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LIST OF ACRONYMS AND ABBREVIATION

ACOX 1	peroxisomal acyl-coenzyme A oxidase 1
AMP	adenosinmonophosphat
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
BCAA	branched chain amino acid
BCAT	branched-chain amino-acid aminotransferase
BLVRA	biliverdin reductase A
BMI	body mass index
BR	bilirubin
CAD	coronary artery disease
CAT	carnitine translocase
CI	confidence interval
CO	carbon monoxide
CoA	coenzyme A
CPT-1	carnitine palmitoyltransferase I
CPT-2	carnitine palmitoyltransferase II
CVD	cardiovascular disease
DMT2	diabetes mellitus type 2
D_2O	deuterium oxide
FACS	fatty acyl-CoA synthase
FATP1/2	fatty acid transport protein 1 and 2
Fe2+	ferrous ion
FGF21	growth factor 21
FMO	flavin monooxygenases
GC-MS	gas chromatography mass–spectrometry
GS	Gilbert`s syndrome
^1H	hydrogen-1
HDL	high-density lipoprotein
HMB	β-hydroxy-β-methylbutyrate
HMG	3-hydroxy-3-methyl-glutaryl

HO	haem oxygenase
LC-MS	liquid chromatography–mass spectrometry
LDL	low density lipoprotein
LOD	limit of detection
mTORC1	mammalian target of rapamycin complex 1
mRNA	messenger ribonucleic acid
NMWL	nominal molecular weight limit
NMR	nuclear magnetic resonance
PFK2	phosphofructokinase 2
PPAR	peroxisome proliferator-activated receptor
QC	quality control
OPLS-DA	orthogonal partial least squares-discriminant analysis
PCA	principal component analysis
PGC-1α	peroxisome proliferator-activated receptor-gamma coactivator-1alpha
SAS	Statistical Analysis System
SIRT1	sirtuin 1
TMA	trimethylamine
TMAO	trimethylamine N-oxide
TSP	3-(trimethylsilyl)-2,2',3,3'-tetradeuteropropionic acid
UCB	unconjugated bilirubin
UGT1A1	uridine diphosphate glucuronosyltransferase 1A1
VIP	variable important in projection
VLDL	very low density lipoprotein

1. INTRODUCTION

Gilbert's syndrome (GS) is an inherited benign disorder of bilirubin metabolism (Clementi et al., 2006), characterized by mutation (UGT1A1*28) of the uridine diphosphate glucuronosyltransferase 1 family polypeptide A1 (UGT1A1) gene (Bosma et al., 1995) and a slight elevation of unconjugated bilirubin (UCB) levels (>17.1 µmol/L) (Wagner et al., 2015). The increased UCB level is based on the decreased expression of the UGT1A1 gene, which leads to a reduction of UGT1A enzyme (encoded by gene) activity of approximately 25%, resulting in a declined bilirubin conjugation and excretion (Black and Billing, 1969). According to total bilirubin concentration, 2-10% of the Caucasian population are affected by this syndrome (Wagner et al., 2018).

Bilirubin is a bile pigment deriving from haem catabolism in mammals. This catabolite has been long associated with hepatobiliary and neurotoxic diseases. Hence, bilirubin was assumed to be biologically useless. In the late 80s, Stocker and colleagues suggested bilirubin's antioxidative activity as a first possible physiological role of UCB based on *in vitro* experiments (Stocker et al., 1987). A possible explanation for the antioxidative function of UCB are the double bounds of the methene and vinyl groups in the UCB structure, which are susceptible to oxidations (Vítek and Ostrow, 2009). Additionally, recent investigations suggest anti-inflammatory and lipid-lowering effects of bilirubin *in vivo*. (Hwang et al., 2011; Bulmer et al., 2013) However, beneficial effects are only attributed to moderately unconjugated hyperbilirubinemia of up to 102.6 µmol/L (6 mg/dL) UCB which is observed in subjects with GS (Strassburg, 2010).

In 1994 Schwertner et al. reported that subjects with endogenously elevated UCB were protected from coronary artery disease (CAD) (Schwertner et al., 1994). Moreover, the data of the Framingham Heart Study including 4276 participants showed an inverse association between bilirubin levels and cardiovascular events (Djoussé et al., 2001). Additionally, a subset of 1780 subjects were followed up for 24 years and genotyped for UGT1A1*28. The homozygous

genotype with high bilirubin level had a lower risk of cardiovascular disease (CVD) (Lin et al., 2006). Most Caucasian GS subjects are homozygotes for UGT1A1*28 (Bosma et al., 1995, Monaghan et al., 1996).

The cardio protecting effect was also demonstrated in a case-control study on subjects with GS. Compared to the general population, these subjects had a 10 % lower prevalence for ischemic heart disease (Vítek et al., 2002).

Furthermore, a moderately elevated bilirubin concentration is associated with lower risk of diabetes mellitus type 2 (DMT2), CVD, some cancer types, respiratory disease and all-cause mortality (Horsfall, 2011; Wagner et al., 2015; Yang et al., 2019; Inoguchi et al., 2021).

Beside elevated UCB levels, GS subjects reveal a significantly lower body mass index (BMI) and an ameliorated lipid and glucose profile. These metabolic biomarkers could indicate alterations of the energy metabolism resulting in lower risk for chronic disorders. Indeed, Mölzer et al. recently showed a higher peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α), adenosinmonophosphat activated protein kinase (AMPK), peroxisome proliferator-activated receptor α and γ (PPARα/γ) activity in fasting GS subjects compared to controls of the Bilihealth study, suggesting a boosted energy turnover (Mölzer et al., 2016).

This master thesis was aimed at investigating the metabolomics profile of subjects with GS and providing further mechanistic understanding for the repeatedly observed beneficial health effects. Especially, metabolites of lipid metabolism are of utmost interest. Therefore, a comprehensive metabolomics approach is used to compare the metabolome of GS subjects with their matched healthy controls. Serum samples of 112 subjects were analysed by using ^{1}H NMR spectroscopy.

2. LITERATURE REVIEW

Bilirubin and Bilirubin metabolism

Bilirubin is a brownish yellow bile pigment deriving from haem catabolism in mammals. Haem degrades in two steps in the reticulo-endothelial system. Initially, haem is converted into biliverdin due to haem oxygenase (HO) by releasing iron and carbon monoxide (Ryter et al., 2006). Afterwards, the enzyme Biliverdin reductase A (BLVRA) further reduces the biliverdin into bilirubin (Singleton JW and Laster, 1965). As it makes its way through the bloodstream before reaching the liver, the lipophile unconjugated bilirubin (UCB) is mainly bound to albumin (90%), but also to high-density lipoprotein (HDL) and apolipoprotein D (Odell, 1973; Suzuki et al., 1988; Goessling and Zucker 2000). In the hepatocytes UCB binds to ligandin to reach the endoplasmic reticulum (Listowsky et al., 1978), where it undergoes conjugation with uridine diphosphate glucuronosyltransferase (UGT1A1). At this point, this bilirubin glucuronide complex is hydrophile and capable to enter the bile canaliculus (Iyanagi et al., 1998). The conjugated bilirubin is henceforth part of the bile and can be excreted in the gut. Subsequently, the majority is reduced into urobilinogen and oxidised to stercobilin by the intestinal floral enzymes (see Figure 1). The minor remaining part can be reabsorbed and become part of the circulating bilirubin pool again (Hamoud et al., 2018).

