VLDL particles from the liver to the extrahepatic tissues. At the same time, insulin fails to properly regulate hepatic metabolism, resulting in continued glucose production via gluconeogenesis even upon feeding and despite

increased lipid synthesis, a paradoxical condition due to hepatic insulin resistance (Santoleri and Titchenell, 2019). When persistent, hepatic insulin resistance also promotes dyslipidemia, i.e., elevated circulating levels of FAs and TAGs (Birkenfeld and Shulman, 2014). Studies have estimated that people with NAFLD have approximately 30% higher rates of hepatic gluconeogenesis and as much as 50% higher rates of adipose lipolysis (Sunny *et al.*, 2011). Moreover, excessive glucagon may be produced in people with impaired glucose tolerance and diabetes, leading to diminished ability to store glycogen in liver as well as elevated gluconeogenesis (Adeva-Andany *et al.*, 2019). Hepatic gluconeogenesis is therefore a target for treating T2D. For instance, the antidiabetic drug metformin at least partially works by reducing glucose production through inhibition of gluconeogenesis, as well as increasing uptake of glucose by cells (Almeda-Valdes, Cuevas-Ramos and Aguilar-Salinas, 2009; Lee and Dong, 2017). Several different molecular pathways have also been reported to contribute to the progression of NAFLD, which make the disease highly heterogeneous (Friedman *et al.*, 2018). Further research is needed to improve our understanding of the different mechanisms involved in the pathogenesis of NAFLD.

Regulation and dysregulation of PDC via PDK4

Pyruvate derived from catabolism of AAs and other precursors can be further converted to acetyl-CoA by the pyruvate dehydrogenase (PDH) complex (PDC) in hepatocytes. PDC activity determines whether the cells are to prioritize oxidation of FAs or glucose. Thus, appropriate regulation of the PDC activity via PDH kinases such as PDK4 is critical to maintain energy homeostasis during various nutritional conditions, including high-energy diets, energy deprivation, exercise, diseases and drugs (Figure 9).

In the fed state, insulin suppresses PDK4 expression and PDC is active (unphosphorylated), favoring glucose utilization over oxidation of FAs (Zhang *et al.*, 2014). Mechanistically, PDC catalyzes the conversion of pyruvate to acetyl-CoA and NADH via pyruvate decarboxylation. Acetyl-CoA can further be completely oxidized to generate ATP through the TCA cycle and oxidative phosphorylation (Jeong *et al.*, 2012). During fasting when glucose availability is limited and insulin levels are low,

PDK4 expression is no longer inhibited. PDK4 will phosphorylate PDC, switching PDC from an active to an inactive state, which favors oxidation of FAs (Zhang *et al.*, 2014). In that way, PDK4 reduces the utilization of pyruvate to generate ATP and pyruvate (derived from for example AA oxidation) is instead directed towards other pathways including gluconeogenesis (to generate glucose). By these mechanisms the liver will increase glucose production in fasting and transport it to the circulation, to supply extrahepatic tissues with energy. Simultaneously, lipolysis occurs in adipose tissue to help meet energy demand, contributing with FFAs and glycerol as energy sources. Additionally, breakdown of muscle glycogen contributes to blood glucose indirectly, through glycolysis and formation of lactate, which is released into the circulation and can used by the liver for gluconeogenesis (Rui, 2014).

PDK4 expression in the liver has been described as a sensitive marker of fatty acid β-oxidation (Pettersen *et al.*, 2019). Inactivation of the PDC by increased expression of PDK4 is observed under diabetic conditions, and loss of regulatory flexibility of PDC is seen in NAFLD (Zhang *et al.*, 2014; Jeon *et al.*, 2021).

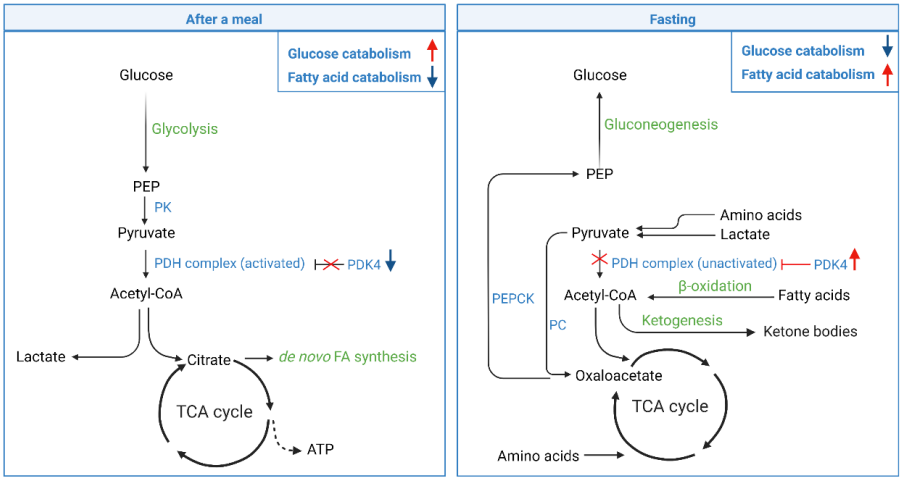

Figure 9. PDK4 regulates the utilization of glucose and fat in the liver. Created with BioRender.com. PC, Pyruvate carboxylase; PDH complex: Pyruvate dehydrogenase complex; PEP, Phosphoenolpyruvate; PEPCK, Phosphoenolpyruvate carboxykinase; PK, Pyruvate kinase; PDK4, pyruvate dehydrogenase kinase 4.

1.4 Amino acids

In the following section uptake and synthesis of amino acids will be briefly described before focusing mainly on the branched-chain amino acids (BCAAs). BCAA metabolism and functions will be described mainly focusing on adipose tissue and liver as well as the role of BCAAs in disease risk.

1.4.1 Dietary uptake and synthesis of protein

Dietary proteins are broken down by hydrolyzation into smaller polypeptides in the stomach, before being further broken down into AAs, dipeptides and tripeptides by the enterocytes of the small intestine. The AAs are transported through the enterocytes via a largely sodium-dependent co-transport system (Bröer, 2008). Once released into the intestinal capillaries, the AAs are transported to the liver via the portal vein. The liver has an important role in catabolism of AAs as the first internal organ that the dietary AAs enter before they are delivered to the systemic circulation. About 60-70% of the AAs present in the portal vein are taken up by the liver and are completely or partially broken down and/or used as building blocks to synthesize other proteins to be used both by the liver and in other parts of the body (Frayn, Keith N. Evans, 2019). As previously mentioned, hepatic protein synthesis is for instance important for producing the blood protein albumin (Spinella, Sawhney and Jalan, 2016) and the liver also plays a unique role in handling the potentially toxic nitrogen that is produced from oxidation of AAs by synthesizing urea. The remaining AAs are transported through the hepatic veins and delivered to the systemic circulation, available for extrahepatic tissues. For instance, insulin's anabolic effect following a protein-rich meal initiates proteins synthesis especially in skeletal muscle (Fujita *et al.*, 2006). Dietary AAs are used by the liver as substrates to produce accessible energy in form of glucose, FAs and ketones bodies for rest of the body, and also contribute to the hepatic energy requirements. Importantly, the BCAAs, in contrast to other AAs, may largely escape first-pass metabolism in the liver and instead transported more directly in their original form to extrahepatic tissues (Brosnan and Brosnan, 2006; Frayn, Keith N. Evans, 2019).

1.4.2 Branched-chain amino acids (BCAAs)

Molecular structures and functions of BCAAs

The BCAAs, which include valine, leucine and isoleucine, are among the nine essential AAs in humans, along with histidine, lysine, methionine, phenylalanine, threonine, and tryptophan. This means that these AAs cannot be made by *de novo* synthesis of AAs, but have to come from a dietary protein source. BCAAs account for about 20-25 % of total protein intake (Brosnan and Brosnan, 2006) and more than half of the splanchnic AA output (i.e., the release of AAs from the abdominal gastrointestinal organs) (Wahren, Felig and Hagenfeldt, 1976).

As all AAs, the BCAAs contain a central carbon atom, a carboxyl group (COOH), an amino group (NH_2), and a specific side chain group, which determines the chemical properties (size, pH, and polarity) for each AA (Figure 10). The BCAAs differ from other AAs by having a branched aliphatic side chain, making them non-polar (no positive or negative charge) and hydrophobic (lacking affinity for water). Since the BCAAs are neutral, the cellular uptake of these AAs occur through NA^+-independent transport, but depends on other AAs such as glutamine to serve as exchange factors (Zhang *et al.*, 2018; Hewton, Johal and Parker, 2021).

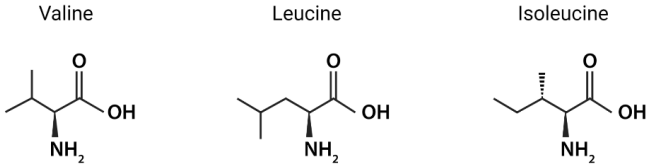

Figure 10. Molecular structure of the three BCAAs; valine, leucine and isoleucine. Created with BioRender.com.

The small size and hydrophobicity of BCAAs offer essential structural properties for using them as substrates in protein synthesis. Additionally, these properties also make the BCAAs important in regulating various metabolic processes such as cell growth (Shao *et al.*, 2018). For instance, proliferating adipocytes mainly use glucose and

glutamine as energy sources (i.e., >80% of the generated acetyl-CoA comes from catabolism of glucose and glutamine), whereas differentiating adipocytes increase their utilization of both essential and non-essential AAs where 1/3 of the generated acetyl-CoA originates from BCAA catabolism (Green *et al.*, 2016). Furthermore, other studies have shown that BCAAs are largely incorporated into proteins in the early stages of adipogenesis, while they are mostly incorporated into lipids later in adipogenesis (Crown, Marze and Antoniewicz, 2015; Estrada-Alcalde *et al.*, 2017). Thus, the BCAAs are important in adipogenesis in part by providing carbon for *de novo* lipogenesis in adipocytes.

It has also been demonstrated that circulating BCAAs participate in regulating the release of hormones and insulin secretion, and act as signaling molecules themselves (Nair and Short, 2005; Yoon, 2016). Furthermore, BCAAs serve as regulators of protein turnover (i.e., the continual renewal of protein). For instance, the BCAAs (especially leucine) can activate the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway, which stimulates protein synthesis and thereby promotes and maintains muscle mass (Drummond and Rasmussen, 2008). BCAAs also participate in regulating food intake, immune responses, gut function and fetal development (Zhang *et al.*, 2017). Moreover, BCAAs are involved in different processes in the liver such as hepatocyte proliferation (Tajiri and Shimizu, 2018). Not least, the interplay between BCAAs and FAs may be critical for the coordinated regulation of various processes in energy metabolism, including insulin- and inflammatory signaling and mitochondrial function (Newgard, 2012; Ye *et al.*, 2020).

It is now well documented that BCAA concentrations in the blood are typically elevated in obesity, insulin resistance, T2D and NAFLD (Huffman *et al.*, 2009; Newgard *et al.*, 2009; Kalhan *et al.*, 2011; Wang *et al.*, 2011; Shah *et al.*, 2012; Menni *et al.*, 2013; Wurtz *et al.*, 2013; Lynch and Adams, 2014; Iwasa *et al.*, 2015; Flores-Guerrero *et al.*, 2018). To dissect the role of altered BCAA metabolism in these conditions requires biochemical knowledge of BCAA catabolism in general as well as an understanding of the whole-body BCAA metabolism.

