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Expression of Hepatic Lipogenesis, Bile Acid Synthesis and Inflammation Genes in Obese NAFLD Patients

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Chapter 1

1. Introduction

1.1.a Outline of the work

NAFLD is a serious hepatic malfunction and a growing public health problem with dramatically increasing costs for health care systems worldwide. Current literature indicates a strong link between reduced hepatic and metabolic health, which seems to be reflected in NAFLD co-morbidities such as obesity and T2DM.

With this study I aim to investigate the metabolic link between liver DNL, insulin sensitivity, inflammation and bile acid metabolism.

Using gene analysis from liver tissue samples from 96 obese diabetic NAFLD patients and their respective controls undergoing bariatric surgeries at the UKE Adiposity Center as well as their blood and anthropometric parametes, we discovered obesity, liver function and metabolic health to be linked. All individuals were morbidly obese with a BMI of 53 to 56 kg/m². For most of the genes under investigation (CXCL10, TNF-α, COL1A1 TREM-2, FASN, FGF21, CYP7A1, CYP7B1, CYP8B1 and SHP1) statistically significant associations were observed for at least one of the disease parameters addressed, namely degree of liver steatosis, diagnosis of T2DM or presence of liver fibrosis.

Together, the general finding of the present study confirms the concept of healthy obesity claiming that despite equal adiposity, metabolic health can vary substantially among obese patients. The study supports growing evidence for a tight unit of liver metabolism, insulin responsive organs and the fatty tissue. Imbalances influence and aggravate each other mutually in the metabolic unit and open targets for medical treatments. Data from the bile acid synthesis gene analysis support the growing hope of effective hepatoprotective pharmacons by stimulating BA synthesis.

1.1.b Überblick der Arbeit

NAFLD ist eine schwere Leberfunktionsstörung und ein zunehmendes Gesundheitsproblem mit dramatisch steigenden Kosten in der medizinischen Versorgung weltweit. Die aktuelle Literatur deutet eine enge Verbindung zwischen reduzierter Leber- und Stoffwechselfunktion an. Diese scheint sich in NAFLD-Komorbiditäten wie beispielsweise der Adipositas und dem T2DM zu zeigen.

Im Rahmen der vorliegenden Arbeit ziele ich darauf ab, die metabolische Verknüpfung von DNL in der Leber, Insulinempfindlichkeit, Entzündung und Gallensäurenstoffwechsel aufzuzeigen. Dafür habe ich genetischen Daten aus den Leberbiopsien von 96 morbide adipösen diabetischen NAFLD-Patientinnen und Patienten, die sich einer bariatrischen Operation am Adipositas Centrum des UKE unterzogen, sowie den entsprechenden Kontrollen genutzt. Für die Beantwortung meiner Fragestellung habe ich ebenfalls die Blutwerte und anthropometrischen Parameter der Studienkohorte ausgewertet. Dabei zeigte sich, dass Adipositas, Leberfunktion und die metabolische Gesundheit miteinander verbunden sind. Alle Patientinnen und Patienten waren morbide adipös mit einem BMI zwischen 53 und 56 kg/m². Die meisten der untersuchten Gene (CXCL10, TNF-α, COL1A1, TREM-2, FASN, FGF21, CYP7A1, CYP8B1 und SHP1) zeigen statistisch signifikante Assoziationen mit den ausgewählten Krankheitsparametern Grad der Leberverfettung, Diagnose eines Typ 2 Diabetes Mellitus und Leberfibrose.

Zusammenfassend ist zu sagen, dass das Gesamtergebnis der vorliegenden Studie das Konzept der gesunden Adipositas bestätigt und Anspruch auf die neue Betrachtungsweise erhebt, nach welcher die metabolische Gesundheit der adipösen Patientinnen und Patienten unabhängig von der Fettleibigkeit grundlegend stark variieren kann. Die Arbeit stützt die vielfachen Nachweise darüber, dass der Leberstoffwechsel, die Insulin empfindlichen Organe und das Fettgewebe eine enge metabolische Einheit miteinander bilden. Ungleichgewichte beeinflussen und stören die einzelnen Komponenten dieser metabolischen Einheit. Sie eröffnen jedoch auch mögliche Angriffspunkte für medikamentöse Therapiestrategien. Die Datenanalysen der Gallensäuren-Synthese nähren die wachsende Hoffnung auf effektive hepatoprotektive Medikamente, welche die Gallensäuren-Synthese stimulieren.