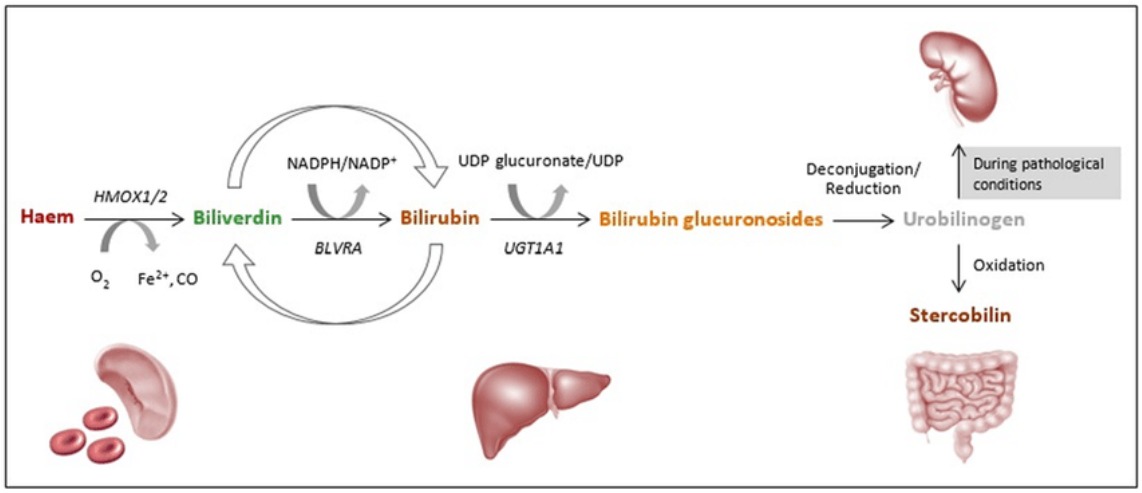

Figure 1: Haem catabolism – from haem to bilirubin (Wagner et al. 2015)

Around the end of the 80s, Stocker and colleagues discovered for the first time bilirubin's antioxidative activity as a possible physiological role of UCB based on *in vitro* experiments (Stocker et al., 1987; Stocker et al., 1987a). The antioxidative characteristic is particularly attributed to UCBs double bonds in the methyl and vinyl group, which are very unstable and susceptible to oxidations (Vítek and Ostrow, 2009). Moreover, the two enzymes involved in haem degradation HO and BLVRA, as well as Bilirubin's precursor Biliverdin were mentioned to have further extended biological importance. On the one hand, HO, which is partly induced by oxidative stress, is involved in defending the cardiovascular system against cellular stress, probably due to the production of carbon monoxide (CO) and Bilirubin which is known to play an important role as cytoprotectant against oxidative stress (Tomaro et al., 2002). Furthermore, HO is predicted to have many functions in the mammalian system including iron homeostasis, oxygen sensing, regulation of inflammatory responses, cell proliferation or innate and adaptive immunity (Ayer et al., 2016). These effects might also be attributed to CO and ferrous ion (Fe^{2+}) (by-products of Haem degradation), which are known as important signalling molecules influencing cell functions (Wegiel et al., 2013). Another mechanism that has been suggested to play a role in the antioxidant defence is the redox system between Bilirubin and Biliverdin through BLVRA, a notable redox system influencing the antioxidant defence (Baranano et al., 2002).

This can partially explain the negative association between UCB levels and CVD, since oxidation plays a crucial role in CVD. (Horsfall, Nazareth and Petersen, 2012). In general, bilirubin is negatively associated with all-causes mortality as well as chronic diseases, including CVD, diabetes mellitus and cancer (Horsfall, 2011; Horsfall et al., 2013; Wagner et al., 2018; Inoguchi et al., 2021).

Hyperbilirubinemia

Hyperbilirubinemia is a state of unusual and multifactorial high bilirubin levels in the blood. Basically, this state is a result of an imbalance between bilirubin production and bilirubin clearance. This can be caused by several liver diseases,

including hepatotropic hepatitis, cirrhosis, alcoholic/non-alcoholic fatty liver disease, or hepato-biliary malignancy. Moreover, hyperbilirubinemia also occurs due to impaired haem metabolism seen in Crigler-Najjar, Dubin-Johnson, Rotor or Gilbert's syndromes (GS). The latter causes a mild hyperbilirubinemia. While severe hyperbilirubinemia is mostly toxic, mild hyperbilirubinemia is not harmful, but even health protective.

A large epidemiological study for example has shown a negative association between UCB levels and cardiovascular events. (Horsfall, Nazareth and Petersen, 2012) This might be attributed to UCBs antioxidative ability, but the underlying biochemical mechanisms are not yet fully understood.

Gilbert's Syndrome

Almost 120 years ago, GS was first described by Gilbert and Lereboullet and is defined by a mildly elevated bilirubin level in absence of any liver diseases and normal red blood cell counts (Gilbert and Lereboullet, 1901; Strassburg et al., 2008). According to Owens and Sherlock, Gilbert's syndrome is diagnosed by a total bilirubin concentration higher than 17.1 µmol/L or 1 mg/dL. (Owens and Sherlock, 1973) The high UCB level occurs because of decreased bilirubin degradation in the liver (Billing et al., 1964; Berk et al., 1970). This is explained by reduced hepatic activity of the UGT1A1, an enzyme of the xenobiotic metabolism that is responsible for the clearance of bilirubin (Black and Billing, 1969). A polymorphism with an insertion of 2 additional TAs ($(TA)_7TAA$ instead of $(TA)_6TAA$) repeats in the TATA sequence, located in the promoter of the UGT1A1 (UGT1A1*28) is referred to be the underlying cause for the decreased UGT1A1 expression and activity. The inheritance of this polymorphism is autosomal recessive (Bosma et al., 1995; Monaghan et al., 1996). Additionally, it is worth noting that an increased haem production and upregulation of haem oxygenase activity can also increase UCB levels in GS subjects (Kadakol et al., 2000; McCarty, 2013; Wallner et al., 2013). UCB concentrations in subjects can also vary depending on fasting status, degree of light exposure, stress, disordered sleep, enterohepatic bilirubin reabsorption/secretion and gastrointestinal motility (Kotal et al., 1996).

The prevalence of GS varies between individuals of different ethnicities. For instance, populations of Eastern Asia and Latin America (Gwee et al., 1992; Mendez et al., 2013) reveal minor bilirubin concentrations, whereas Middle East countries have the highest level (Hemmati et al., 2010; Kamal et al., 2019). In some studies, the prevalence of GS in Caucasian is estimated around 2-10% (Sieg et al., 1987; Wagner et al., 2018).

Beneficial effects of Gilbert's Syndrome

The epidemiological data of the Framingham Heart Study has shown that homozygote UGT1A1*28 allele carriers, as GS subjects are described, are associated with decreased risk for CVD (Lin et al., 2006). The cardioprotective effect was also demonstrated in a case control study on subjects with GS. Compared to the general population, subjects with GS had a 10 % lower prevalence for ischemic heart disease (Vítek et al., 2002). According to a meta-analysis, an increase of each 1 µmol/L total bilirubin level was associated with a reduction of 6.5% risk for CVD (Novotný and Vítek, 2003; Vítek, 2012).

Many studies repeatedly observed a reduction in total cholesterol, low density lipoprotein cholesterol (LDL-cholesterol), triacylglycerol, LDL subfractions (LDL-1 and LDL-2), apolipoprotein B (Apo-B) and Apo-B/Apo-A1 ratio in subjects with mildly elevated bilirubin level (Wallner et al., 2013; Bulmer et al., 2013; Seyed Khoei et al., 2018).

A comprehensive analysis of human and rodent lipid profile during a case control study have shown favourable conditions in GS subjects and hyperbilirubinemic Gunn rats compared to their matched controls. Beside beneficial lipid profile in aged GS subjects, Wallner et al. have found significant lower BMI level in older GS subjects compared to matched controls. Remarkable thereby is that no differences in age-related parameters were noticed between old and young GS subjects. (Wallner et al., 2013)

In addition, a cross-sectional study showed that the lower body mass index (BMI) was more pronounced between GS and controls older than 35 years than between GS and controls younger than 35. In consistence to Wallners results,

this older GS subjects also revealed an improved lipid profile, a smaller hip circumference and lower fat mass compared to their age and gender matched controls. (Khoei et al., 2018). All together, these results suggest that GS subjects are protected in gaining body fat and weight during ageing which are known risk factors for CVD.

A recent study of diet-induced obesity in rats confirms this idea of preventive effects of hyperbilirubinemia in weight and fat gaining. Rats fed a diet high in fats and sugar were injected intraperitoneal with bilirubin or vehicle for 14 days. Bilirubin treatment prevented a deterioration in glucose tolerance and suppressed weight gain. In addition, compared with control mice, bilirubin-treated mice had reduced total cholesterol and leptin and increased adiponectin levels.

The favourable aging condition was also seen at molecular level. GS subjects had longer telomeres, confirming less telomere oxidation and therefore have less abrasion throughout aging. This was proved *in vivo* in human peripheral blood mononuclear cells and Gunn rat's liver tissue (Tosevska et al., 2016).