1.4.3 Whole-body BCAA metabolism

The steps of the BCAA catabolism

Biochemically, BCAA catabolism is relatively well established (Figure 11). The first step of BCAA catabolism includes two branched-chain aminotransferase (BCAT) isoenzymes: BCAT1 which is active in the cytosol and BCAT2 which is localized in mitochondria of the cells. The activity of the BCAT enzymes transform each of the BCAAs into their respective branched-chain α-ketoacids, i.e., α-ketoisovalerate (KIV) from valine, α-ketoisocaproate (KIC) from leucine and α-keto-β-mehylvalerate (KMV) from isoleucine by transamination (removal of their NH_2 group). Next, these branched-chain α-ketoacids are irreversibly decarboxylated (removal of their COOH group and releases of CO_2) by branched-chain α-ketoacid dehydrogenase (BCKD), the active component of the BCKDH complex. This complex acts similarly to the pyruvate dehydrogenase complex (PDC), which catalyzes the conversion of pyruvate to acetyl-CoA, as the activity is regulated by a phosphorylation-dephosphorylation mechanism mediated by a specific kinase. While the two first steps in the catabolism are common for all three BCAAs, the series of enzyme reactions that follows differs. The carbon products of the resulting enzyme reactions are mainly succinyl-CoA for valine (making valine more glucogenic), acetyl-CoA and acetoacetate for leucine (making leucine more ketogenic) and propionyl-CoA and acetyl-CoA for isoleucine (making isoleucine both glucogenic and ketogenic). Both succinyl-CoA and acetyl-CoA can enter the TCA cycle (Harper, Miller and Block, 1984; Holeček, 2018). Acetyl-CoA can also be used to synthesize FAs or cholesterol (Halama *et al.*, 2016). Of note, the need for producing glucose or ketone bodies does not seem to be the main driver to catabolize the specific BCAAs since the BCAAs share the first steps in the degradation pathway (Brosnan and Brosnan, 2006). Through unique catabolism in the consequent steps, however, each individual BCAA can contribute to specific metabolic functions (Zhang *et al.*, 2017).

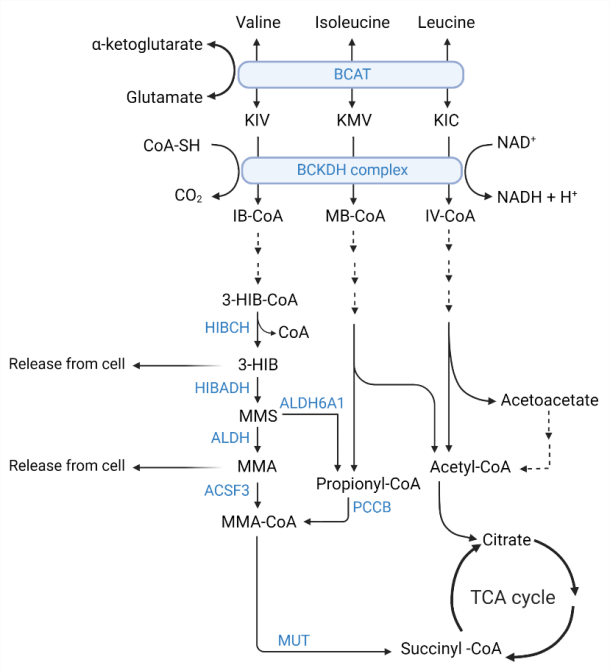

Figure 11. General overview of cellular BCAA catabolism. The figure is based on KEGG Pathway: Valine, leucine and isoleucine degradation - Reference pathway. Enzymes are marked in blue. Created with BioRender.com. ACSF3, Acyl-CoA Synthetase Family Member 3; ALDH, Aldehyde dehydrogenase; ALH6A1, Aldehyde Dehydrogenase 6 Family Member A1; BCAT, Branched-chain aminotransferase, BCKD, Branched-chain α-ketoacid dehydrogenase; HIBADH, 3-hydroxyisobutyrate dehydrogenase; HIBCH, 3-hydroxyisobutyryl-CoA hydrolase; MUT, Methylmalonyl-CoA mutase; PCCB, Propionyl-CoA carboxylase beta chain.

Tissue contributions to whole-body BCAA metabolism

The coordination of whole-body BCAA metabolism is less well understood. For a long time, it has been thought that the BCAAs deriving from dietary sources are hardly oxidized in the liver (Wahren, Felig and Hagenfeldt, 1976). Therefore, the BCAA concentration increases quickly in the systemic circulation after a meal and the BCAAs are dependent on being catabolized by extrahepatic tissues to lower the circulating BCAA concentrations (Matthews, 2005; Brosnan and Brosnan, 2006; Herman *et al.*, 2010). The reason for this thought mainly arose because the liver has low activity of the first enzymes in BCAA catabolism, the BCAT isoenzymes. Skeletal muscle have

in contrast been found to have high BCAT activity, and therefore the breakdown of the BCAAs has been thought to mainly initiate in muscle (Holeček, 2018).

Brosnan and Brosnan examined the role of different tissues in BCAA degradation and utilization in human by using the reported activity of BCAT and BCKD from a previously published study (Sitryawan *et al.*, 1998), thereby calculating the enzyme capacities of each tissue based on the mass of the different tissues of a 70 kg human (Brosnan and Brosnan, 2006). This showed that more than half of the total BCAT activity takes place in skeletal muscle, but a substantial part of the BCAT activity also occurred in adipose tissue. The liver also had BCAT activity, though to a lesser extent than these tissues. Interestingly, when calculating the activity of BCKD, the liver showed considerably higher BCKD activity than BCAT activity (Brosnan and Brosnan, 2006). Another study measuring the BCKD activity in rat tissues found that the liver showed the highest BCKD activity of all tissues measured (Harper, Miller and Block, 1984), but if the total weights of individual tissues are considered, both muscle, adipose tissue and liver all appeared to contribute substantially to BCAA catabolism. Of note, the liver can also receive α-ketoacids originating from the BCAA oxidation in extrahepatic tissues and catabolize them further by BCKD, but the extent of cycling of the BCAA and their derivatives among the different tissues are still uncertain (Shin *et al.*, 2014; Holeček, 2018).

A recent study investigated the metabolic fate of the BCAAs by *in vivo* isotopic tracing combined with metabolite analyses in mice (Neinast *et al.*, 2019). Firstly, this revealed rapid oxidation of the BCAAs in most tissues, with the greatest quantitative oxidation in skeletal muscle, brown adipose tissue (BAT), liver, kidneys and heart. Secondly, most of these tissues oxidized the BCAAs to obtain carbons for the TCA cycle, and the pancreas supplied as much as 20% of its TCA carbons from BCAA oxidation. Of note, the overall TCA turnover was lower in the pancreas than in highly metabolically active tissues. Finally, their quantitative analysis showed that skeletal muscle had the greatest contribution to whole-body BCAA oxidation, accounting for approximately 59%, followed by BAT (19%) and liver (8%). Furthermore, the liver, pancreas and skeletal

muscle were the tissues that showed the greatest utilization of BCAAs for protein synthesis, accounting for about 27%, 24% and 24% of isoleucine oxidation and disposal into protein, respectively (Neinast *et al.*, 2019).

Although skeletal muscle seems to be the tissue that contributes the most to whole-body BCAA catabolism (Neinast *et al.*, 2019), the data by Neinast *et al* also indicate that altered BCAA catabolism in different tissues contributes to the elevated blood BCAA concentrations seen in obesity and insulin resistance (Neinast *et al.*, 2019). Specifically, they found that insulin resistance led to a redistribution of BCAA oxidation, with reduced BCAA oxidation in the liver and adipose tissues and an increased oxidation of BCAA in skeletal muscle, accompanied by systemic elevations in the BCAA concentrations (Neinast *et al.*, 2019). This mouse study suggests that BCAA oxidation in adipose tissue and liver plays a unique role in whole-body energy homeostasis and the altered metabolic state in obesity and insulin resistance. Several studies have demonstrated that the elevation of the BCAAs in these conditions at least partly reflects impaired BCAA catabolism in different tissues, including skeletal muscle, adipose tissue and liver (Herman *et al.*, 2010; White *et al.*, 2016, 2018; Wang *et al.*, 2019; Zhou *et al.*, 2019), although the underlying mechanisms are unclear.

1.4.4 BCAAs and disease risk

A diet rich in BCAAs has frequently been reported to be beneficial for metabolic health, including body weight management, muscle protein synthesis, energy expenditure and energy homeostasis (Shimomura *et al.*, 2004; Qin *et al.*, 2011; Lynch and Adams, 2014). Dietary intake of BCAAs also seems to have positive effects in patients with liver diseases (Marchesini *et al.*, 2003; Holecek, 2010) as well as in various hypermetabolic states (e.g., trauma, burn injury and sepsis) (De Bandt and Cynober, 2006). Notably, these positive effects of BCAA supplementation are mainly seen in conditions with enhanced BCAA oxidation and/or increased demand of BCAAs (Holeček, 2018).

Despite the positive metabolic health effects of BCAA intake, elevated circulating concentrations of BCAAs are associated with obesity, insulin resistance, T2D (Newgard *et al.*, 2009; Mccormack *et al.*, 2013; Lynch and Adams, 2014; Halama *et al.*, 2016) as well as NAFLD (Kalhan *et al.*, 2011; Cheng *et al.*, 2015; Iwasa *et al.*, 2015). Additionally, elevation in circulating BCAAs may independently predict future insulin resistance and T2D, and may serve as biomarkers of these diseases states (Newgard *et al.*, 2009; Mccormack *et al.*, 2013). Furthermore, dietary interventions, bariatric surgery and treatment with antidiabetic drugs that result in weight loss and/or improved metabolic health in humans lower the circulating concentrations of BCAAs (Magkos *et al.*, 2013; Walford *et al.*, 2013; Iwasa *et al.*, 2015; Zheng *et al.*, 2016) and/or restores the BCAA catabolism in different tissues (Zhou *et al.*, 2019). Several studies have also shown positive effects of BCAA-reduced diets (Fontana *et al.*, 2016; White *et al.*, 2016; Cummings *et al.*, 2018) and negative effects of BCAA-rich diets (Newgard *et al.*, 2009; Solon-Biet *et al.*, 2019) in both humans and in animal studies. These seemingly contradictory observations regarding health effects of BCAA intake and disease associations with BCAA concentrations in blood raise important questions that need to be resolved.

1.4.5 The valine degradation product 3-HIB

While most previous studies have considered BCAAs as a group of AAs, to fully understand the role of these AAs in metabolic regulation we need to also consider the downstream pathways that are unique to the different BCAAs. The valine degradation pathway is unique by giving rise to the intermediate 3-hydroxyisobutyrate (3-HIB) (Figure 11 and 12). 3-HIB is produced in the mitochondria of cells and the release of 3-HIB from the mitochondria into the extracellular fluid is dependent on the hydrolyzation of 3-HIB-CoA by the enzyme 3-hydroxyisobutylryl-CoA hydrolase (HIBCH), which acts on the thioester binding and releases the CoA that normally prevents CoA-bound AA degradation products from leaving the cell (Letto, Brosnan and Brosnan, 1986).

3-HIB was first characterized in the urine of humans (Landaas, 1975). That study reported considerably higher concentrations of 3-HIB in urine samples of patients with ketoacidosis, along with higher serum concentrations of the BCAAs (Landaas, 1975). 3-HIB was then later linked to T1D in 1989 by measuring its concentration in human plasma samples (Avogaro and Bier, 1989). Here they reported higher 3-HIB concentrations in people with T1D and after a 72-hour fast (Avogaro and Bier, 1989). 3-HIB has later also been measured in the saliva of humans and salivary 3-HIB levels were found to correlate strongly to serum 3-HIB levels, suggesting that the salivary levels reflected the serum levels (Miyazaki *et al.*, 2015). In addition, patients with liver cirrhosis (part of the latest stages of NAFLD) were reported to have higher salivary 3-HIB concentrations compared to healthy controls.

More recent studies have reported elevation in circulating 3-HIB concentrations in insulin resistance and future risk of developing T2D and gestational diabetes (Harris *et al.*, 2017; Haufe *et al.*, 2017; Andersson-Hall *et al.*, 2018; Mardinoglu *et al.*, 2018). Therefore, 3-HIB might be a potential new biomarker for development of these disease conditions, reflecting altered downstream valine catabolism possibly in specific tissues.

Little is known about the function of 3-HIB and whether 3-HIB is directly involved in processes that contribute to the development of insulin resistance and T2D. Release of 3-HIB from muscle, heart and preadipocytes has been reported (Kedishvili *et al.*, 1994). Like other BCAA intermediates, 3-HIB can serve as a gluconeogenic substrate for hepatocytes when glucose is needed as well as being used as a substrate for *de novo* FA synthesis in differentiating adipocytes (Letto, Brosnan and Brosnan, 1986; Hu, Jaskiewicz and Harris, 1992; Kedishvili *et al.*, 1994). Interestingly, 3-HIB has also been found to stimulate uptake of FAs by acting as a paracrine factor on endothelial cells and to promote insulin resistance in skeletal muscles of mice (Jang *et al.*, 2016), while another study found no effect of 3-HIB on fatty acid uptake in human adipose tissue-derived endothelial cells (Mardinoglu *et al.*, 2018). Later, another study has also demonstrated that 3-HIB is involved in insulin signaling (indicated by decreased

activation of Akt) in myotubes, suggesting another mechanism of how increased 3-HIB may contribute to development of insulin resistance in muscle (Lyon *et al.*, 2019). However, the role of the valine degradation pathway in different adipocyte subtypes, and whether 3-HIB is secreted by differentiating and/or mature adipocytes, has remained unknown. Little is also known about the valine/3-HIB pathway in the liver.

