

DISSERTATION

Role of Bile Acid Signalling in Non-alcoholic Fatty Liver Disease and Progression to Non-alcoholic Steatohepatitis in Morbid Obesity

submitted by

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Declaration

I hereby certify that this thesis leading to the award of PhD is entirely my own original work and contains no results which have been accepted for the award of any other degree or diploma in any university or other institution. Additionally, I certify the full acknowledgement of co-workers and their institutions who contributed to the research of this thesis. For content that has been previously published or written by another person, due reference has been made in the text. Also due acknowledgement is stated in the text for used materials. Throughout the thesis and in all associated publications I followed the guidelines of “Good Scientific Practice”.

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Ich habe gelernt, dass der Weg des Fortschritts weder kurz noch unbeschwerlich ist.

Marie Curie (1867 – 1934)

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Abbreviations

NAFLD, non-alcoholic fatty liver disease; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; IR, insulin resistance; FFA, free fatty acid; vWAT, visceral white adipose tissue; TNF α , tumor necrosis factor alpha; IL6, interleukin 6; iRS-1, insulin receptor substrate-1; ROS, reactive oxygen species; ATP, adenosine triphosphate; ER, endoplasmic reticulum; PNPLA3, patatin-like phospholipase domain-containing protein 3; TG, triglyceride; NAS, NAFLD activity score; SAF, steatosis, activity and fibrosis; ALT, alanine aminotransferase, AST, aspartate aminotransferase; GGT, gamma-glutamyltransferase; FA, fatty acid; LPL, lipoprotein lipase; FATP, fatty acid transport protein; FAT, fatty acid translocases; FABP, fatty acid binding protein; DNL, *de novo* lipogenesis; SREBP-1c, sterol responsive element-binding protein-1c; ChREBP, carbohydrate response element-binding protein; PPAR γ , peroxisome proliferator-activated receptor gamma; LXR, liver X receptor; IRE, insulin response element; ACC, acetyl-coenzyme A carboxylase; FASN, fatty acid synthase; ELOVL, elongation of long-chain fatty acids; DGAT, diacylglycerol acyltransferase; SCE, stearoyl-coenzyme A desaturase; ACS, acetyl-coenzyme A synthetase; ATGL, adipose triglyceride lipase, HSL, hormone sensitive lipase; MGL, monoacylglycerol lipase; cAMP, cyclic adenosine 3',5'-monophosphate; PKA, protein kinase A; VLDL, very low-density lipoproteins; MTP, microsomal TG transport protein; CETP, cholesteryl ester transfer protein; LDLR, low density lipoprotein receptor; HNF4, hepatocyte nuclear factor 4; APOB, apolipoprotein B; CYP4A, cytochrome P450; acyl-CoA, acyl-coenzyme A esters; CPT-1, carnitine palmitoyl transferase-1, CACT, carnitine acyl-carnitine translocase; LCFA, long chain FA; VLCFA, very long chain FA; AOX, fatty acyl-CoA oxidase; RXR α , retinoid X receptor alpha; RYGBP, Roux-en-Y gastric bypass; UDCA, ursodeoxycholic acid; T2D, type 2 diabetes; HMG-CoA, β -hydroxy- β -methylglutaryl CoA; HMGCR, HMG-CoA reductase; SREBP2, sterol regulatory element-binding protein 2; BA, bile acid; MCA, muricholic acid; CDCA, chenodeoxycholic acid; CA, cholic acid, DCA, deoxycholic acid; LCA, lithocholic acid; CYP7A1, cytochrome P-450 enzyme cholesterol 7 α -hydroxylase A1; BSEP, bile salt export pump; ABC, ATP-binding cassette; MRP2, multidrug resistance-associated protein 2;

ASBT, apical sodium,dependent bile salt transporter; NTCP, Na⁺-taurocholate co-transporting polypeptide; OATP, organic anion transporting polypeptide, FXR, farnesoid X receptor; SHP, small heterodimer partner; LRH-1, liver receptor homolog-1; FGF19, fibroblast growth factor 19; JNK, c-Jun N-terminal kinase; PXR, pregnane X receptor; CAR, constitutive androstane receptor; TGR5, G protein-coupled receptor 5; VDR, vitamin D receptor; SOCS3, cytokine signalling 3; PBC, primary biliary cirrhosis; miRNA, micro RNA; RISC, RNA-inducing silencing complex; UTR, untranslated region; HCV, hepatitis C virus; SIRT1, sirtuin; U/L, unit/liter; MA, myristic acid; PA, palmitic acid; SA, stearic acid; OA, oleic acid; TFA, total fatty acid;

Zusammenfassung

Die Ansammlung von Lipiden in der Leber ist ein Merkmal der nicht-alkoholischen Fettlebererkrankungen (NAFLD). Das Krankheitsbild von NAFLD kann sich von einer Fettleber (Steatose) über eine nicht-alkoholische Leberentzündung (Steatohepatitis), Vernarbung des Lebergewebes (Fibrose) bis hin zum Endstadium der chronischen Lebererkrankungen, einer Zirrhose, und zum Leberkrebs erstrecken. Fettleibigkeit gepaart mit Insulinresistenz führen zu einem erhöhten Transport von Fettsäuren aus dem Fettgewebe in die Leber und tragen so zur Entstehung und dem Fortschreiten der Krankheit bei.

Gallensäuren sind durch ihre Detergenzwirkung primär für ihre Rolle in der Fettverdauung bekannt. Nach neuesten Kenntnissen sind sie auch potente Signalvermittler. So aktivieren sie z.B. den nukleären Gallensäure-Rezeptor Farnesoid-X-Rezeptor (FXR) und hemmen dadurch ihre eigene Synthese sowie die Lipidsynthese. Ursodeoxycholsäure (UDCA), eine hydrophile sekundäre Gallensäure verbesserte die Insulinresistenz und die Steatose bei Mäusen. Nichtsdestotrotz sind noch zahlreiche Fragen bezüglich ihrer Wirkmechanismen bei NAFLD-PatientInnen offen. Daher untersuchten wir potentielle UDCA-Mechanismen und deren Einflüsse auf Stoffwechselvorgänge in adipösen NAFLD-PatientInnen. Während die Interventionsgruppe für drei Wochen mit UDCA behandelt wurde, erhielt die Kontrollgruppe keine medikamentöse Therapie. Zur weiteren biochemischen Analyse wurden den PatientInnen vor Beginn und nach Ende der UDCA-Applikation Blutproben abgenommen. Ebenso wurden Leber- und Fettbiopsien den PatientInnen beider Gruppen nach Ende der Interventionsphase entnommen. Die Alters- und Geschlechterverteilung wie auch der Body-Mass-Index beider PatientInnengruppen waren ohne signifikante Unterschiede. Hinsichtlich der Leberhistologie zeigte sich eine Zunahme des Steatose- und NAFLD-Aktivitätsgrades unter UDCA. Im Gegensatz dazu verbesserte die Gabe von UDCA Serumleberwerte wie z.B. Aspartat-Aminotransferase und Gamma-Glutamyltransferase.

Die Serumkonzentration des Fibroblasten-Wachstumsfaktors 19 (FGF19), einem Inhibitor der Gallensäuresynthese, war in der UDCA-Gruppe verringert, was mit einer gesteigerten Gallensäuresynthese, erhöhten Gallensäurepräcursor- und

primären Gallensäurenkonzentrationen korrelierte. So hatten UDCA-behandelte PatientInnen im Vergleich zu PatientInnen der Kontrollgruppe am Ende der Studie eine um ca. 10-fach höhere Gallensäurekonzentration im Serum.

In weiterer Folge analysierten wir Gene, die die Gallensäurebiosynthese regulieren. Die mRNA- und Proteinexpression von CYP7A1, einem Schlüsselenzym in der Gallensäurebiosynthese, waren erhöht, während die Expression von FXR und dem transkriptionellen Repressor SHP unverändert blieben. Wir untersuchten daher die FXR-Aktivität und stellten eine reduzierte Rezeptoraktivierung als Folge der UDCA-Behandlung fest. Weitere Serumanalysen innerhalb der UDCA-Gruppe zeigten Veränderungen im Cholesterin- und Fettstoffwechsel: Die Proteinexpression der HMG-CoA Reduktase, dem Schrittmacherenzym in der Cholesterinbiosynthese und ihrem transkriptionellen Regulator Stearoyl response element-binding protein-2, war induziert. Zudem beobachteten wir einen Expressionsanstieg des LDL-Rezeptors, was auf eine erhöhte Cholesterinaufnahme aus der Zirkulation hindeutet. Der somit gesteigerte Cholesterinimport in die Leber, kombiniert mit der zusätzlich induzierten Cholesterinbiosynthese, lieferten dabei das Substrat für die Gallensäuresynthese. Im Gegensatz zu Vorstudien bei PatientInnen mit Gallensteinleiden, fanden sich bei den adipösen PatientInnen unter UDCA keine Unterschiede in der Transporterexpression.

Die Lipidverteilung entlang der Fettgewebs-Leber-Achse war durch eine Akkumulation von Triglyceriden in der Leber und im Fettgewebe in der UDCA-Gruppe gekennzeichnet. Für die vermehrte Triglyceridbildung konnte die erhöhte Expression der Stearoyl-CoA-Desaturase (SCD) identifiziert werden, jenes Enzym, das die Synthese einfach-ungesättigter Fettsäuren wie z.B. der Ölsäure katalysiert.

Das Fettsäure-Profil des visceralen Fettgewebes zeigte unter UDCA im Pool der totalen Fettsäuren eine Akkumulation der Ölsäure, die präferentiell in Triglyceriden gespeichert wird. Zudem verringerte sich die Konzentration mehrerer Fettsäurearten im Pool der freien Fettsäuren. Aus diesen Daten schließen wir, dass freie Fettsäuren vermehrt in Triglyceride eingelagert wurden. Im Gegensatz dazu zeigte die Fettsäurezusammensetzung der Leber einen unveränderten Anteil an freien Fettsäuren und eine Zunahme an Fettsäuren im Pool der totalen

Fettsäuren, was auf eine erhöhte Einlagerung von Lipiden in die Leber unter UDCA hindeutet.

Micro-RNAs (miRNAs), kurze nicht-kodierende RNA-Sequenzen, regulieren die Genexpression auf Translationsebene, indem sie an mRNA binden und deren Abbau einleiten. Sie üben eine Schlüsselrolle in der Homöostase von Stoffwechselvorgängen, wie z.B. Fett- und Cholesterinstoffwechsel aus und sind daher in NAFLD relevant. Basierend auf einer Versuchsreihe in primären Rattenhepatozyten wurde gezeigt, dass nach Verabreichung von Fettsäuren eine erhöhte Expression von miR-34a und die dadurch aktivierten pathologischen Signaltransduktionswege mittels UDCA unterdrückt werden konnten. Allerdings hatte UDCA in adipösen PatientInnen keine Auswirkung auf die Expression von miR-34a und die damit in Zusammenhang stehenden apoptotischen Signalwege.

Zusammengefasst zeigen diese Daten, dass UDCA die Aktivierung von FXR verringert, wodurch die Gallensäurebiosynthese angeregt wird, die auf das Cholesterin der Leber zurückgreift. Folglich wird die Cholesterinbiosynthese sowie die Cholesterinaufnahme aus der Zirkulation durch eine erhöhte LDL-Rezeptor-Expression stimuliert. Die erniedrigte FXR-Aktivität steigert die SCD-Expression in der Leber und dadurch auch die Triglyceridbildung. Prinzipiell stellt die induzierte SCD-Aktivität in der Leber und im Fettgewebe einen möglichen Schutzmechanismus dar, der die Freisetzung und Zirkulation von potenziell toxischen Fettsäurespezies vermeiden könnte.

Summary

Non-alcoholic fatty liver disease (NAFLD) is characterized by hepatic lipid accumulation. NAFLD represents a spectrum of disease ranging from simple steatosis, non-alcoholic steatohepatitis (NASH), fibrosis to cirrhosis or even hepatocellular carcinoma (HCC). Obesity and insulin resistance (IR), by mediating an elevated fatty acid (FA) flux from white adipose tissue (WAT) to the liver, play a central role in disease development and progression.

Bile acids (BAs) are potent signalling molecules implicated in the regulation of multiple metabolic processes. Via the activation of the nuclear BA receptor farnesoid X receptor (FXR), BAs regulate a host of metabolic processes including lipid biosynthesis. Furthermore, Ursodeoxycholic acid (UDCA) a hydrophilic secondary BA with cytoprotective properties improves IR and steatosis in mouse models. However, its efficacy in human NAFLD is under debate.

Therefore, we endeavoured to uncover the mechanisms and efficacy of UDCA in treating morbid obesity in a randomized clinical study. In this study, serum and biopsies from liver and visceral WAT (vWAT) were obtained from NAFLD patients after three weeks of UDCA treatment. Patient groups were without significant differences in gender, age or body mass index. Histological investigations revealed a higher steatosis score and NAFLD activity score in UDCA treated patients. Liver injury was improved upon UDCA reflected by decreased concentrations of serum liver enzymes such as aspartate aminotransferase and gamma-glutamyl transferase. Further serum analysis showed decreased fibroblast growth factor 19 levels, a BA biosynthesis inhibitor. An approximately ten fold induction of the total BA pool was observed due to elevated serum BA precursors and primary BA levels. Analysis of positive and negative regulators of BA biosynthesis in liver tissue revealed the induction of CYP7A1 expression, the major enzyme in BA biosynthesis, on mRNA and protein level. Interestingly, expression of negative regulators of BA biosynthesis such as FXR and SHP remained unchanged on mRNA and protein level. Consequently, FXR activity was assessed and analysis demonstrated repressed FXR activation upon UDCA

treatment. Further serum analysis within the UDCA group before and after treatment, revealed changes in parameters associated with cholesterol metabolism (decreased total and LDL cholesterol, increased cholesterol precursors) and lipid metabolism (elevated triglyceride (TG) and reduced free FA levels). Hepatic stearyl response element-binding protein-2 expression and HMG-CoA reductase activity, the two major factors in cholesterol *de novo* biosynthesis, were induced upon UDCA. Moreover, LDL receptor (LDLR) expression was stimulated upon UDCA suggesting increased cholesterol uptake from the circulation to fuel together with cholesterol generation *de novo* BA biosynthesis. UDCA affects hepatic transporter expression in hepatobiliary disorders. However, in obesity, UDCA had no regulatory effects on transporter expression. Examination of the role of UDCA in lipid partitioning along the WAT-liver-axis indicated the accumulation of TGs in both vWAT and liver: This accumulation was via the induction of stearyl CoA desaturase (SCD) activity, the enzyme generating monounsaturated FAs such as oleic acid (OA). FA profiling of vWAT indicated several changes upon UDCA treatment: While the accumulation of OA, the preferential substrate for the incorporation into TGs, was observed in the total FA pool, the FFA pool showed a reduction of several FA species suggesting a shift in lipid metabolism towards FFA incorporation into TGs. However, hepatic FA profiling uncovered a slightly different pattern compared to vWAT: While the FFA pool remained unchanged, several FA species accumulated in the total FA pool suggesting elevated lipid storage in the liver upon UDCA treatment.

Micro RNAs (miRNAs) are small non-coding RNAs regulating gene expression at the level of translation by binding to mRNAs. MiRNAs are suggested to have a crucial function in metabolic homeostasis thus distinct miRNAs play a role in NAFLD. Induced miR-34a expression and activation of pathological downstream pathways have been shown to be repressed via UDCA in rat primary hepatocytes in NASH. However, UDCA had no impact neither on miRNA expression nor on apoptotic downstream signalling pathways in morbidly obese patients.

In summary, UDCA, by decreasing FXR activity, increases BA generation, thereby depleting liver cholesterol and subsequently inducing cholesterol *de novo* biosynthesis and uptake from the periphery via LDLR. Deficient FXR activation increases SCD mediated hepatic TG formation. In general, induced SCD activity in liver and vWAT might depict a mechanism to prevent the accumulation of potentially toxic FA species.

CHAPTER 1

INTRODUCTION

The proportion of obese people has dramatically increased in recent years. The liver harbours a central function in obesity-associated disorders (1). Unsurprisingly, also the prevalence of non-alcoholic fatty liver disease (NAFLD) as hepatic manifestation and complication of obesity and diabetes, also termed “diabesity”, has dramatically increased over the past decades (1, 2). NAFLD describes a spectrum of hepatic pathologies including simple steatosis or non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), which can progress to advanced fibrosis/cirrhosis and might ultimately lead to hepatocellular carcinoma (HCC) (3).

According to current estimations, up to 30% of the general adult population worldwide has developed NAFLD and in the obese population that rate increases up to 74% (4, 5). Therefore, NAFLD is currently the major chronic liver disease in the western society (6). 30% of NAFLD patients develop NASH, the more aggressive form of the disease characterized by inflammation and fibrosis (collagen deposition) (3). At the time point of diagnosis up to 67% of the patients already display fibrosis. Fibrosis is known to further progress to cirrhosis in about 14% of NAFLD patients (7, 8). 4-22% of HCC cases are associated with NAFLD (9).

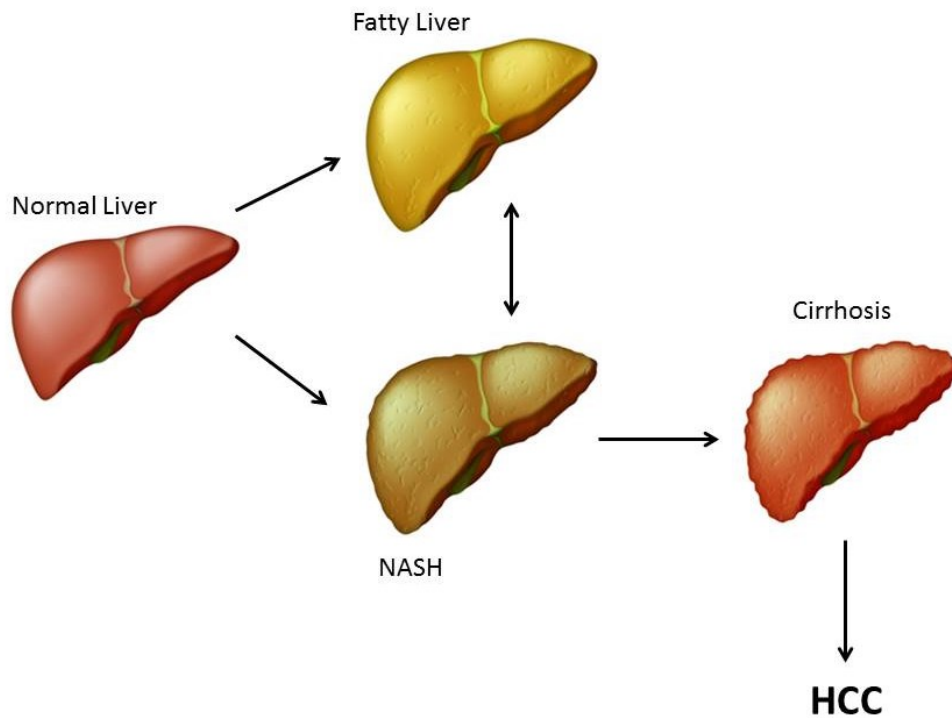


Figure 1. Spectrum of NAFLD. Simple steatosis can progress into more aggressive non-alcoholic steatohepatitis (NASH), fibrosis/cirrhosis or even hepatocellular carcinoma. Adapted from Jonathan C. Cohen et al. (10) and <http://www.bidmc.org/Centers-and-Departments/Departments/Digestive-Disease-Center/Liver-Center/Nonalcoholic-Fatty-Liver-Disease-Program.aspx>.

Recent studies revealed an increased risk for HCC development in non-cirrhotic NAFL/NASH livers, which was attributed to obesity, diabetes and visceral fat accumulation (11-15). In contrast, other reports claim that the severity of underlying liver disease is the major risk factor in HCC formation in NAFLD, which was described to be a rare event in the Japanese society (16). Ethnicity and metabolic factors impact on the prevalence of the disease: Up to 16% Asians, 33% African-Americans and Caucasians and 45% Hispanics are affected by NAFLD. This, to a certain extent, might be explained by the differential distribution of visceral adipose tissue (17).