However, beneficial effects on at least secondary diseases, as CVD and diabetes mellitus is assigned to, can only be attributed indirectly to bilirubin as energetic misbalance might be the more predominant cause (Lee et al., 2006); and GS subjects are well known to have a lean body composition and lower BMI pointing to an altered energy homeostasis (Wallner et al., 2013; Khoei et al., 2018). Bilirubin's protective effects are strongly suggested to work due to its antioxidative character and the ability to influence fat metabolism. Hence, it is of importance to evaluate the underlying effect of bilirubin on human energy metabolism, especially, on lipid metabolism.

Energy metabolism

The human metabolism is constantly responding to fed and fasting periods. While usually carbohydrates and fat are utilized, only fat depots serve as fuel during periods of energy deprivation. In prolonged fasting periods, ketone bodies provide sustenance to the brain in substitution for glucose. Additionally, the insulin level decreases and glucagon, glucocorticoids as well as nor- and

adrenalin increases. Consequently, lipolysis of triglycerides in adipocytes is triggered by the latter hormones and free fatty acid (FFA) level in plasma rises. In the liver, FFA undergoes beta-oxidation or is transported in very low density lipoprotein (VLDL). Once the FFA enters the cytosol, the fatty acyl-CoA synthase (FACS) adds a CoA to the acyl chain. Thus, yet the fatty acyl-CoA can be transferred into the mitochondrial matrix by the membrane-bound carnitine acyltransferase system. Therefore, carnitine palmitoyltransferase I (CPT1) transfers 1 acyl from acyl-CoA to carnitine. Subsequently, acylcarnitine access the mitochondrial matrix in exchange to a free carnitine supported by the carnitine translocase (CAT) in the inner membrane of the mitochondria (Figure 2). Inside the mitochondrial matrix carnitine palmitoyltransferase II (CPT2) separates the acyl-CoA from carnitine which again can bind a new acyl molecule in the cytosol. Meanwhile, the acyl-CoA molecule is ready for ß-oxidation.

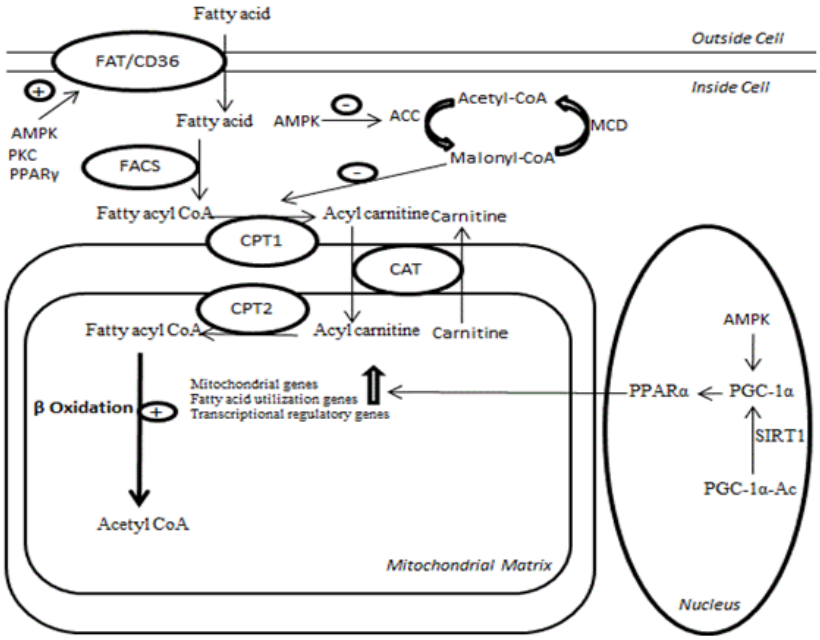

Figure 2: The fatty acid Beta-oxidation pathway (Fillmore et al. 2021)

Beta-oxidation is a multistep process to break down fatty acids to acetyl-CoA in the mitochondria. Therefore, the long-chain acyl-CoA undergo repeated cycles including two oxidations, hydration, and shortening of the acyl-CoA chain by two carbons by subtraction of one acetyl-CoA each time. The number of the cycles is

depending on the length of the acyl-CoA chains and results in the formation of one remaining acetyl-CoA or propionyl-CoA molecule at the end. Propionyl-CoA is formed at the last oxidation cycle of odds numbered acyl-CoA chains, while even numbered acyl-CoA chains are completely oxidised to acetyl-CoA.

As seen in Figure 3, acetyl-CoA as the result of beta-oxidation either attend locally the Krebs cycle or condense to ketone bodies.

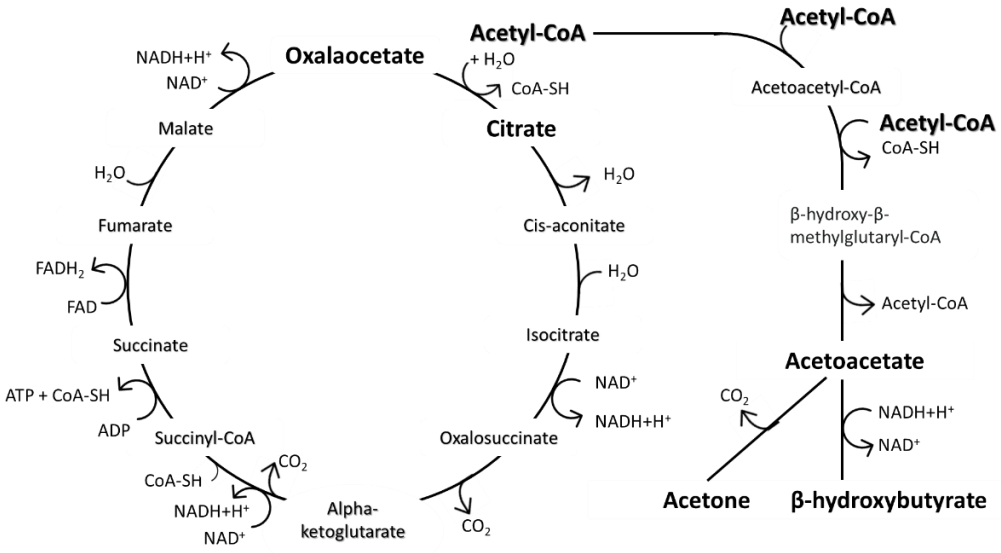

Figure 3: The pathway of Acetyl-CoA: Krebs cycle and ketogenesis

During fasting periods, the peroxisome proliferator-activated receptor alpha (PPARα) plays an import role in catabolic metabolism by inducing the transcription of various genes involved in glucose and lipid metabolism (Kersten et al., 1999) This includes the expression of CPT1, a crucial player for ß-oxidation and growth factor 21 (FGF21), a metabolic adapter to fasting condition inducing adipose tissue lipolysis and liver ketogenesis (Lundåsen et al., 2007; Ribet et al., 2010).

Indeed, Bilirubin is structural similar to ligands of peroxisome proliferator-activated receptor alpha (PPARα) (Figure 4). Stec et al. 2016 proved *in vitro* that bilirubin is able to bind to PPARα (Stec et al., 2016). In addition, bilirubin influences the expression of PPARα which was shown in mice with higher bilirubin levels (Hinds et al., 2017). Thus, bilirubin possibly enhances

transcriptional activity of PPARα and therefore increases the expression of CPT1 and FGF21.

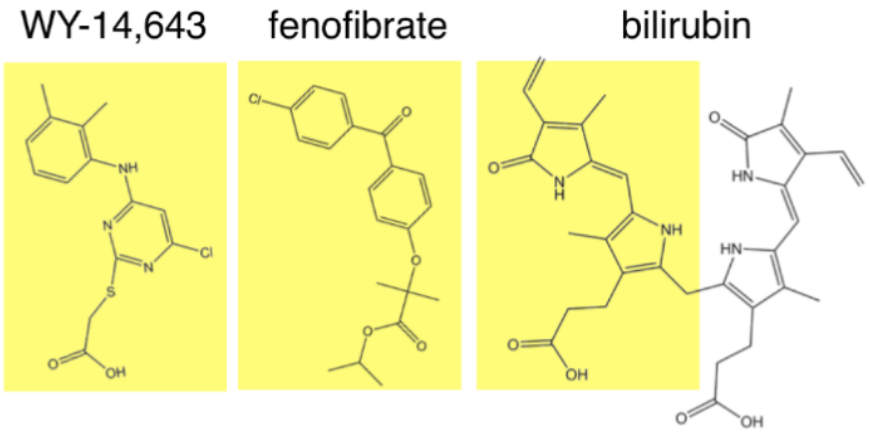

Figure 4 Comparison of standard lipid-lowering drugs (PPARα agonist) and bilirubin (Stec et al., 2016)

AMPK is a further key factor for catabolic metabolism which is associated with enhanced autophagy, fatty acid oxidation, glucose uptake and utilization as well as reduction of protein synthesis by inhibition of mammalian target of rapamycin complex 1 (mTORC1). Therefore, AMPK activates phosphofructokinase 2 (PFK2), a catalyser of glycolysis. Simultaneously, AMPK inhibits the activation HMG-CoA-reductase and acetyl-CoA-Carboxylase which are key enzymes of cholesterol and fatty acid formation. Interestingly, FGF21 increases the activation of AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1) in mice (Chau et al., 2010). Mölzer et al. found AMPK and PPARα activation to be higher in peripheral blood mononuclear cells of GS subjects, endorsing the impact of bilirubin on PPAR expression in human and revealing a distinct boosted catabolic metabolism in fasted GS subjects. In term of FGF21 level, no significant differences were observed between GS subjects and controls. Apart from that, the mechanistic understanding regarding bilirubin's impact on energy metabolism is still poorly understood. There is currently a growing interest in how the metabolome is affected by bilirubin and its metabolism. In this context, this work seeks to investigate lipid catabolism by investigating the metabolomics profile of GS subjects.