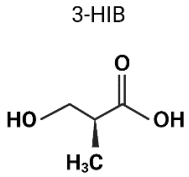

Figure 12. Structure for (S)-3-Hydroxyisobutyrate. Created with BioRender.com

2. Aims

The overall aim of this project was to determine roles of the valine–3-HIB pathway in energy metabolism in metabolically active tissues including adipose tissue and liver, towards improved prediction, prevention and treatment of fat storage and obesity-related diseases.

The specific aims were to:

1. Assess the relationship between the plasma concentrations of the valine-derived metabolite 3-HIB and incidence of hyperglycemia and type 2 diabetes, as well as degree of adiposity and fatty liver, in humans (Paper I, Paper III).
2. Determine whether changes in *HIBCH* expression and extracellular 3-HIB concentrations regulate energy metabolism and/or lipid accumulation in cultured white and brown adipocytes (Paper I) and hepatocytes (Paper III).
3. In an *in vivo* rat model, determine the dependency of circulating 3-HIB concentrations on fatty acid oxidation status and on the expression of *Hibch* mRNA in adipocytes, hepatocytes and/or myocytes (Paper II).
4. Identify specific pathways and metabolic substrates involved in HIBCH-dependent regulation of lipid metabolism in hepatocytes relevant for NAFLD (Paper III).

3. Methodological considerations

The work presented in this thesis was based on data from different sources, including human cohorts, animal studies and *in vitro* cultured human and mouse cell cultures. The combination of different models has strengthened the basis for drawing reliable conclusions in the studies. However, each of these sources of data has limitations that will be discussed here. A detailed description of the material and methods used in this thesis can be found in the methods section of the separate papers, while the most central parts of the methodology are presented and discussed below.

3.1 Clinical cohorts/patient samples (Paper I and III)

Three independent human cohort studies (HUSK, WNOB-1 and WNOB-2) were used to analyze circulating 3-HIB concentrations in Paper I. Additionally, *HIBCH* mRNA expression data were obtained from human adipose tissue samples from bariatric surgery patients and kidney donors. In Paper III, liver *HIBCH* and *PDK4* mRNA expression was measured in tissue samples from donors and correlated to the degrees of liver fat content, NAFLD and NASH (Liver cohort). Moreover, in additional cohorts of people with different degrees of adiposity and fatty liver, *HIBCH* mRNA expression was measured in visceral and subcutaneous adipose tissue (Leipzig obesity biobank cohort) and 3-HIB concentrations were measured in plasma (CARBFUNC cohort). The studies were approved by the respective Regional Ethical committees in Norway, Sweden and Germany, and all participants gave written informed consent.

A key strength of using human cohorts, to complement cell culture studies, is to obtain *in vivo* insight into disease-related changes (e.g., physiological, biochemical and molecular) associated with a specific factor of interest in living subjects, including comparison of different tissues and organs in the body. The information retrieved from analyzing for instance circulating concentrations of metabolites in the blood of patients will support the clinical relevance of observations made in cell cultures, such as measurements of metabolite concentrations in a human or mouse cell line. Furthermore, we also analyzed human tissue samples, including perirenal WAT and

BAT surgical biopsies collected from healthy human kidney donors (Svensson *et al.*, 2014) to compare the results with the immortalized WAT and BAT cells (see details below).

There are however limitations with human cohort studies. A cohort study follows people over a period of time and some people will typically withdraw before the study is finished. For instance, when we analyzed circulating 3-HIB concentration before and one year after bariatric surgery in WNOB-2 (Paper I) we only had samples from 15 out of 45 participants one year after bariatric surgery. This lessens the statistical power and may introduce some bias that influences the study outcome. Validating findings across different cohorts will therefore be important. We therefore analyzed several complementary cohorts (including WNOB-1 and WNOB-2) to confirm results and draw conclusions with greater confidence. It is also important to be aware of individual variation in a heterogenous group of people, which requires inclusion of a sufficient number of people to draw reliable conclusions. Other aspects to consider in cohort studies include the influence of confounding factors. For example, participants may have several other diseases than the one being studied as well as use different medications, which may influence the data. Depending on the study, other confounding factors can be lifestyle-related such as smoking, exercise, alcohol consumption etc. Sex differences are also important to consider when interpreting the results.

Another limitation of human cohort studies is that we cannot, for safety and ethical reasons, do all the same types of mechanistic/experimental studies on humans in the same way as we do in animal models and/or cell culture experiments. By combining data from human cohorts with cell culture experiments and/or animal models, the data can however together help to advance our knowledge of the human body and diseases.

3.2 Rat models (Paper II)

In paper II, we utilized data and materials from two previous rat experiments to compare metabolite concentrations and gene expression levels between treated and

control animals. Both studies were organized and conducted at The Laboratory Animal Facility by our collaborator, Rolf Kristian Berge, and contributions by members of his group at the Department of Clinical Sciences, University of Bergen, Norway. The animal studies were approved by The Norwegian State Board of Biological Experiments with Living Animals. I was not directly involved in organizing the animal experiments. These studies will be briefly presented below, along with a discussion of some ethical considerations regarding animal research in general.

Animal study with 1-triple TTA

Male Wistar rats (5 weeks of age) were housed in open cages in groups and kept in 12 h light/dark cycles at 22±2°C and relative humidity conditions of 55± 5%. The rats had *ad libitum* access to standard chow diet and water. 0.5 mL 0.5 % methylcellulose with or without 0.02 g 1-triple TTA was given once a day to the rats in their respective groups over 3 weeks. The weight of the rats was registered daily, and feed consumption was logged weekly. After euthanization, muscle, liver, epididymal white adipose tissue (eWAT) and EDTA-blood were collected.

Animal study with TTA and BCMTD

Male Wistar rats (weighing 150-200 g) were housed in pairs in wire cages. The rats were kept in 12 h light/dark cycles at 20±3°C and had unlimited access to standard chow diet and water. Palmitic acid (control), TTA and BCMTD, dissolved in 0.5% sodium carboxymethyl cellulose (CMC), were given daily for 1 week at doses of 150 and 300 mg/kg body weight to the rats in their respective groups. 0.5% CMC was used as a second control in some experiments. The rats were weighed daily and feed consumption was registered weekly. After euthanization, liver tissue samples were collected.

The Wistar rats we used in paper 2 is an outbred strain of *Rattus norvegicus* and is a frequently used animal model in biomedical research. Animal models are useful to study whole-body phenotypes and much of our knowledge (in medicine etc.) today depends on the use of animals in research. Mice and rats make up about 95% of all

laboratory animals and are popular to use in research due to their comparable physiology, anatomy and genetics with humans, high reproductive rate, short life cycle, small size (for housing and maintenance), and the possibility to create genetically engineered animal models corresponding to human diseases (Medicine and Bryda, 2013; Hickman *et al.*, 2017).

The ethical aspect of the use of animal models has become more important over the years, especially since the introduction of the three R's became a part of the guidelines involving the use of animals in research. The 3Rs (replacement, reduction, refinement) refer to three basic parts of the concept (NC3Rs, 2020). The first R is *replacement* and is about making an effort to prevent the use of animals if possible by using alternative ways to answer a research question, either by avoiding the use of animals completely or partially by changing to a type of animal that is considered less capable of suffering or by using primary cells, tissues or other relevant material/models. The second R is *reduction* and implies that we should try to decrease the number of animals used in an experiment and, additionally, to plan the experiment in a way that increases the information we get from each animal in the study. The third R is *refinement* and refers to all methods that can be performed to decrease the animals' pain and suffering (MacArthur Clark, 2018; NC3Rs, 2020).

As paper II in this thesis was based on collected material and data from two previously performed animal experiments, the principle of reduction is of high relevance. By using "leftovers" of the already collected tissue from the previous studies, we were able to do gene expression analyses of enzymes that were not studied before in these experiments, and the previously collected plasma samples were used to analyze the metabolite 3-HIB, which was not done when the animal studies originally were conducted. This illustrates how data for new metabolites and from technologies can become available in the future, making previously collected materials from animal experiments relevant for use in new analyses.

3.3 Cell cultures (Paper I and III)

The human adipose tissue samples used in this thesis were collected with approval from the respective Regional Ethical committees and all participants gave written consent. An important strength of this study is that we have used both the 3T3-L1 cell line and freshly isolated as well as immortalized preadipocytes from different adipose tissue depots from mice and humans. It is more challenging to obtain primary human hepatocytes, and we chose the well-characterized human hepatoma cell line Huh7 for the *in vitro* studies, as this cell line has a relevant metabolism for studying fatty liver disease.

Primary preadipocytes, derived from fat tissues of mice and humans, can be isolated and differentiated into mature adipocytes *in vitro*, with or without immortalization. In paper I, we mainly used immortalized preadipocytes from interscapular WAT and BAT collected from C57Bl/6 male mice, immortalized using SV40 virus. Additionally, we used immortalized human preadipocytes obtained from subcutaneous neck adipose tissue from a patient undergoing thyroidectomy (Cypess *et al.*, 2013). We also used the immortalized preadipocyte cell line 3T3-L1. Immortalized cell lines are genetically manipulated to proliferate indefinitely and are quick and easy to culture (White and Tchoukalova, 2014). Our knowledge of molecular pathways and transcriptional mechanism in adipogenesis largely comes from well-characterized cellular *in vitro* model systems such as the 3T3-L1 cell line. Immortalized cells can, on the other hand, differ more genetically from unmanipulated cells *in vivo* and hence be less similar to *in vivo* cells and freshly isolated primary cells. The reason for this includes that clonal selection/competition in culture may have selected certain subtypes of cells or changed the cells after culturing over longer periods of time. For example, the cells that were more prone to becoming virally immortalized and/or grow fastest will come to dominate the culture. Unfortunately, these effects are not easily controlled. After some trypsinizations of cells (passages), the cells may have changed the characteristics that they originally possessed (Armani *et al.*, 2010) and may consequently not be entirely representative of the original preadipocytes *in vivo*. In culture over time, cells can become continually proliferating cancer-like cells with reduced differentiation

capacity. Therefore, maximum 10-12 passages of a cell batch is typically a limit before rebooting with a new cryovial (Geraghty *et al.*, 2014). To ensure that the culture consisted entirely of immortalized cells before freezing, the primary mouse WAT and BAT cell cultures were cultured for at least 3 months. To obtain cell cultures with high differentiation capacity, preadipocytes were immortalized from six different adipose depots from three mice with the same genetic background (siblings), and cultures that were found to have good and comparable adipocyte differentiation capacity were selected.

Primary preadipocytes will retain most of their native cellular functions and may be closer to reflecting adipose function in the body compared to immortalized cells. To confirm our findings, we therefore included primary human preadipocytes cultures isolated from the stromal vascular fraction (SVF) that was obtained from abdominal subcutaneous liposuction aspirate, and the preadipocytes were differentiated as described previously (Veum *et al.*, 2012). However, as with tissue samples, primary cells have some important challenges and limitations. Although often both time-consuming and costly, collection of primary cultures from different people and repeating experiments in these can be important to obtain representative data, as donor variability in cellular characteristics occurs (Mohamed-Ahmed *et al.*, 2018). Other factors that can influence the results of primary cell culture experiments include the anatomical site of the fat depot, and the age of the donor which has a reducible effect on the replicative ability of preadipocytes (Djian, Roncari and Hollenberg, 1983). This is likewise a relevant, although less common, consideration for immortalized cell lines, where there is also a single original donor with unique characteristics (sex, age, BMI, etc.). Importantly, the immortalized WAT and BAT adipocytes were collected from mice of the same age and genetic background.

To increase our knowledge about the valine pathway we also investigated the same processes in the human liver cell line Huh7. Huh7 cells are immortalized cells that have a stable phenotype and unlimited growth potential. The cells exhibit several characteristics of normal hepatocytes and have thus been extensively used as an

alternative to primary hepatocytes (Soret *et al.*, 2021). The cells show increased lipid accumulation when exposed to FFAs and have been used successfully to reveal new mechanisms relevant to development of (Chavez-Tapia, Rosso and Tiribelli, 2012; Khamphaya *et al.*, 2018). Although primary human hepatocytes may have a phenotype closer to the human liver, these cells have weak cell viability and findings may be more difficult to reproduce (Soret *et al.*, 2021). It should however be noted that Huh7 is a well-differentiated hepatoma cell line originally derived from liver tumor from a 57-year old Japanese man (Nakabayashi *et al.*, 1982), and may not be representative of other genetic and epigenetic backgrounds, including females.