1.1. Pathogenesis

The main feature of NAFLD is the accumulation of lipids and free cholesterol in the cytoplasm of hepatocytes (10, 18). Besides this, insulin resistance (IR) in liver and adipose tissue is considered the key pathophysiological characteristic of disease development and progression (19). In NAFLD patients, impaired insulin signalling is not limited to liver and adipose tissue, which is indicated by lacking suppression of endogenous hepatic glucose generation and free fatty acid (FFA) flux from adipose tissue. These patients exhibit decreased whole-body insulin sensitivity (20, 21). Since NAFLD is accompanied by a deregulation of several metabolic processes, it is difficult to trace back the onset of IR to one site. However, according to liver transplantation studies, peripheral IR is crucial for the recurrence of NAFLD (22, 23). Impaired peripheral insulin signalling results in uninhibited lipolysis in adipose tissue and an elevated FFA flux to the liver. In turn, the organism compensates high FFA levels with further insulin secretion, promoting hyperinsulinemia, which then induces hepatic *de novo* lipogenesis (3, 22, 23).

Adipose tissue, especially visceral white adipose tissue (vWAT) constitutes the largest endocrine organ. Hormones produced and secreted from adipose tissue are termed adipokines or adipocytokines. Similar adipose derived cytokines (including tumor necrosis factor alpha [TNF α] and interleukin 6 [IL6]), adipokines such as leptin, adiponectin, resistin, apelin, vaspin, visfatin and zinc- α 2-glycoprotein have pro- and anti-inflammatory characteristics (24). Consequently, adipose tissue and its associated hormones have garnered interest within the context of NAFLD research (3, 25).

High serum concentrations of leptin were observed in several stages of NAFLD. Particularly, progressive NASH is associated with hyperleptinemia (26). Leptin-deficient mice, a genetically modified mouse model of NAFLD, spontaneously develop fatty liver, which in some cases progresses to NASH. In turn, administration of leptin, reverses the phenotype (27). It has been shown that insulin secretion as well as tissue responsiveness to insulin is under the regulation of leptin (28, 29). This adipokine exerts complex effects on insulin signalling in the liver, effecting both glucose transport and turnover. Additionally, leptin represses

insulin-mediated phosphorylation of multiple intracellular signalling molecules such as insulin receptor substrate-1 (IRS-1) and decreases hepatic gluconeogenesis. Therefore, leptin potentially influences the development of insulin resistance (28). Furthermore, high leptin levels are described to mediate steatosis via i) the promotion of insulin resistance and increasing serum insulin concentrations and ii) the induction of hepatic *de novo* lipogenesis (29).

While obese and steatotic patients have similar adiponectin serum concentrations, NASH patients have decreased adiponectin levels. Moreover, adiponectin negatively correlates with disease severity: increased scores of inflammation and fibrosis were accompanied by decreased adiponectin concentrations (30-32). However, determination of adiponectin concentrations as an approach to distinguish between NAFL and NASH patients is controversial. It was demonstrated in one study that the decrease in adiponectin serum concentrations

was relative to the reduction of insulin sensitivity and induction of fat content in the liver but was not associated with a higher grade of necroinflammation and fibrosis. In contrast, it was also reported that hypoadiponectinemia was found in patients with more pronounced necroinflammation and fibrosis (33-35).

Resistin is linked to the pathogenesis of obesity-mediated insulin resistance and type 2 diabetes mellitus. It also induces the secretion of proinflammatory cytokines including TNF α and IL12 (36). Noteworthy, increased serum resistin levels are associated with hepatic fat accumulation. However, the function of resistin in disease progression requires further elucidation (37).

Proinflammatory cytokines such as TNF α and IL6 are considered important factors in the development of obesity-associated insulin resistance and steatosis. The adipose tissue is a major site of TNF α production. Serum TNF α levels as well as hepatic and adipose tissue TNF α concentrations were shown to be elevated in animal models of obesity (38, 39). While TNF α administration in vitro or in vivo resulted in impaired insulin signalling, a lack of TNF α or its receptor improved insulin signalling in genetically as well as dietary-induced mouse models of obesity

(40). In humans, TNF α serum concentrations positively correlate with the grade of insulin resistance. Moreover, TNF α administration represses insulin-stimulated disposal of glucose (41).

In addition to TNF α , IL6 is considered to play a role in impaired insulin signalling and the development of steatosis. In animal models of obesity as well as in human patients with NAFLD, IL6 serum concentrations are induced (42). IL6 expression analysis in hepatocytes and Kupffer cells revealed enhanced expression levels in human NAFLD patients (43). Notably, acute and chronic IL6 secretion has opposing effects in the liver: While acute IL6 improves hepatic repair and regeneration processes, chronic IL6 exposure to liver tissue renders the liver more susceptible to injury and apoptosis (44). Indeed, the liver represents the preferential target tissue for IL6 actions consequently leading to induced gluconeogenesis, hyperglycemia and hyperinsulinemia (45).

An additional metabolic abnormality in the pathogenesis of NAFLD and in particular in the progression to NASH is the formation of bioactive products from lipid peroxidation (46). Excess oxidation of FAs leads to the formation of reactive oxygen species (ROS). Elevated ROS levels damage membranes via lipid peroxidation, adenosine triphosphate (ATP) depletion, DNA damage and secretion of proinflammatory cytokines (47). Decreased capacity in mitochondrial oxidation leads to FA deposition in the cytosol, which consequently induces oxidation pathways in other cell compartments such as peroxisomes or microsomes (48). Along with the pro-oxidant conditions in the liver, a repression of super oxide dismutase activity was observed in NAFLD patients (49). Several cell types in liver tissue, including hepatocytes, Kupffer cells, hepatic stellate cells and neutrophils are known to release ROS. Antioxidants have been proven very efficient in the amelioration of oxidative stress in mouse models of NASH. However, efficacy of antioxidant therapy in human NASH is under debate (46, 50).

An additional clinical concern in NAFLD progression is the unfolded protein response and the endoplasmic reticulum (ER) stress. It has been reported that liver and adipose tissue disruption of ER homeostasis has been observed human NAFLD (51).

1.1.1. Genetic Risk Factors in the Development of NAFLD

In several independent studies, the missense variant rs738409 in the patatin-like phospholipase domain-containing protein 3 (PNPLA3) was identified to be associated with hepatic steatosis, irrespective of ethnicity, obesity or diabetic status (52-54). The substitution of methionine for isoleucine at position 148 (I148M) may result in either a loss or gain of function mutation (52, 55). PNPLA3, also termed adiponutrin, is highly expressed in liver and adipose tissue and exerts phospholipase, triglyceride (TG) lipase and acylglycerol transacylase activities (56). Besides its association with NAFLD, the polymorphism in PNPLA3 was related to portal and lobular inflammation as well as fibrosis (57). Other polymorphisms associated with NASH are rs2569190 in the monocyte differentiation antigen CD14 and rs4140528 in GCLC, the gene encoding glutamate-cysteine ligase (58, 59).

1.1.2. NAFLD Definition and Diagnosis

Steatosis or fatty liver, the first stage in the disease, is defined by the amount of TG deposition in cytoplasmic lipid droplets in more than 5% of hepatocytes or TG levels exceeding 55 mg/g liver (60). In addition to the aforementioned characteristics of NASH, such as inflammation and fibrosis, disease progression is also represented by hepatocyte ballooning and cell death (60, 61). Diagnosis of NASH and disease staging are based on tissue biopsies and depicted in the NAFLD activity score (NAS) (62). Due to defined criteria, NAS mirrors the disease progression and is therefore the preferential descriptive evaluation to distinguish between NAFL and NASH and is used to facilitate the comparability and reproducibility of clinical trials (61, 63).

The scoring system includes several histological features, of which four underlie a semi-quantitative evaluation:

- steatosis (0-3)
- lobular inflammation (0-2)
- hepatocellular ballooning (0-2)
- fibrosis (0-4) (61).

Scoring of NAS ≥ 5 correlates with diagnosed NASH and NAS < 3 is categorized as “not NASH” (61).

Bedossa et al. (64) introduced an additional histopathological system in which steatosis, activity and fibrosis (SAF) is scored (64).

Besides histological analysis of hepatic biopsies, NAFLD is diagnosed via imaging examinations such as ultrasonography, in which fatty liver causes increased echogenicity, computed tomography or magnetic resonance imaging, allowing the quantitative assessment of liver fat content. Other diagnostic tools are the analysis of serum concentrations of the liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) or gamma-glutamyltransferase (GGT) (4, 65). However, normal ALT levels were found in the entire spectrum of histologically proven NAFLD, which renders liver enzyme concentrations not sensitive enough

for disease staging (66, 67). So far, histological scoring of liver biopsies provides the most accurate tool for assessing disease stage. Nonetheless, due to limitations in obtaining liver biopsies due to invasiveness and variability in tissue samples, the development of non-invasive diagnostic methods in clinical research gained interest (68, 69). With respect to non-invasive strategies, two different approaches namely liver stiffness measurements via transient elastography and/or serum biomarker-based analysis are currently investigated in their efficacy in distinguishing simple steatosis from NASH (70-72).

1.1.3. Two Hit Hypothesis

In 1998 Day and James presented the currently accepted NAFLD-pathogenesis model, which is based on a “two-hit” hypothesis. According to the proposed model, steatosis in combination with IR are considered the “first hit” (73). The first hit renders the liver more vulnerable to injury. The second hit such as mitochondrial dysfunction, pro-inflammatory cytokines, endotoxin-induced damage, ischemia or ER stress, causes disease progression to NASH (73-75). In line with the “two-hit” hypothesis, mouse models of obesity developed more severe hepatic damage compared to lean controls after administration of “second hits” (e.g. endotoxin or pro-oxidants) (76, 77). Over the past years, the “two-hit”

hypothesis was expanded to a “multiple-hits” model because following the first hepatic injury, further liver damage may be caused by several additional hits (75).

Recent studies have proposed that steatosis may represent a protective mechanism against the development of a more progressive form of the disease. This finding has also contributed to speculations about so called “good and bad fat storers”. While “good fat storers” can easily accommodate an elevated fatty acid (FA)-flux from the periphery via FA incorporation into hepatic lipid droplets, “bad fat storers” are not able to manage the excess and directly develop NASH due to hepatic lipotoxicity. However, liver disease of “good fat storers” may progress to NASH in the presence of a “second hit” (e.g. viral hepatitis, drugs, alcohol) (3, 52, 78).

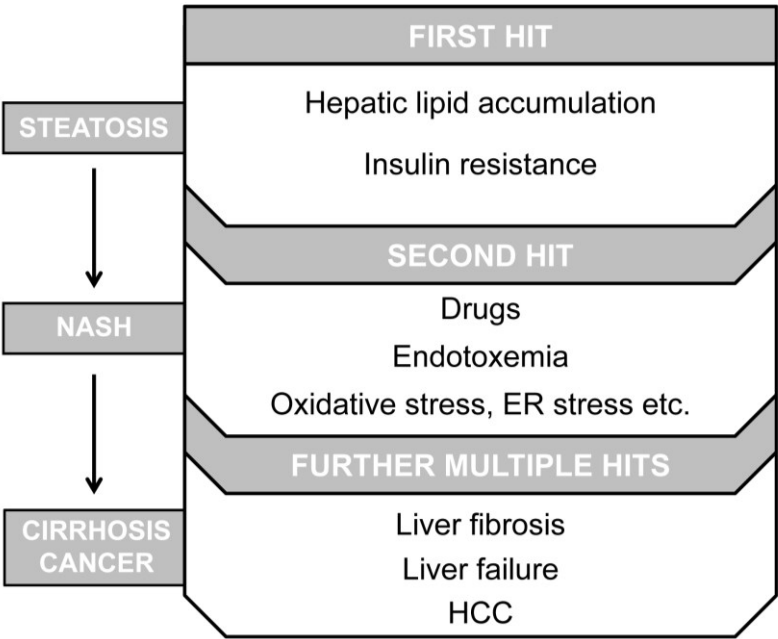


Figure 2. Two/multiple hit hypothesis. Lipid accumulation accompanied by IR represents the first hit, followed by a second hit (e.g. drugs, endotoxins, oxidative or ER stress) which causes NASH development and thereby renders the liver susceptible to further liver injury and disease progression. Figure adapted from Yilmaz Y. (75).

1.1.4. Potential Mechanisms for Hepatic Fat Accumulation

NAFLD arises from an imbalance between uptake and removal of TGs, which are neutral lipids composed of three long-chain fatty acids coupled to a glycerol backbone (10, 79). The fatty acids incorporated into hepatic lipid droplets can derive from several sources:

1. Diet associated increased lipid up-take
2. Induced hepatic *de novo* lipogenesis
3. Fatty acid flux from lipolysis in adipose tissue
4. Reduced export of TGs via very low-density lipoproteins
5. Decreased fatty acid oxidation (80).

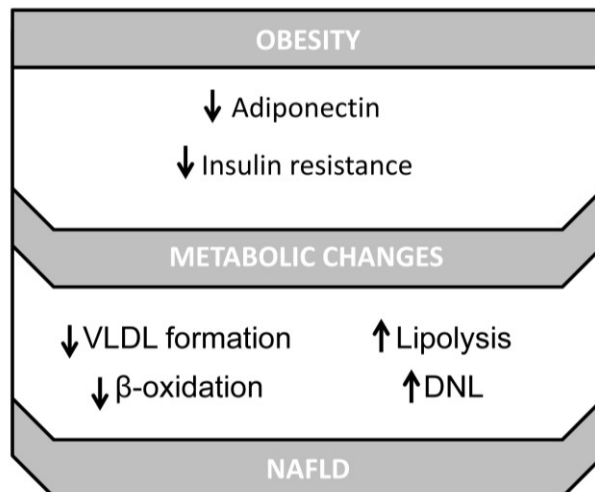


Figure 3. Sources for hepatic TG accumulation and NAFLD development.

Increased calorie intake leading to obesity and elevated dietary fat content are major risk factors in NAFLD development, which can result in decreased hepatic β -oxidation and VLDL-formation as well as induced hepatic *de novo* lipogenesis and lipolysis in adipose tissue. Subsequently high levels of free fatty acids translocate from adipose tissue to the liver. Adapted from Utzschneider et al. (22).

1.1.4.1. Diet Associated Increased Lipid Up-take

The fate of FAs taken up by the liver is divided into three pathways: They are either, stored, oxidized or secreted in the form of TGs (81). Fats derived from the diet are absorbed in the intestine, packaged into chylomicrons and released into the circulation to be hydrolysed by the lipoprotein lipase (LPL). Subsequently, FFAs are delivered to adipose tissue for storage (82). Under conditions of chronic FFA excess, the capacity of adipose tissue to clear the circulation from FFA is exceeded. Consequently, FFAs circulate to other tissues such as liver, where they are retained and incorporated into storage lipid depots. These events can result in fatty liver (81, 83). Isotope labelling studies revealed that FAs in serum originating from lipolysis in WAT constitute up to 60% of TG depositions in NAFLD patients (84). Moreover, NAFLD-patients preferentially consume diets enriched in carbohydrate, sucrose, fructose and have reduced amounts of poly-unsaturated FAs. Reduction in carbohydrate intake significantly improved the grade of steatosis, inflammation and fibrosis (25, 85, 86). Besides the quantity of fat intake, the composition of dietary fat impacts on NAFLD development. While saturated FAs induce hepatic steatosis, monounsaturated FAs improve the lipid profile by reducing concentrations of TGs, cholesterol and low density lipoproteins. Moreover, elevated ω -3 FA consumption ameliorates steatosis due to a reduction of serum TGs, fasting glucose levels and serum liver enzymes (87-89).

FFAs taken up from the circulation are another source of hepatic FA accumulation. Approximately 90% of the total FA import into the liver is performed by three transport protein families: fatty acid transport proteins (FATPs), fatty acid translocases (FATs/CD36) and fatty acid binding proteins (FABPs). Six isoforms are known of FATPs and have varying tissue expression patterns while nine isoforms are described for FABPs (90). FATP2 and FATP5 are abundantly expressed in the liver and they might account for the majority of FAs imported into the liver. A further isoform, FATP1, has been described to be mainly expressed in muscle and adipose tissue (91). FAT/CD36, a transmembrane protein, induces FA translocation into the liver via facilitated diffusion. Under normal conditions, FAT/CD36 is moderately expressed, however expression levels increase in diet-induced obesity (92). Interestingly, FAT expression levels positively correlate with the TG deposition in the liver in NAFLD patients and were shown to be increased

in NASH patients (93, 94). FABPs are located in the cytosol and facilitate intracellular FA transport. Among the nine isoforms, FABP1, FABP4 and FABP5 are predominantly expressed in the liver. Of note, NAFLD patients exhibit higher FABP4 and FABP5 expression (95). The transporters FATP1, FATP5, FAT/CD36, FABP1, FABP4 and FABP5 have come into focus with regard NAFLD-research on account of their tissue distribution and their regulation by peroxisome proliferator activator receptor α and insulin (91, 96).

1.1.4.2. Induced Hepatic *De Novo* Lipogenesis

Hepatic *de novo* lipogenesis (DNL) accounts for about 25% of TGs in human NAFLD and is mediated by the up-regulation of critical transcription factors: sterol responsive element-binding protein-1c (SREBP-1c), carbohydrate response element-binding protein (ChREBP) and peroxisome proliferator-activated receptor (PPAR)- γ (97).

In the liver, SREBP-1c expression is induced via glucose, sterols and saturated FA. In contrast, polyunsaturated FAs repress its expression (98). Insulin elevates SREBP-1c at the level of transcription and translation mediated by a signalling cascade via AKT2, liver X receptor (LXR) and mTOR (99). A multicomponent insulin response element (IRE) is integrated in the SREBP-1c gene promoter, which rapidly induces SREBP-1c expression upon insulin stimulation (100). Under hyperinsulinemic conditions, as observed in obese patients, circulating high levels of insulin enhance expression of SREBP-1c. Consequently, expression of other lipogenic genes such as acetyl-coenzyme A carboxylase (ACC), fatty acid synthase (FASN), elongation of long-chain fatty acids (ELOVL) family member 6, diacylglycerol acyltransferase (DGAT) and stearoyl-coenzyme A desaturase (SCD) is elevated (101-103). An additional pathway for hepatic DNL induction mediated via SREBP-1c is driven by the increase of acetyl-coenzyme A synthetase (ACS) resulting in elevated concentrations of acetyl CoA, which serves as building molecule in FA synthesis (104, 105).

ACC catalyses the formation of malonyl-CoA. FASN, converting dietary carbohydrates into saturated FAs, processes malonyl-CoA to palmitic acid (C16:0) - the first step in FA synthesis (106, 107). ELOVL6 encodes the enzyme mediating the elongation of palmitic acid (C16:0) to stearic acid (C18:0) (108). SCD converts saturated FAs into monounsaturated FAs such as palmitoleic acid (C16:1) or oleic acid (C18:1) (109). The specificity of saturated FAs for SCD in rats are as follows C18:0 > 17:0 > 16:0 > 19:0 > 14:0 > 15:0 (110). In general, SCD activity is determined according to the ratio of C18:1/C18:0 or C16:1/C16:0 (111). Oleic acid (C18:1), the product of SCD, has been shown to be the preferential substrate for TG formation. In turn, SCD activity positively correlates with TG storage and liver fat content in humans (112, 113). Furthermore, SCD has been associated with cholesterol metabolism because SCD provides important monounsaturated FA for cholesterol ester biosynthesis (114).

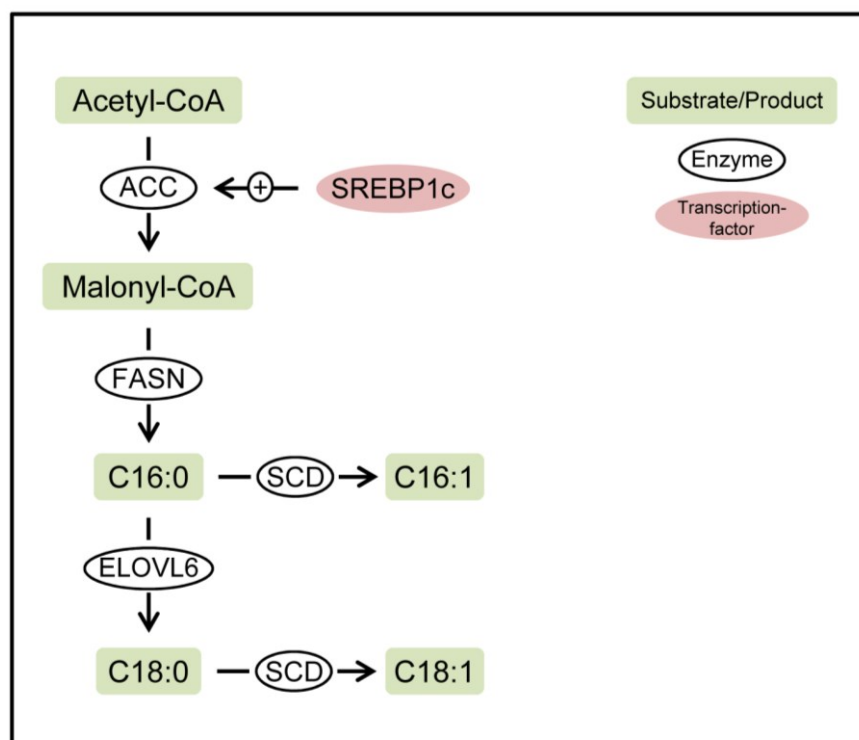


Figure 4. Fatty acid synthesis pathway. The transcription factor SREBP-1c positively regulates enzymes involved in FA synthesis such as ACC, FASN, SCD and ELOVL6. After C16:0 synthesis via FASN and the elongation to C18:0 via ELOVL6, SCD converts saturated FAs into monounsaturated FAs.