Metabolomics

Metabolomics is a valuable tool to investigate comprehensive metabolic patterns. It is basically used for the analysis of molecules, particularly metabolites, equal or smaller than 1500 daltons. Metabolites are intermediates of biochemical pathways, including processes of energy homeostasis, signal transduction and apoptosis. Among others, small metabolites are lipids, fatty acids, amino acids, phenolic compound, and alkaloids. Clinical studies take advantage of this robust method to identify biomarkers and establish mechanistic understanding. Thereby, samples across biological systems including plasma, urine, saliva, and tissues can be analysed. Beside gas chromatography coupled to mass spectrometry (GC-MS) and liquid chromatography coupled with single-stage mass spectrometry (LC-MS), nuclear magnetic resonance (NMR) is one of the three primary analytical techniques used for metabolomics analysis (Emwas et al., 2019). The advantage of NMR analysis is the simple sample preparation, the easily and fast quantification (Röhnisch et al., 2018), the high level of reproducibility (Li-Gao et al., 2019), and the suitability for high-throughput application (Soininen et al., 2009; Guennec et al., 2014). However, compared to MS, NMR analyses are less sensitive. MS approaches are ten to hundred times more sensitive by having lower limits of detection. Thus, only metabolites in abundance can be identified with NMR. On the other hand, NMR has the capacity to identify highly polar molecules. This includes carboxylic acids, sugars, alcohols and polyols. Several nuclei can be recorded by NMR (such as ^{1}H, ^{13}C, ^{15}N, and ^{31}P) although proton (^{1}H) NMR spectroscopy (^{1}H NMR) is the most applied approach since nearly all metabolite contains ^{1}H atoms (Emwas et al., 2019). Hence, many epidemiological and clinical, including large-scaled, studies use this reliable and efficient method to explore knowledge gaps in metabolism. For instance, to determine signature molecular markers of disease risk during fasting as wells as postprandial condition (Cheng et al., 2017; Holmes et al., 2018; Müllner et al., 2021). Furthermore, NMR is likely applied to investigate metabolic differences in subjects with different diets or after several food challenge (Schmidt et al., 2021; Rådjursöga et al., 2018; Shi et al., 2017). Principal component analysis (PCA) is often used to visually reveal different metabolic pattern.

3. MATERIALS AND METHODS

Study design and subjects

The aim of the present master thesis was to investigate metabolic differences between subjects with GS and healthy controls and to reveal mechanisms responsible for the beneficial health condition in GS subjects. Therefore, a case control study was implemented and the metabolic profile in serum was determined by using ^{1}H NMR spectroscopy.

Study population

A total number of 128 subjects between 20 and 80 years were recruited. Thereof, 112 were included and assigned in two paired matched groups, each consisting of 56 participants, which were coded with a continuing number of 2 digits (Figure 5).

Pregnant or smoking subjects, as well as subjects with excess drinking habits, constant intake of nutritional supplements and medicines, liver diseases, acute or chronic diseases, cancer and organ transplants were excluded.

Objects and Assignment

Cases (GS):

Total plasma bilirubin > 1 mg/dL or 17.1 µmol/L

Unconjugated (indirect) bilirubin ≥ 1 mg/dL

Control (non-GS):

Total plasma bilirubin < 1 mg/dL or 17.1 µmol/L

Unconjugated (indirect) bilirubin < 1 mg/dL

After screening, according to the indirect bilirubin level, participants were assigned in GS group and non-GS group (Wallner et al., 2012). GS subjects were recruited first and then healthy control (non-GS) subjects with matching age and gender; each group includes 20 females and 36 males. The distribution of age was also considered; therefore, two age groups (< 35 and 35 year) were stratified to achieve a 1:1 match within the groups. Resulting in 31 subjects under 35 years and 25 subjects equal or higher than 35 years.

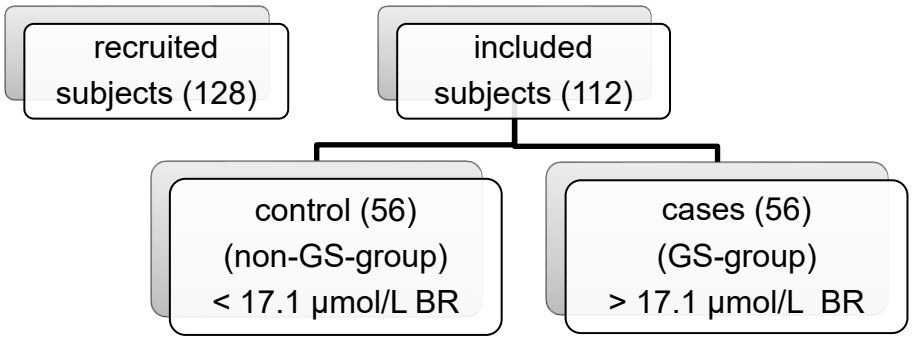

Figure 5: Distribution of all recruited subjects

Sample collection

All 112 participants completed a specific fasting program. On the day before the survey, participants could take a usual breakfast until 9 am. During the rest of the day, no later than 6 pm, a light meal was allowed. From 6 pm until the first blood sampling on the next morning, only water was allowed. After an initial screening (questionnaires, blood pressure, BMI, fasting blood biochemistry including unconjugated bilirubin (UCB) and liver enzymes level), serum samples were collected for the metabolomics analysis.

NMR metabolomics analysis

In -80°C freezer stored serum samples of 56 GS and 56 healthy controls (age and gender matched) were sent on dry ice to the Swedish University of Agricultural Sciences (Department of Molecular Sciences) in Uppsala. Until the analyses were performed, the samples were stored at -80°C.

Depending on the available volume of serum, samples were prepared according to two different methods, the 5 mm NMR tube method and the 3 mm NMR tube method (Table 1). For the 5mm method, at least 400 µL serum were required, whereas 60 µL were sufficient for the 3 mm method. Thus, two different extraction methods were implemented. Thereby, case and corresponding healthy control samples were filtered and later analysed on the same day, to avoid batch wise systematic error within a pair.

Table 1: Distribution of serum samples submitted for NMR analysis

	5mm protocol		3mm protocol	
	GS	control	GS	control
Serum samples	33	33	23	23
Serum samples total	66		46	
QC	12		8	
Total	78		54	

Sample preparation for NMR analysis

5mm method

Serum samples with a minimum volume of 400 µL were prepared by a previously described method with slight modifications. Samples were extracted to reach a cut off of 3 kDa. Therefore, prewashed Amicon® filter (Merk Millipore Ltd.) system with a pore size of 3.000 NMWL was used. To remove glycerol from the filter membrane, the filter system was washed with 9 mL Millipore water for 2 hours at 1500 g and 36°C. Afterwards, the remaining water was removed and immediately replaced by 400 µL serum to avoid dehydration and breaking of the membrane. Serum samples were extracted at 10,000 × g and 4°C until the volume of filtrate and residue remain constant (155-180 minutes). In addition, 12 quality control (QC) serum samples from 6 aliquots based on pooled serum samples were extracted in the same way, to assess intra- and inter batch performance. In total, serum and QC samples were extracted in 9 batches. Each batch included maximum 30 samples. Filtrates were stored at -20°C until preparation of NMR analysis.