In vitro cellular models, either primary or immortalized cells, also have other limitations. For instance, adipocytes *in vitro* typically exist as a monolayer of a single, homogenous cell type. In contrast, *in vivo* adipocytes are embedded in a three-dimensional environment comprising several other cell types and factors such as signaling molecules within the specific extracellular matrix.

3.4 Targeted metabolite analyses (Paper I-III)

In all the three papers included in this thesis, we performed targeted metabolite analyses (GC-MS/MS) at Bevital AS to precisely quantify the concentrations of AAs and metabolites in various types of samples, including human plasma and serum samples, plasma samples of rats, and cell culture medium and total cell lysates collected from different types of adipocytes and hepatocytes.

By measuring the concentration of 3-HIB, BCAAs and other relevant metabolites in the cell culture medium collected during different 48 h periods throughout differentiation, and in cell culture medium from cells with and without different treatments, we obtained new insight into changes in AA consumption/flux and metabolism during treatment and/or differentiation of adipocytes and hepatocytes. Furthermore, combined with complementary analyses such as qPCR and functional assays, we collected information on the metabolic pathways involved upon gene knockdown or specific treatments. In our studies, we considered the cell culture

medium as an extracellular environment (i.e., outside the cells). Nevertheless, it is important to be aware that cell culture medium is not fully comparable to the extracellular environment within an organism, given the absence of, e.g., other cell types, tissues and signals/molecules/factors that are present *in vivo* (White and Tchoukalova, 2014). Additionally, the experiments were limited to specific windows of short- and longer-term treatment, showing time-sensitive responses. Therefore, findings of *in vitro* studies of rodent cells have limited transferability for making inferences on whole-body phenotypes (White and Tchoukalova, 2014).

Of note, we also obtained some intracellular metabolite measurements in total cell lysates in Paper I, which often corresponded to the change of the intracellular concentrations, but we were not able to do this in all the cell culture experiments because it required at least six 15-cm dishes per timepoint pooled together to obtain enough lysate for one replicate to be analyzed. This was in other words too time-consuming and costly to be performed for all experiments.

Another aspect that is important to be aware of is how the samples are collected before metabolite analysis and the importance of good sample storage and handling. For instance, to accurately analyze metabolite consumption and release over several days during differentiation/treatments, the conditioned medium must be collected at the same timepoint each day in order to calculate the flux of metabolites per time unit. We collected the medium within a tight time schedule (± 1 hour) to standardize this.

Repeated freeze-thaw cycles of samples are generally not recommended because this can affect the stability of the metabolites to sample degradation and precipitation. We therefore analyzed the cell culture medium samples we wanted to compare at the same time and ran the analyses shortly after collecting them to avoid such technical effects. However, it should also be noted that most of the measured metabolites are stable through repeated freeze-thaw cycles as tested by Bevital AS (bevital.no), and the plasma samples from rats and humans were nonetheless not previously used in other analyses before.

3.5 Gene expression analyses (Paper I-III)

Quantitative polymerase chain reaction (qPCR)

To investigate the gene expression of selected target genes, purified RNA from cultured cells or tissues was used as templates for the synthesis of complementary DNA (cDNA) via reverse transcription followed by real-time qPCR using SYBR Green and Roche LightCycler® 480. During a qPCR run, specific primers targeting a region of the gene of interest are amplified, the fluorescent dye SYBR Green binds to the double-stranded DNA, and the DNA amplification is detected by the fluorescence signal in each PCR cycle. The obtained cycle threshold (Ct-values) defines the number of PCR cycles that are required for the fluorescent signals to exceed the threshold of the background level. The Ct-value is inversely proportional (2^{-Ct}) to the amount of target cDNA (here corresponding to mRNA) present in the original sample, as the amplicon amount doubles per PCR cycle. Because fewer PCR cycles are needed for a strong fluorescent signal to be detected, a low Ct value represents high mRNA expression in the sample. Empirically, the optimal range for target genes is approximately 20-35 PCR cycles. The amplicon (mRNA) levels were estimated by the ΔΔCt method and finally, the gene expression was normalized to a suitable reference gene.

Generally, it is important to have in mind that even though a gene seems to be highly affected by treatment, showing increased or decreased mRNA expression compared to control, this does not necessarily mean that the mRNA is translated into protein. This warrants some caution when interpreting gene expression data in isolation. Post-translational modifications may also be required to make a functional or active protein. To confirm that a change in mRNA of a specific gene reflects something of biological significance, we often perform Western immunoblotting to measure protein expression. We combined these methods particularly to confirm that siRNA-mediated knockdown of a gene affected both mRNA and protein expression. For example, we found that Hibch knockdown in adipocytes reduced *Hibch* mRNA by 80-90% and HIBCH protein level by 30-40%, which resulted in functional effects corresponding to

the change of the protein level. This illustrates the importance of interpreting the results of gene expression analyses together with other relevant methods.

RNA sequencing and gene ontology analyses

In paper III, we used RNA sequencing (RNA seq) to measure the global gene expression in Huh7 liver cells 24h after treatments. An advantage of using RNA seq instead of qPCR is that we obtain expression values for all genes at the same time. All samples were treated with DNAse during RNA purification to avoid contamination with genomic DNA before sequencing. The sequencing was done using the Illumina platform with a depth of approximately 40 million reads per sample. Genes in the dataset that were found to have a significant differential expression between the control and treatments were categorized based on whether they were significantly up- or down-regulated and were then ranked based on fold change. These ranked gene lists were used further in gene ontology (GO) analyses using the freely available online bioinformatic tool PANTHER (Thomas *et al.*, 2003) and KEGG database (Kanehisa and Goto, 2000) as well as Gene Set Enrichment Analysis (GSEA)(Subramanian *et al.*, 2007). Analyzing transcriptome data by these analyses helps us to group genes in our lists based on which pathways they are known to participate in (based on an updated database) and provides an overview of which biological processes and cellular pathways are affected the most by the specific treatment. Another strategy we used was to correlate *HIBCH* mRNA expression to mRNA levels of all other genes across samples in a dataset and consequently perform GO analyses, which showed up- and down-regulated pathways associated with *HIBCH* expression. Together, these analyses helped us to characterize different gene expression patterns depending on the treatments. After these analyses, we could also identify specific genes in each pattern that were strongly affected. As mentioned earlier, analyses on protein expression and/or functional assays should also be performed to validate the biological relevance of the results of the gene expression analyses.

3.6 Cell culture-based functional assays (Paper I and III)

In Paper I and III, several functional assays were performed, measuring mitochondrial respiration, uptake of FAs, lipid accumulation, generation of reactive oxygen species (ROS) and/or radiolabeled glucose uptake.

Oil red o lipid staining

Staining of intracellular lipids in cells was performed to evaluate the cell's capacity to store lipids. The oil red o dye binds to neutral lipids in the cells (e.g., triacylglycerols and cholesterol) and the stained lipid droplets can be quantified by fluorescence measurements. This assay is useful in many ways. For instance, we 1) evaluated the differentiation potential of preadipocytes into mature adipocytes, 2) phenotypically characterized brown adipocyte multilocular versus white adipocyte unilocular lipid droplets *in vitro* by studying the lipid-stained cells in the microscope, 3) confirmed that our fatty liver cell culture model was successful, and 4) assessed whether different treatments affected lipid metabolism by quantification of the accumulated intracellular oil red o content. The disadvantage of this method is that it is not precise enough to capture small differences/changes in lipid accumulation, in part due to some unspecific binding to cell plate walls. Additionally, the method only gives information on general lipid content, but not lipid composition or the diversity of lipids. Another issue is that cells must be fixed with formaldehyde before staining, which makes it impossible to look at dynamic biological processes later since the cells die. After staining and quantification, it is furthermore difficult to normalize the results to cell numbers or protein content, but replicating the experiments several times to confirm the result of this method should provide reliable data of the response to treatments.

Fatty acid uptake assay

As a complementary method to oil red o lipid staining, we also measured the uptake of FAs in live cells spectrophotometrically at 37°C. This assay measures the uptake of a fluorescent fatty acid substrate from the cell culture medium. Unfortunately, we often observed considerable variation in measurement values between replicates, which made it necessary to include a relatively high number of replicates.

Mitochondrial respiration

To evaluate whether treatments affected mitochondrial function, the Seahorse XF Cell Mito Stress Test Assay was performed. To measure aspects of mitochondrial function, this assay combines measurements of oxygen consumption and pH change in live cells, with use of mitochondrial modulator compounds (oligomycin, CCCP, rotenone and antimycin A) that are sequentially added to each well. Measurements of oxygen consumption rate (OCR) in response to the different additions is used to calculate basal respiration (baseline energy demand), ATP production, maximal respiration (the maximal respiratory rate the cells can achieve by rapid substrate oxidation during metabolic challenge), spare respiratory capacity (the cells' ability to respond to increased energy demand) and non-mitochondrial respiration (persisting oxygen consumption after last injection due to some remaining enzymes that promote oxygen consumption in the cells). An advantage of using this assay is that results can be normalized to cell number per well by fixation of cells by Hoechst staining after the measurements. Hoechst is a blue fluorescent dye that stains DNA (i.e., nuclei of the fixed cells), and cell count data is obtained by automated imaging using a High-Content Imager BD Pathway 855 microscope combined with AttoVision 103 CellProfiler software.

Generation of reactive oxygen species (ROS)

To assess oxidative stress in the live cells, the production of reactive oxygen species (ROS) was quantified spectrophotometrically at 37°C by using the fluorescent probe CM-H2DCFDA. Fluorescence of the oxidized probe was measured once every 10 minutes until a steady-state ROS signal was obtained. Data were normalized to cell number per well by Hoechst staining.

Radioactive glucose uptake

In Paper I, basal and insulin-stimulated glucose uptake was performed in differentiated adipocytes to investigate whether 3-HIB supplementation affected the insulin-dependent uptake of glucose. The cells were incubated with Deoxy-D-[14C]-Glucose for 30 minutes before lysing the cells. Lysates were transferred to Ultima Gold fluid cartridges and radiolabeled glucose levels were measured as counts per minute (CPM) using a Tri-Carb 4910 TR scintillation counter. Counts were normalized to protein content using DC Protein Assay Kit.

4. Summary of results

4.1 Paper I: "3-Hydroxyisobutyrate, A Strong Marker of Insulin Resistance in Type 2 Diabetes and Obesity That Modulates White and Brown Adipocyte Metabolism"

In this study, we sought to investigate the valine-derived metabolite 3-HIB as a marker of hyperglycemia and type 2 diabetes (T2D), and to investigate whether 3-HIB is directly involved in regulating the metabolism of white and brown adipocytes, as a novel mechanism implicated in disease development.

In several human cohorts, including the Hordaland Health Studies (HUSK) and Western Norway Obesity Biobank (WNOB), we found that circulating 3-HIB was increased in people with hyperglycemia and established T2D. Additionally, we observed a positive association between 3-HIB and BMI. Furthermore, we observed a marked decrease in 3-HIB concentrations 1 year after bariatric surgery and weight loss. Interestingly, we saw a temporary increase in 3-HIB concentrations 1 week after bariatric surgery, which may indicate a highly dynamic role for 3-HIB in the regulation of energy metabolism.

To evaluate whether 3-HIB has a role in regulating key adipocyte functions, we analyzed AA and metabolite fluxes and the gene expression of enzymes involved in BCAA catabolism during adipogenesis. Both white and brown adipocytes increased BCAA utilization and released an increasing amount of 3-HIB throughout mid/late differentiation, accompanied by an increase in BCAA enzyme expression. Additionally, we observed increased expression of BCAA catabolic enzymes in brown compared to white adipocytes.

Knockdown of the enzyme 3-hydroxyisobutyryl-CoA hydrolase (HIBCH), which is responsible for the formation of 3-HIB from 3-HIB-CoA, resulted in decreased 3-HIB release with a concomitant reduction in lipid accumulation, while supplementation of 3-HIB to the adipocyte cultures increased fatty acid uptake. We also observed that 3-

HIB modified insulin-stimulated uptake of glucose in a time-dependent manner. Lastly, we found that 3-HIB supplementation resulted in downregulation of mitochondrial oxygen consumption and ROS generation in white adipocytes, while these processes were increased by the supplementation in brown adipocytes.