1.2 Non-alcoholic fatty liver disease

1.2.1 Current state of research

Non-communicable diseases or disorders like cardiovascular diseases, T2DM and obesity are a growing burden and account for the highest mortality in the industrial world. They are rooted in a combination of harmful, sedentary life-styles with unhealthy dietary habits like the consumption of simple sugars, hight-fat and salted and micronutrient poor foods, and genetic and environmental factors (WHO 2017).

Current literature indicates that NAFLD is the hepatic manifestation of the so-called metabolic syndrome. The metabolic syndrome in turn is associated with obesity and T2DM, which manifest in co-morbidities such as diabetic retinopathy with loss of sight, diabetic neuropathy with chronic foot ulcer and limb amputations, hepatocellular carcinoma, liver failure and ultimately, death (Adviti *et al.* 2013, WHO 2017, Lazaridis and Tsochatzis 2017). NAFLD is globally present with increasing prevalence and incidence in the United States, Asia and Europe since the last 30 years resulting in a high economic burden to healthcare systems (Adviti *et al.* 2013, WHO 2017). Today, it is the most common liver disease worldwide, and therefore of great interest for researchers, physicians and health economists all over the world to gain broad and profound knowledge about the pathogenic nature of NAFLD (Adviti *et al.* 2013, WHO 2017).

Increasing evidence points to obesity *per se* having high impact on the fatty degeneration of the liver, in the meanwhile even higher than alcohol. This assumption has been supported with data from researchers, who conducted a cross-sectional study in Northern Italy in 2000, in which they presented a prevalence for *steatosis hepatis* of 75.8 % in obese study subjects and comparatively only 46.4 % of study subjects with liver steatosis in the group of classified heavy drinkers. They presented an increased risk for liver steatosis in obese study subjects compared to controls by 5.8-fold and only a 2.8-fold increased risk for liver steatosis in heavy drinkers compared to the controls. This study emphasizes only once more the compelling need of gaining profound knowledge about fat metabolism and liver disease in obesity (Bellentani *et al.* 2000).

The non-alcoholic fatty liver disease *per definition* is a significant fatty degeneration of the liver parenchyma (cut-off value > 5 % steatosis) without history of significant alcohol consumption (see threshold values under paragraphs 1.4.2 to 1.4.3) or evidence for secondary pathogenesis (Roeb *et al*. 2015).

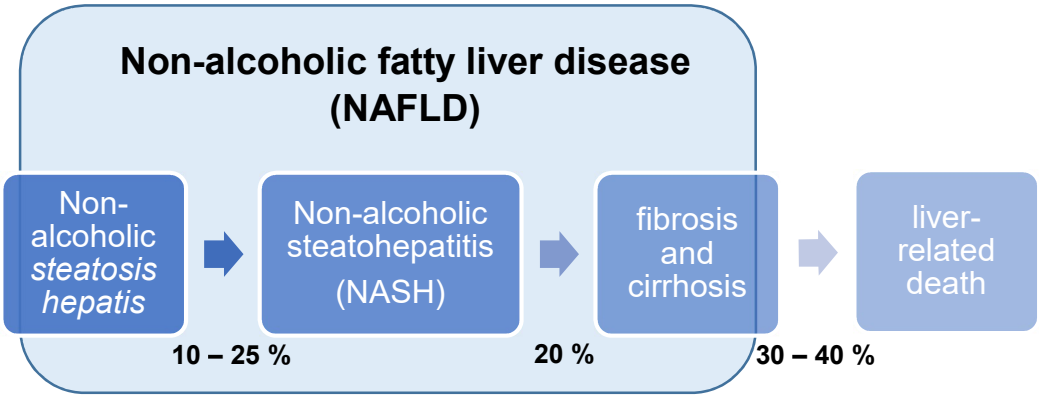

***Figure 1 – Histopathological entities of NAFLD**. Transitions between the histopathological entities are fluid and were quantified with data from 40 patients in the U.K. by Day et al. in 1995.*

1.2.2 Histopathology

The precise diagnosis of the fatty liver (lat.: *steatosis hepatis*) in general is not easily made in clinical routine as the current way of making a precise and final diagnosis needs to be performed histologically (Lazaridis and Tsochatzis 2017, Parekh *et al.* 2015, Pinto *et al.* 1996). This includes an invasive procedure for the patient with considerable side-effects in the form of a liver biopsy of which the standard techniques are the percutaneous one, first performed in 1923 by Bingel, and the transjugular liver biopsy, which was first reported in 1973 by Rösch *et al.*, and then recently emerging, the endoscopic ultrasound-guided liver biopsy (EUS-LB) (Bingel 1923, Grant and Neuberger 1999, Lazaridis and Tsochatzis 2017, Parekh *et al.* 2015, Rösch *et al.* 1973). Histologically, a *steatosis hepatis* is defined by the amount of so called fatty hepatocytes exceeding a number of 5 % of the total liver parenchyma, even though this consulted cut-off value of 5 % still has to be fully justified as described above (Roeb *et al.* 2015). Fatty hepatocytes in turn are defined by accumulation of lipids, mostly triglycerides (TG), in the hepatocellular cytoplasm under bright field microscopy, either in a macro or micro vesicular appearance. (Lazaridis and Tsochatzis 2017, Roeb *et al.* 2015).