For the preparation of the NMR analysis, 310 µL serum filtrate were defrosted on ice and was mixed with 65 µL Millipore water, 150 µL sodium phosphate buffer (0.4 mol/L, pH=6.98), 45 µL D_2O and 30 µL TSP ((3-(trimethylsilyl)-2,2',3,3'-tetradeuteropropionic acid), 5.8 mmol/L, Cambridge Isotope Laboratories). Thereafter, 560 µL of the mixture was transferred into a 5mm NMR tube and were used for the NMR analysis (Table 2). NMR analyses were performed in 3 runs and samples were analysed in a random order.

Table 2: Sample preparation for 5mm NMR method

Volume (µL)	Solutions
310	Serum filtrate
150	Sodium phosphate 0.4 mol/L
65	Millipore water
45	D_2O
30	TSP 5.8 mmol/L

3mm method

Nanosep centrifugal filters (Pall Life Science) were used for the ultra-filtration. Details to this approach have been previously described (Röhnisch et al., 2018). In brief, glycerol was removed from the filter by washing eight times with 500 µL Millipore water for 8 minutes at 2000 g and 36°C. After that, all remaining water in the filter was removed and replaced with 60 µL serum one by one. Serum samples were extracted at 10,000 × g and 4°C until the volume of filtrate and residue remain constant (25-35 minutes). Filtrates were stored at -20°C.

To prepare the NMR analysis, 40 µL, on ice defrosted, serum filtrate was mixed with 55 µL Millipore water, 50 µL sodium phosphate buffer (0.4 mol/L, pH=6.98), 15 µL D2O and 10 µL TSP (5.8 mmol/L). 170 µL of the mixture was transferred into a 3 mm NMR tube and were used for the NMR analysis (Table 3). Additionally, 8 QC serum were analysed, as mentioned before. In total, serum and QC samples were extracted in 4 batches. NMR analyses were performed in 2 batches and randomized in sample order.

Table 3: Samples preparation for 3mm NMR method

Volume (µL)	Solutions
40	Serum filtrate
55	Sodium phosphate 0.4 mol/L
50	Millipore water
15	D_2O
10	TSP 5.8 mmol/L

H NMR spectroscopy?

All H NMR spectra were acquired as previously described (Röhnisch et al., 2018; Moazzami et al., 2014; Tiziani et al., 2008; Hwang and Shaka, 1995) at a 600 MHz proton frequency on Bruker Advance III spectrometer, equipped with cryogenically cooled probe and auto sampler, using zgesgp pulse sequence (Bruker Spectrospin Ltd.) at 25°C and a relaxation delay of 4.0s. Free induction decays (FIDs) were obtained after 128 scans for samples of 5mm method or 512 scans for samples of 3mm method and 65,536 data points over a spectral width of 17,942.58 Hz before transforming into a 1H NMR spectra by using the software TopSpin 3.1, supplied by Bruker Biospin AG. Further processing steps, including phase correction, line broadening and shim correction were performed manually by using the software NMR Suite 8.3 (ChenomX Inc., Edmonton, Canada). Signals were principally identified by using NMR Suite 8.3 library, Biological Magnetic Resonance Data Bank, and Human Metabolome Data Base. In total, a set of 58 metabolites were identified. Metabolites showing ≤ 20% occurrence were defined as data below the detection limit (LOD, limit of detection) and were excluded. Thereof 7 were below the LOD; and hence, they were excluded from further statistical analysis. This includes 1,2-propandiol, 1-methylguanidine, 2-propanol, aspartic acid, isocaproic acid, sarcosine, and propionic acid. The remaining 51 metabolites were then quantified by using the Automated Quantification Algorithm (AQuA) (Röhnisch et al., 2018). Final serum concentrations were obtained after consideration of the dilution factor.

Statistical Analysis

Prior any statistical analysis, the total concentrations were normalized to enable comparability between the two different NMR methods. Therefore, every single concentration was divided with its median consulted from all concentration within a metabolite within a method. This calculation was conducted for all 51 metabolites.

$$\frac{\text{sample concentration of metabolite x of 5mm method}}{\text{median of all concentration of metabolite x of 5mm method}}$$

For multivariate statistical analyses, the evaluated and normalized concentrations were imported to the SIMCA 14 software (Umetrics, Umeå, Sweden). All variables were UV scaled prior principal component analysis (PCA) and orthogal partial least-squares-discriminant analysis (OPLS-DA). The PCA model reduced the set of data to a few numbers of principal components and provides a visual overview of the data. Thereby, trends, groups and outliers can be recognized. The presence of outliers was examined by using a Hotelling T2 Ellipse (95% CI). The OPLS-DA is a regression model for multivariate data, calculated to describe the most discriminating metabolites between GS and control. The significance of the OPLS-DA model was tested by CV-ANOVA and validated by using overall cross-validation R2. Metabolites contributing to distinguish cases and controls can be visualized in the corresponding loading plots. Additionally, the variable importance for the projection (VIP) based on OPLS-DA was used to rank and determine the most discriminative metabolites. VIPs greater than 1 for which the corresponding jack-knife-based CI (95%) were greater than 0 were regarded discriminative. This calculation was conducted for all 51 metabolites.

In addition to multivariate analysis, univariate statistics were performed of all 51 metabolites by Statistical Analysis System (SAS, 9.3, SAS institute, Cary NC, USA). A paired t-test was applied to compare between matched GS and controls. Histogram and normal probability plots were visually checked for normal distribution. If not normally distributed, log-transformations were conducted, and all normality tests were repeated. For not normal distributed data Wilcoxon-Mann-Whitney test was used as a non-parametric test. A p-value <0.05 was defined as statistically significant. Bonferroni correction was performed to account for multiple testing. Thereby, the number of metabolites under statistical investigation (metabolites above the LOD), was used as number of variables, resulting a=0.001.

4. RESULTS

The OPLS-DA model was fitted to evaluate the metabolic difference in the fasting serum of GS subjects versus healthy controls. As shown in the score plot (Figure 6) and based on R2Y and Q2 values, the OPLS-DA model was found to be reliable and to reveal significant differences between the two groups according to cross-validated ANOVA (p=0.0004).

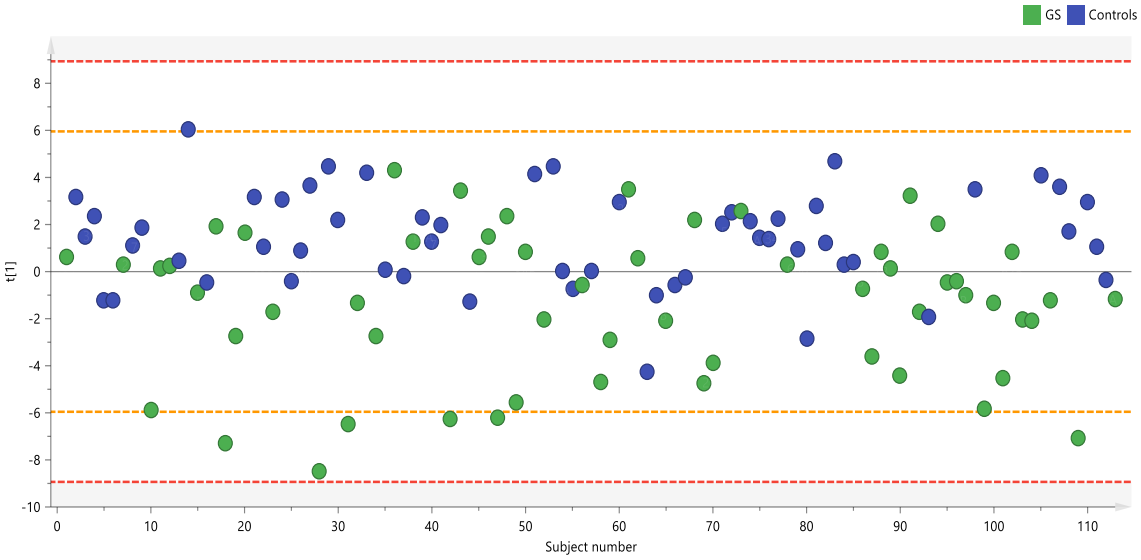

Figure 6: Score plot of the supervised OPLS-DA model of NMR data of fasting serum extracts in GS subjects (green) and healthy controls (blue). Data set derived from 112 subjects and 51 identified metabolites were normalized, UV scaled, and log transformed before fitting and cross validation (P-value of CV-ANOVA =0.0004)

The corresponding loading scatter plot (Figure 7) demonstrates metabolites influencing the area showed on the score plot. Metabolites on the lowest area are also reflected in the evaluation of VIPs.