ChREBP, a glucose-sensitive transcription factor, is involved in the basal and carbohydrate-induced expression of genes controlling glucose metabolism as well as FA and TG synthesis: ChREBP increases pyruvate kinase expression, a key enzyme in glycolysis, which in turn delivers precursors for lipogenesis and thereby induces the expression of hepatic lipogenic enzymes such as ACC, FASN and SCD (115, 116). Disruption of ChREBP represses expression of aforementioned lipogenic genes and a liver specific inhibition of ChREBP in a mouse model of genetic obesity improves hepatic steatosis (117). ChREBP is directly regulated via the nuclear receptor liver X receptor (LXR), which mediates lipogenesis directly through stimulation of ACC and FASN gene expression and indirectly through SREBP-1c (118).

PPAR- γ expression and activity is mediated by insulin. IR, dyslipidemia and steatosis stimulate PPAR- γ expression in the liver. Two PPAR- γ isoforms exist: PPAR- γ 1 and PPAR- γ 2 (80). While PPAR- γ 1 is expressed in the liver and has been described to stimulate TG accumulation, PPAR- γ 2 promotes adipocyte differentiation and is exclusively expressed in adipose tissue (119, 120).

1.1.4.3. FA Flux from Lipolysis in Adipose Tissue

Uninhibited lipolysis in vWAT due to induced whole-body insulin resistance (IR) is believed to have a major impact in NAFLD development and progression (3). IR describes the bodies inability to respond to insulin and dispose of blood glucose in muscle and WAT, the failure to stop gluconeogenesis in the liver and to adapt insulin secretion from pancreatic beta cells (121). A recent study demonstrated that almost 60% of circulating FFAs accounted for the overall hepatic TG formation in NAFLD patients. 26% of stored TGs are derived from DNL and only 15% from the diet. This data provide strong evidence for the importance of vWAT lipolysis in human hepatic TG deposition (84).

Fat is mobilized from vWAT via lipolysis: The catabolism of TGs into FAs is mediated via several enzymes and is tightly regulated via enzymatic co-factors and hormones (122). The major enzymes catalysing lipolysis are adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase (MGL) (123).

Catecholamines induce lipolysis in adipocytes under conditions of increased energy expenditure via the β -adrenergic receptors. They bind to G protein-coupled receptors and activate a signalling cascade resulting in elevated formation of cyclic adenosine 3',5'-monophosphate (cAMP) and the subsequent induction of cAMP-dependent protein kinase A (PKA) activity (124, 125). PKA phosphorylates HSL and the lipid droplet coating protein perilipin A. Phosphorylation of perilipin A strengthens its interaction with HSL, which is suggested to accelerate enzymatic activity. HSL phosphorylation promotes its translocation from the cytosol to the surface of the lipid droplet (126, 127). Similarly to HSL, ATGL was hypothesized to translocate from the cytosol to the lipid droplet but a recent study identified ATGL as lipid droplet associated protein – in the stimulated and non-stimulated state (123). However, ATGL activation is a PKA-indirect mechanism. ATGL-mediated lipolysis, representing the initial step in TG breakdown, depends on the co-localization of positive or negative regulators. Upon stimulation, comparative gene identification-58 (CGI-58), the co-activator, is released from perilipin A and associates with ATGL. More precisely, PKA phosphorylates perilipin A on Ser492 or Ser517 thereby resulting in the dissociation of the CGI-58/perilipin A-complex and providing “free” CGI-58 for ATGL activation (128-130). G₀/G₁ switch gene 2 (G0S2) was described as a selective ATGL inhibitor sharing 78% homology between mouse and human orthologs. G0S2 directly binds to CGI-58-coupled or -uncoupled ATGL and inhibits its hydrolytic activity (131).

While catecholamines stimulate TG hydrolysis in adipocytes, insulin signalling exerts antilipolytic effects. Considering that NAFLD patients exhibit impaired insulin signalling, ATGL might offer a potential target to counteract uninhibited lipolysis in adipose tissue (123, 132).

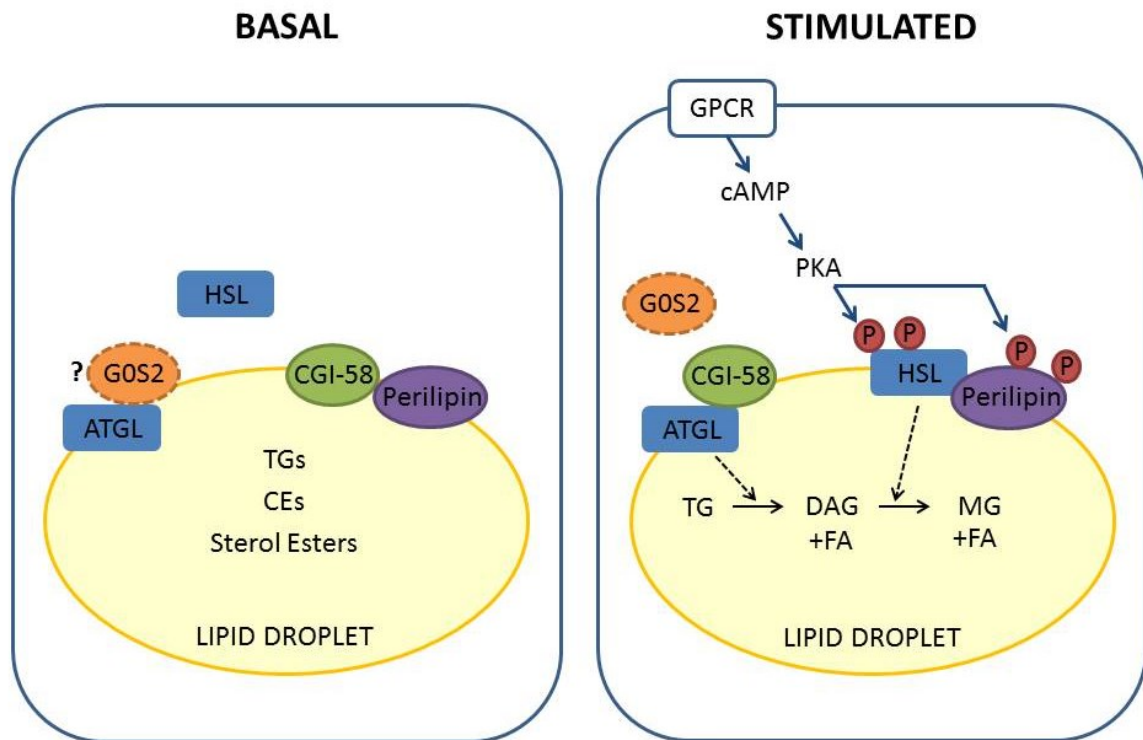


Figure 5. Triglyceride hydrolysis. In the basal state, ATGL is located at the LD and might be bound to its inhibitor G0S2. Inactive HSL remains in the cytosol and the CGI-58/perilipin A complex is on the surface of the lipid droplet. Upon stimulation, PKA phosphorylates perilipin A resulting in the dissociation of the CGI-58/perilipin A complex allowing CGI-58 translocation and ATGL activation on the one hand and induction of HSL hydrolytic activity on the other. Adapted from Granneman et al. (130).

1.1.4.4. Reduced Export of TGs via Very Low-density Lipoproteins

Disturbed lipoprotein metabolism is a characteristic of NAFLD (133). Under normal conditions, non-catabolised lipids are stored in the form of TGs in cytosolic lipid droplets in the liver. Upon increased energy demands in peripheral tissues, TGs are assembled into very low-density lipoproteins (VLDL) and secreted into the circulation. Therefore, TGs are mobilized from lipid droplets via lipolysis and re-esterified via DGAT2. Subsequently, they are translocated via the microsomal TG transport protein (MTP) into the ER for lipidation of apoB100 to form VLDLs and to be finally incorporated into VLDLs. Alternatively, TGs can return to cytosolic lipid droplets. This process is mediated by insulin leading to the repression of VLDL

secretion and an increase of hepatic TG concentrations (134). When released into the circulation via FABP, LPL reduces the TG content of VLDLs to form intermediate-density lipoproteins and low-density lipoproteins. Cholesteryl ester transfer protein (CETP) transfers TGs from VLDL to LDL and cholesteryl esters from LDL to VLDL, thereby enriching VLDLs in cholesterol. Circulating LDL is taken up by the liver via its receptor (LDLR) (135-137).

Steatosis is characterized by the overproduction of large TG-rich VLDLs resulting in hyperlipidemia. Elevated VLDL secretion provides a compensatory mechanism to export TGs in the form of VLDLs from the liver to maintain hepatic lipid homeostasis (135). Since VLDL secretion is inhibited by insulin, obese patients with impaired insulin signalling exhibit elevated VLDL production and abnormal serum lipoprotein concentrations (138). In contrast to steatosis, NASH patients exhibit disturbances in VLDL synthesis and export. Recently it has been reported that liver lipid profiling did not reveal any differences between NAFL and NASH patients. However, VLDL assembly and secretion were deregulated in NASH (139). This may be explained by apoB100 degradation due to ER and oxidative stress following excess FA delivery to the liver and hence a decline in VLDL-secretion (140). Suppression of MTP, mediating the key step in VLDL formation, was reported to be indirectly regulated via FoxO1, hepatocyte nuclear factor (HNF)4 or PPAR α (140). Furthermore, a single nucleotide polymorphism in the MTP-promoter or mutations in apolipoprotein (APO)B can lead to hepatic steatosis. Subjects carrying mutations for these genes exhibit reduced VLDL formation rates and in turn have a three-fold induction in hepatic TG accumulation compared to healthy subjects (141, 142). In addition, reduced LPL activity or induction of the LPL inhibitor apoC3 contribute to less efficient TG hydrolysis and can further exacerbate hyperlipidemia (135).

1.1.4.5. Decreased FA Oxidation

FA oxidation is, besides storage and secretion, a further pathway to handle hepatic lipid accumulation. The liver exhibits a high energy demand to accomplish various metabolic processes. The metabolic rate of the liver reaches approximately the rate of the brain, is twenty times higher than skeletal muscle at resting state and fifty times higher than adipose tissue (143). Although liver mass

comprises only 2.5% of total body mass in lean subjects, hepatic energy consumption rises up to 20% of total spent resting energy, which is mainly caused (up to 90%) by hepatic lipid oxidation (143, 144).

Lipid oxidation takes place in three different compartments: β -oxidation in mitochondria and peroxisomes and ω -oxidation in the ER (81). FAs with up to 20 carbon chains undergo mitochondrial oxidation. Peroxisomal oxidation handles rather complex and toxic FAs with ≥ 20 carbon chains as well as bile acid intermediates. Ω -oxidation in microsomes is responsible for FA hydroxylation by cytochrome P450 (CYP4A), which thereafter might enter mitochondrial or peroxisomal oxidation processes. For instance, toxic dicarboxylic acids resulting from ω -oxidation are substrates for peroxisomal β -oxidation (145).

During mitochondrial and peroxisomal oxidation acyl-coenzyme A esters (acyl-CoA) are dehydrogenated, hydrated and finally undergo thiolitic cleavage (19). During mitochondrial β -oxidation oxygen is entirely reduced to water, whereas hydrogen peroxide is produced by peroxisomal β -oxidation due to the transfer of only two electrons to oxygen. Peroxisomes manage the resulting hydrogen peroxide via resident catalases. Reactive oxygen species bypassing the catalase-pathway are eliminated by the glutathione peroxidase, representing a cytosolic antioxidant (146).

Although oxidative processes are similar in mitochondria and peroxisomes, preferential substrates for oxidation already indicate differences in enzymatic regulation. Indeed, defects in either mitochondrial or peroxisomal oxidation have different metabolic consequences (147, 148).

The mitochondrial β -oxidation can be divided into two major processes: i) transport of acyl-groups into mitochondria for oxidation and ii) intra-mitochondrial oxidative FA-chain shortening by two carbon groups (149). Acyl-CoA esters require a complex transportation system to pass the inner and outer mitochondrial membrane. To pass the outer membrane the acyl-group is attached to carnitine via carnitine palmitoyl transferase-1 (CPT-1). Carnitine acyl-carnitine translocase (CACT) then facilitates transport to reach the inner membrane, where it is then transported via the carnitine palmitoyl transferase-2 (CPT-2) into the mitochondrial

matrix (149). Acetyl-CoA resulting from chain-shortening can enter different pathways depending on the tissue. Acetyl-CoA produced in liver is used for ketone body-formation for the export to peripheral tissues. In heart and skeletal muscle, acetyl-CoA is introduced into the tricarboxylic acid cycle for ATP production (150).

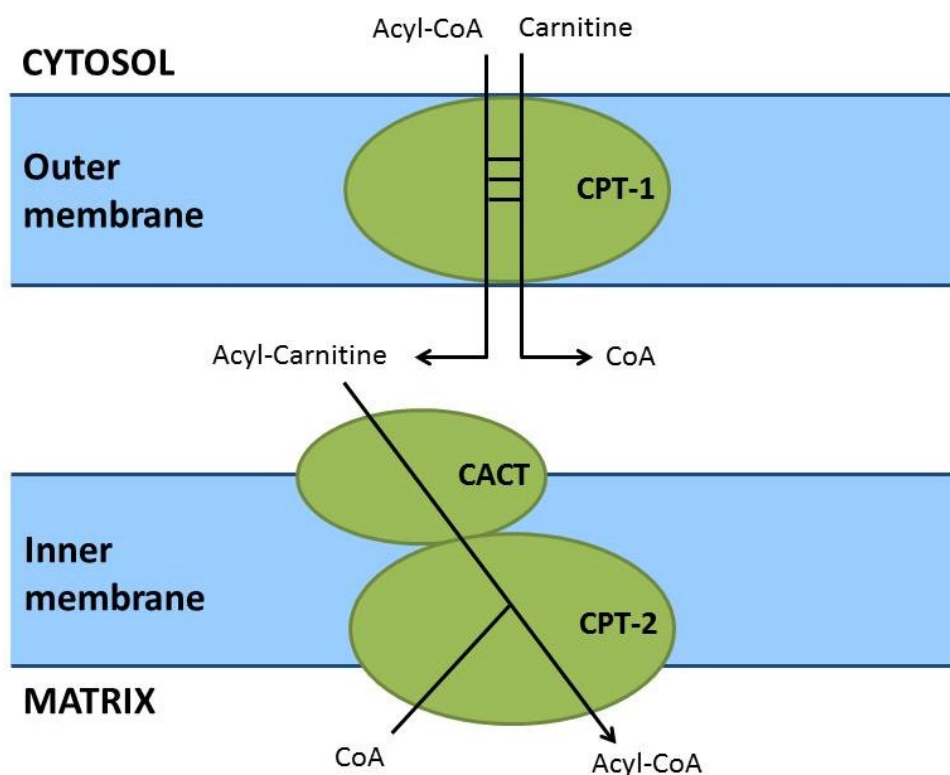


Figure 6. Mitochondrial β -oxidation. Translocation of acyl-CoA across the outer and inner mitochondrial membrane by carnitine palmitoyl transferase-1 (CPT-1), carnitine acyl-carnitine translocase (CACT) and carnitine palmitoyl transferase-2 (CPT-2). Adapted from Bartlet et al. (149).

Long chain FAs (LCFAs) and very long chain FAs (VLCFAs) preferentially enter peroxisomal β -oxidation (151). The oxidation of LCFAs and VLCFAs is catalysed via fatty acyl-CoA oxidase (AOX) and enoyl-CoA hydratase/L-3hydroxyacyl-CoA dehydrogenase, which is a bi-functional enzyme performing hydration and dehydrogenation (152). AOX is a target of the PPAR α , which after activation due to binding to its ligands and co-activator retinoid X receptor alpha (RXR α) stimulates genes involved in peroxisomal, microsomal and mitochondrial FA oxidation. AOX deficiency was reported to promote severe microvesicular

steatohepatitis (145). Moreover, inhibition of CPT-1, translocating acyl-groups across the outer mitochondrial membrane, suppresses FA oxidation and induces hepatic TG accumulation (153). Noteworthy, in mouse models of NAFLD PPAR α expression was significantly reduced and in turn, PPAR α agonist feeding resulted in the regression of hepatic lipid deposition (78).

A further player in FA oxidation is adiponectin exerting anti-inflammatory, anti-diabetic, anti-atherogenic and insulin-sensitizing effects. Adiponectin is primarily synthesized by adipocytes. The relatively high serum levels (5-30 $\mu\text{g/ml}$) inversely correlate with obesity (154-156). Previously it was generally accepted that adiponectin is produced and secreted from adipocytes only. However, recent reports indicate adiponectin secretion from liver cells which were exposed to fibrotic stimuli (157, 158). In the liver, adiponectin binds to its receptor ADIPOR2. The receptor interacts with the adaptor protein APPL-1 and stimulates 5-AMPK and PPAR α . Apart from decreased gluconeogenesis, ACC and FASN expression, adiponectin stimulates CPT-1 activity and thereby induces β -oxidation. In addition, FFA influx is reduced via CD36 repression (154).

1.2. NAFLD Treatment

Recently, the main focus in developing treatment strategies was on NASH patients because of their potential to develop more severe liver disease. Although the exact factors promoting steatosis progression to NASH and end-stage liver disease are not fully understood, the common feature of NAFLD patients is obesity predominantly in the abdominal region. Currently, there is no established treatment for steatosis and the progression to more aggressive disease forms (22, 159). However, weight loss interventions (e.g. low-fat/low-carbohydrate diet, physical exercise, bariatric surgery etc.) are a central approach in the treatment of NAFLD. Importantly, weight reduction needs to be monitored, because massive weight loss within a short period of time leading to an enhanced FFA flux to the liver might worsen liver function and histology (3, 160, 161).

1.2.1. Lifestyle Interventions

The strong link between obesity and impaired insulin signalling on the one hand and development of NAFLD on the other, led to the hypothesis of weight reduction representing the first line therapy for NAFLD patients (25).

Diet based modifications in the lifestyle (e.g. low fat diet) leading to weight loss improved liver transaminases (162). It was previously shown in 1990 that every 1% reduction in body weight improved levels of the liver injury indicator alanine transaminase (ALT) by 8.1% (163). Although abdominal adiposity is a common characteristic in NAFLD, it was observed that before body weight reduction, liver TGs positively correlated with dietary fat but not with visceral fat content. Moreover, reductions in hepatic lipid content upon weight loss were not related to alterations of visceral fat (164). Moderate weight reduction by restricting overall calorie intake improved insulin signalling, liver transaminase levels, liver histology and lowered hepatic TG loads (165-167). In general, weight loss up to 7% is considered safe. A study, comparing 5% and 7% weight loss revealed that only 7% reduction in body weight achieves improvements in necroinflammation and NAS, whereas 5% weight loss showed reduction in steatosis grade and improvement of insulin signalling but was insufficient to change inflammation parameters (159).

Exercise was observed to prevent diet-induced fatty liver in rats, which provided a new approach to treat human NAFLD or at least to prevent disease progression (168). Four weeks of mildly intensity exercise reduced overall body fat, body weight and improved insulin sensitivity (169). Furthermore, a six-month exercise trial with obese diabetic patients revealed reduced VLDL secretion, which was hypothesized to be a consequence of induced lipid peroxidation and the repression of hepatic *de novo* lipogenesis (170).