Three levels of histological hepatic parenchymal fatty degeneration classify the steatosis and assess illness severity ranging from mild steatosis with a fatty degeneration of less than 33 % of the parenchyma, to average steatosis with a fatty degeneration between 33 to 66 % and finally to severe steatosis with a fatty degeneration of more than 66 % of the liver parenchyma, see also **table 1** (Roeb *et al.* 2015). However, besides specific study designs, patients barely undergo liver biopsy in daily clinical routine merely for the benefit of verifying a

likely *steatosis hepatis* as this may be of huge basic scientific interest but is not in accordance with directives. Also, the risk-reward-ratio remains ineffective and at least, biopsies show a certain sampling variability (Parekh *et al*. 2015). In contrast, in particular populations, such as in obese individuals, some experts propose to establish liver biopsy as a standard diagnostic procedure (Souhed *et al*. 2017). This population naturally is more likely to be treated with bariatric surgery and therefore needs to be staged and graded in NAFLD precisely as obese bariatric surgery patients have shown a significant peri- and postoperative morbidity and mortality due to undiagnosed cirrhosis preoperatively (Shouhed *et al*. 2017). For this purpose, it is also of high importance to apply novel non-invasive techniques of diagnosis making in clinical routine and screening of obese and T2DM patients.

Currently, specialists suggest to characterize and thus stage NAFLD histologically in a standardized way using three different scoring systems: The Brunt's and Kleiner's classifications and the NAFLD activity score, which differ, yet show a certain level of agreement (Ballestri *et al.* 2011, Santiago-Rolón *et al.* 2015). The Brunt's scoring system is the oldest established in 1999 on the base of 51 liver biopsies of 50 patients and works with four histopathological features, which result, once applied on histological specimen, in three grades and four stages of severity of the underlying liver disease (Brunt *et al.* 1999). The histopathological features of NAFLD in the Brunt criteria are shown in **table 1**.

Grading and staging occur independently one from another as they reflect different aspects of liver injury (Brunt *et al.* 1999). In 2000, the Non-alcoholic Steatohepatitis (NASH) Clinical Research Network extended the Brunt criteria into the NAFLD activity score (NAS), of which the most important difference is the cut-off value of > 5 % fatty hepatocytes of the total liver parenchyma, which defines a significant *steatosis hepatis* (Brunt 2005, Brunt *et al.* 2011, Kleiner *et al.* 2005, Roeb *et al.* 2015). In addition, fibrotic stages again are proposed to be staged according to another grading system, suggested by Kleiner *et al.* in the same year (Kleiner *et al.* 2005). All three systems have not been fully established in clinical practice yet and need to be merged.

NAFLD grading and staging system according to the Brunt Criteria

(modified after Brunt *et al.* 1999 and Torres and Harrison 2016)

Steatosis	grade	
< 0 %	0	
0 – 33 %	1	
33 – 66 %	2	
> 66 %	3	
Ballooning	grade	
Minimal	1	
Present	2	
Marked	3	
Inflammation	lobular	portal
	grade	grade
None	0	0
Mild	1	1
Moderate	2	2
Severe	3	3
Total Score Grading	score	**grade**
Mild	4 – 5	**1**
Moderate	6 – 9	**2**
Severe	9 – 12	**3**
Fibrosis Staging		**stage**
Zone 3 perisinusoidal fibrosis	0 1 2 3	**1**
+ portal fibrosis	0 1 2 3	**2**
+ bridging fibrosis	0 1 2 3	**3**
cirrhosis	4	**4**