Figure 7: Loading scatter plot showing metabolites influencing the area showed on the score plot (Figure 6) of the supervised OPLS-DA model of NMR data of fasting serum extracts in GS subjects (green) and healthy controls (blue). Data set derived from 112 subjects and 51 identified metabolites were normalized, UV scaled, and log transformed before fitting and cross-validation (P-value of CV-ANOVA =0.0004).

Table 4 ranks all VIP scores indicating 20 metabolites with largest discriminatory power from the OPLS-DA modelling. In other words, these 20 metabolites were found to be different in serum of GS subjects versus healthy control based on their VIP greater than 1.

Table 4: Variable importance for the projection (VIP)

Metabolites	VIP	CI	VIP - CI
2-Oxoisocaproic acid	**2.03354**	0.679622	1.353918
3-Hydroxybutyric acid	**1.88514**	1.0844	0.80074
Leucine	1.82738	0.401693	1.425687
Acetylcarnitine	**1.77915**	0.867235	0.911915
2-Hydroxybutyric acid	1.74041	0.852252	0.888158
2-Aminobutyric acid	1.72682	0.313305	1.413515
Acetone	**1.66071**	0.961789	0.698921
Acetoacetic acid	**1.63103**	1.30002	0.33101
Citric acid	**1.4728**	1.18989	0.28291
Valine	1.42896	0.538552	0.890408
2-Ketoglutaric acid	1.32079	0.647511	0.673279
3-Hydroxyisovaleric acid	1.31665	0.632746	0.683904
Isoleucine	1.27325	0.855402	0.417848
Acetylglycine	**1.25451**	0.777787	0.476723
Myoinositol	1.19172	0.749788	0.441932
2-Hydroxyisovaleric acid	1.10824	0.967401	0.140839
Glutamine	1.03689	0.526627	0.510263
Histidine	1.03479	0.717664	0.317126
Trimethylamine	**1.03457**	0.76337	0.2712
Trimethylamine N oxide	1.00094	0.431925	0.569015

VIP and CI values derived from OPLS-DA model (CV-ANOVA cross-validated, p=0.0004) of NMR data of fasting serum extracts in GS subjects versus healthy controls. Metabolites with VIP greater than 1 and VIP-CI greater than 0 were regarded discriminative. VIPs in **bold** were also found significant by using t-test after Bonferroni correction

For further investigation, univariate statistic was used to compare the NMR output in GS subjects versus healthy controls. Accordingly, a significant increased level of ketone bodies (acetoacetate, 3-hydroxybutyric acid and acetone), trimethylamine, acetylglycine, citric acid, acetylcarnitine, 2-oxoisocaproic acid in GS subjects compared to healthy controls during fasting was observed after Bonferroni correction (Figure 8).

The concentration of ketone bodies in serum was significantly and approximately 2-fold higher in GS subjects compared to controls, in detail the median concentrations of 3-hydroxybutyric acid was 132.5 % (p=0.00001), of acetoacetic acid 94.8 % (p=0.0007) and acetone 46.1 % (p=0.00001) higher. Beside ketone bodies, trimethylamine was also significantly higher (34.8 %) in GS with a p-value of 0.00001. Moreover, acetylglycine was 20.4 % higher in GS with a significance of p=0.0002. With reference to citric acid, the median serum concentration in GS subjects was 149.8 µM versus 130.7 µM in controls and thus 14.7 % higher in GS subjects (p=0.0002). Furthermore, the median concentration of acetylcarnitine acid was 20.4 % higher in GS subjects with a p-value of 0.0003. Regarding 2-oxoisocaproic acid, the median serum concentration in GS subjects was 11.98 % higher compared to controls (p=0.0006) (Table 5).

By using the Bonferroni correction, a very conservative approach was conducted to counteract the problem of multiple comparison. However, the univariate result is in line with the multivariate analysis.

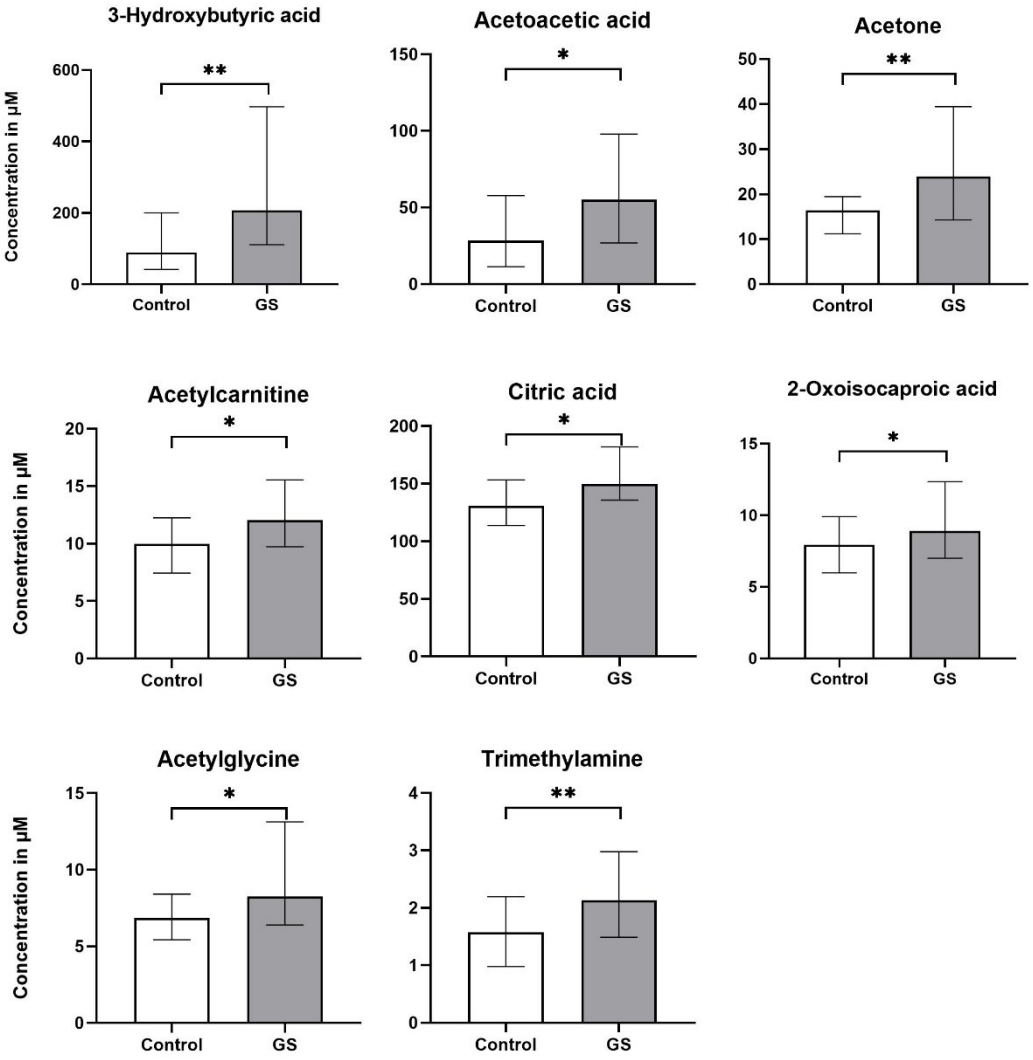

Figure 8: Concentration of significant metabolites after Bonferroni correction. Median ± IQR (n=112). Concentration of 3 Hydroxybutyric acid, Acetoacetic acid, Acetone, Acetylcarnitine, Citric acid, 2-Oxoisocaproic acid, Acetylglycine and Trimethylamine. *p<0.0001; **p=0.00001