In conclusion, we identified 3-HIB as a novel important metabolic mediator produced by adipocytes, with regulatory functions in an adipocyte subtype-dependent manner, and linked to the development of insulin resistance, T2D and potentially other obesity-related diseases.

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

There is evidence that BCAAs and fatty acids (FAs) are interlinked in the regulation of energy metabolism, mitochondrial biogenesis and insulin sensitivity, with implications for the development of metabolic diseases. Having identified 3-HIB as a regulatory signaling molecule in adipocytes linked to insulin resistance and type 2 diabetes, we further aimed to determine if alterations in mitochondrial fatty acid β-oxidation and lipid metabolism in the liver could be reflected by specific markers such as 3-HIB and its downstream metabolite methylmalonic acid (MMA).

Rats were treated with synthetic mitochondria-targeted fatty acid analogues (TTA, 1-triple TTA, BCMTD), which robustly increase mitochondrial activity and oxidation of FAs in the liver. Gene expression analyses revealed an upregulation of key genes that influence the flux of FAs into the liver by 1-triple TTA treatment, with a concomitant decrease in TAG, total FAs and NEFA in plasma and improved hepatic insulin sensitivity. Interestingly, we found that the treated rats had reduced circulating 3-HIB and increased MMA concentrations in plasma, while the BCAA concentrations were unaffected.

Moreover, we looked at the expression of genes encoding BCAA catabolic enzymes in the liver, epididymal WAT and muscle. This showed that the hepatic mRNA of *Hibch,* the gene responsible for converting 3-HIB-CoA to 3-HIB, was increased 3-fold by fatty acid analogue treatment. The effect of 1-triple TTA on tissue 3-HIB formation appeared to be specific for the liver since no changes of gene expression of *Hibch* were seen in samples from muscle and adipose tissue. We also observed that the mitochondrial fatty acid oxidation increased by 1-triple TTA administration, and the plasma concentrations of 3-HIB were inversely correlated with mitochondrial fatty acid oxidation. Additionally, we found that 3-HIB correlated positively with circulating triacylglycerols, FFAs and insulin/glucose-ratio.

To gain further insight into the degradation of 3-HIB, we analyzed various short chain acyl-CoA derivatives in liver fractions and found that the concentrations of several of these intermediates (free CoASH, propionyl-CoA and methylmalonyl-CoA) were increased in the liver of rats treated with TTA or BCMTD. This was accompanied by an increase in the expression and/or activity of enzymes that couple BCAA catabolism with the TCA cycle and an increased plasma concentration of succinate.

In conclusion, activation of fatty acid oxidation with these liver-targeting fatty acid analogues decreased circulating concentrations of the valine-derived catabolite 3-HIB, while increasing the concentrations of MMA. The data indicate an important contribution of the liver to changes the circulating concentrations of these metabolites, which may serve as novel biomarkers of altered hepatic fatty acid oxidation.

4.3 Paper III: "The valine-3-hydroxyisobutyrate (3-HIB) pathway is associated with fatty liver disease and metabolic flexibility in human hepatocytes"

Elevated circulating concentrations of BCAAs, including valine, are associated with non-alcoholic fatty liver diseases (NAFLD) and non-alcoholic steatohepatitis (NASH), which is strongly linked to other adiposity-related conditions including insulin resistance and type 2 diabetes (T2D). Additionally, we have found that elevated circulating concentrations of the valine-derived catabolite 3-HIB, dependent on HIBCH, are strongly associated with development of insulin resistance and T2D, and reflect adipose lipid storage. Having observed that pharmacologic activation of hepatic mitochondrial fatty acid β-oxidation lowered plasma 3-HIB concentrations, while affecting *Hibch* mRNA specifically in liver and not adipose tissue or skeletal muscle, we hypothesized that hepatic expression of HIBCH plays a role in hepatic lipid accumulation and NAFLD.

Human cohort data were used to correlate liver expression of *HIBCH* mRNA and plasma 3-HIB concentrations with measures related to NAFLD, obesity and insulin resistance. In people with a range of adiposity, liver *HIBCH* mRNA was positively correlated to liver fat, NASH score, visceral and subcutaneous fat area and BMI. Accordingly, liver *HIBCH* mRNA expression was elevated in people with NASH and obesity. Additionally, in another cohort of people with obesity plasma 3-HIB concentrations were significantly correlated to the degree of liver fat.

Human Huh7 hepatocytes were supplemented with free fatty acids (FFAs) to induce lipid accumulation, as a model to investigate possible HIBCH- and 3-HIB-dependent mechanisms in the regulation of hepatocyte energy- and lipid metabolism. Transient *HIBCH* overexpression and siRNA-mediated HIBCH knockdown, RNA sequencing, targeted metabolite analyses and functional assays were performed. mRNA expression of *HIBCH* as well as *PDK4*, encoding a key stimulator of fatty acid oxidation at the expense of glucose utilization, were upregulated in the liver of people with NAFLD and in FA-treated Huh7 cells. Overexpression of *HIBCH* increased the release of 3-

HIB from the hepatocytes along with increasing the fatty acid uptake and efflux of methylmalonic acid (MMA) downstream in the BCAA pathway, while decreasing expression of genes involved in oxidative phosphorylation. Knockdown of *HIBCH* in Huh7 cells decreased cellular 3-HIB release as well as ROS generation while increasing the mRNA expression of *PDK4*, cellular oxygen consumption and release of the short-chain fatty acid acetate. Inhibition of PDK4 in the Huh7 cells in turn upregulated the mRNA expression of *HIBCH* and lowered the efflux of 3-HIB, suggesting an interdependent relationship between HIBCH and PDK4. Finally, supplementation of Huh7 cells with 3-HIB lowered *HIBCH* mRNA expression in association with a lowering of FA uptake and increased ROS generation.

Taken together, the data implicate the 3-HIB generating enzyme HIBCH in the regulation of hepatic energy- and lipid metabolism, in part by affecting PDK4 expression which is a marker of fatty acid oxidation, and with possible implications for the development of NAFLD.

5. Generall discussion

5.1 3-HIB and BCAAs in obesity, insulin resistance, NAFLD and type 2 diabetes

5.1.1 Evaluating 3-HIB as a biomarker for disease pathogenesis

Overall, our human cohort studies reveal positive associations for circulating 3-HIB concentrations with hyperglycemia, T2D and measures of insulin resistance and adiposity/obesity. Moreover, circulating 3-HIB concentrations were further elevated among those with T2D who require medications to control their blood glucose levels, suggesting that 3-HIB reflects the severity of the underlying metabolic changes. In the CARBFUNC cohort, where we also measured liver density and visceral fat volume by CT imaging, we found liver density (reflecting the degree of liver fat), fasting insulin and HOMA-IR to have the strongest correlations with 3-HIB among several other parameters such as BMI, waist circumference, glucose and HbA1c. The relationship between 3-HIB, obesity and insulin resistance was further strengthened by a marked reduction in circulating 3-HIB concentrations 1 year after bariatric surgery compared to before surgery. Of note, all correlations of 3-HIB with the different traits were adjusted for age and sex. It could be interesting to explore if 3-HIB concentrations differ between males and females and by age. Moreover, our population samples were recruited within a single county in Norway, which may limit the generalizability of our results to other populations/ethnicities.

Only a few other studies on the potential role of 3-HIB in disease have been performed so far. Harris *et al.* demonstrated that plasma 3-HIB concentrations are decreased when insulin and glucose availability increases during the hyperinsulinemic-euglycemic clamp procedure (HECP), but this effect was abolished by protein intake which reduced insulin-mediated glucose disposal, suggesting that 3-HIB is linked to changes in diet as well as being involved in the development of insulin resistance (Harris *et al.*, 2017). Consistent with our data, it has also been reported that changes in 3-HIB correlate with insulin resistance/HOMA-IR, also after controlling for changes in BMI, age, sex and diet (Haufe *et al.*, 2017). 3-HIB concentrations have moreover been associated with

future risk of incident T2D (Mardinoglu *et al.*, 2018) and evaluated as a potential predictor of metabolic risk after pregnancy complicated by gestational diabetes mellitus (Andersson-Hall *et al.*, 2018).

In our large population-based HUSK cohort, we saw a greater change in circulating concentrations of 3-HIB than in the BCAAs when comparing people with normoglycemia to those with hyperglycemia and T2D. The result of our logistic regression with diabetes as outcome and predictors valine, 3-HIB, BMI, TAG, and HDL-C subjected to a stepwise selection procedure showed that 3-HIB, but not valine, was an independent predictor of diabetes. Therefore, our findings suggest that 3-HIB is a more specific biomarker than its precursor valine. These findings are in line with the study of Haufe *et al*. reporting that metabolic improvements following weight loss were associated with changes in 3-HIB rather than BCAAs (Haufe *et al.*, 2017). Overall, the data indicate that measurement of circulating 3-HIB concentrations may reflect changes in cellular processes related to insulin resistance more accurately than the circulating BCAAs.

More accurate biomarkers that reflect the specific changes in tissues during disease progression could have great value, as this could help us identify specific metabolic changes in tissues and cellular processes that may underlie disease development in a given person. This could in turn help inform the development and administration of more precise treatment. Additionally, it is desirable to identify biomarkers that predict disease risk early before disease onset, thereby having the opportunity to apply preventive strategies as early as possible. Our studies uncover 3-HIB as a potentially useful marker that can detect people with a higher degree of fatty liver and concomitant insulin resistance. As such, measuring 3-HIB in a blood sample could help detect people at risk of fatty liver disease and future diabetes.

5.1.2 3-HIB as a signaling molecule/metabolic regulator

Our data indicate that 3-HIB may not only serve as a reflector of disease progression but may directly act as a signaling molecule with paracrine/endocrine/exocrine effects.

The first evidence that 3-HIB may have a functional role in signaling is that this metabolite is one of the few catabolic intermediates of the BCAA degradation that is not trapped in the mitochondria by a CoA group, as HIBCH removes the CoA that is bound to 3-HIB. This allows release of 3-HIB from the mitochondria to the extracellular space and potentially to the circulation for interaction with other cells and organs. Our different studies indicate that both adipocytes and hepatocytes may be sources of circulating 3-HIB dependent on the expression level of *HIBCH*, adding to previous literature showing release of 3-HIB from skeletal muscle (Jang *et al.*, 2016).

Specifically, we demonstrated that adipocytes increase the consumption and catabolism of BCAAs and release increasing amounts of 3-HIB during adipogenesis. We also showed that liver cells produce and release 3-HIB, both in response to increased FA availability and increased *HIBCH* expression. Moreover, demonstrating direct metabolic effects of 3-HIB, we found that supplementation of 3-HIB increased FA uptake in both white and brown adipocytes as well as altering insulin-stimulated glucose uptake, mitochondrial respiration and ROS generation. In hepatocytes, we found effects of 3-HIB treatment on FA uptake (reduction) and ROS production (stimulation). In total, these findings suggest that 3-HIB may serve as a signaling molecule between cells and potentially between tissues. Our data extend the work of Jang *et al* (Jang *et al.*, 2016), who demonstrated that induction of Peroxisome proliferator-activated receptor-γ coactivator (PGC-1α), a key regulator of energy metabolism (Liang and Ward, 2006), in myotubes increased catabolism of valine and the production of 3-HIB (Jang *et al.*, 2016). The 3-HIB generated from the myotubes stimulated the uptake of FAs in endothelial cells and promoted insulin resistance in muscle. Moreover, siRNA-mediated knockdown of *Hibch* in myotubes blocked the trans-endothelial fatty acid import into the myotubes (Jang *et al.*, 2016).

Although we and others have demonstrated that 3-HIB is transported in and out of cells, a relevant question is exactly how 3-HIB is transported across the cell membrane (i.e., is 3-HIB dependent on active or passive transport?). Some clues can be obtained by looking at the molecular structure of 3-HIB and thus its properties (Figure 12). 3-HIB

is a hydroxy monocarboxylic acid anion (or more specifically a β-hydroxy acid), consisting of 3 carbons (National Center for Biotechnology Information, 2022). Monocarboxylic acids are weak acids with one COOH functional group, which can be deprotonated. Because 3-HIB has less than 5 carbons and the –COOH group is polar, it makes this molecule hydrophilic and soluble in water. Other monocarboxylic acids (e.g., pyruvate, lactate and ketone bodies) are dependent on facilitated transport by membrane proteins called MonoCarboxylate Transporters (MCTs), which are expressed in most cells, to be transported across the cell membrane (Felmlee *et al.*, 2020). Glycolate is an example of a hydroxy monocarboxylic acid anion like 3-HIB, and is a metabolite derived from acetate and found to be dependent on AA transport (Baker *et al.*, 2004). It therefore appears likely that 3-HIB is transported similarly, motivating studies of 3-HIB transport to understand all mechanisms involved in regulating its concentrations in blood.