Table 1 – NAFLD grading and staging system according to the Brunt Criteria. *Shows the proposed grading and staging system for NAFLD by Brunt in 1999, which uses four histopathological features:* ***Steatosis*** *means the percentage of fatty hepatocytes in the biopsy.* ***Ballooning*** *means the number of ballooned hepatocytes in the biopsy.* ***Inflammation*** *is defined by two parameters namely lobular (= intra-acinar) and portal. Inflammatory characteristics are defined by the cellular components of the biopsy specimen, which can be polymorphonuclear leukocytes, lymphocytes and other mononuclear cells, eosinophils, microgranulomas and plasma cells. Lobular inflammation means the number of lobular inflammatory herds, which in turn means the number of inflammatory foci per 20 X with a 20 X ocular.* ***Fibrosis*** *is defined as zone 3 perisinusoidal (= pericellular) fibrosis, portal fibrosis, bridging fibrosis and finally cirrhosis. Non-alcoholic fatty liver disease (NAFLD) (Brunt et al. 1999).*

Nevertheless, transitions between the histological subgroups remain fluid and challenging to distinguish. Attempts to quantify the progression percentages along the NAFLD stages have been conducted by several researchers, including C. P. Day and colleagues in the U. K., who recruited 40 patients from the Freeman Hospital and enrolled them in a seven years long-term follow-up study, which was published in 1995 (Teli *et al.* 1995). Their data indicate that 10 % to 25 % of the patients with non-alcoholic hepatic steatosis transform into non-alcoholic steatohepatitis, from which 20 % progress into fibrosis respectively non-alcoholic hepatic cirrhosis and 30 to 40 % of the patients with liver cirrhosis suffer from liver-related death (see **figure 1**) (Engin 2017a, Teli *et al.* 1995). 10 years later the German Society for Gastroenterology, Digestion and Metabolism Disorders published data in the updated guideline of the specialist union of physicians and clinicians indicating that 10 % to 20 % of NAFLD patients progress into NASH and 5% of NASH patients progress into cirrhotic stages (Roeb *et al.* 2015). Meanwhile, it is important to keep in mind that literature hasn't agreed yet on a uniform nomenclature of non-alcoholic fatty liver diseases and so one can still find various ways of describing the same disease, even though there have been attempts

to agree on one spelling and nomination (Araújo *et al.* 2018, Hashimoto *et al.* 2013, Sanal 2011).

1.2.3 Epidemiology

NAFLD is highly prevalent worldwide with an increasing incidence in the United States, Asia and Europe, finally a globally spreading disease with a high economic burden since the last 30 years (Shouhed *et al.* 2017). And yet, only a limited number of studies elucidate the exact incidence and prevalence values of NAFLD, which is due to multiple reasons. First, NAFLD is happening asymptomatically for the majority of patients, which means it is simply overlooked in clinic due to lack of indications (Torres and Harrison 2016). And second, the applied diagnostic criteria for NAFLD vary broadly amongst countries and even within countries, which may also explain the discrepancy in numbers as the diagnostic modalities vary in their sensitivity powers and may detect NAFLD earlier or later, worse or better (Shouhed *et al.* 2017, Torres and Harrison 2016). So, it happens that prevalence values still vary between 2.8% and 46%, to give only one example (Torres and Harrison 2016). Nevertheless, the World Gastroenterology Organization published prevalence values for NAFLD of 20 to 30 % in Europe and the Middle East, 40 to 90 % in obese populations worldwide and 90 to 95 % in morbidly obese patients worldwide (Shouhed *et al.* 2017). In 2016, Younossi *et al.* have conducted a meta-analytic assessment of prevalence, incidence and outcomes for NAFLD from cross-sectional studies worldwide (Younossi *et al.* 2016). Data from this overview and attempt to catch NAFLD in concrete numbers are presented in **figure 2**.