1.2.2. Bariatric Surgery

Morbidly obese patients were observed as having difficulties in losing weight and maintaining the body weight reduction for a longer period of time (42). Therefore, bariatric surgery is an effective surgical intervention supporting morbidly obese patients in losing and maintaining body weight (25). Roux-en-Y gastric bypass (RYGBP) surgery, replaced the jejuno-ileal bypass, which was the first applied

bariatric intervention and could lead to post-surgical complications such as bacterial overgrowth (171). RYGBP can be performed by two procedures: i) open or ii) laparoscopic. The stomach is divided at the junction of the body and the antrum to form a tube-shaped gastric pouch followed by the connection of a loop of the jejunum with the created gastric pouch (172).

1.2.3. Pharmacological Interventions

Currently prescribed drugs in the treatment of NAFLD are metformin, glitazones, vitamin E, betaine, fibrates and ursodeoxycholic acid (UDCA) (22).

1.2.3.1. Metformin

Metformin is widely used in the treatment of patients with type 2 diabetes (T2D). Metformin exerts insulin-sensitizing actions via the reduction of hepatic glucose output and FFA oxidation as well as induced insulin-mediated glucose uptake in muscle and adipose tissue (173). It also represses the expression of TNF α (174). Metformin treatment in NAFLD resulted in improved liver histology including reduced hepatic TG content, inflammation and fibrosis. Whereas treatment of NASH patients revealed improved transaminase levels and insulin signalling, however, inflammation and fibrosis remained unchanged (175, 176).

1.2.3.2. Glitazones (Thiazolidinediones)

Glitazones activate PPAR γ , which is predominantly expressed in adipose tissue. PPAR γ stimulation induces the differentiation of adipocytes, mediates FA uptake into adipocytes and thereby reduces FA flux to the liver and improves insulin sensitivity (177). Glitazone treatment in NAFLD patients is under debate. While in some studies serum parameters and histology improved, recurrence of elevated liver enzymes and worsening of histology scores were reported upon completing six months of therapy (178-180). Rosiglitazone treatment in NAFLD patients improved steatosis within the first year of treatment, however extension of the therapy had no further beneficial effects (181).

1.2.3.3. Orlistat, Sibutramine and Incretin Mimetics

Medication with Orlistat, Sibutramine and incretin mimetics aims to improve patient's condition via weight reduction. While Orlistat represses gastric and

pancreatic lipases thereby decreasing fat absorption from the diet, Sibutramine acts on the central nervous system by stimulating feelings of satiety (182). Incretins, including glucagon like peptide 1 or gastric inhibitory peptide, are secreted in the gastrointestinal tract upon food intake. Besides weight loss, incretin mimetics have been reported to reduce serum liver enzyme levels and liver TG deposition (183).

1.2.3.4. Statins and Cholesterol Biosynthesis

Cholesterol is an important structural component of higher eukaryotic membranes. Moreover, cholesterol is involved in the organization, dynamics and function of cell membranes. According to the 'Bloch hypothesis', the biosynthesis pathway of cholesterol evolved out of a natural selection process, which optimized eukaryotic cell membranes taking over several biological functions (184, 185). In humans, cholesterol biosynthesis accounts for approximately two thirds of the whole body cholesterol content (186). Cholesterol synthesis is a complex process including about 20 enzymes. β -hydroxy- β -methylglutaryl CoA (HMG-CoA) reductase (HMGCR) is the key enzyme in the cholesterol biosynthesis pathway. HMGCR catalyzes the reduction of HMG-CoA to mevalonate (185). HMGCR is positively regulated by the transcription factor sterol regulatory element-binding protein 2 (SREBP2). However, reductase activity depends on the degree of protein dephosphorylation (187). Along with HMGCR phosphorylation, serum levels of desmosterol, which is a cholesterol precursor, are indicative for elevated cholesterol biosynthesis (188). Statins are structural analogues of HMGCR and hence act as HMGCR inhibitors, thereby decreasing cholesterol biosynthesis and circulating cholesterol concentrations (189).

1.3. Bile Acid Metabolism

Besides phosphatidylcholine, bilirubin and cholesterol, bile acids (BAs) are a main component of bile. Their function is to facilitate uptake and emulsification of dietary derived lipids and fat soluble-vitamins including A, D, E and K. Additionally, BA biosynthesis represents the pathway for excess cholesterol removal from the body (190).

BAs are C-24 molecules with 5 α or 5 β cholic acid as basic ring structure with detergent properties. In bile or blood, BAs circulate in an anionic salt form, are therefore negatively charged and referred to as bile salts representing the major transport form of glycine and taurine conjugated BAs (191). Most BAs are synthesized via oxidation from cholesterol in the liver via the classical or so-called neutral pathway (192). In humans, most of the newly synthesized BAs derive from the classical pathway and only approximately 10% are generated via the alternative pathway (193). Of note, the BA pool of human and mice are quite different: The BA pool of mice predominantly consists of muricholic acid (MCA) and cholic acid (CA), whereas the human BA pool is mainly comprised of chenodeoxycholic acid (CDCA), cholic acid (CA) and deoxycholic acid (DCA). In general, BAs such as the primary BAs CA and CDCA as well as the secondary BAs including lithocholic acid (LCA) and DCA are maintained at low concentrations not reaching the critical micellar concentration, which describes the concentration limit at which BAs exert their tensioactive activities (194).

The different composition of the BA pool of mice and humans is reflected in the BA properties. While the murine BA pool is more hydrophilic, CDCA, CA and DCA render the human BA pool more hydrophobic. The differences in hydrophilicity/hydrophobicity can be attributed to different hydroxylation processes (195).

The BA biosynthesis pathway comprises of a variety of sequentially acting enzymes. The most important and rate limiting enzyme in the neutral pathway catalysing the initial step is the microsomal cytochrome P-450 enzyme cholesterol 7 α -hydroxylase A1 (CYP7A1). CYP7A1 synthesizes 7 α -hydroxycholesterol (7 α -OH-cholesterol), which is then processed to the primary BAs CDCA and CA (196). A characteristic of the alternative pathway is the synthesis of several oxysterols before 7 α -hydroxylation. Interestingly, oxysterols undergo processing by CYP7A1. Moreover, 7 α -hydroxylation of oxysterols has been shown to repress their function in inhibiting SREBP, which is an important transcription factor of lipogenic gene expression (197). As a consequence, 7 α -hydroxylation of oxysterols impacts on lipid metabolism via the induction of SREBP-regulated gene expression including LDLR, ACC, FASN or SCD (193, 198).

The synthesis ratio of CDCA/CA in humans and MCA/CA in mice depends on the activity of sterol 12 α -hydroxylase (CYP8B1), which is the enzyme generating CA. Finally, BAs are conjugated with glycine or taurine via the bile acid-CoA synthetase (BACS) and the BA CoA amino acid N-acyltransferase (BAAT) which transform BAs into bile salts. Furthermore, in a knock out mouse model, the conjugation of secondary BAs was shown to involve FATP5 (199-201). BAs are secreted into bile canaliculi via the bile salt export pump (BSEP), an ATP-binding cassette (ABC) transporter located at the canalicular or apical site of the hepatocyte or via the canalicular multidrug resistance-associated protein 2 (MRP2) (202). Besides BA transport, MRP2 regulates the export of several anions such as bilirubin, chemotherapeutics, antibiotics, toxins, leukotrienes and heavy metals (203). An additional exporter for sulfated conjugates in humans is ABCG2. Along with BAs, cholesterol is secreted into bile via the apical transporters ABCG5 and ABCG8. Additionally, ABCA1 exports cholesterol derived from macrophages at the basolateral site of hepatocytes (204). Subsequently, bile is collected in the gallbladder. Cholecystokinin, a hormone secreted into the duodenum, mediates gallbladder contraction and bile secretion via MRP3 into the duodenum. BA absorption in the upper part of the intestine is prevented by their negative charge, which increases the retention time in the intestine and induces their efficacy in emulsifying FA from the diet. BAs are actively absorbed in the terminal ileum via the apical sodium-dependent bile salt transporter (ASBT) and return back to the liver via MRP3, located at the basolateral site in enterocytes. BAs are taken up by the liver via Na⁺-taurocholate co-transporting polypeptide (NTCP) at the basolateral site of hepatocytes. Na⁺-independent BA import into the liver is conducted via organic anion transporting polypeptides (OATPs) (193). This circuit is referred to as the “enterohepatic circulation”. Approximately 95% of BAs secreted into the liver enter the enterohepatic circulation up to twelve times and are transported back to the liver by either passive diffusion or active transport via BA transport proteins (205, 206).

Via bacterial transformation in the intestine, the primary BAs CDCA and CA are converted into the secondary BAs LCA and DCA (196, 207). The two main microorganism families present in the human intestine are Lactobacillaceae and Bacteroidaceae and to a smaller extent microorganisms such as *Escherichia coli*,

other coliforms and *Clostridia*. The 55 genera identified in the intestine are strictly anaerob (208, 209).

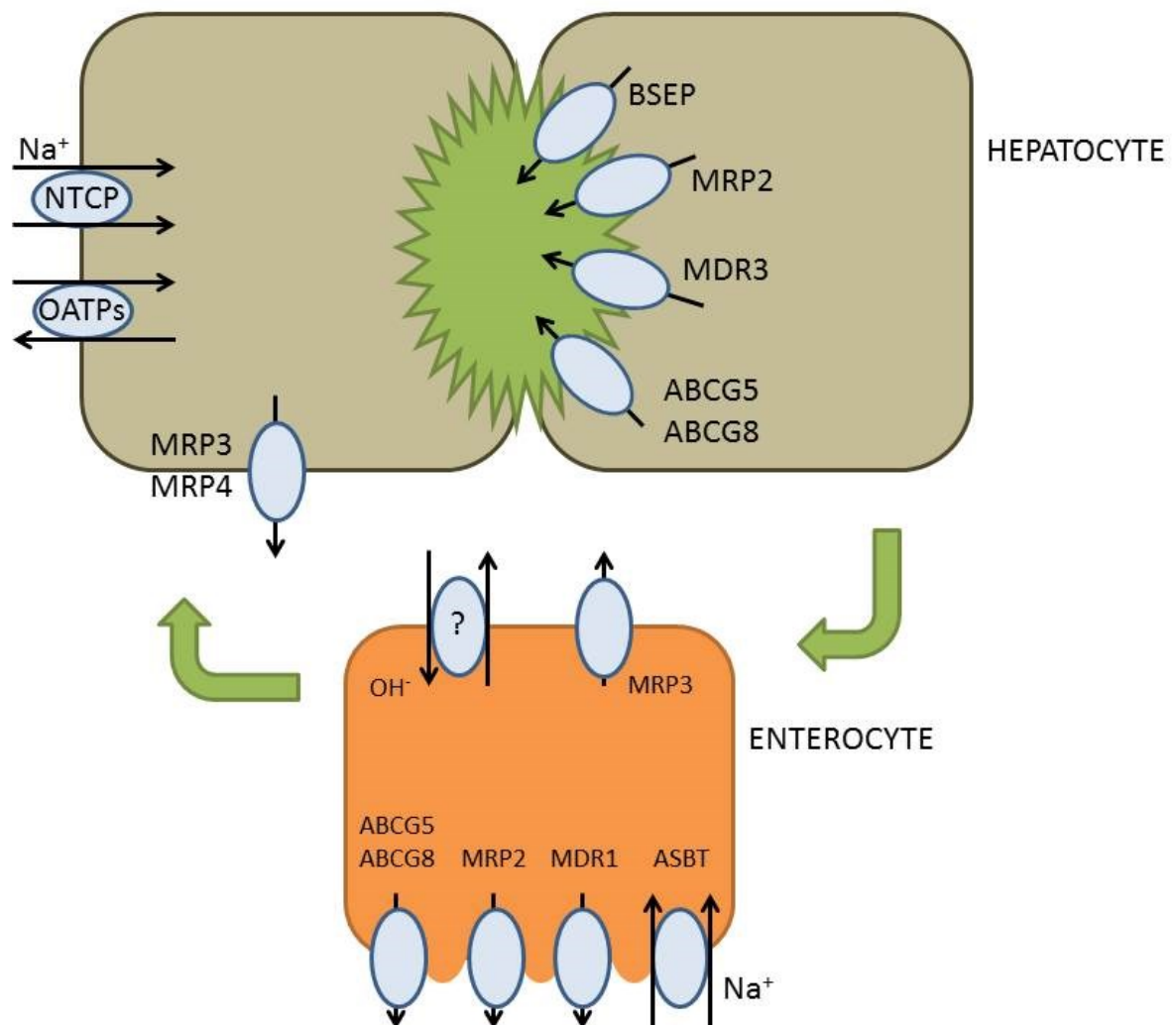


Figure 7. BA transporters in human hepatocytes and enterocyte. At the canalicular membrane of the hepatocyte, BA export is mediated via BSEP. Along with non-bile molecules, MRP2 secretes sulfated or glucuronidated BAs. Phospholipids and cholesterol are exported via MDR3 and ABCG5/ABCG8, respectively. MRP3, located at the basolateral membrane exhibits low affinity for monovalent BAs. MRP4 exports anionic conjugates such as sulfated BAs. ASBT is a Na^+ -dependent BA import pump at the luminal membrane. In enterocytes, MRP3 represents the basolateral efflux system. MDR1 exports xenobiotics. At the basolateral hepatocyte membrane, BA uptake is mediated via NTCP and OATPs. Adapted from Kullak-Ublick et al. (193).

1.3.1. Regulation of Bile Acid Metabolism

Cholesterol represents the building block for BA biosynthesis. When cholesterol reaches the liver via the so called “reverse cholesterol transport”, BA biosynthesis is stimulated (210). In the liver, a fraction of the cholesterol is processed to oxysterols, which in turn stimulate CYP7A1 expression via LXR activation in mice but not in humans (211, 212). Further regulation of BA biosynthesis is conducted via the nuclear farnesoid X receptor (FXR): BAs reaching a certain concentration in the liver repress CYP7A1 expression via FXR activation. FXR, thereafter, stimulates the expression of the atypical nuclear receptor small heterodimer partner (SHP) leading to the repression of CYP7A1-activators such as liver receptor homolog-1 (LRH-1), LXR and HNF-4 α . The inhibition of the aforementioned nuclear receptors via SHP/LRH-1 interaction ultimately decreases BA biosynthesis and also BA accumulation in the liver (213-215). The BA composition depends on CYP8B1 activity, which is regulated by LRH-1 and HNF-4 α (216). Intestinal activation of FXR stimulates fibroblast growth factor 19 (FGF19) production and secretion (FGF19: referred to as Fgf15 in mice). After FGF19 secretion into the circulation, it translocates to the liver, where its uptake is mediated by the FGF receptor 4 (FGFR4). Hepatic FGF19 uptake results in tyrosine kinase and c-Jun N-terminal kinase (JNK) activation, thereby repressing CYP7A1 mediated BA *de novo* biosynthesis (217, 218). BAs regulate their own biosynthesis via the activation of two complementary negative feedback loops (FXR/SHP in the liver and FGF19 via the intestine) preventing the hepatic accumulation of excess BAs, which would exert cytotoxic functions if left unregulated (219).

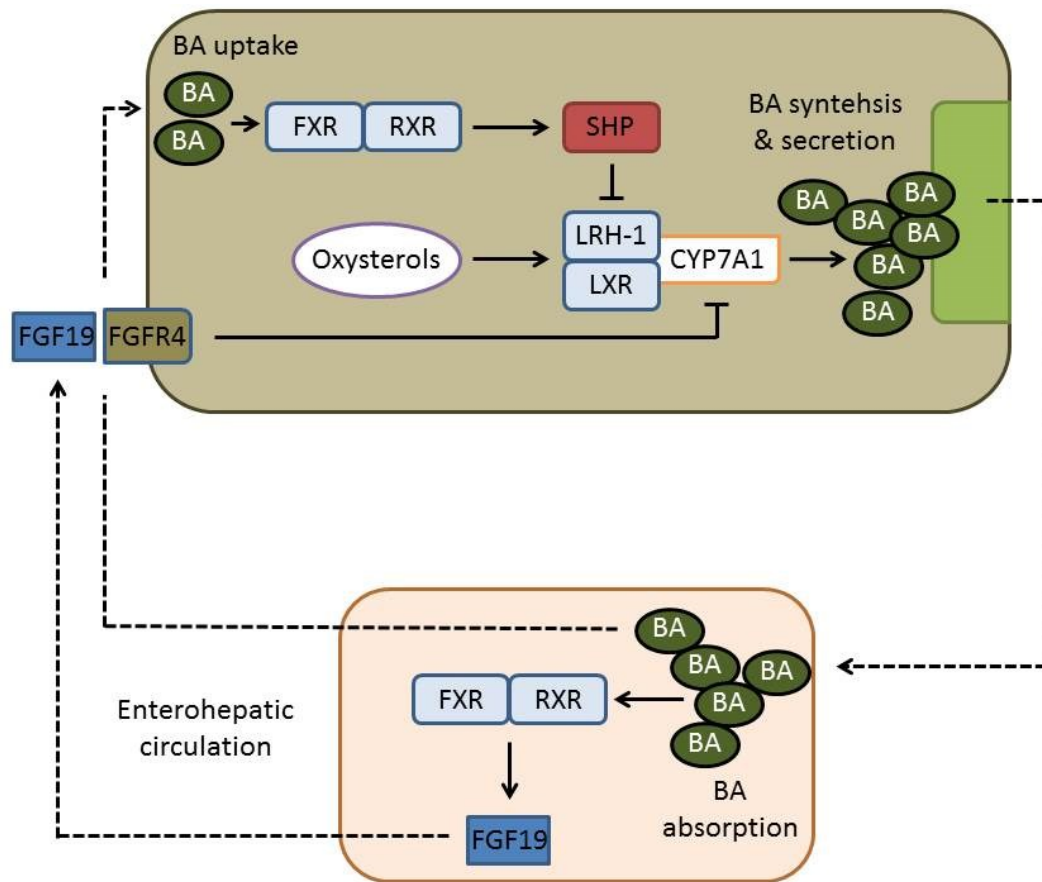


Figure 8. Regulation of BA biosynthesis. CYP7A1 is the key enzyme in BA *de novo* biosynthesis. After BA generation in the liver and release into the circulation, BAs activate intestinal FXR and stimulate FGF19 synthesis and secretion into the enterohepatic circulation. BAs and FGF19 returning to the liver stimulate a negative feedback loop via FXR and SHP and via FGF19 receptor mediated signalling. Adapted from Garruti et al. (220).

1.4. Farnesoid X Receptor

This receptor was named according to its ligand farnesol which activates FXR under supra-physiological conditions. Further analysis identified CDCA and CA as endogenous FXR ligands. FXR expression analysis revealed its presence in liver, intestine, kidney and adrenal glands and to a lower extent in heart, adipose tissue and blood vessels (221, 222). FXR and RXR form a heterodimer. Along with FXR ligands, the FXR/RXR heterodimer is also activated by RXR ligands. Interestingly, BAs additionally activate pregnane X receptor (PXR), constitutive androstane

receptor (CAR), G protein-coupled receptor 5 (TGR5) and the vitamin D receptor (VDR) (222).

1.4.1. FXR in Lipid Metabolism

Along with the regulation of BA biosynthesis, FXR exerts relevant actions in lipid, triglyceride and glucose metabolism. FXR deficiency in mouse models revealed an impact on TG metabolism. The physiological relevance of FXR gene disruption is displayed by hypercholesterolaemia, hypertriglyceridaemia and induced cholesterol uptake from the intestine in FXR knockout mice (223, 224). This phenotype arises from FXR-mediated regulation of lipoprotein metabolism. FXR stimulates APOC2 expression, which coordinates the interaction of LPL with VLDLs and chylomicrons thereby leading to a reduction in serum TG concentrations (225). Furthermore, FXR regulates lipogenic gene expression including SREBP1C. SREBP1C is suggested to be repressed via FXR-mediated SHP induction because SREBP1C repression was lacking in shp-null mice. Of note, FAS promoter analysis revealed an inverted repeat site in the promoter region to which FXR binds. Therefore, FAS is suggested to represent a direct transcriptional target of FXR (226, 227). Interestingly, in human but not in mouse cell lines, FXR stimulates PPAR α expression, which in turn results in an elevated oxidation of FAs (228). FXR expression is activated by PPAR γ co-activator 1 α (PGC1 α), a regulator of genes involved in energy metabolism (229). In addition, FXR reduces TNF α production from adipocytes and circulating FFA levels (230).