Is the effect of 3-HIB dose-dependent?

When deciding which doses to use for the experiments with 3-HIB supplementation, we relied on findings from recent studies including cell culture experiments with 3-HIB (Jang *et al.*, 2016; Mardinoglu *et al.*, 2018; Lyon *et al.*, 2019), 3-HIB measurements in animal studies (Neinast *et al.*, 2019), as well as our own measurements of 3-HIB concentrations in conditioned medium during *in vitro* differentiation of adipocytes. Jang *et al.* demonstrated increasing FA uptake in Human Umbilical Vein Endothelial Cells (HUVECs) in a dose-response manner using 3-HIB concentrations of 0.1, 1, 10 and 100 mmol/L for 1 hour (Jang *et al.*, 2016), while Mardinoglu *et al.* stimulated human adipose tissue– and cardiac-derived microvascular endothelial cells (a-MVECs and c-MVECs) as well as HUVECs with 5 mmol/L 3-HIB for 24 hours, and found increased FA uptake in c-MVECs and HUVECs (Mardinoglu *et al.*, 2018). Later, another study treated C2C12 myotubes with 3-HIB using what they defined as physiological (25-100 µmol/L) and supraphysiological (5 mmol/L) concentrations for up to 48 hours, and reported alterations in mitochondrial and glycolytic metabolism, including reduced pAkt expression during stimulation with insulin (Lyon *et al.*, 2019). The highest doses they tested in these studies seem rather

high compared to physiological 3-HIB concentrations in the human blood ranging from approximately 15-100 μmol/L in plasma, depending on the metabolic condition: healthy individuals/normoglycemica (~15-20 μmol/L), individuals with dysglycaemia/T1D (~25-40 μmol/L), and 3-days fasted individual ~97 μmol/L) (Avogaro and Bier, 1989; Harris *et al.*, 2017; Lee *et al.*, 2021). In our study, the circulating 3-HIB concentrations ranged between 8-44 μmol/L. However, 3-HIB levels were found to be significantly higher in all tissues measured by Neinast *et al* compared to the 3-HIB values in blood of mice (Neinast *et al.*, 2019), supporting that the cells are probably exposed to higher 3-HIB concentrations than those we measured in the circulation. Furthermore, our measurement of 3-HIB concentrations in conditioned cell culture medium (extracellular 3-HIB concentration) showed that 3-HIB concentrations ranged between 2-200 μmol/L (depending on cell type and state of the cells, e.g., undifferentiated vs. differentiated). Based on this, we chose to test both physiological (25-100 μmol/L) and supraphysiological (10 mmol/L) 3-HIB doses when treating the adipocytes, to see if effects could be observed also in the more physiological range. In white and brown adipocytes we found that 3-HIB affected FA uptake, insulin-stimulated glucose uptake, mitochondrial respiration and ROS production, and the results partly indicated a dose-dependent effect of 3-HIB. For the liver cells we chose to treat the cells with physiological 3-HIB dose (25 μmol/L), which was sufficient to affect FA uptake and ROS generation in FA-treated hepatocytes.

Is the effect of 3-HIB time-dependent?

As we observed that circulating 3-HIB concentrations were increased 1 week after bariatric surgery while 3-HIB was decreased 1 year after the surgery, we were prompted to test if effects of 3-HIB treatment were time-dependent. Moreover, the study by Neinast *et al.* (Neinast *et al.*, 2019) showed that approximately 20% of radiolabeled 3-HIB was observed in the blood of the mice just 3 minutes after an intravenous bolus injection of radiolabeled valine, revealing very rapid uptake and oxidation of the BCAAs as well as fast release of 3-HIB from the tissues into the circulation. We began to test 24 hour 3-HIB treatment, which was representative of the observed effect on adipocyte lipid accumulation during adipogenesis. This led to

increased insulin-stimulated glucose uptake, suggesting that chronically elevated 3-HIB results in greater glucose transport into adipocytes. Excess glucose can be oxidized to substrates used for TAG synthesis (instead of ATP production) and as a result increase fat storage. Moreover, we observed that short-term 3-HIB treatment increased cellular FA uptake while the insulin-stimulated glucose uptake was decreased, indicating that other substrates than glucose, such as pyruvate from AAs, contributed to glyceroneogenesis and thereby incorporation of the FAs into intracellular TAGs. We also observed an acute effect of ROS generation by 3-HIB treatment. Furthermore, we found that both short- and long-term 3-HIB treatment similarly affected mitochondrial respiration, observed both in white and brown adipocytes. For the liver cells we chose to treat the cells with 3-HIB for 24 hours (same treatment time as with FA treatment which increased fat storage). Future studies should test possible time-dependent effects of 3-HIB as well as changes in HIBCH expression also in those cells.

Is the effect of 3-HIB cell-dependent?

We found that 3-HIB treatment decreased mitochondrial respiration in white adipocytes, while brown adipocytes showed increased mitochondrial respiration. Interestingly, another study found that chronic physiological 3-HIB treatment for 48 hours increased oxygen consumption in C2C12 myotubes (Lyon *et al.*, 2019). Studies have shown that muscle and brown adipocytes have common developmental origins and share important features such as specialized energy utilization through thermogenesis (Seale *et al.*, 2008), which may explain the similar findings of Lyon *et al.* in myocytes and our findings in brown adipocytes. The different effects of 3-HIB supplementation between white and brown adipocytes may also be explained by higher BCAA usage in brown adipocytes, as we observed increased mRNA expression of BCAA catabolic genes in brown compared to white adipocytes. Accordingly, a study has reported that brown adipocytes increase their BCAA utilization during cold exposure to enhance thermogenesis, resulting in reduced circulating BCAA concentrations concomitant with enhanced whole-body homeostasis (Yoneshiro *et al.*, 2019).

We also found effects on 3-HIB addition to hepatocyte cultures. Interestingly, hepatocytes had a similar response as brown adipocytes, increasing ROS generation especially in liver cells that were treated with FA. In contrast to adipocytes, 3-HIB supplementation decreased FA uptake in FA-treated hepatocytes. The effects of 3-HIB on liver cells should be tested further, especially combined with FA treatment since our data indicated that there seems to be an interplay between 3-HIB and the availability of FAs.

5.2 Manipulation of *HIBCH* alters lipid storage

We found increased utilization of the BCAAs and increasing release of 3-HIB as well as upregulated gene expression of enzymes of the BCAA catabolism during adipogenesis, indicating that the BCAAs serve as important substrates in this process. These findings are in line with other studies showing that proliferating adipocytes change from mainly utilizing glucose and glutamine to increasing their utilization of AAs (especially BCAAs) during differentiation, and that the carbons originating from BCAA oxidation are incorporated into lipids and stored as TAGs (Crown, Marze and Antoniewicz, 2015; Green *et al.*, 2016; Estrada-Alcalde *et al.*, 2017). Thus, the BCAAs are important for adipocyte *de novo* lipogenesis and fat storage. Our study, however, also provides new knowledge about the metabolite 3-HIB, whose levels appear to correspond to lipid storage in adipocytes as well as in hepatocytes. Consistently, we demonstrated that knockdown of HIBCH reduced the production and release of 3-HIB concomitant with reduced lipid storage in white and brown adipocytes. In line with our results of adipocyte lipid storage, we found increased *HIBCH* expression and increased release of 3-HIB in hepatocytes with increased lipid storage due to treatment with FAs. These data suggest that the BCAAs may also play a role in *de novo* lipogenesis in hepatocytes, and strengthen the conclusion that 3-HIB promotes lipid storage in different lipid-storing cell types.

Interestingly, our RNA sequencing analyses revealed that genes related to oxidative phosphorylation were reduced by *HIBCH* overexpression. Consistent with these findings, we found that *HIBCH* knockdown increased mitochondrial respiration both

in the absence and presence of exogenous FAs concomitant with increased expression of *PDK4*, which is a marker of increased FA β-oxidation (Pettersen *et al.*, 2019). Together, these data suggest that HIBCH may promote lipid storage in hepatocytes by suppressing mitochondrial respiration. Interestingly, we found that the hepatocytes increased FA uptake upon *HIBCH* knockdown, while this effect was lost in FA-treated hepatocytes, possibly because the hepatocytes had already reached their maximum capacity of FA uptake by FA treatment. In light of some inconsistent effects of HIBCH manipulation on FA uptake (e.g., increased both with HIBCH overexpression and knockdown in the Huh7 hepatocytes), it needs to be considered that changes in the FA oxidation capacity may be more important for net lipid storage than changes in FA uptake, as an increase in FA uptake could provide substrate for FA oxidation as well as for FA storage.

5.3 Tissue contributions to whole-body BCAA metabolism

5.3.1 Associations of altered BCAA catabolism in a rat model of increased hepatic FA oxidation

Animal models can be useful to evaluate how BCAA catabolism in different tissues is involved in the development of disease conditions such as insulin resistance. We investigated BCAA catabolism in rats treated with mitochondria-targeted FA analogs (1-triple TTA, TTA and BCMTD), which are known to increase mitochondrial activity and FA β-oxidation in the liver, leading to decreased TAGs in plasma and liver and improved hepatic insulin sensitivity (Berge *et al.*, 1989; Lindquist *et al.*, 2017). We found that the plasma concentration of 3-HIB was decreased by 1-triple TTA and that plasma 3-HIB showed an inverse correlation with hepatic β-oxidation. Surprisingly, however, we found that the hepatic *Hibch* expression was increased 3-fold by 1-triple TTA concomitant with this reduction in plasma 3-HIB concentrations. The suggested effect of 1-triple TTA on tissue 3-HIB formation appeared to be specific for liver, since no changes in expression of *Hibch* were seen in samples from muscle and adipose tissue. The apparent discrepancy between increased hepatic *Hibch* mRNA and decreased plasma 3-HIB might be explained by an increased metabolic flux of 3-HIB towards downstream intermediates including MMA, suggested by the increased MMA

concentration in plasma of the 1-triple TTA-treated rats as well as in the HIBCH-overexpressing Huh7 hepatocytes. Nonetheless, our data overall suggest that 3-HIB may serve as a marker of reduced fatty acid metabolism in the liver.

Although our findings reflect the whole-body effect of the FA analogs in the rats, these FA analogs target primarily the mitochondria in the liver (Lindquist *et al.*, 2017), which may explain why we only observed an effect on the BCAA catabolism in the liver compared to muscle and adipose tissue. Unfortunately, we were not able to measure the concentration of 3-HIB in the different tissues or perform whole-body isotopic tracing of valine, 3-HIB and other metabolites. This would have given us more information on the 3-HIB formation and fluxes through 3-HIB in the different tissues in this animal model. Nonetheless, our results overall indicate that BCAA catabolism plays an important role in a state of increased β-oxidation and improved insulin sensitivity in the liver.

5.3.2 Genetic evidence linking valine catabolism to metabolic functions

Conditions of heritable changes in BCAA catabolism have been described, which may provide further insight into specific BCAA-dependent metabolic effects. Rare inborn errors that result in HIBCH deficiency strongly reduce HIBCH activity, depending on the specific gene mutation and leads to toxic accumulation of 3-hydroxyisobutyryl-CoA (3-HIB-CoA) and methacrylyl-CoA (the metabolite upstream of 3-HIB-CoA) in several tissues, causing similar clinical features as Leigh syndrome and ketoacidosis (Yamada *et al.*, 2014). The first patient was reported in 1982 by Brown *et al.*, and they found that HIBCH activity in the liver of the patient was around 10% of normal activity in control livers (Brown *et al.*, 1982). Based on their findings, they hypothesized that methacrylyl-CoA reacts with thiol compounds such as cysteine and glutathione (GSH) (Brown *et al.*, 1982), which have been found to cause defects in oxidative phosphorylation by reducing the activities of multiple enzymes of the respiratory chain as well as the pyruvate dehydrogenase complex (PDC) (Loupatty *et al.*, 2007; Ferdinandusse *et al.*, 2013; Peters *et al.*, 2015). Some studies also report elevated circulating lactate and/or ammonia concentrations in these patients (Ferdinandusse *et*

al., 2013; Reuter *et al.*, 2014) The condition results in cell damage by markedly reducing the cellular reduction state and decreasing ATP production (Ferdinandusse *et al.*, 2013). Since HIBCH deficiency is a rare condition with less than 10 cases reported worldwide, little is still known about the disease (Karimzadeh *et al.*, 2019), but the links to the respiratory chain and the PDC support the observations in our studies of hepatocytes.