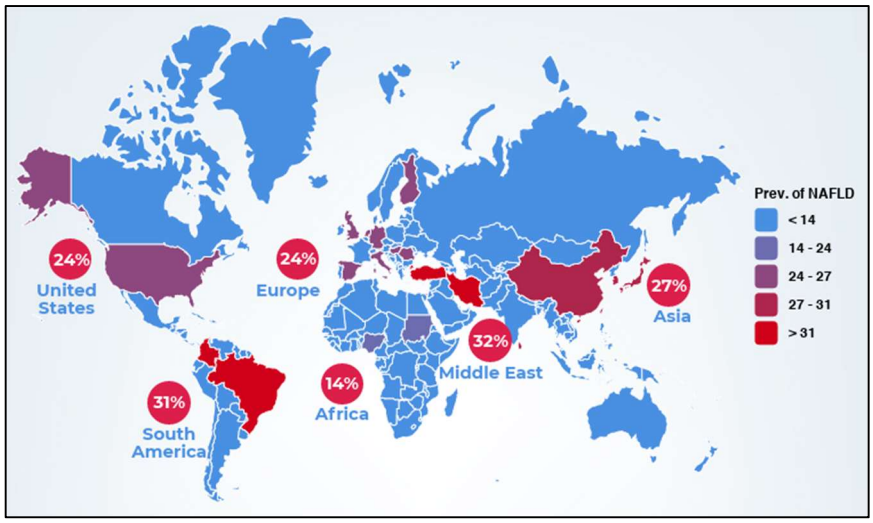

Figure 2 – Global prevalence of NAFLD. *In the Middle East, 32 % of the study population are estimated to have NAFLD (data collected in Iran, Israel and Turkey), in South America 31 % (data collected in Brazil and Colombia), in Asia 27 % (data collected in China, Japan, Korea, Sri Lanka and Taiwan), in Europe 24 % (data collected in Finland, Germany, Hungary, Italy, Netherlands, Romania, Spain and the UK), in North America*

24 % (data collected in the U.S.) and in Africa 14 % (data collected in Nigeria and Sudan) of the local population (Younossi et al. 2016). Figure has been created with © 2018 Piktochart. All Rights Reserved. Malaysia Incorporated Company. Non-alcoholic fatty liver disease (NAFLD).

A large cohort study to date has been carried out in Texas, U.S., with 400 patients enrolled, which were recruited from the Brooke Army Medical Center between 2007 and 2010, using a baseline questionnaire, laboratory data and ultrasound to diagnose *steatosis hepatis* and then verify and stage NAFLD histologically. Williams and colleagues established a prevalence rate for NAFLD of 46 % (151 out of 328 patients with a positive ultrasound) and for NASH of 12.2 % (40 out of 328 patients with a positive ultrasound) in middle-aged adults (54.6 +/- 7.35 years old) (Williams *et al.* 2011). In addition, William *et al.* as well as Browning *et al.* found a clear association between NAFLD and ethnicity introducing a higher prevalence of NAFLD in Hispanics (Williams *et al.* 58.3% and Browning *et al.* 45 %) compared to Caucasians (Williams *et al.* 44.4 % and Browning *et al.* 33%) and African Americans (Williams *et al.* 35.1 % and Browning *et al.* 24 %) (Williams *et al.* 2011, Browning *et al.* 2004). Important factors for this ethnicity dependency seem to be lifestyle and therefore differences in physical activity and diet as well as genetic influences (Torres and Harrison 2016).

1.2.4 Diagnosis

The clinical diagnosis of NAFLD is performed in two steps: First, by ruling out aetiologies, which result in liver disease of secondary pathogenesis. Second, by measuring the risk factors and co-morbidities of NAFLD that current literature indicates to be obesity, hypertension, dyslipidaemia and hyperglycemia respectively T2DM, which all together form the metabolic syndrome (see **figure 3)** (Engin 2017b, Roeb *et al.* 2015).

The metabolic syndrome shows a highly significant association with the prevalence of NAFLD, although NAFLD also occurs in patients not diagnosed with the metabolic syndrome (Roeb *et al.* 2015). Clinicians perform, accordingly to the current recommendations of the respective specialist boards, physical examination, blood tests and imaging techniques (Roeb *et al.* 2015). The recommended diagnostic algorithm attempts to roughly picture the severity of liver disease and, most importantly, to catch the turning point where the simple and benign steatosis of the liver parenchyma progresses into an inflammatory yet reversible

stage, the non-alcoholic steatosis hepatitis (NASH). If overlooked, in the course of many years, NASH may progress into more severe liver diseases such as fibrosis, cirrhosis and – with or without precursory fibrosis or cirrhosis – malignant dignities such as the hepatocellular carcinoma, which is the malignant liver tumour of highest prevalence (Bellentani *et al.* 2000, Lazaridis and Tsochatzis 2017, Lee and Lee 2017, Leitlinienprogramm Onkologie der AWMF *et al.* 2013, Teli *et al.* 1995, Zoller and Tilg 2016). It also needs to be ruled out, whether a history of significant alcohol consumption is present. In that case, diagnosis may also not be precise – therefore histology is always needed – yet, more easily performed as it requires parameters that the clinician can simply and quickly access from detailed medical history taking. Important to take in account is that the cut-off values for the parameters described differ between countries. In that sense, valid cut-off values in Germany have been set up as a consensus recommendation by the German Society of Gastroenterology, Dyspepsia and Metabolic Disorders (Deutsche Gesellschaft für Gastroenterologie, Verdauungs- und Stoffwechselkrankheiten e.V.) and defined as an alcohol intake per day of 10 g for women and 20 g for men (Roeb *et al*. 2015). 10 g of alcohol correspond to a german standard glass of beer (250ml) with an average alcoholic content of 4,8 vol.-%, of wine or prosecco (100ml) with an average alcoholic content of 11 vol. -% and to a shot of Schnaps (4cl) with an average alcoholic content of 33 vol.-% (Deutsche Hauptstelle für Suchtfragen e.V. 2017). This in turn refers to a weekly consumption of 70 g of alcohol equalling 7 glasses of beer, wine, prosecco or 7 shots accounting for women and 140 g of alcohol equalling 14 glasses of beer, wine, prosecco or 14 shots accounting for men. The corresponding cut-off values for a significant alcohol use in the United States have been collected and defined in the U.S. Guideline for NAFLD written by the American Association for the Study of Liver Diseases, the American College of Gastroenterology and the American Gastroenterological Association as a current or recent alcohol consumption of more than 14 drinks per week for female and more than 21 drinks per week for male patients (Younossi *et al*. 2016). *Steatosis hepatis* of secondary genesis originates from primary diseases like virus infections, toxic effects from drugs and metals, hereditary metabolic disorders, intestinal diseases, pregnancy and structural disorders of yet unsolved origin (Kneeman *et al.* 2012, Roeb *et al.* 2015, Torres and Harrison 2016).