Table 5: Univariate metabolomics profile of GS vs control individuals

Metabolites	GS subjects		Control		p-value
3-Hydroxybutyric acid	207.6	84.6	89.3	44.18	0.000
Acetone	23.99	25.2	16.42	8.24	0.000
Trimethylamine	2.13	1.49	1.58	1.22	0.000
Acetylglycine	8.26	6.73	6.86	2.98	0.000
Citric acid	149.84	46.09	130.68	39.58	0.000
Acetylcarnitine	12.03	5.82	9.99	4.79	0.000
2-Oxoisocaproic acid	8.9	5.36	7.95	3.92	0.001
Acetoacetic acid	55.24	71.16	28.36	46.58	0.001
Glutamine	443.74	122.99	415.19	75.64	0.002
2-Hydroxybutyric acid	50.23	43.32	39.38	27.04	0.002
Creatine	15.11	10.69	19.21	20.94	0.003
Leucine	124.85	48.15	112.75	32.93	0.005
2-Aminobutyric acid	29.34	13.27	26.07	10.74	0.007
2-Ketoglutaric acid	12.07	6.3	11.81	4.25	0.009
Creatinine	74.61	20.46	71.36	20.37	0.013
Histidine	79.98	27.33	78.42	17.64	0.018
3-Hydroxyisovaleric acid	2.44	0.31	2.35	0.22	0.023
Tyrosine *	57.34	3.08	61.38	3.27	0.037
Myoinositol	32.61	9.24	31.05	6.96	0.044
Dimethylsulfone	3.18	2.99	4.41	3.24	0.044
Methanol	362.92	772.76	261.01	774.68	0.056
Glycine	228	90.41	205.07	76.3	0.062
Proline	162.69	57.65	181.22	63.66	0.089
Asparagine	52.6	14.88	52.42	11.81	0.090
Glucose	3062.4	788.59	3235.2	777.63	0.107
Acetic acid	40.01	14.6	36.09	20.45	0.111
Lysine	156.08	54	152.33	44.98	0.111
Betaine	35.81	11.78	37.15	12.68	0.133
Pyruvic acid	59.86	25.1	54.63	24.59	0.152
Valine	237.42	17.54	228.81	11.98	0.161
Methionine	28.97	8.61	29	7.19	0.192
2-Hydroxyisovaleric acid	9.06	5.68	8.74	5.17	0.216
Dimethylglycine	3.16	1.28	3.07	1.4	0.253
Isoleucine *	66.76	4.43	64.4	3.88	0.266
O phosphocholine	2.17	1.22	2.31	1.32	0.282
Trimethylamine N oxide *	31.53	2.93	29.7	2.35	0.310
Formic acid	16.14	6.16	17.26	6.88	0.439
Choline	10.51	3.7	9.78	3.18	0.459
Ornithine *	58.2	4.31	56.27	4.13	0.504
Alanine	330.39	92.45	335	98.25	0.653
Butyrate	6.84	5.75	5.72	5.78	0.664
Carnitine	38.69	14.7	37.25	10.13	0.674
Glutamic acid	47.92	21.95	41.53	27.87	0.676
Threonine *	121.82	6.75	123.52	5.9	0.683
Arginine	96.67	53.24	94.12	38.68	0.741
Glycerol	679.2	361.87	735.96	336.69	0.747
Lactic acid	1849.84	517.79	1829.8	572.55	0.772
Ethanol	40.97	241.68	32.94	141.73	0.847
Phenylalanine	48.62	10.16	49.91	8.76	0.847
Succinic acid	7.67	3.17	7.75	3.24	0.877
Serine	130.35	42.99	127.99	35.16	0.962

Median/IQR or mean/SD (*) concentration in μM. The corrected p-value after Bonferroni is 0.001. n=56 per group (creatine, tyrosine and acetic acid n=55 per group).

5. DISCUSSION

Previous studies have shown that bilirubin can bind to PPARα, inducing the transcription of genes involved in lipolysis and liver ketogenesis under catabolic condition. To furthermore investigate into the metabolome of subjects with mildly elevated bilirubin levels as observed in GS subjects, NMR based metabolomics was used. This comprehensive approach is conducted to reveal metabolic differences between subjects with GS and healthy controls. All samples were originated from fasted individuals. Moreover, PCA and OPLS-DA models were created for multivariate statistical analysis. Thereby, 20 metabolites were found discriminative via VIP >1. Additionally, a paired t-test or Wilcoxon-Mann-Whitney test was performed for univariate analysis, showing eight metabolites which were significantly different between GS individuals and controls after Bonferroni correction including, acetylcarnitine, acetoacetate, 3-hydroxybutyric, acetone, citric acid, 2-oxoisocaproic acid, trimethylamine and acetylglycine.

Acetylcarnitine is an established indicator of fasting and an important marker for lipid catabolism as it is an end product of Beta-oxidation (Kondoh et al, 2020). In this work, acetylcarnitine is significantly higher in GS subjects compared to healthy controls. Carnitine can derive from both nutrition and endogenous synthesis and is mainly synthesized in kidney and liver but also in the brain. (Hoppel 2003, Siliprandi 1989). The presence of carnitine is crucial for the oxidation of long chain fatty acids because carnitine acts as a transporter for long chain fatty acids to pass the inner membrane of the mitochondria, paving the way for the mitochondrial ß-oxidation. After ß-oxidation, carnitine can again bind to acetyl-CoA, the end product of ß-oxidation, forming acetylcarnitine. The accumulation of acetylcarnitine in plasma is suggested to occur particularly, when the level of acetyl-CoA exceeds the acetyl-CoA utilization capacity in the Krebs cycle (Muoio et al., 2012). In this context, acetylcarnitine is accomplished to provide extrahepatic tissue with substrate for energy metabolism. Especially, the brain can call on acetylcarnitine. While ketone bodies have to be converted to acetyl-CoA in order to be utilized by the brain (Owen et al., 1969), acetylcarnitine

can directly supply acetyl-CoA to the brain. Thus, a higher level of acetylcarnitine in serum of GS subjects may observed due to a higher acetyl-CoA availability due to a distinct Beta-oxidation in GS subjects.

Congruently, ketone bodies were observed to be significantly higher in GS subjects compared to healthy controls. As mentioned, Beta-oxidation leads to an excessive acetyl-CoA production. In consequence, the liver cell is forced to convert those excess intramitochondrial acetyl-CoA to 3-hydroxybutyrate, acetoacetic acid and acetone. On demand, 3-hydroxybutyrate and acetoacetic acid can be easily transformed back to acetyl-CoA in extrahepatic tissues and enter the Krebs cycle. In contrast, acetone is highly volatile and hence, it is unsuitable for further utilization by the body. These entities basically derive from lipid metabolism and serve as alternative energy source to glucose in periods of energy scarcity and are well known marker of catabolic metabolism, for example, during prolonged exercise, fasting, starving, and ketogenic diets (Evans et al., 2017; Kondoh et al, 2020). In short, elevated levels of 3-hydroxybutyrate, acetoacetic acid and acetone in fasting serum samples of GS subjects additionally indicate a pronounced ß-oxidation during fasting. This result is consistent to a recent mouse study of Hinds and colleagues revealing an increased expression of PPARα messenger ribonucleic acid (mRNA) as well as its targeted genes fatty acid transport protein 1/2 (FATP1/2) and peroxisomal acyl-coenzyme A oxidase 1 (ACOX1) in liver tissue after bilirubin administration (Hinds et al., 2020). The fact that bilirubin affects lipid metabolism is in accordance with a mouse study showing that bilirubin decreases body fat PPARα-dependently (Stec et al., 2016). Consequently, Hinds et al. found for the first time the PPARα downstream metabolite 3-hydroxybutyrate to be increased in plasma by bilirubin (Hinds et al., 2020). Mölzer et al. confirmed the increased PPARα and AMPK activation of mildly elevated bilirubin levels in human (Mölzer et al., 2016), while the association of ketone bodies with bilirubin is novel in this thesis.

Another way for the mitochondria to cope with excessive acetyl-CoA is the use of the citrate-shuttle to pass the short chain acetyl-CoA throughout the mitochondrial membrane. Briefly, citrate is formed by the addition of acetyl-CoA

to oxaloacetate in the mitochondrial matrix. As citrate, acetyl-CoA can be mobilized outwards the mitochondrial inner membrane by the citrate-shuttle, a tricarboxylate transporter. In the cytoplasm, citrate is available for reconversion to acetyl-CoA. In the present work, citric acid was observed to be significantly higher in serum of subjects with GS compared to healthy controls. This is a further indication for an increased ß-oxidation accompanied by an increased Krebs cycle flux in the mitochondria.