6. Conclusions

In the present thesis, we found that increased activity of the valine-3-HIB pathway in adipocytes and hepatocytes, as well as increased 3-HIB concentrations in the blood, reflect increased lipid accumulation, insulin resistance, hyperglycemia and fatty liver, in association with decreased fatty acid oxidation.

Addressing the specific aims, we may draw the following conclusions:

1. In humans, we identified a strong relationship between plasma concentrations of the valine-derived metabolite 3-HIB and incidence of hyperglycemia and type 2 diabetes, as well as degree of adiposity and fatty liver (Paper I, Paper III).
2. Altered *HIBCH* mRNA expression and extracellular 3-HIB concentrations are involved in regulating energy metabolism and lipid accumulation in cultured white and brown adipocytes, partly with cell-specific effects (Paper I).
3. Circulating 3-HIB concentrations were lowered upon pharmacologic stimulation of hepatic FA oxidation *in vivo* in rats, associated with increased *Hibch* mRNA expression in the liver (Paper II).
4. *HIBCH* overexpression and knockdown in human hepatocyte cultures affect pathways of mitochondrial respiration (e.g., oxidative phosphorylation and oxygen consumption rate) and ROS generation, in association with altered extracellular concentrations of metabolic substrates, including 3-HIB, MMA and the short-chain fatty acid acetate (Paper III).

Thus, we determined novel roles of the valine–3-HIB pathway in energy metabolism in important metabolically active tissues, with possible implications for improving the prediction, prevention and treatment of adipose and liver fat storage and related diseases.

7. Future perspectives

In this thesis, we have revealed novel functions of the HIBCH enzyme, and identified 3-HIB as an important mediator and regulator of metabolic processes in adipocytes and hepatocytes. Our study has raised several questions that need to be further explored.

It would be interesting to study the role of HIBCH in whole-body BCAA metabolism in an animal model. Although some other animal studies have investigated the interplay between BCAA catabolism in different tissues, no studies have as far as we know manipulated HIBCH specifically in a specific tissue and examined consequent effects on gene expression, AA and metabolite flux between different tissues, and as well as effects on lipid storage and whole-body metabolic homeostasis. It would in this context be particularly interesting to perform adipose tissue-, muscle- and liver-specific manipulation of HIBCH (i.e., knockout as well as overexpression) in transgenic mouse models, and measure effects of dietary challenge (e.g., obesity- and insulin resistance-promoting diets). Moreover, our animal study only included male rats, and future experiments need to be performed to determine effects in female animals as well as in different strains. Additionally, future studies should be performed in an animal model of obesity/insulin resistance, since the study was performed in lean insulin sensitive animals. It would also be interesting to test if 3-HIB supplementation inhibits β-oxidation and ketogenesis in the liver *in vivo*.

In vitro, it would be relevant to perform a similar study with muscle cells for comparison with adipocytes and hepatocytes (i.e., *HIBCH* knockdown followed by different downstream analyses).

There is also a great potential for using genetic information to uncover molecular mechanisms that regulate regional fat storage and disease pathogenesis. Genome wide association studies (GWAS) have identified many genomic regions associated with increased metabolic disease risk. A previous study has reported that a specific single-nucleotide polymorphism (SNP) in the *HIBCH* locus (2q32.2) changes the circulating

of concentrations MMA (Molloy *et al.*, 2016), a metabolite immediately downstream of 3-HIB which reflects vitamin B12 status and mitochondrial activity (Molloy *et al.*, 2016; Dalmia *et al.*, 2019). Furthermore, the study reported that the allele variants of the SNP (*HIBCH* rs291466; c:2T>C) affected the protein structure as well as gene expression of HIBCH (Molloy *et al.*, 2016). It would therefore be relevant to evaluate gene-regulatory mechanisms that may be affected by this specific SNP in the *HIBCH* locus, and thereby gain new insight into the transcriptional regulation of *HIBCH* mRNA expression via novel enhancers that may be active in adipocytes, myocytes and/or hepatocytes. We have recently started to investigate this by measuring the enhancer activity of different non-coding DNA segments in the *HIBCH* locus, by cloning the respective segments into luciferase reporter plasmids. Our preliminary results show enhancer activity of the segment containing rs291466, with increased enhancer activity with the T compared to the C allele, while other segments in the *HIBCH* locus had minimal enhancer activity. We have begun to explore this SNP further by performing single-nucleotide substitution (prime editing, an alternative to CRISPR/Cas9) to edit the protective to risk rs291466 allele (and the other way around) in human adipocyte cultures, with the aim to determine cellular consequences of this single-nucleotide substitution (possible effects on, e.g., mitochondrial respiration, lipid accumulation and release of 3-HIB). These studies may later be extended to other cell types including different adipocyte subtypes, hepatocytes and myocytes.

Since it has been reported that activation of brown adipocytes by cold exposure increases their cellular BCAA uptake to enhance thermogenesis and as a result decreases circulating BCAA concentrations (Yoneshiro *et al.*, 2019; Sun *et al.*, 2022), it would also be interesting to explore 3-HIB functions *in vitro* and *in vivo* in a setting of activation of BAT and browning of white adipocytes, such as by treatment with β3-adrenergic receptor agonists. We have also started to explore this by using the β3-adrenergic receptor agonist CL-316243, which triggers an acute thermogenic response. Here we have preliminary results showing that CL-treatment increases *Hibch* mRNA expression in both white and brown adipocytes as well as the plasma concentrations 3-HIB and ketone bodies (β-hydroxybutyrate and acetoacetate) in mice fed a casein-rich

diet (unpublished). We already have supporting data showing that BAT has considerably higher mRNA expression of key BCAA catabolic genes than WAT in mice and humans (Paper I). Despite this, however, white and brown adipocytes seem to have similar intra- and extracellular 3-HIB levels (Paper I), suggesting that 3-HIB may be used by BAT to a greater extent in metabolic processes that are specific to BAT functions. This will also need to be clarified in future studies.

Lastly, it would be interesting to further investigate the link between hepatic HIBCH and PDK4. A valuable experiment would be to manipulate HIBCH expression (*HIBCH* knockdown and overexpression) in hepatocytes and investigate if PDC activity, which is largely regulated by PDK4, is affected. For instance, we could quantify the phosphorylation of the PDH complex by measuring p-PDH-E1α and total PDH-E1α protein, as well as PDK4 protein by Western immunoblotting.

Measuring the circulating 3-HIB concentrations in blood samples from NASH and NAFLD patients could also provide valuable information. It would be highly clinically relevant to determine if 3-HIB could serve as a predictive biomarker associated with increased hepatic lipid storage in NAFLD and NASH.

8. References

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9. Appendix

II

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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].

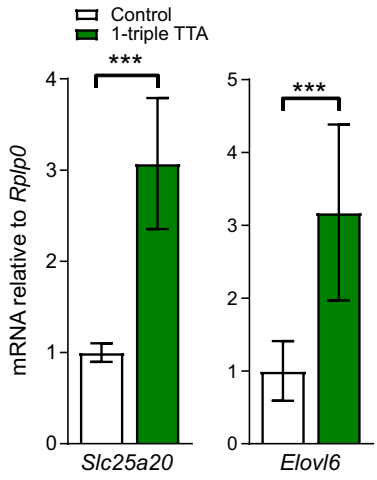

Fig. 1. Effects of 1-triple-TTA on genes regulating fatty acid flux into the liver in vivo.
Relative hepatic mRNA expression of *Slc25a20* and *Elovl6*, relative to *Rplp0* in control (n = 7) and 1-triple TTA-treated Male Wistar rats (n = 7). The results are presented as mean ± SD.
*$p < 0.05$, **$p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

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

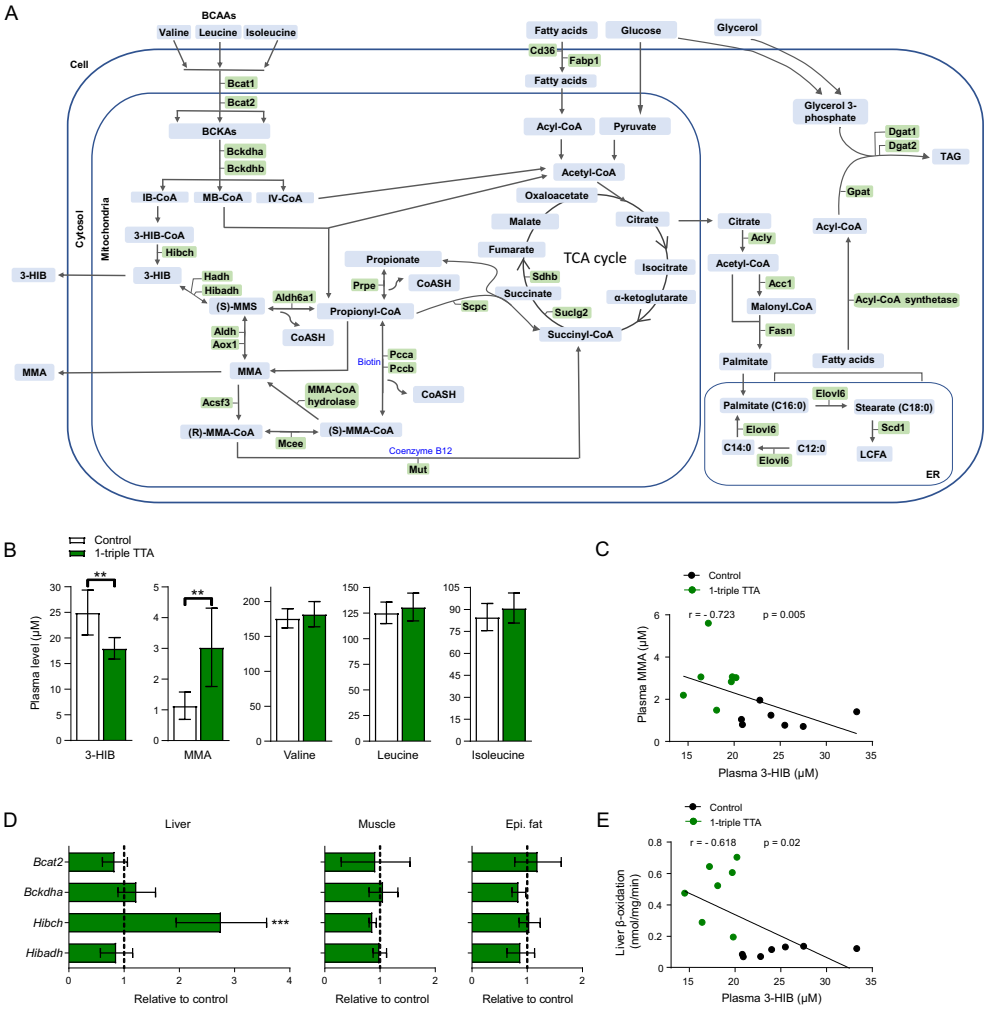

Fig. 2. Comparison of liver, muscle and fat in BCAA metabolism and the effect of 1-triple TTA on fatty acid oxidation in the liver in vivo.
(A) Overview of cellular BCAA catabolism and TCA cycle. Amino acids, metabolites and intermediates are marked in blue and relevant enzymes in green. (B) Plasma levels 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