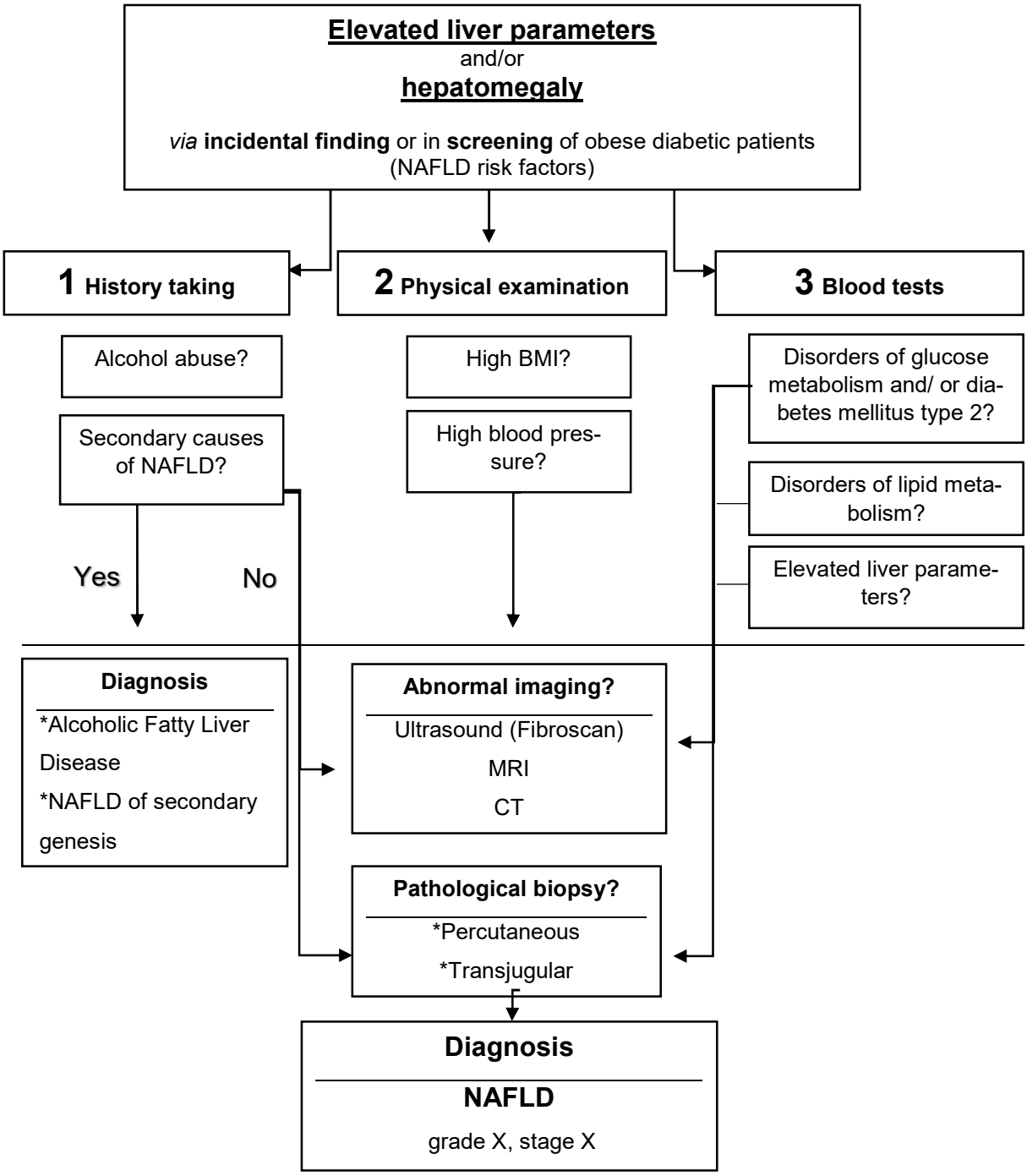

Figure 3 – Diagnostic algorithm for NAFLD*. The diagnostic algorithm had been suggested by the German and American guidelines for NAFLD in order to diagnose NAFLD correctly. Upon incidental findings of elevated serum liver parameters and/ or hepatomegaly as well as the occurrence of co-morbidities associated with NAFLD such as obesity and diabetes mellitus type 2, physicians should consider to follow the given algorithm to detect and thus grade and stage liver injury. Secondary causes of NAFLD: Chemical compounds, drugs, intestinal associated, acquired and hereditary metabolic disorders, metals, plant-based, pregnancy, structural disorders and virus infections. Disorders of glycolytic metabolism: Fasting serum blood glucose and HbA1c. Disorders of lipid metabolism: Fasting serum TG, HDL-cholesterol and LDL-cholesterol. Liver parameters: Serum transaminases (ALT, ALP), γ-GT, Quick-value, albumin and the cholinesterase. Alanine aminotransferase*

(ALT), aspartate aminotransferase (AST), high density lipoprotein (HDL), gamma glutamyl tranpeptidase (γ-GT), glycated haemoglobin (HbA1c), low density lipoprotein (LDL).