Furthermore, the metabolite 2-oxoisocaproic acid was observed to be significantly higher in GS subjects compared to healthy controls. The intermediate 2-oxoisocaproic acid derives from degradation of the branched chain amino acid (BCAA) leucine. Leucine catabolism is primarily important in muscle cells, where branched-chain amino-acid aminotransferase (BCAT) is highly expressed (Suryawan et al., 1998). In the mitochondria, 2-oxoisocaproic acid is oxidized to isovaleryl CoA, resulting in β-hydroxy-β-methylglutaryl-CoA formation, and contributing to acetoacetate and acetyl-CoA production (Hyun et al., 2007). Hence, these end metabolites can directly enter the Krebs cycle and serve as energy substrate. Alternatively, 2-oxoisocaproic acid can be released into the blood stream and transported to other tissues. The higher accumulation in serum of GS subjects during fasting can again be explained by an acetyl-CoA and ketone body saturation due to enhanced Beta-oxidation.

Moreover, 2-oxoisocaproic acid and its catabolite β-hydroxy-β-methylbutyrate (HMB) were discussed to have beneficial impact on protein and lipid metabolism. *In vivo*, 2-oxoisocaproic acid was observed to suppress proteolysis in skeletal muscle tissue (Nakashima et al., 2007). The same was observed after supplementation of 2-oxoisocaproic acids metabolite HMB (Holecek et al., 2009; Noh et al., 2014). Interestingly, this advantage is also reported during catabolic conditions (Wilkinson et al., 2013; Duan et al., 2016). Both 2-oxoisocaproic acid and HMB enhance mitochondrial biogenesis and fatty acid oxidation *in vivo* in C2C12 myotubes (Stancliffe, 2012). However, there is no sufficient evidence to exclude that the role of 2-oxoisocaproic acid in protein and energy metabolism is not depending on the reconversion of 2-oxoisocaproic acid to leucine. In contrast, HMB is not undergoing reconversion, therefore all effects can be exclusively

attributed to HMB. The discussed effects of 2-oxoisocaproic acid may contribute to the lean body composition seen in GS.

Additionally, acetylglycine was significantly higher in the GS group. Acetylglycine is formed by the reaction of acyl-CoA with glycine. Therefore, the mitochondrial glycine acyltransferase, mainly expressed in liver and kidney, catalyses the transfer of the acetyl of acetyl-CoA to glycine (Adeva-Andany et al., 2018). In general, acylglycine is associated with ß-oxidation (Li et al., 2020). Glycine is lately inversely associated with incidence of CVD and DMT2 (Floegel et al., 2013; Ding et al., 2015).

Interestingly, trimethylamine (TMA) which is suggested to have an impact on metabolic diseases is significantly higher in GS subjects. Trimethylamine derives from microbiotic degradation of choline, lecithin, and L-carnitine in the gut. In the liver, TMA is further metabolized to trimethylamine N-oxide (TMAO) by flavin monooxygenases (FMO). TMAO is a novel metabolite, just detected over the last years in the metabolomics field and is associated with cardiovascular diseases (Naghipour et al., 2021).

6. CONCLUSION

The significantly higher serum level of acetylcarnitine, ketone bodies, and citric acid in GS subjects during fasting showed a pronounced catabolic condition in mildly hyperbilirubinaemic subjects, which is marked by enhanced lipid oxidation and furthermore explains the underlying mechanism of bilirubin's beneficial influence on metabolic diseases by reducing the risk for CVD and DMT2. The significantly higher serum level of leucine's catabolite 2-oxoisocaproic acid indicates for the first time an altered leucine metabolism in mildly hyperbilirubinaemic subjects.

7. SUMMARY

Mildly elevated bilirubin levels, which are characteristic of subjects with Gilbert's Syndrome (GS), were repeatedly associated with decreased risk of CVD, diabetes mellitus type 2 and all-cause mortality. Additionally, subjects with GS were observed to have lower BMI, lean body composition and a favourable lipid profile. Previous studies have shown that bilirubin can bind to PPARα, inducing the transcription of genes involved in lipolysis and liver ketogenesis under catabolic condition. Mölzer et al. found activated PPARα and PPARγ but also phosphorylated AMPK and PGC-1α elevated in subjects with GS during fasting, suggesting a boosted energy metabolism through mildly elevated bilirubin level. The aim of this thesis was to broaden the current knowledge of bilirubin's link to energy metabolism. Therefore, a comprehensive NMR approach was used to investigate the metabolome of GS subjects. Acetylcarnitine, ketone bodies and citric acid were observed to be significantly higher in GS subjects compared to healthy controls. These metabolites are well known proxies of acetyl-CoA, which are excessively produced during ß-oxidation. The high level of acetylcarnitine, ketone bodies and citric in GS subjects is in accordance with increased AMPK and PPAR activity. This was already shown in mice but is a novel finding in humans. Furthermore, 2-oxoisocaproic acid, an intermediate of leucine's degradation, was found to be significantly higher in GS subjects compared to healthy controls. This entity is known to enter the Krebs cycle or alternatively contribute to acetyl-CoA production during catabolic condition. The metabolic shift towards enhanced lipid metabolism could explain the favourable body composition and lipid profile as well as the low risk for metabolic diseases of GS subject. In accordance, acetylglycine, a glycine-based metabolite was significantly higher in GS subjects and glycine has recently been inversely associated with a reduced incidence of CVD and DMT2. Investigations into antioxidative agents have received a lot of attention over the last years. Bilirubin particle or bilirubin enhancing agents might play a promising role in future therapies of metabolic diseases.

8. ZUSAMMENFASSUNG

Ein moderat erhöhter Bilirubinspiegel wird in der Literatur oft mit einem niedrigeren Risiko für Herz-Kreislauf-Erkrankung und Diabetes Typ 2, sowie einer niedrigeren Gesamtmortalität assoziiert. Dieses Merkmal ist typisch für Personen mit Gilbert-Syndrom (GS), weshalb diese Population gerne in Bilirubin-Studien als Referenzgruppe herangezogen wird. Außerdem wird bei diesen Personen wiederholt ein niedriger BMI, sowie ein vorteilhaftes Lipidprofil beobachtet. Dies äußert sich insbesondere durch niedrigere Cholesterin-, Triglycerid-, sowie Lipoproteinspiegel. Studien konnten zeigen, dass Bilirubin an das Peroxisom-Proliferator-aktivierte Rezeptor α (PPARα) binden kann, welches für die Transkription von Genen der Lipolyse und der Ketogenese verantwortlich ist. Mölzer et al. haben herausgefunden, dass Personen mit GS eine höhere Aktivität von Adenosinmonophosphat-aktivierte Proteinkinase (AMPK), Peroxisom-Proliferator-aktivierter Rezeptor Gamma Koaktivator 1 α (PGC1α), PPARα und PPARγ während eines Fastenstoffwechsels aufweisen. Diese Ergebnisse suggerieren, dass ein moderat erhöhter Bilirubinspiegel zu einem verstärkten Energie- und Fettmetabolismus führen kann. Um das Verständnis über den Zusammenhang zwischen Bilirubin und dem Energiemetabolismus auf metabolischer Ebene zu erweitern, wurde in dieser Masterarbeit die Kernresonanzspektroskopie (NMR)-Analyse angewendet, um das Metabolom von 112 Personen mit GS gegenüber einer Kontrollkohorte zu untersuchen. In Übereinstimmung mit Tierstudien, konnte eine signifikant höhere Acetylcarnitin-, Ketonkörper- und Zitronensäurekonzentration bei Personen mit GS festgestellt werden. Diese Metabolite sind wohlbekannte Vertreter des Acetyl-CoA, welches während einer Beta-Oxidation vermehrt produziert wird. Zusätzlich ist die 2-Oxo-isocapronsäure, welche ein Zwischenprodukt des Leucinabbaus ist, signifikant höher in der GS Gruppe. Bekanntlich ist sie ein Metabolit im Citratzyklus oder trägt zur Produktion von Acetyl-CoA während einer katabolen Phase bei. Der verstärkte Lipidstoffwechsel bei Personen mit GS, insbesondere im Katabolismus, könnte zum Teil eine Erklärung für das vorteilhafte Lipidprofil und das geringere Körpergewicht liefern und Aufschluss über den Zusammenhang

von Bilirubin und dem niedrigeren Risiko für Stoffwechselerkrankungen geben. Infolgedessen könnte die Supplementierung von Bilirubin bzw. die endogene Bilirubinerhöhung in Zukunft eine Rolle bei der Prävention oder Therapie von Stoffwechselerkrankungen spielen.

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10. APPENDIX

OPLS analysis

	SS	DF	MS	F	p	SD
Total corr.	112	112	1			1
Regression	14.6927	2	7.34636	8.30461	0.000437525	2.71042
Residual	97.3073	110	0.884612			0.940538