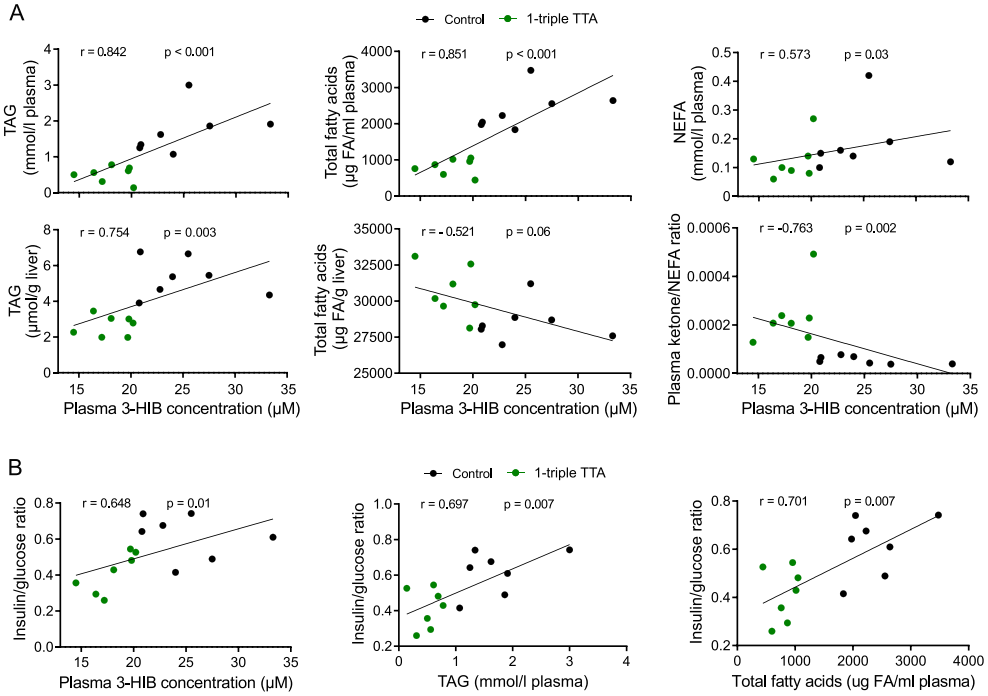

Fig. 3. Plasma 3-HIB levels correlates with changes in mitochondrial functions and parameters linked to lipid and glucose metabolism.
(A–B) Spearman correlations of plasma 3-HIB concentrations and parameters related to lipid and glucose metabolism in control (n = 7) and 1-triple TTA-treated Male Wistar 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

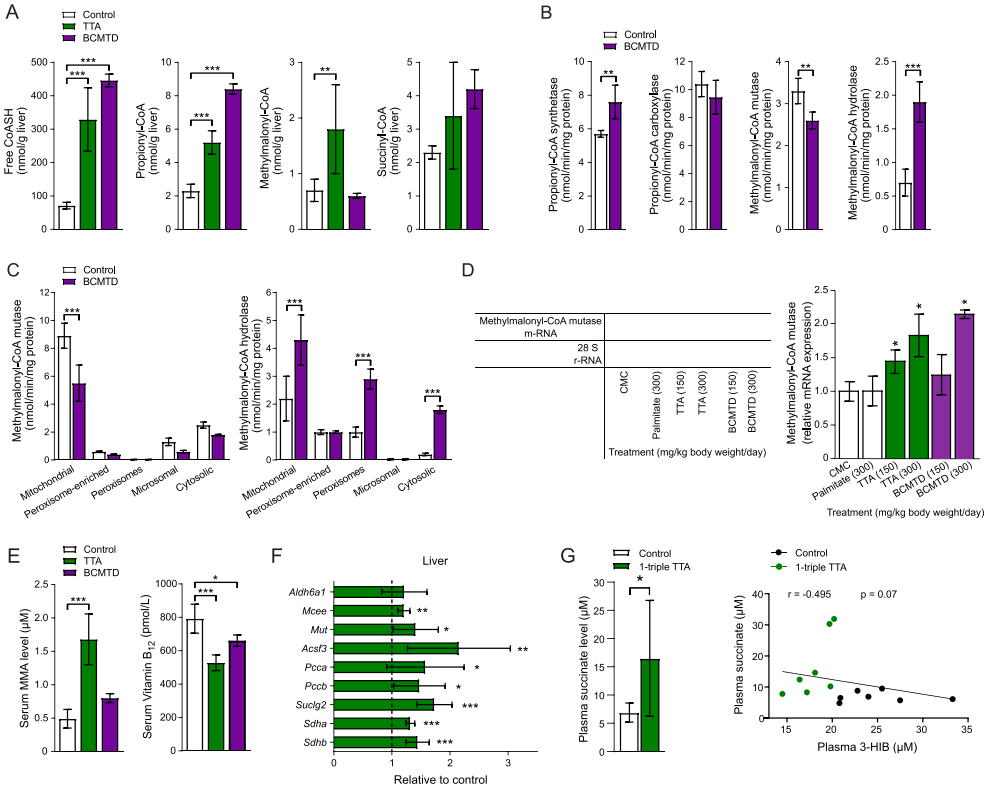

Fig. 4. 3-Thia fatty acids affect pathways of hepatic BCAA catabolism and 3-HIB degradation.
(A) Levels of various short chain acyl-CoA derivatives in liver homogenates of rats treated with TTA (n = 5) or BCMTD (n = 5) for 7 days. The results are presented as mean ± S.D. (One-way ANOVA, Sidak's). (B) 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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbalip.2021.158887.

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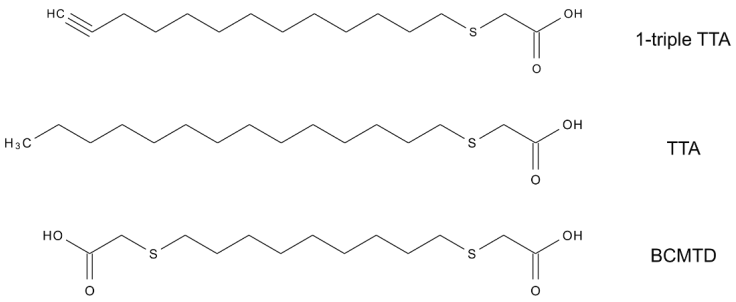
SUPPLEMENTARY MATERIALS

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



Supplementary Figure 1. Chemical structures of the synthetic fatty acids, (2-(tridec-12-yn-1-ylthio)acetic acid (1-triple TTA), tetradecylthioacetic acid (TTA) and 3-thiadicarboxylic acid (BCMTD).

Supplementary Table 1. Probes and primers for quantitative real-time PCR (TaqMan).

Assay	Gene name	Assay ID
18S	18S ribosomal RNA	Eurogentec 18S rRNA Control Kit RT-CKFT-18S
Acsf3	acyl-CoA synthetase family member 3	Applied Biosystems TaqMan Assay Rn01431293_m1
Aldh6a1	aldehyde dehydrogenase 6 family, member A1	Applied Biosystems TaqMan Assay Rn00579182_m1
Bcat2	branched chain amino transaminase 2, mitochondrial	Applied Biosystems TaqMan Assay Rn00574455_m1
Bckdha	branched chain keto acid dehydrogenase E1, alpha polypeptide	Applied Biosystems TaqMan Assay Rn01457724_m1
Slc25a20	Solute carrier 25 (carnitine/acylcarnitine translocase), member 20	Applied Biosystems TaqMan Assay Rn00588652_m1
Elovl6	ELOVL fatty acid elongase 6	Applied Biosystems TaqMan Assay Rn01522299_m1
Hibadh	3-hydroxyisobutyrate dehydrogenase	Applied Biosystems TaqMan Assay Rn01763063_m1
Hibch	3-hydroxyisobutyryl-CoA hydrolase	Applied Biosystems TaqMan Assay Rn01437588_m1
Mcee	methylmalonyl-CoA epimerase	Applied Biosystems TaqMan Assay Rn01478033_m1
Mut	methylmalonyl-CoA mutase	Applied Biosystems TaqMan Assay Rn01512343_m1
Pcca	propionyl-CoA carboxylase, alpha polypeptide	Applied Biosystems TaqMan Assay Rn01234283_m1
Pccb	propionyl-CoA carboxylase, beta polypeptide	Applied Biosystems TaqMan Assay Rn00566764_m1
Rplp0	ribosomal protein, large, P0	Applied Biosystems TaqMan Assay Rn03302271_gh
Sdha	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	Applied Biosystems TaqMan Assay Rn00590475_m1
Sdhb	succinate dehydrogenase complex, subunit B, iron sulphur (Ip)	Applied Biosystems TaqMan Assay Rn01515728_m1
Suclg2	succinyl-CoA ligase, GDP-forming, beta subunit	Applied Biosystems TaqMan Assay Rn01425947_m1

Supplementary Table 2. Primers for quantitative real-time PCR (SYBR Green).

Primer	Gene name	Sequence (5 → 3)
Bcat2	branched chain amino transferase 2, mitochondrial	F: AGATGTGTGTCCTCCAACTTCA R: GGGGCTGGCTTCTTCTGT
Bckdha	branched chain keto acid dehydrogenase E1, alpha polypeptide	F: TGGGATGAGGAACAGGAGAA R: AGAAGAGGAGGCTTGGGTTG
Hibadh	3-hydroxyisobutyrate dehydrogenase	F: GGTGGATTTGGGACAACACT R: TCTTTGAGTAGCCCTTTGAACAC
Hibch	3-hydroxyisobutyryl-CoA hydrolase	F: GGGAATCGCCACACACTTT R: AGACACCAGCAACATCCTCA
Rplp0	ribosomal protein lateral stalk subunit P0	F: GATGCCCAGGGAAGACAG R: GAAGCATTTTGGGTAGTCATCC

Primers used for qPCR for muscle and epididymal adipose tissue from rats were designed with Universal ProbeLibrary Assay Design Center from Roche. Primer directions are indicated as F (Forward) and R (Reverse).

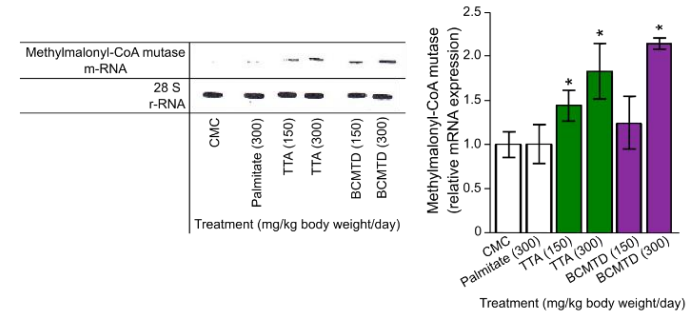
Supplementary Table 3. Spearman correlations for serum 3-HIB, MMA and 3-HIB/MMA ratio and parameters related to lipid and glucose metabolism in rats treated with or without 1-triple TTA.

	Spearman's rho	P value	
3-HIB vs plasma TAG	0.842	<0.001	***
MMA vs plasma TAG	-0.811	<0.001	***
3-HIB/MMA vs plasma TAG	0.859	<0.001	***
3-HIB vs total fatty acids (plasma)	0.851	<0.001	***
MMA vs total fatty acids (plasma)	-0.785	0.001	**
3-HIB/MMA vs total fatty acids (plasma)	0.837	<0.001	***
3-HIB vs NEFA (plasma)	0.573	0.03	*
MMA vs NEFA (plasma)	-0.529	0.05	ns
3-HIB/MMA vs NEFA (plasma)	0.562	0.04	*
3-HIB vs hepatic TAG	0.754	0.003	**
MMA vs hepatic TAG	-0.820	<0.001	***
3-HIB/MMA vs hepatic TAG	0.802	<0.001	***
3-HIB vs total fatty acids (liver)	-0.521	0.06	ns
MMA vs total fatty acids (liver)	0.297	0.30	ns
3-HIB/MMA vs total fatty acids (liver)	-0.376	0.19	ns
3-HIB vs insulin	0.763	0.002	**
MMA vs insulin	-0.745	0.003	**
3-HIB/MMA vs insulin	0.793	0.001	**
3-HIB vs glucose	0.194	0.50	ns
MMA vs glucose	-0.376	0.18	ns
3-HIB/MMA vs glucose	0.304	0.29	ns
3-HIB vs insulin/glucose ratio	0.648	0.01	*
MMA vs insulin/glucose ratio	-0.604	0.02	*
3-HIB/MMA vs insulin/glucose ratio	0.692	0.008	**
3-HIB vs hepatic β-oxidation	-0.618	0.02	*
MMA vs hepatic β-oxidation	0.648	0.01	*
3-HIB/MMA vs hepatic β-oxidation	-0.613	0.02	*
3-HIB vs ketone body/total plasma FA ratio	-0.763	0.002	**
MMA vs ketone body/total plasma FA ratio	0.899	<0.001	***
3-HIB/MMA vs ketone body/total plasma FA ratio	-0.890	<0.001	***

Correction of Figure 4D in Paper II

The published article did not show the bands of the slot blot. These are shown below:

D



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