1.4.2. FXR in Glucose Metabolism

Another unexpected observation in the past was that FXR substantially impacts on liver carbohydrate metabolism. It has been shown in several studies that FXR activity exerts a plasma glucose lowering effect and decreases gluconeogenesis. FXR activation attenuates the expression of pyruvate kinase and ACC. Moreover, FXR agonist treatment in diabetic mice reduced gluconeogenic gene expression including glucose-6-phosphatase and in turn induced glycogen synthesis and overall glycogen content (231, 232). FXR-associated changes in gene expression were related to reductions in plasma glucose, TG and cholesterol levels, as these alterations were not observed in FXR knockout mice (233). Additionally, FXR deletion caused TG accumulation in the liver as well as impaired insulin signalling

in mice. Interestingly, the FXR target FGF19 in humans and Fgf15 in mice, represses gluconeogenesis by inhibiting CREB and down-regulating PGC1 α (231, 234).

1.4.3. FXR in Inflammation

FXR activation via the natural ligand CDCA improves hepatic inflammation, reduces transaminase serum levels, represses proinflammatory cytokines such as IL-6 and TNF α and blocks STAT3 phosphorylation. In addition, FXR induces the expression levels of suppressor of cytokine signalling 3 (SOCS3), which negatively regulates cytokine-mediated STAT3 signalling (235).

1.5. UDCA

Originally, the use of UDCA was widely spread in the traditional Chinese medicine for the therapy of jaundice. The name “urso” arises from the Latin word for “bear” because UDCA comprises the major BA in bears. In the early 1960s, UDCA found its way into western medicine - first described in Japan (236).

UDCA is a 3 α ,7 β -dihydroxy-5 β -cholanoic acid or in other words it is a BA carrying a hydroxy group (-OH) at position 3 and 7. The β -orientation at position C-7 renders the BA more hydrophilic than its epimer chenodeoxycholic acid (CDCA) - an important characteristic ascribing UDCA less cytotoxic effects due to its decreased ability to interfere with and thereby damage lipid membranes (194).

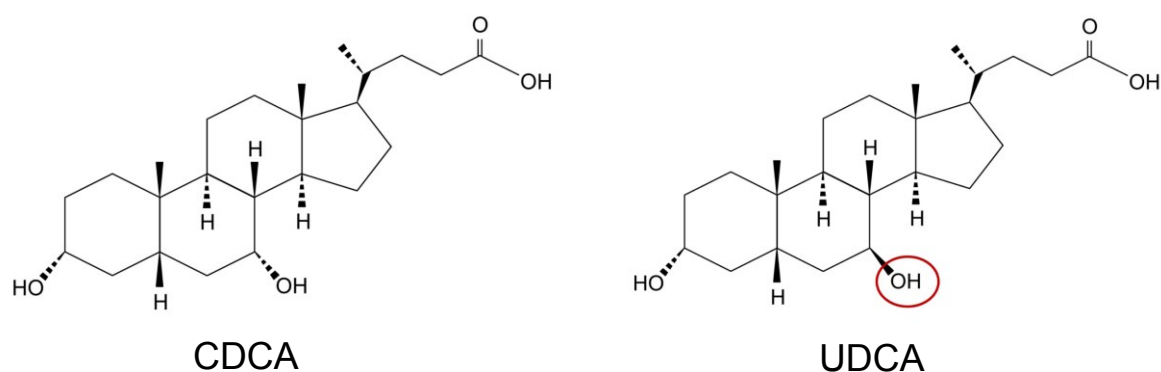


Figure 9. Structure of the primary BA CDCA and the secondary BA UDCA. CDCA and UDCA are different in the orientation of the hydroxy-group at C-7 (red circle). Adapted from Im et al. (237).

Under normal conditions, only 4% of UDCA is found in the total BA pool. Besides its hydrophilic properties, UDCA and/or its conjugated forms have proven beneficial by exerting anti-apoptotic effects. Of note, apoptosis can be induced by BAs at concentrations of about 25 μ M, which are achieved under cholestatic conditions (238).

So far, UDCA administration is established for the clinical treatment of hepatobiliary disorders such as primary biliary cirrhosis (PBC) (239). In addition, UDCA has been shown to decrease serum liver enzymes in several studies with different duration periods (three to twelve months). However other studies could not reveal beneficial effects of UDCA neither on serum biochemistry parameters nor on liver histology (240).

1.5.1. UDCA Effects on Hepatic Transporter Expression

UDCA's hydrophilic properties are relevant in its therapeutic application because UDCA dilutes the potentially hydrophobic/cytotoxic BA pool. In addition, it facilitates the urinary excretion of other toxic cholephilic substances via i) repression of the basolateral BA uptake, ii) overexpression of BSEP and iii) stimulation of BA hydroxylation (241). UDCA induces the expression of basolateral export pumps including Mrp3 and Mrp4 on mRNA and protein level.

Stimulated expression leads to the secretion of amidated, glucuronidated and sulfated BA into the circulation resulting in elevated serum BA concentrations and their elimination via the urine. This was observed in healthy and PBC patients upon UDCA treatment (242, 243). UDCA exerts no or very weak FXR activating effects but has been described to be a strong PXR agonist. Interestingly, these two nuclear receptors regulate the expression of genes involved in lipid, lipoprotein and BA metabolism in a complementary manner (244). UDCA therapy in gallstone patients revealed a UDCA-mediated induction of BSEP and MRP4 expression but not of OATP1, MRP2 and MRP3 (245). UDCA administration to healthy patients has no effect on transporter expression levels. However, early-stage PBC patients suffering from MRP2 down-regulation show normalized MRP2 expression levels upon UDCA therapy (246). Of note, UDCA counteracts cholestatic factors rather than changing MRP2 expression because UDCA exerts beneficial effects in early-stage PBC patients, however, only in patients with pro-inflammatory cytokines as predominant cause for cholestatic effects (247).

1.5.2. UDCA in NAFLD

Several clinical studies have been published presenting data on UDCA treatment in NAFLD and NASH patients. The fact that different UDCA dosages over different periods of time were applied, makes any study comparison complicated. For some studies NASH was even not histologically proven (248).

However, in histology proven studies using UDCA at a dosage between 12 and 28 mg/kg/day with a treatment time between 18 and 24 months, analysis revealed increased ALT and AST serum levels. In the high-dose UDCA study, treatment improved lobular inflammation and reduced GGT levels. Nevertheless, there was also a trend towards increased fibrosis scores. Therefore, high-dose UDCA therapy in NASH should be prescribed with caution (249-251).

In a mouse model of T2D, UDCA administration improved hepatic but not peripheral IR. Despite induced expression of lipogenic genes including FASN, other parameters such as HMGCR expression, liver TG and cholesterol concentrations were decreased. In addition, UDCA treatment reduced fasting

glucose and insulin levels. However, ER stress markers remained unchanged upon UDCA in this mouse model of T2D (252).

Another clinical study with 27 female obese participants ascribes UDCA beneficial effects: In this six weeks trial, UDCA reduced steatosis (determined by abdominal ultrasound) as well as AST and ALT serum concentrations and induced weight loss (253).

Since hydrophobic BAs might cause complications upon liver transplantation, UDCA was considered to prevent BA mediated adverse effects. UDCA administration (13-15mg/kg/day for 4 weeks) improved serum biochemistry after liver transplantation in 56 patients reflected by reduced ALT, AST and GGT levels (254).

1.6. microRNA

MicroRNAs (miRNAs) have been discovered in *Caenorhabditis elegans* in 1993 by Ambros and colleagues and are characterized as small noncoding RNAs composed of 18 to 24 nucleotides (255). miRNAs control gene expression at the level of translation due to their interference with mRNAs. So far identified miRNAs are collected in the miRBase, which is under the administration of the Sanger Institute. The miRBase is fully publicly available via <http://microrna.sanger.ac.uk/>. According to bioinformatic approaches and computational calculations, a single miRNA might interfere simultaneously with more than 100 mRNAs. To date, approximately 1000 miRNAs are transcribed from the human genome. Of note, about 30-60% of protein coding genes are under the control of human miRNAs (256). The location of miRNAs in the genome can vary: intergenic, intronic, the far more rare exonic miRNAs as well as miRNAs within long non-coding RNAs have been found. Their expression can follow sense and antisense direction (257).

There are several steps of miRNA maturation: Genes encoding miRNAs are transcribed in the nucleus from DNA to primary transcripts (pri-miRNA). Pri-miRNAs are cleaved by the enzymes Drosha and Pasha in the nucleus.

The resulting shorter precursor miRNA (pre-miRNA) is exported into the cytoplasm via exportin-5, where it assembles in a protein complex, the so called RNA-inducing silencing complex (RISC) (258, 259). RISC is composed of i) Dicer, an RNA-specific endonuclease mediating the maturation of pre-miRNA to mature miRNA; ii) argonaute (Ago) protein, which is also known as “slicer” and cleaves the target mRNA; iii) the P-body protein PW182; iv) the trans-activating response RNA-binding protein (TRBP) mediating the translocation of Ago2 to the complex; and v) the fragile X mental retardation protein (FMRP1), which binds to polyribosomes (260-263). miRNA synthesis is regulated at the transcriptional and post-transcriptional level by transcription factors and changes in mRNA processing, respectively. Furthermore, single nucleotide polymorphisms in miRNA genes are suggested to regulate miRNA activity and function (264).

It was generally accepted that miRNAs inhibit the expression of their target genes. However, recent studies report that the site of miRNA interaction with the target gene is crucial for miRNA function in repressing or activating gene expression. While the interaction in the 3' untranslated region (UTR) represses target gene expression, the interaction in a non-3' UTR-dependent manner results in the stimulation of target genes. Of note, miRNAs, which target sequences out of the 3'-UTR, are less frequent. A single 3'-UTR might be regulated by several miRNAs. The impact of miRNA on gene expression is reflected by target gene RNA levels (258, 259, 265). According to computational analysis, nucleotides two to eight from the 5'-end of the miRNA are most critical and essential for the interaction of the miRNA with its target. If the base-base interaction is established between the 3'-end of the miRNA and the target sequence under conditions of strong complementarity, then nucleotides on position two to five are important for the interaction (266).

1.6.1. miRNAs in the Liver

miRNAs mediate the regulation of several biological processes in different organs including the liver. Moreover, multiple studies revealed the influence of miRNAs on various liver diseases such as viral hepatitis, autoimmune liver disease and NAFLD (267). It has been shown that miRNAs are important factors in metabolic homeostasis (268).

In the liver, miR-122 accounts for up to 70% of miRNAs with an estimated copy number ranging from 15.000 to 25.000 copies per 10 pg of total liver RNA (269). miR-122 is highly conserved over different species indicating a significant function for this miRNA that has been under selective pressure throughout evolution (256). It regulates several genes implicated in hepatic lipid and cholesterol metabolism. Interestingly, miR-122 repression led to a decrease of plasma cholesterol levels in mice. Reduced miR-122 expression levels were observed in human and animal models of NASH. Deletion of the gene encoding miR-122 resulted in steatohepatitis, fibrosis and liver cancer (270-273). Since miR-122 does not exhibit toxic effects, it was identified as promising therapeutic target in the treatment of hypercholesterolemia. Administration of miR-122 inhibitors strongly repressed genes regulating cholesterol synthesis, including 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1), HMGCR and 7-dehydrocholesterol reductase (DHCR7). However, the mechanism underlying the regulation of lipid and cholesterol metabolism remains to be elucidated (270). Noteworthy, several miR-122 target genes have been discovered as regulators of glucose, iron homeostasis and members of the Crebs cycle, including aldolase A. Furthermore, miR-122 is involved in the propagation of hepatitis C virus (HCV). The binding of miR-122 to two positions in the 5'UTR of the HCV genome is required for the accumulation of the virus and its propagation in infected hepatocytes. Recently, a study uncovered that miR-122 silencing reduced the accumulation of HCV virus and strongly improved liver pathology without the generation of viral resistance (274-277).

Further regulators of hepatic lipid and cholesterol metabolism are miR-33, miR-34, miR-103, miR104 and miR-370 (268). MiR-33a and miR-33b are intronic miRNAs embedded in the genomic sequence of SREBP1 and SREBP2, which are co-transcribed with their reference gene. Consequently, miR-33a and miR-33b are involved in the regulation of cholesterol and FA metabolism. Overexpression of miR-33 represses ABCA1 at the transcriptional and the translational level and, therefore, reduces cellular cholesterol export. MiR-33 repression induces cholesterol efflux, which in vivo results in elevated plasma HDL concentrations and improvement of atherosclerosis (278, 279). Moreover, CPT1 α , carnitine O-octanoyltransferase (CROT), hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (HADHB), SIRT6 and AMPK, genes involved in

β -oxidation of FAs, are regulated via miR-33a and miR-33b. Additionally, miR-33a and miR-33b also target an important component of the hepatic insulin-signalling pathway, namely the insulin receptor substrate 2 (IRS2). Besides lipid and glucose metabolism, miR-33 plays an important role during cell cycle progression as demonstrated via miR-33 silencing, which improves hepatic regeneration upon partial hepatectomy (280, 281).

Another important player in liver disease in the context of cancer formation is miR-21. MiR-21, targeting PTEN, has been reported to be highly expressed in HCC resulting in induced cell proliferation and migration of HCC cell lines (282, 283). Besides lipid/cholesterol metabolism, miR-34a is implicated in apoptosis signalling. MiR-34a represses hepatic sirtuin 1 (SIRT1) expression, which results in the activation of the pro-apoptotic protein p53 in mouse models and human NAFLD. Interestingly, this pathway has been shown to be inhibited by UDCA when administered to primary rat hepatocytes (284).

1.6.2. miRNAs in Adipose Tissue

Drosophila represents the first in vivo model used for investigations on miRNA function in adipocyte metabolism (285). Mutations in the miR-278 encoding gene resulted in a leaner phenotype with higher circulating glucose concentrations in flies (286). MiR-335 represents a miRNA involved in lipid metabolism and adipocyte differentiation. Recently it has been shown that miR-335 is also implicated in adipose tissue inflammation, an important signalling pathway in mediating insulin resistance in obesity (287). Obese subjects and subjects suffering from obesity-associated type 2 diabetes have higher circulating leptin levels. Leptin, a hormone and cytokine secreted from adipocytes, has been shown to induce miR-335 expression. This might account for the induced basal inflammatory state. Additionally, resistin, another adipocyte-derived hormone, has been associated with IR. Resistin administration in mice stimulated miR-335 expression, suggesting that resistin and miR-335 are involved in the development of obesity (288, 289). Previously it was suggested that IL-6 induces miR-335 expression. Furthermore, other cytokines such as TNF- α and/or IL-1 β might up-regulate miR-335 expression by the release of inflammatory signalling molecules

(288). Interestingly, miR-335 as well as adipogenic gene expression, including PPAR γ and aP2, were induced after the stimulation of adipocyte differentiation. These data suggest that miR-335 might play a role in the differentiation process and in lipid accumulation (290).

CHAPTER 2

AIM OF THE STUDY

2.1. PART 1: UDCA in the Regulation of Bile Acid, Cholesterol and Lipid Metabolism in Morbid Obesity

UDCA is established for the clinical treatment of hepatobiliary disorders. It has been shown to improve IR and steatosis in mouse models. However, the efficacy and possible effects of UDCA treatment in human NAFLD is under debate. Therefore, in the present study we aimed to investigate the potential effects and underlying mechanisms of short-term high-dose UDCA on the interplay of hepatic and vWAT metabolism in (i) BA and cholesterol homeostasis, (ii) biliary transporter expression and (iii) FA/lipid partitioning in morbidly obese patients with NAFLD/NASH awaiting bariatric surgery.

2.2. PART 2: UDCA in the Regulation of miRNA Expression in Morbid Obesity

miRNAs are implicated in the development of numerous human diseases. It is known that miRNA expression is deregulated in NAFLD. UDCA represses apoptosis via the down-regulation of miR-34a expression in rat primary hepatocytes.

Therefore, our aim was to delineate potential UDCA mediated regulatory effects on miRNA expression and down-stream signalling in liver and adipose tissue of morbidly obese patients after three weeks of UDCA treatment.

CHAPTER 3

MATERIAL AND METHODS

3.1. PART 1: UDCA in the Regulation of Bile Acid, Cholesterol and Lipid Metabolism in Morbid Obesity

3.1.1. Study Population

Patients scheduled for laparoscopic Roux-en-Y gastric bypass surgery for morbid obesity were recruited for the clinical study of metabolic and molecular effects of UDCA. All candidates completed a clinical research file consisting of a detailed questionnaire about the patient's history and life-style. A total of 40 well matched patients were divided into two study groups: control and UDCA treated group. They were randomized by drawing lots to treatment with UDCA (Ursofalk®, 20 mg/kg/day, for three weeks) or no medication (controls) three weeks before surgery. UDCA was administered open-label in two daily doses until the day before surgery. Liver, kidney, intestinal or metabolic diseases other than NAFLD/NASH (ALT/AST/GGT < 3xULN) in addition to medication known to affect liver function and metabolism, for example lipid or glucose lowering drugs, P450 enzyme inducers, and serotonin reuptake inhibitors were classified as exclusion criteria. Tissue samples from liver and vWAT were obtained during surgery. Based on standard criteria (63), fatty liver and steatohepatitis were determined from liver biopsies after surgery.

All participants provided written informed consent. The study protocol (ClinicalTrials.gov NCT01548079) was approved by the Ethics Committee at Karolinska Institutet (Dnr 2008/2:3) and the Swedish Medical Products agency (EudraCT 2007-005531-28).

3.1.2. Serum Clinical Chemistry, C4, and FGF19/21

Cholesterol (total, HDL and LDL), triglycerides, as well as liver and kidney function tests were analyzed using standard clinical biochemical procedures. Serum 7 α -hydroxy-4-cholesten-3-one (C4) was measured by HPLC in comparison to the internal standard 7 α -hydroxy-4-cholesten-3-one (Steraloids, RI, USA). Serum FGF19 and FGF21 were measured in the fasting state at 8:00 AM by ELISA according to the manufacturer's handbook (R&D Systems, Oxon, UK).

3.1.3. Serum Bile Acids

Serum bile acids were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using electron ionization in negative mode on a Waters LC-MS/MS Quattro Micro, equipped with a C18 reversed-phase column. Column flow was 0.8 mL/min. Mobile phase started with 29% water containing 0.01% formic acid and ammonium acetate at 5 mM and 71% methanol containing 0.01% formic acid and ammonium acetate at 5 mM. The gradient ended after 24 minutes at 100% methanol containing 0.01% formic acid and ammonium acetate at 5 mM. Capillary voltage was 2.7 V. Cone voltage was 60 V for unconjugated bile acids, 53 V for glycine- and 85 V for taurine-conjugated bile acids. Collision energy was 10 V for unconjugated bile acids, 40 V for glycine- and 63 V for taurine-conjugated bile acids. Stable isotope-labeled reference compounds (D4-CA, D4-UDCA, D4-LCA, D4-GCA, D4-GUDCA, D4-GLCA) were from Qmx laboratories (Essex, UK), unlabeled unconjugated (LCA, DCA, CDCA, HDCA, UDCA, CA, HCA) and glycine- or taurine-conjugated reference bile acids (GLCA, GDCA, GCDCA, GCA, GUDCA, TLCA, TDCA, TCDCA, TCA, TUDCA) were from Sigma-Aldrich (St Louis, MO, USA) and Steraloids.

3.1.4. Serum Sterols

Serum desmo-, and sitosterols as well as 7-hydroxysterols were analyzed by gas-chromatography-mass spectrometry (GCMS) as previously described (291).

3.1.5. mRNA Expression Analysis

Biopsies from liver and vWAT were immediately frozen in liquid nitrogen. RNA was extracted according to the manufacturer's protocol using peqGOLD TriFast™ reagent (PEQLAB). Complementary DNA (cDNA) was synthesized in a reverse transcription (RT) reaction from 1.5 µg RNA via PCR using SuperScript® II Reverse Transcriptase (Invitrogen), random nonamers and deoxynucleotide sets (Sigma). Quantitative real time-PCR (qRT-PCR) was performed on a 7500 Fast

Real-Time PCR System (Applied Biosystems) using SYBR®Green Master Mix (Applied Biosystems): 20 sec at 95°C., followed by 40 amplification cycles (3 sec at 95°C, 30 sec at 60°C). Samples without template served as negative control determining the specificity of qRT-PCR primers. mRNA cycle threshold (Ct) values were normalized to the housekeeping gene 18S and relative quantity of target mRNA was calculated using integrated 7500 Software v2.0.5.

3.1.6. Protein Extraction and Western Blot Analysis

Protein extraction for hepatobiliary transporter analysis was performed as previously described (245, 292, 293).