1.2.5 Aetiology and pathogenesis

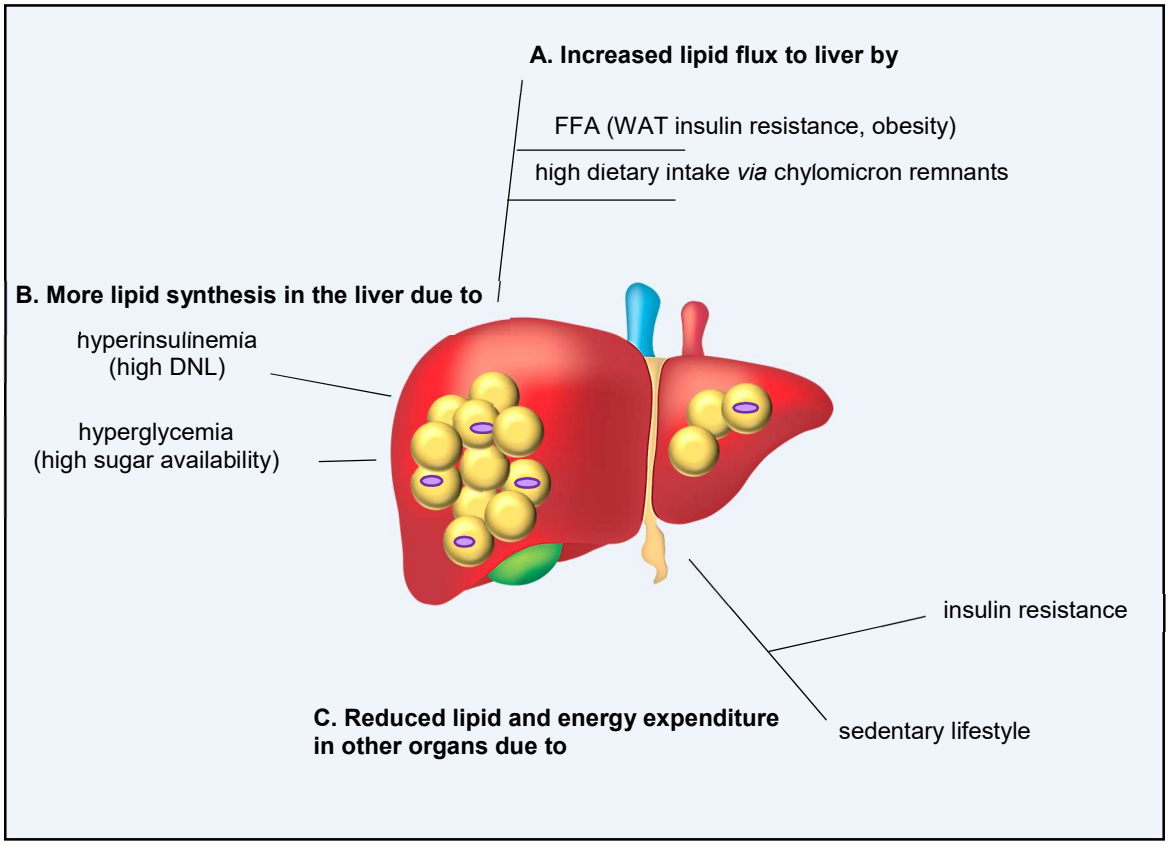

Figure 4 – Pathogenesis of NAFLD. *Where does the fat in the liver originate? The underlying mechanisms of the pathogenesis of NAFLD are highly complex, but are characterized by parameters such as obesity, hyperinsulinemia, insulin resistance, hyperglycaemia, T2DM and de novo lipogenesis (DNL), which is the synthesis of fatty acids from non-lipid precursors like glucose and amino acids and is catalyzed by the cytoplasmic multidimensional homodimeric enzyme complex fatty acid synthase (FASN) (Ameer et al. 2014, Postic and Girard 2008). Due to peripheral insulin resistance, WAT mobilizes free fatty acids (FFA) and secretes them into the blood stream, which the liver uses as substrates for generation of very low density lipoproteins (VLDLs), which are again secreted back into the blood. A vicious circle evolves resulting in elevated serum lipids and the accumulation of fat in the liver in form of triacylglycerides packed in fat droplets in the hepatocytes (Scheja and Heeren 2016). Free fatty acid (FFA), very low density lipoprotein (VLDL).*

Aetiology and thus true natural history of NAFLD are a highly researched subject in the past decade, even though the discussion had started already in the early 60's (Thaler 1962). Some

of the pioneers describing the correlation between the fatty liver disease and obesity were two medical doctors from the Sinai School of Medicine in New York in 1979, Michael Adler and Fenton Schaffner. They participated in the exploration of the causes for the observed liver steatosis and cirrhosis in patients lacking any history of significant alcohol consumption (Adler and Fenton 1979, Lee 1989, Ludwig *et al.* 1980, Pinot *et al.* 1996, Thaler 1962). Hereafter, the first to describe NASH as a clinical entity were Ludwig *et al.* in 1980 (Ludwig *et al.* 1980). So far, there is high evidence throughout the literature that insulin resistance plays a pivotal role in the pathogenesis of NAFLD (see **figure 4**) (Feldman *et al.* 2016, Sanders and Griffin 2016, Scheja and Heeren 2016). Insulin resistance and compensatory hyperinsulinemia, through mechanisms which are not fully understood and described yet, cause reduced DNL in the WAT and increased DNL in the liver (Feldman *et al.