To extract cytosolic fractions from liver, micropestles (Eppendorf®) were used to homogenize 50-70 mg of tissue in 10 mM HEPES, 10 mM KCl, 1.5 mM MgCl, pH 7.5, followed by 15 min incubation on ice. After adding 10% Surfact-Amps NP-40 Detergent Solution (Thermo Scientific) and brief vortexing, samples were centrifuged (10000 g x 30 sec, 4°C). The supernatant comprised the cytosolic fraction. To extract nuclear fractions, pellets were re-suspended in 20 mM HEPES, 420 mM NaCl, 1.5 mM MgCl, 25% glycerol, pH 7.5, incubated on ice for 45 min and centrifuged (10000 g x 10 min, 4°C). Proteins were quantified according to Lowry (294), separated on 7.5% glycine gels and transferred to nitrocellulose membranes for 1 hr 15 min at 100 V in Trans-Blot Protein Transfer Systems (BioRad). After membrane blocking and overnight incubation with primary antibodies, membranes were washed and probed with HRP-conjugated secondary antibodies, which were detected using Pierce ECL 2 Western Blotting Substrate. For quantification of relative expression levels, blots were scanned using a densitometer (BioRad) and quantified with QuantityOne soft-ware (BioRad). Protein

signals detected were normalized to β -Actin for cytosolic extracts or Fibrillarin for nuclear extracts and the relative expression levels were calculated accordingly.

3.1.7. Avidin Biotin Complex DNA-Assay

The avidin, biotin, complex DNA (ABCD)-Assay was used to immobilize the FXR/RXR heterodimer/DNA-complex via binding of the biotinylated FXR/RXR target DNA sequence to streptavidin coated beads. The assay was performed according to the protocol in Baumann et al. (295).

3.1.8. TG and Cholesterol Determination

Frozen tissue samples were used for total lipid extraction according to the method of Folch (296). Dried lipids were dissolved under sonication in 0.1% Triton-X 100. TG concentrations were determined with a kit from Diagnostic Systems International (Holzheim, Germany). Cholesterol content was quantified with the Cholesterol/Cholesteryl Ester Quantitation Kit from Abcam.

3.1.9. Adipose Tissue and Liver Lipid Profiling

GC-NICI/MS of free fatty acids

For lipid profiling, about 50 mg of human white adipose tissue or liver were homogenized in 2.5 ml of methyl-tert-butylether (MTBE) and 0.7 ml of methanol. Each sample was spiked with 4.5 nmol of FA 15:0 and 1.8 μ mol FA 17:0 as internal standards immediately. Then, lipids were extracted according to Matyash et al. (V. Matyash, JLR 2008). Lipid extracts were dried and dissolved in 50 μ l of a pentafluoro benzyl bromide solution (3.4 % in acetonitrile) and 10 μ l of N, N-diisopropyl ethanolamine. After 10 min of incubation at room temperature samples were evaporated under a stream of nitrogen and suspended in 50 μ l of hexane.

A Trace-DSQ GC-MS (Thermo Scientific, Austin, TX) equipped with a TR-FAME 30m column was used in splitless mode with 1 ml/min helium as carrier gas and 300°C injector temperature. The initial oven temperature of 150°C was held for 1min and then increased to 200°C at a rate of 25°C/min followed by 325°C at a rate of 12.5°C/min and held for 2min. The mass spectrometer was run in negative ion chemical ionization (NICI) mode. Fatty acids were detected in full scan as carboxylates after loss of the pentafluoro benzyl moiety. Methane was used as CI

gas. Source temperature was set to 250°C and the transfer line temperature to 330°C. Quantitation of fatty acids was performed by correlating integrated areas of fatty acids versus the integrated area of FA 15:0.

GC-El/MS of total fatty acids (free and esterified)

Dried lipid extracts from above were suspended in 1 ml methanol NaOH. After 10 min incubation at 80°C, samples were cooled for 5 min on ice. Then, 1 ml BF₃ was added and samples were incubated for 10 min at 80°C. Fatty acid methyl esters were extracted with 1 ml saturated NaCl and 2 ml hexane. The hexane phase was dried and methyl esters dissolved in 1.5 ml hexane. A Trace-DSQ GC-MS equipped with a TR-FAME 30m column was used for analysis. Helium was used as carrier gas at a flow of 1.3 ml/min, in split mode, at 250°C injector temperature. The initial oven temperature of 150°C was held for 0.5 min and then the temperature was increased to 180°C at a rate of 10°C/min. This was followed by a further increase to 190°C at a rate of 0.5°C/min and then increased to 250°C at a rate of 40°C/min and kept for 3 min. The mass spectrometer was run in electron impact mode and fatty acids were detected in full scan of m/z 80 - 400. Source temperature was set to 250°C and the transfer line temperature to 200°C. Peak areas for FAs were calculated by Xcalibur QuanBrowser and related to FA 17:0 internal standard peak areas.

3.1.10. Statistical Analysis

Data are expressed as mean values $\pm$ standard deviation (SD). Differences were calculated with Mann-Whitney-U-Test analysing unequally distributed parameters using the SigmaStat® statistic program (Jandel Scientific, San Rafael, CA. USA). A p value of < 0.05 was considered significant.

3.2. PART 2: UDCA in the Regulation of miRNA Expression in Morbid Obesity

3.2.1. mRNA Expression Analysis

Performed according to Material and Methods PART 1.

3.2.2. Protein Extraction and Western Blot Analysis

Performed according to Material and Methods PART 1.

3.2.3. Micro-RNA Analysis

Micro-RNA (miRNA) analysis was performed using TaqMan®MicroRNA Assays available from Applied Biosystems. RNA from liver and vWAT was extracted according to the manufacturer's protocol using peqGOLD TriFast™ reagent (PEQLAB). To evaluate the expression level of several mature miRNAs from human liver and vWAT, RT reactions for each sample and each miRNA to be analyzed were performed using TaqMan MicroRNA Reverse Transcription kit from Applied Biosystems. RT reactions and qRT-PCR were performed according to material and methods in Castro et al. (297). Ct values were normalized to the mean values of U6 and U44 for both liver and vWAT. The $2^{-\Delta\Delta Ct}$ method was used for the relative quantification of mature miRNA expression levels in which $\Delta\Delta Ct = \Delta Ct \text{ test sample} - \Delta Ct \text{ calibrator sample}$.

3.2.4. Statistical analysis

Data are expressed as mean values $\pm$ standard deviation (SD). Differences were calculated with Mann-Whitney-U-Test analysing unequally distributed parameters using the SigmaStat® statistic program (Jandel Scientific, San Rafael, CA. USA). A p value of < 0.05 was considered significant.

CHAPTER 4

RESULTS

4.1. PART 1: UDCA in the Regulation of Bile Acid, Cholesterol and Lipid Metabolism in Morbid Obesity

Ursodeoxycholic Acid Exerts Farnesoid X Receptor-antagonistic Effects on Bile Acid and Lipid Metabolism in Morbid Obesity

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4.1.1. Patient Characteristics

Out of 40 randomized patients, 19 finished per protocol in the UDCA and 18 in the control groups. Drop-outs were due to diarrhea in the UDCA, and pregnancy and minor intraoperative bleeding in the control groups. Gender, age, body mass index (BMI), liver function tests and IR (estimated by HOMA-IR) did not differ between groups. Compliance to UDCA (>95% in each patient randomized to treatment) was confirmed by pill counts and UDCA measurements in serum. All participants had been instructed not to change their dietary habits, thus, BMI increased during the study period, both in UDCA ($42.4 \pm 5.1 \text{ kg/m}^2$ to $43.2 \pm 5.2 \text{ kg/m}^2$, $p < 0.05$) and

control ($40.6 \pm 3.9 \text{ kg/m}^2$ to $41.1 \pm 3.7 \text{ kg/m}^2$, $p < 0.05$) groups. Interestingly, histological analysis revealed a higher steatosis grade (1.2 to 1.9, $p < 0.05$) and thereby NAFLD activity score (NAS) (1.9 to 2.5, $p < 0.05$) in the UDCA treated patients compared to untreated controls at the day of surgery. Baseline HOMA-IR classified 18/19 UDCA and 15/18 control patients as insulin resistant. Fasting glucose and HbA1c levels were normal in all patients.

4.1.2. UDCA Increased Bile Acid Synthesis and Cholesterol Turnover

UDCA treatment resulted in a reduction of serum aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), as well as free FA, total and LDL-cholesterol (LDL-C), whereas TGs increased (Table1).

Table 1. Serum Parameters Before and After UDCA Treatment

	UDCA Day 1	UDCA Day21	P-Value
Aspartate aminotransferase [U/L]	31.5 ± 12.0	23.0 ± 5.6	$p < 0.05$
Alanine aminotransferase [U/L]	46.0 ± 26.1	33.4 ± 12.4	n.s.
Gamma-glutamyl transferase [U/L]	48.1 ± 20.1	31.6 ± 12.7	$p < 0.01$
Total Cholesterol [mM]	5.3 ± 1.2	4.9 ± 1.1	$p < 0.05$
LDL-Cholesterol [mM]	3.2 ± 0.9	2.9 ± 1.1	$p < 0.01$
HDL- Cholesterol [mM]	1.2 ± 0.2	1.2 ± 0.3	n.s.
Triglycerides [mM]	1.6 ± 0.8	2.1 ± 1.3	$p < 0.05$
Free Fatty Acids [mM]	0.63 ± 0.30	0.45 ± 0.20	$p < 0.05$
HOMA-IR	6.6 ± 3.9	5.8 ± 3.1	n.s.
FGF19 [pg/L]	83.1 ± 44.1	68.0 ± 46.5	$p < 0.05$
FGF21 [pg/L]	333.7 ± 271.2	352.0 ± 251.5	n.s.
7 α -Hydroxycholesterol [μ M]	0.11 ± 0.03	0.16 ± 0.05	$p < 0.05$
7 α -Hydroxy-4-cholesten-3-one [μ mol/mol cholesterol]	4.81 ± 2.56	8.74 ± 5.47	$p < 0.05$

LDL, low density lipoprotein; HDL, high density lipoprotein; n.s., not significant.

After UDCA treatment, BAs increased 10-fold with UDCA enrichments in the range of recently reported peak concentrations in non-cholestatic subjects (298). UDCA constituted $87.7 \pm 3.7\%$ of total BAs, in equal amounts of unconjugated or glycine-conjugated (Table 2). Of note, the amounts of primary bile acids chenodeoxycholic acid (CDCA) and cholic acid (CA) also increased in their

glycine-conjugate forms (Table 2). Whereas CDCA elevations might have resulted from intestinal and/or hepatic epimerization of UDCA (299), the simultaneous increase of CA indicated enhanced *de novo* synthesis.

Table 2. Serum Bile Acid Before and After UDCA Treatment

BA [μ M]	UDCA Day 1	UDCA Day 21	P-Value
UDCA	0.13 $\pm$ 0.11	5.79 $\pm$ 3.73	p<0.001
CA	0.13 $\pm$ 0.18	0.14 $\pm$ 0.14	n.s.
DCA	0.43 $\pm$ 0.31	0.31 $\pm$ 0.17	n.s.
CDCA	0.23 $\pm$ 0.23	0.35 $\pm$ 0.17	p<0.01
LCA	0.03 $\pm$ 0.01	0.03 $\pm$ 0.03	n.s.
T-UDCA	0.02 $\pm$ 0.02	0.21 $\pm$ 0.20	p<0.001
T-CA	0.04 $\pm$ 0.03	0.06 $\pm$ 0.05	n.s.
T-DCA	0.06 $\pm$ 0.07	0.06 $\pm$ 0.06	n.s.
T-CDCA	0.08 $\pm$ 0.08	0.10 $\pm$ 0.09	n.s.
T-LCA	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	n.s.
G-UDCA	0.12 $\pm$ 0.19	8.79 $\pm$ 7.66	p<0.001
G-CA	0.18 $\pm$ 0.18	0.47 $\pm$ 0.56	p<0.01
G-DCA	0.17 $\pm$ 0.2	0.29 $\pm$ 0.36	n.s.
G-CDCA	0.48 $\pm$ 0.68	1.20 $\pm$ 1.41	p<0.01
G-LCA	0.03 $\pm$ 0.01	0.05 $\pm$ 0.05	n.s.
TOTAL	1.89 $\pm$ 0.85	19.5 $\pm$ 12.2	p<0.001

UDCA, ursodeoxycholic acid; CA, cholic acid; DCA, deoxycholic acid; CDCA chenodeoxycholic acid; LCA, lithocholic acid; T, tauro-; G, glyco-; n.s., not significant.

We first focused on changes in BA metabolism. Serum BA precursors, 7 α -hydroxy-cholesterol and 7 α -hydroxy-4-cholesten-3-one (C4), were increased (Table 1) and mRNA and protein expression levels of CYP7A1 were higher in liver samples of UDCA treated patients compared to controls (Figure 11A and B). Thus, BA biosynthesis was clearly stimulated upon UDCA. Notably, negative feedback regulation of BA homeostasis via hepatic FXR/SHP was not activated as reflected by unchanged SHP mRNA expression (Figure. 11A) and decreased formation of FXR/RXR-DNA sequence complexes in the ABCD-assay (Figure 11C). Rather, BA synthesis was enhanced via decreased circulating FGF19 (Table 1), the inhibitor

of CYP7A1 (300). Further nuclear receptors such as CAR and PXR remained unchanged at the mRNA level upon UDCA (data not shown).

Enhanced BA synthesis should affect cholesterol turn-over. Indeed, we observed elevated hepatic mRNA levels of the transcriptional regulator SREBP2 and its target, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), the rate-determining enzyme in cholesterol synthesis (Figure 11D) (187). This was further substantiated by decreased HMGCR-phosphorylation (Figure 11E). Therefore UDCA treatment thus enhanced cholesterol synthesis. Nevertheless, steady hepatic cholesterol flux was suggested by unchanged mRNA expression of the ATP-binding cassette transporters ABCA1, ABCG1 mediating cholesterol efflux and the biliary cholesterol transporters ABCG5 and ABCG8 (data not shown). Notably, despite unchanged mRNA levels of the LDL-receptor (LDLR), increased LDLR protein expression (Figure 11E) points towards increased cholesterol uptake from blood, which could explain the observed decreases in total and LDL-cholesterol upon UDCA treatment (Table 1).

Taken together, our results indicate that UDCA treatment induced BA synthesis fueled by hepatic cholesterol. The liver compensated cholesterol consumption via two pathways: i) the induction of *de-novo* cholesterol synthesis via SREBP2/HMGCR, and ii) the increase of cholesterol uptake via LDLR.

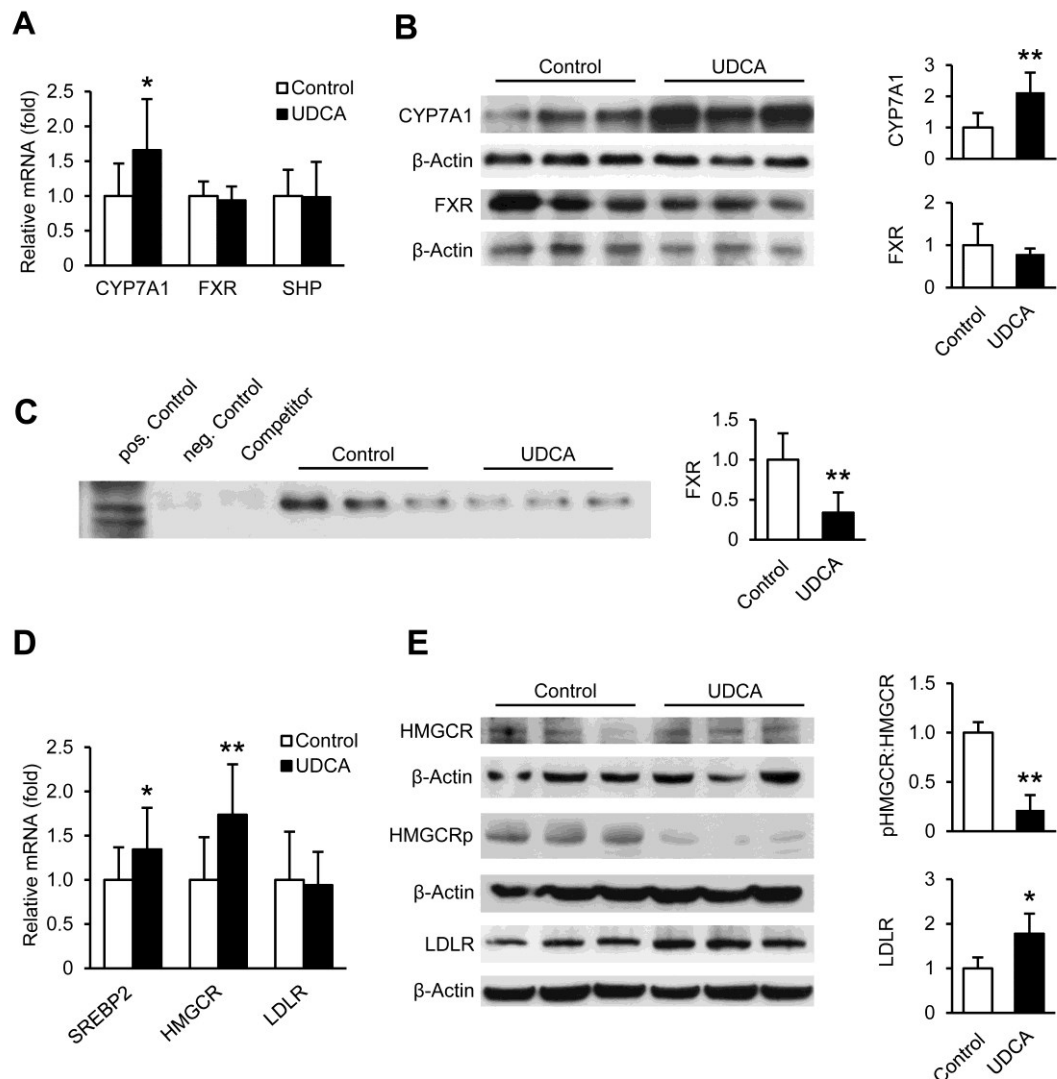


Figure 10. UDCA Alters Key Determinants of Hepatic Bile Acid and Cholesterol Homeostasis. (A) mRNA analysis of bile acid biosynthesis markers. Controls: n=18; UDCA: n=19. (B) Representative Western blots of CYP7A1, FXR and densitometry (all samples) of protein levels relative to β -actin. Controls: n=7; UDCA: n=6. (C) ABCD-Assay indicating FXR activity. Controls: n=7; UDCA: n=6. (D) mRNA analysis of cholesterol biosynthesis markers. Controls: n=18; UDCA: n=19. (E) Representative Western blots of HMGCR, phosphorylation status of HMGCR (HMGCRp), LDLR and densitometry (all samples). Controls: n=7; UDCA: n=6. Mean values $\pm$ SD are expressed for all data. * $p \leq 0.05$, ** $p \leq 0.01$ versus control group.

4.1.3. Highly Enriched UDCA Does Not Alter Hepatobiliary Transporter Expression in NAFLD

Changes in BA flux through the liver may affect BA signalling and in turn, hepatic BA and lipid homeostasis. Therefore, we examined the effects of UDCA on hepatobiliary transporter expression. No differences between untreated or UDCA treated groups were observed from RNA or protein expression of MRP2, MRP3, MDR3 and BSEP (Figure 12A-C). Notably, up-regulation of MRP4 mRNA was not reflected by changes in protein expression (Figure 12A-C). Thus, UDCA did not affect hepatobiliary transporter expression including NTCP and OST-alpha/beta (data not shown) in morbidly obese patients, in contrast to non-obese gallstone patients (245).

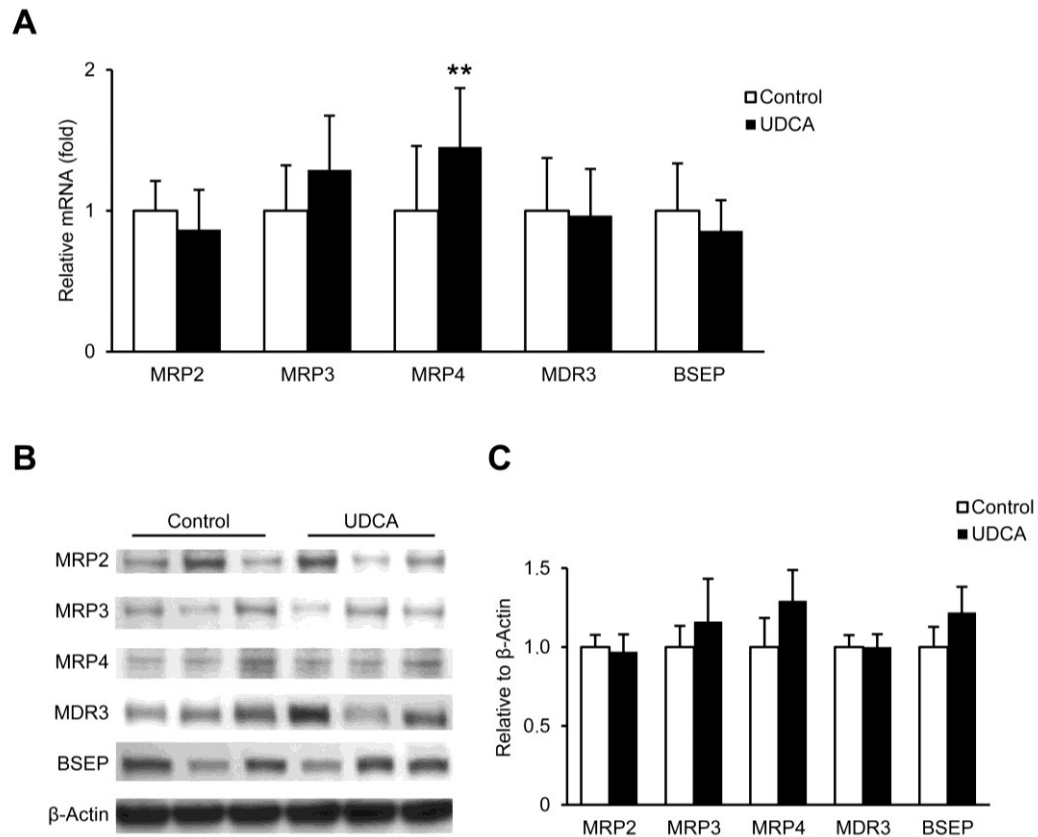


Figure 11. Hepatobiliary ABC transporter expression remains unchanged upon highly enriched UDCA treatment. (A) Gene expression analysis of key hepatobiliary ABC transporters in morbidly obese NAFLD patients via qRT-PCR. Controls: n=18; UDCA: n=19. (B) Immunoblotting of hepatobiliary transporters of steatotic livers. Controls: n=7; UDCA: n=6. (C) Densitometry of protein levels (all samples). Control: n=7; UDCA: n=6. Mean values $\pm$ SD are expressed for all data. * $p < 0.05$ versus control group.

4.1.4. UDCA Affects Triglyceride and Fatty Acid Partitioning in Liver and Visceral White Adipose Tissue

To examine the impact of UDCA on lipid and cholesterol partitioning along the liver-WAT axis, we performed tissue concentration measurements and lipid profiling.

Hepatic cholesterol content (Figure 13A) did not differ in UDCA treated or untreated patients, despite changes in cholesterol synthesis and uptake proteins. However, we observed an increase in hepatic TG levels (Figure 13B). FA profiling of the total liver FA pool revealed an overall accumulation of FA species such as myristic (MA, 14:0), palmitic (PA, 16:0), palmitoleic (16:1n7), stearic (SA, C18:0) and oleic acids (OA, 18:1n9), whereas free FA species were unaltered upon UDCA treatment (Table 3). We thus determined the expression of lipid metabolism regulatory genes. SCD, the enzyme catalyzing the formation of monounsaturated FAs such as OA (109), was induced on mRNA and protein levels upon UDCA, whereas expression of other lipogenic genes such as SREBP1c, FASN and ACC1/2 remained unaltered (Figure 13C- D). Moreover, the microsomal triglyceride transfer protein (MTTP) and apolipoprotein B (ApoB), which are involved in VLDL export, did not differ between the groups (Figure 13E). Thus, UDCA likely stimulated hepatic FA deposition as TG via hepatic SCD upregulation due to the absence of FXR-mediated anti-lipogenic effects.

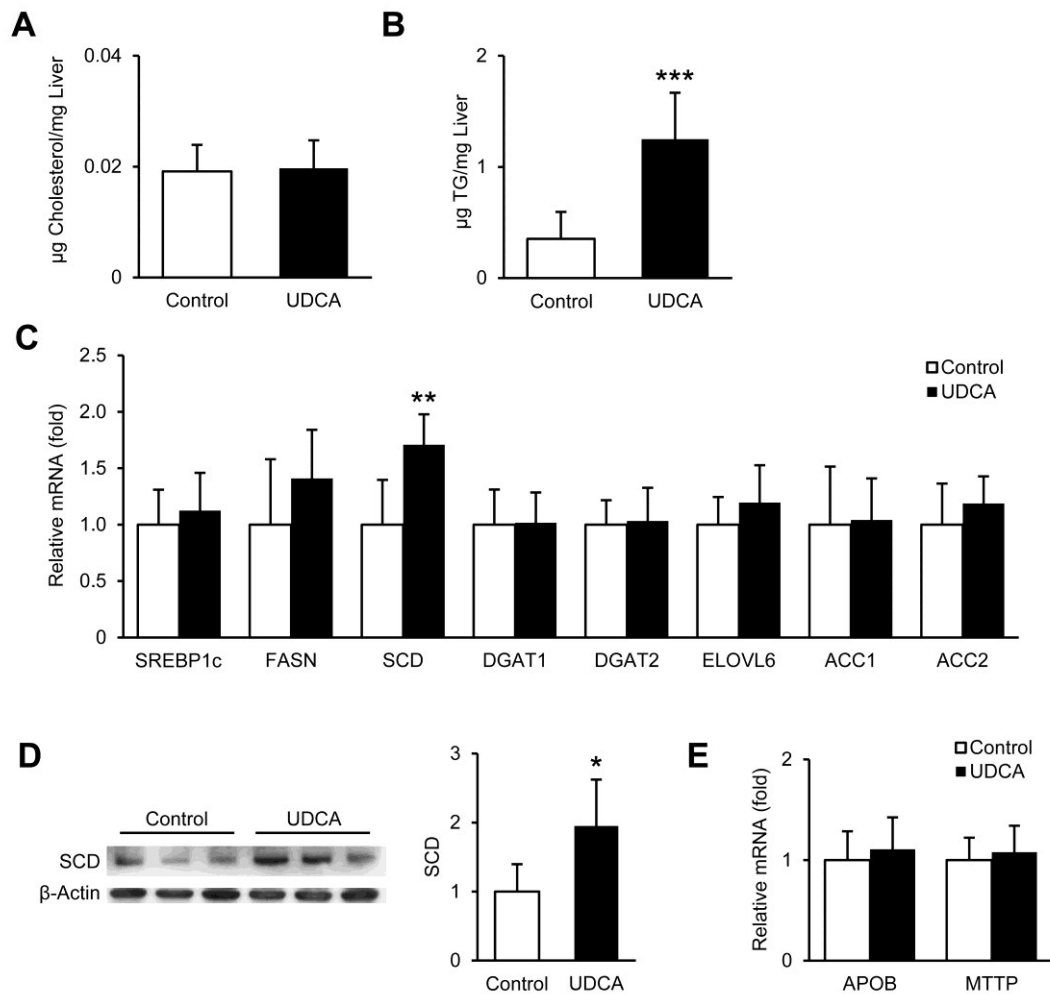


Figure 12. UDCA increases hepatic TG formation and modulates hepatic SCD expression. (A) Hepatic cholesterol and (B) Triglyceride concentrations. (C) Hepatic mRNA expression analysis of markers of lipid metabolism in morbidly obese NAFLD patients. Control: n=18; UDCA: n=19. (D) Representative Western blot and densitometry (all samples) of hepatic SCD relative to β -actin. Control: n=7; UDCA: n=6. (E) mRNA expression of APOB and MTTP in NAFLD patients. Control: n=18; UDCA: n=19. Mean values $\pm$ SD are expressed for all data. * $p \leq 0.05$, ** $p \leq 0.01$ versus control group.

Table 3. Changes of Fatty Acid Species in Liver and vWAT Upon UDCA

Liver						
FA	Control TFA	UDCA TFA	P-Value	Control FFA	UDCA FFA	P-Value
14:0	13.76 ± 6.92	27.22 ± 12.10	p<0.001	2.5 ± 0.77	2.9 ± 0.91	n.s.
16:0	202.44 ± 84.96	312.93 ± 98.31	p<0.01	39.84 ± 13.71	34.29 ± 8.08	n.s.
16:1n7	29.98 ± 17.09	54.60 ± 20.7	p<0.001	2.12 ± 0.88	2.98 ± 0.79	n.s.
18:0	53.91 ± 8.37	63.10 ± 10.43	p<0.01	32.68 ± 13.43	25.70 ± 13.1	n.s.
18:1n9	243.00 ± 113.73	425.02 ± 107.56	p<0.001	19.84 ± 5.55	21.52 ± 6.36	n.s.
vWAT						
14:0	28.5 ± 6.5	28.4 ± 6.9	n.s.	2.2 ± 1.6	0.37 ± 0.36	p<0.01
16:0	215.1 ± 35.4	220.8 ± 27.1	n.s.	28.4 ± 22.3	4.29 ± 4.98	p<0.001
16:1n7	73.9 ± 18.6	81.5 ± 15.2	n.s.	4.1 ± 2.8	2.82 ± 1.65	n.s.
18:0	26.7 ± 6.2	23.7 ± 5.4	n.s.	10.8 ± 7.9	0.65 ± 0.96	p<0.001
18:1n9	430.1 ± 68.5	475.5 ± 56.0	p<0.05	40.0 ± 28.2	19.3 ± 9.73	p<0.001
18:1n11	63.6 ± 15.4	50.3 ± 11.3	p<0.05	2.0 ± 1.4	1.20 ± 0.64	n.s.
Total Fatty Acid (TFA) and Free Fatty Acid (FFA) species are relative to internal standards. n.s., not significant.						

In vWAT of UDCA treated patients an increased TG load, again without changes in cholesterol levels, was found (Figure 14A and B). Notably, UDCA treatment resulted in enrichment of OA in the total FA-fraction (Table 3), in line with up-regulation of SCD mRNA (Figure 14C). As observed in liver, expression of other lipogenic genes such as FASN and SREBP1c did not differ between the groups in vWAT (Figure 14C).

Conversely, analysis of free FAs in vWAT revealed decreased levels of free OA, together with MA, PA and SA upon UDCA treatment (Table 3). Consistently, lower free FA concentrations were also detected in the serum (Table 1). Additionally, FA transport protein FATP1 was reduced on mRNA level (Figure 14C), which in part could be attributed to restricted plasma free FA availability (Table 1). The small amounts of protein in vWAT, limited our expression analysis to mRNA levels. However, as a measure of SCD activity, the ratio of unsaturated and saturated C16 and C18 was calculated from total and free FAs. Except for C16 total FA-ratio, desaturation processes and hence SCD activity were induced upon UDCA treatment (Figure 14D).

Since OA is the preferential substrate for TG storage and prevents lipotoxicity (112), our data suggest a shift in lipid metabolism towards FA incorporation into visceral lipid droplets, which was not related to changes in anti-lipolytic FGF21 serum levels (Table 1). Unfortunately we could not address potential UDCA effects on vWAT lipolysis due to virtually absent vWAT enzymatic activity (data not shown).

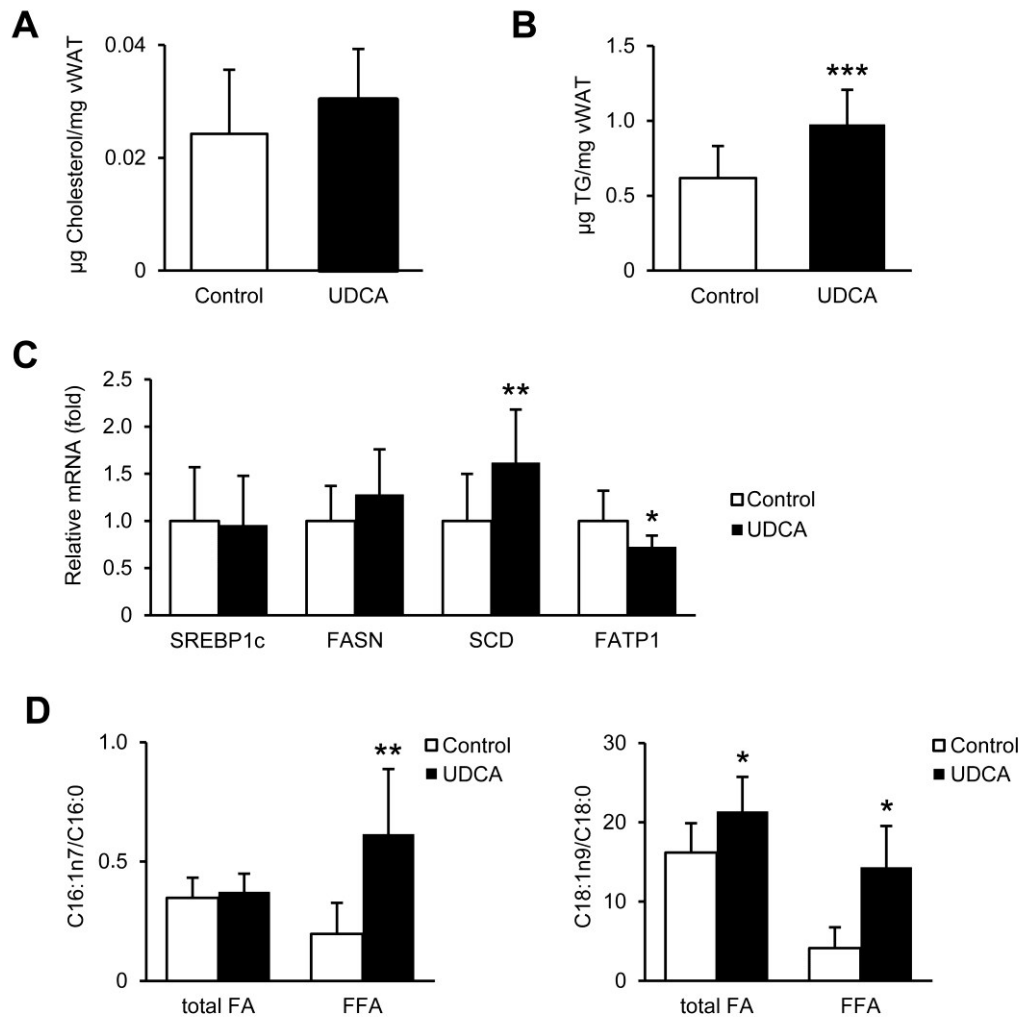


Figure 13. UDCA induces TG formation and lipogenic gene expression in visceral white adipose tissue (vWAT). (A) Measurement of cholesterol and (B) TG content in vWAT. (C) Relative mRNA expression of markers of *de novo* lipogenesis and FA transport in vWAT. (D) SCD activity calculated according to C16:1/C16:0 and C18:1/C18:0 concentrations in total and free FA obtained from lipid profiling. Control: n=16; UDCA: n=14. Mean values $\pm$ SD are expressed for all data. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ versus control group.

4.2. PART 2: UDCA in the Regulation of miRNA Expression in Morbid Obesity

4.2.1. UDCA Had No Impact on Hepatic MiR-34a Expression and its Downstream Signalling Pathway

Since miRNAs are known to regulate several genes involved in hepatic cholesterol and lipid metabolism, they represent potential targets in liver diseases such as alcoholic and non-alcoholic fatty liver disease. We thus compared the expression levels of several miRNA-species in liver and adipose tissue samples of UDCA treated and untreated patients. While hepatic miRNA levels of miR-34a, miR-143, miR-33b, miR-451, miR-16 and miR-34a_rev remained unchanged between the groups, expression of miR-21, miR-33a, miR-122 and miR-335 was reduced in the liver upon UDCA treatment (Figure 15A). Notably, in line with miRNA122 down-regulation, which has been shown to decrease plasma cholesterol levels (301), UDCA patients exhibited lower plasma total- and LDL-cholesterol concentrations (Table 1). Despite unchanged hepatic miR-34a expression, we continued investigating potential alterations of miR-34a upstream and downstream targets. SIRT1 as well as acetylated and therefore activated p53 protein levels were unchanged (Figure 15B).

Adipose tissue also underwent detailed miRNA analysis. However, no changes in the most common miRNAs such as miR-143, miR-378 and miR-335 were revealed (Figure 15C).

Our data suggest that UDCA, however modulating expression in rat primary hepatocytes (284), fails to regulate hepatic miR-34a expression and several miRNA-species in vWAT in morbidly obese patients. As already indicated by unchanged miR-34a expression, miRNA34a-downstream pathways did not show any activation pattern upon UDCA in human NAFLD.

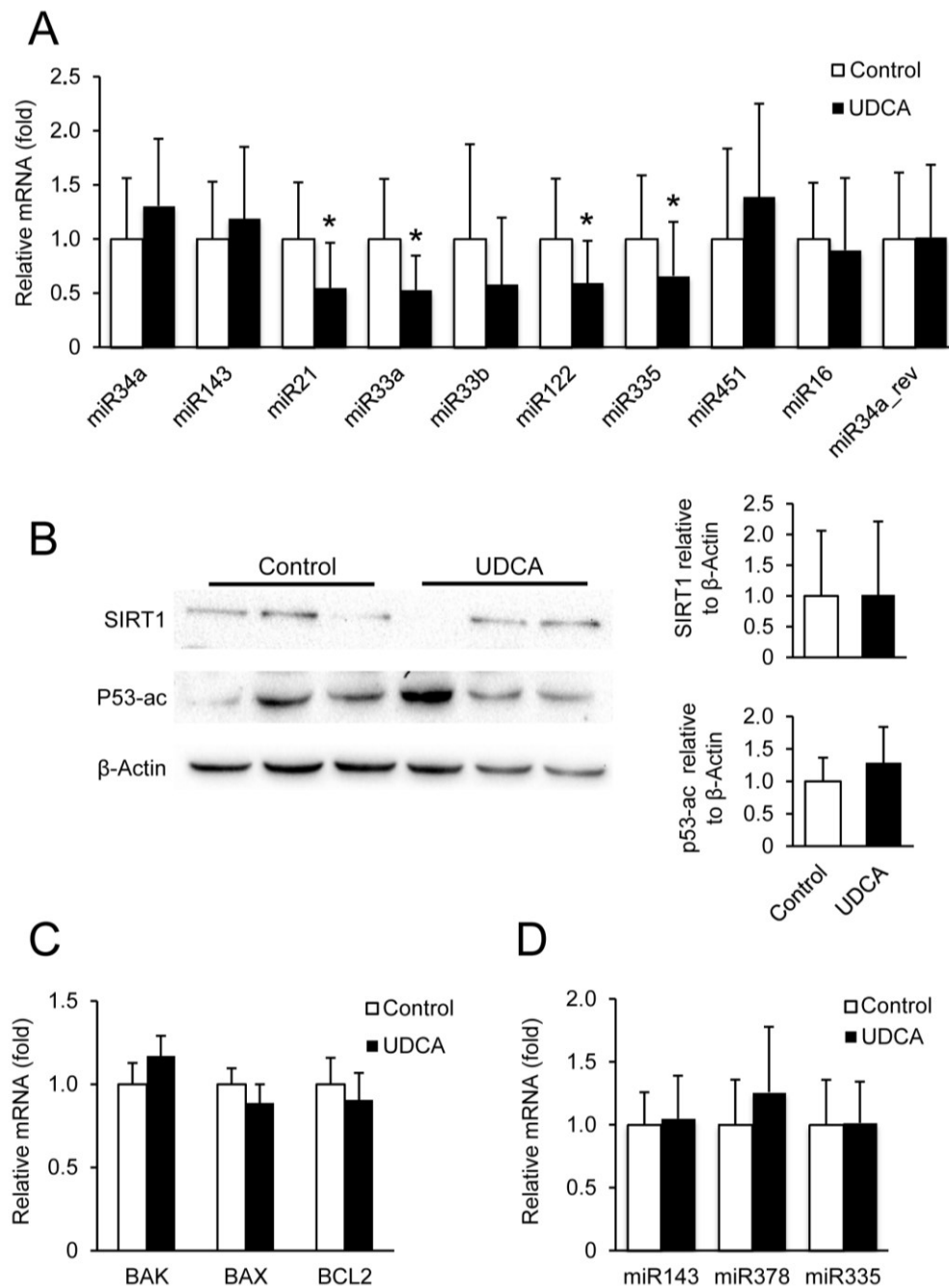


Figure 14. Decreased hepatic miRNA expression has no impact on downstream targets upon UDCA treatment. (A) Expression analysis of key hepatic miRNA species in morbidly obese NAFLD patients via qRT-PCR. Controls: n=18; UDCA: n=19. (B) Immunoblotting of miR-34a downstream targets of steatotic livers, including densitometry of protein levels (all samples) relative to β -Actin. Controls: n=7; UDCA: n=6. (C) MiRNA analysis in vWAT. Control: n=16; UDCA: n=14. Mean values $\pm$ SD are expressed for all data. * $p < 0.05$.

CHAPTER 5

DISCUSSION

In the first part of this pharmacodynamic study, we analyzed liver and vWAT tissue obtained during bariatric surgery from short term UDCA treated and untreated morbidly obese NAFLD-patients leading to decreased FXR activity, stimulated cholesterol and BA synthesis, hepatic lipid accumulation and altered lipid conversion in vWAT. Here, we provide a comprehensive evaluation of mechanistically interrelated metabolic alterations associated with UDCA treatment, which may be most relevant for the development of more effective BA mimetics for the treatment of NAFLD.

Stimulated BA synthesis during UDCA treatment, first described by Einarsson et al (302), was later not consistently confirmed (303). Furthermore, previous studies showed a considerable increase in cholesterol and BA synthesis in obesity (304). We found that highly enriched UDCA enhances *de novo* BA and cholesterol synthesis highlighted by induction of several BA and cholesterol synthetic markers such as C4, 7 α -hydroxycholesterol and CYP7A1 (for BAs) and hepatic SREBP2 and HMGCR (for cholesterol) in morbid obesity. Since enhanced cholesterol synthesis in turn fueled stimulated BA formation, no net effect on hepatic cholesterol levels was observed. BAs suppress CYP7A1 via hepatic FXR/SHP and intestinal FXR/FGF19 signalling pathways (213). However, UDCA, which constituted almost 90% of BAs in our patients, has only low affinity for and hence no agonistic activity on FXR (305-309). Indeed, ABCD-assays suggest a decreased interaction of the FXR/RXR heterodimer with its DNA binding sequence

and hence reduced FXR activation upon UDCA. Notably, this mechanism distinguishes UDCA from the highly potent FXR agonist obeticholic acid recently proven beneficial in NASH (310, 311). Moreover, our data suggest FXR-mediated reduction of intestinal FGF19 synthesis and secretion indicated by decreased FGF19 serum concentrations, which are inverse to hepatic CYP7A1 expression levels. This further supports the concept that UDCA abolishes endogenous FXR effects to an extent even reaching FXR-antagonistic properties, reflected by increased mRNA and protein expression of CYP7A1; by increased serum levels of BA precursors 7 α -hydroxycholesterol and C4, and primary BAs; as well as by

lowered serum FGF19 concentrations, decreased FXR/RXR DNA binding activities and blunted FXR mediated anti-lipogenic actions. While such effects may be beneficial for cholesterol catabolism, FXR-antagonistic properties could also explain UDCA's limited clinical efficiency (249, 251, 312) in comparison to FXR-agonist obeticholic acid, which is associated with increased insulin sensitivity in diabetic NAFLD patients and improved liver histology but not IR in NASH patients (310, 311). Interestingly, UDCA, circulating in the enterohepatic system, had no impact on hepatobiliary transporter expression in morbidly obese patients, in contrast to a previous study in non-obese gallstone patients (245), which might be related to approximately ten-fold higher total BA concentrations in the current study cohort.

Recently, up-regulation of SREBP2 and HMGCR resulting in increased serum LDL-C and accumulation of hepatic free cholesterol and LDL-C was related to the pathogenesis and progression of human NAFLD (18). Despite enhanced cholesterol *de novo* synthesis, our UDCA treated cohort featured decreased serum total- and LDL-C concentrations. Thus, to meet the demands of induced BA generation due to diminished FXR activation, cholesterol was also mobilized from the periphery via LDLR since cholesterol catabolism was compensated only in part by *de novo* synthesis.

Induced de-esterification of cholesterol-esters via neutral cholesterol ester hydrolase (nCEH) has been linked to human NAFLD (18). However, our data suggest that short term UDCA treatment does not affect hepatic free cholesterol

and cholesterol-ester homeostasis, since expression of both acetyl-CoA acetyltransferase 2 and nCEH, the enzymes regulating cholesterol esterification and de-esterification (313), was similar in both groups (data not shown). Based on these results, and consistent with unchanged hepatic cholesterol levels, we conclude that *de novo* synthesized cholesterol is directed towards BA synthesis rather than cholesterol storage.

Since both WAT and liver lipid metabolism are tightly linked, disturbed energy homeostasis in vWAT (e.g. increased FA flux to the liver as a result of IR) may

exert deleterious effects on hepatic FA turn-over (314). Here we show that short term UDCA administration, by replacing hydrophobic FXR-agonistic BAs and thereby reducing FXR anti-lipogenic effects, impacts on lipid conversion processes via elevated hepatic SCD expression. Reduction of serum free FAs and induction of serum TGs resemble lipid conversion events in vWAT and might be a consequence of altered FA conversion processes via up-regulated SCD expression in both liver and vWAT. Despite decreased FXR signalling, direct UDCA effects on SREBP-1c, a known FXR target regulating FASN (226), are missing. Unchanged SREBP-1c expression might be explained by the gene's high sensitivity to insulin (315), which did not change within 21 days of UDCA treatment (data not shown). Thus, we hypothesize that the lack of FXR mediated SREBP-1c repression may represent one of the reasons for UDCA mediated induction of hepatic lipid accumulation. Moreover, blunted FXR mediated anti-lipogenic effects are indicated by elevated histology-proven steatosis scores and increased hepatic TGs along with induced FA species in the total FA-pool upon UDCA. In contrast to other studies showing unaltered NAS, in which obese patients were encouraged to lose weight (249, 316), our participants were instructed not to change their dietary habits - possibly further promoting the adverse liver histology outcome. Besides these potentially harmful properties, these changes could also represent a hepatic mechanism counteracting injury from exogenous lipid overflow, known to mediate liver injury and apoptosis. Notably, conditions when SCD activity was induced and monounsaturated FAs and TGs constituted the major lipid species, were shown to attenuate lipotoxicity (112).

Lipid profiling of vWAT indicates that a combination of increased OA (18:1n9) in the total FA pool and a corresponding reduction of several lipid species in the free FA pool may contribute to a potentially beneficial shift towards improved lipid storage and reduced lipotoxicity upon UDCA. Increased expression of SCD appears to be the mechanism behind elevated concentrations of monounsaturated OA (18:1n9) in the total FA-pool. OA (18:1n9) accumulation along with the observation that except for SCD other lipogenic genes did not differ between the groups, suggests an increase in FA synthesis, which was not detected via mRNA analysis but by the accumulation of final products. The conversion of PA (16:0)

and SA (18:0), deriving from the free FA-pool and representing preferential substrates for SCD (317), could explain both their reduction in the free FA-pool and the elevation of OA (18:1n9) loads in the total FA-pool. Due to TG accumulation in vWAT, we hypothesize that rising OA (18:1n9) levels derived from the free FA-pool might be preferentially stored as TGs in lipid droplets in vWAT and thereby prevent lipotoxic effects of other potentially harmful lipid species.

Elevated SCD activity in vWAT could be explained by surplus of substrate for lipogenesis from hepatic lipid spill-over into the circulation. However, the exact mechanism behind SCD activation in vWAT upon short term UDCA treatment awaits further experimental elucidation. Apart from increased OA (18:1n9), we identified a decrease of vaccenic acid (VA, 18:1n11) in the total FA pool, which is mainly provided by ruminant meat and dairy products (318) and might be caused by less efficient micelle formation and dietary lipid absorption in the UDCA treated cohort (319).

Besides its function as FA transport protein, FATP1 possesses acyl-CoA synthetase activity, generating substrates for β -oxidation (320). Therefore, the reduction of FATP1 mRNA levels in vWAT suggests blunted visceral long chain free FA uptake and decreased very long chain FA activation. However, genes linked to β -oxidation such as CPT1 α and AOX, did not differ between groups in liver or vWAT (data not shown). Of note, loss of FATP1 function in mice redistributes lipids from WAT and muscle towards the liver leading to hepatic TG accumulation (321). This might reflect an additional mechanism inducing hepatic

TG accumulation upon UDCA. Induction of alternative FA transport proteins in vWAT such as CD36 and FATP4, potentially restoring FA transport homeostasis, was not observed (data not shown).

The limitations of our study are the lack of placebo, of biopsies before UDCA treatment and of feces sampling for BA measurements. Due to restricted biopsy material, distinct investigations are based on mRNA analysis; however key findings are validated on protein level and are also supported by different biochemical methods.

In summary, this unique pharmacodynamic study in morbid obesity sheds new light on the therapeutic mechanisms and potential limitations of UDCA in liver and vWAT and provides novel mechanistic insights in the regulation of metabolic pathways in uncomplicated NAFLD outlined in Figure 16. Short term UDCA treatment, by blunting FXR activity, increases BA generation, thereby increasingly utilizing hepatic cholesterol and subsequently inducing cholesterol *de novo* biosynthesis and LDLR expression. Deficient FXR activation also increases SCD mediated hepatic TG formation (310). Understanding SCD activation and the cytoprotective mechanism behind UDCA action, counteracting accumulation of lipotoxic FA species in vWAT, may facilitate the development of novel BA-based therapeutic strategies and/or expanding the use of UDCA.

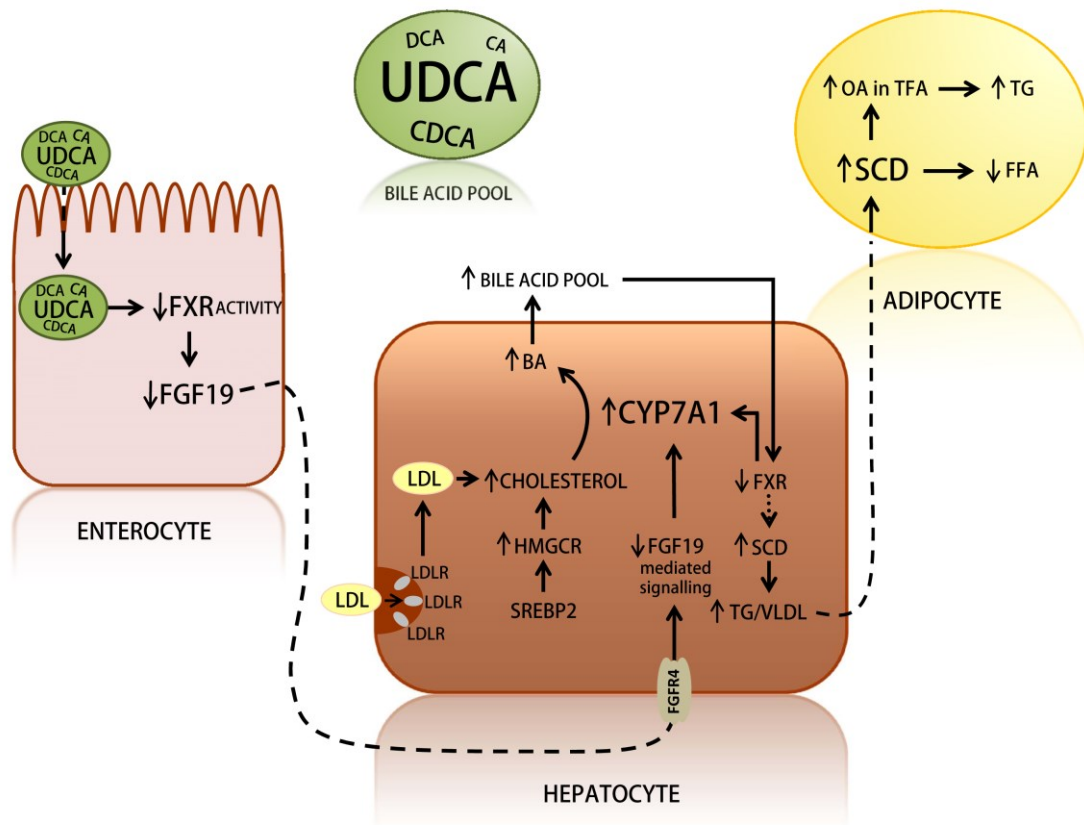


Figure 15. Overview of UDCA mediated effects in morbid obesity. UDCA decreases intestinal FXR activation. Reduced circulating FGF19 levels induce CYP7A1 activity and BA formation. Compensation of stimulated cholesterol conversion into BAs is regulated via LDL-C import and cholesterol *de novo* biosynthesis. Elevated BA concentrations repress hepatic FXR activity, thereby decreasing FXR mediated anti-lipogenic effects resulting in induced SCD expression and TG accumulation. Hepatic TG-overload is secreted into the circulation and stored in vWAT. Delivered free FAs (FFA) are converted into OA in the total fatty acid (TFA) pool and TG formation occurs due to elevated SCD activity in vWAT.

In the second part of the study, we further analyzed miRNA expression in liver and vWAT tissue of our study cohort. While investigations on hepatic miRNA expression revealed UDCA mediated repression of distinct miRNA species, further miRNA analysis in vWAT showed an unaltered miRNA expression pattern in these patients.

Several studies have highlighted the significant role of miRNAs in NAFLD. In human NASH, miRNAs have been demonstrated to be differentially expressed and their dysregulation is often associated with cell proliferation, apoptosis and inflammation (322). Although elevated miR-34a serum concentrations and hepatic expression have been detected in NAFLD patients and the administration of UDCA to primary rat hepatocytes repressed miR-34a expression (284, 323), UDCA had no impact on hepatic miR-34a expression in our NAFLD patient cohort. Unfortunately no miR-34a serum data are available to delineate potential differences in expression patterns comparing liver tissue and serum of UDCA treated and untreated patients. Interestingly, a recent study linked disease progression and severity to deregulated cholesterol metabolism, which was mediated via miR-34a induced dephosphorylation of HMGCR, known to cause accumulation of free cholesterol and the worsening of liver histology in NAFLD (18). In fact, cholesterol metabolism was up-regulated in the patient cohort upon UDCA treatment and not, as one would expect according to previous findings, in the untreated NAFLD group. However, serum analysis revealed decreased serum total- and LDL-C levels as well as increased LDLR expression, which together - despite elevated cholesterol synthesis - points towards mechanism(s) even stronger which stimulate cholesterol catabolism (see part 1).

A recent work by Castro et al. (284), claims a correlation between hepatic SIRT1 expression, acetylated and hence activated p53 protein levels and miR-34a expression as well as apoptosis in NAFLD patients (284). Since neither of the aforementioned parameters were changed, including gene expression levels of apoptosis and cell survival markers, we suggest a minor role of UDCA on miR-34a expression in our morbidly obese patient cohort. We speculate that UDCA concentration and duration of administration could be adapted to achieve beneficial results as demonstrated in rats (284).

The miRNA duplex consists of a miRNA forward and a miRNA reverse (miRNA*) strand and although little is known about miRNA* strands, they may exert regulatory actions distinct from miRNA strands (324, 325). In fact, analysis to elucidate the potential mechanistic impact of miR-34a*, demonstrated unchanged expression levels between the groups. This indicates no UDCA efficacy on miR-34a and miR-34a* in morbidly obese patients.

Besides miR-34a, over 46 miRNAs have been identified to be differentially regulated in patients with metabolic syndrome and NASH compared to patients with unobtrusive liver histology (326). However, data on miRNA expression in NASH in particular for miR-122 is controversial (323, 326, 327). It has been reported, for instance, that an inhibition of miR-122 results in decreased serum cholesterol and FA synthesis (257). However, it has also been shown that miR-122 expression correlates with the severity of fibrosis and inflammation (323). Therefore interpretation of UDCA action on hepatic miR-122 expression is complicated, which is in particular due to focus on serum analysis and limited analysis of expression levels in tissue. As mentioned before, differential expression in serum and tissue is likely since miR-122 has been found up-regulated in serum and down-regulated in tissue samples of HCC patients (257).

MiR-33a/b are known promoters of cholesterol and FA biosynthesis in mouse and human because they are expressed from an intron-located SREBP2 sequence. They are up-regulated in steatosis and NASH. Therefore, they are considered promising targets for the development of miRNA-specific antagonists in NAFLD (328, 329). Since inhibition of miR-33a/b was demonstrated to decrease plasma TG and VLDL particles (330), reduced miR-33a expression upon UDCA may exert beneficial effects. Nevertheless, we failed to demonstrate reported favorable effects associated with miR-33a/b inhibition as for example induced insulin receptor substrate 2 (data not shown) and reduced FASN and SREBP1 expression (330). This may be attributed to miR-33b compensatory events. A further possibility could be that differential miR copy numbers may be crucial for promoting anti-steatotic effects, however no data on miR-33a/b copy numbers are available to date.

A further candidate representing a crucial factor in NAFLD and strongly implicated in cancer development is miR-21. In cancer, miR-21 is secreted by cancer cells mediating IL-6 and TNF release and thereby inducing proliferation and metastatic potential of cancer cells (331, 332). In NAFLD, stimulated miR-21 expression via FAs such as OA or PA promotes phosphatase and tensin homolog (PTEN) down-regulation, AKT activation, and thus leads to the development of steatosis by inducing FA synthesis (333, 334). Despite controversial results in another study showing no difference between serum miR-21 levels in NAFLD and healthy patients (323), reduced miR-21 expression in tissue upon UDCA treatment might represent a mechanism counteracting lipid accumulation and steatosis, however potential regulation via PTEN and its downstream targets awaits further elucidation. This might also account for miR-335 reduction in morbidly obese patients upon UDCA treatment, since miR-335 expression was up-regulated in mouse models of obesity in leptin deficient and leptin receptor deficient mice (335).

CHAPTER 6

CONCLUSION

UDCA, a hydrophilic secondary BA with anti-apoptotic properties, has been proven beneficial in gallstone and PBC patients (194, 239, 247). However, data describing its effects in NAFLD are controversial (249-251).

This study reports novel UDCA effects in NAFLD patients providing a new framework to understand UDCA mechanisms in morbid obesity. While upon three weeks of UDCA treatment, key parameters in BA, cholesterol and lipid metabolism were changed, biliary transporters and cytokine signalling remained unaffected. Induction of cholesterol metabolism provided substrates for BA *de novo* biosynthesis, which was stimulated due to UDCA's antagonistic properties affecting FXR activity. Besides inducing BA metabolism, UDCA had strong impact on lipid metabolism via SCD upregulation in vWAT and liver. In vWAT, lipid conversion processes were modulated driving FA production towards free FA incorporation into lipid droplets and OA formation, which is considered anti-lipotoxic (112). However, analysis of hepatic tissue revealed increased TG accumulation and worsening of histology parameters upon UDCA treatment, thus leading to the conclusion that while UDCA exerts beneficial effects on vWAT, while being potentially harmful for liver tissue in morbid obesity by promoting NAFL progression to NASH.

MiRNAs have been extensively studied in various human diseases over the past decade. Their dysregulation has been reported as major driving force in disease severity (332). MiRNAs are attracting considerable interest as potential future non-

invasive biomarkers, since their serum concentrations provide representative fingerprints for distinct diseases (336). Here we investigated UDCA action on several miRNA-species. MiR-34a, an important mediator of apoptosis, which is up-regulated in NASH patients, was repressed in rat primary hepatocytes upon UDCA (284). We found that UDCA treatment had no impact on either hepatic miR-34a or miR-34a* expression nor on any miR-34a downstream targets in our morbidly obese cohort. However, it has to be considered that absence of UDCA efficacy might be related to treatment duration and/or UDCA dosage. Data on miRNAs such as miR-21, miR-33a and miR-122 are controversial (322, 323, 337). Controversy may arise from the fact that human data are predominantly available from serum but not tissue, whereas expression data from mice are usually from both serum and tissue. It also has to be taken into account that miRNA levels vary in an organ and tissue dependent manner and they even show differential expression patterns according to the cellular context (332). An absence of downstream target modulation represents a substantial challenge associated with data interpretation of decreased miRNA expression. However, we conclude that downregulation of distinct miRNAs may display in part a compensatory mechanism dealing with changes in BA, lipid and cholesterol metabolism upon UDCA treatment.

Collectively, UDCA's future clinical application in NAFLD requires further elucidation especially with respect to its regulatory action(s) on lipid metabolism. Combination therapies improving liver histology and avoiding hepatic lipid accumulation might support the development of treatment strategies in NAFLD. Moreover, UDCA mediated regulation of miRNA expression in human tissue could change in a time and dose dependent manner.

CHAPTER 7

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