* 2016, Scheja and Heeren 2016). In the metabolic dysregulated stage of hyperinsulinemia, insulin resistance and obesity, the adipocytes from the central (visceral or omental) and peripheral (subcutaneous) adipose tissue seem to be saturated and upon this, alterations in the liver take place as the WAT and the liver communicate in a very tight metabolic unit regarding the lipid metabolism (see **figure 4**) (Scheja and Heeren 2016). The underlying molecular mechanisms are subject of the present work.

1.3 Metabolic inflammation

1.3.1 The inflammatory nature of obesity and T2DM associated NAFLD

The purpose of any inflammatory process in general is tissue protection and it therefore has beneficial effects in single cells, organs and the whole body system *via* eliminating pathogens and restoring the physiological organ function and homeostasis (Brenner *et al.* 2013). Typically, only when the inflammatory immune response is exaggerated or the resolving arm of the immune response is dysfunctional and thus the inflammation becomes chronic, is has deleterious effects on tissue, causes cell death and severe irreversible organ damage (Brenner *et al.* 2013). This also accounts for inflammation in liver disease, which will be looked at by measuring the hepatic expression levels of CXCL10, TREM-2, TNF-α and COL1A1 in the liver tissue biopsy specimen from three steatosis groups (I, II and III) of non-diabetic and diabetic obese NAFLD patients.

1.3.2 Expression and regulation of inflammatory and fibrotic genes

C-X-C motif chemokine ligand 10 (CXCL10)

The human CXCL10 gene codes for the gene product with the official full name C-X-C motif chemokine ligand 10, also known under the names interferon-γ-inducible protein 10 (IP-10) and C7 (Bishara 2012, Ruffilli *et al.* 2014). CXCL10 is member of the CXC subfamily and binds as a ligand to the G protein coupled C-X-C motif receptor 3 (CXCR3) expressed on immune cells like T lymphocytes and natural killer (NK) cells; and some epithelial cells (Ruffilli *et al.* 2014). CXCL10 secretion by T helper 1 lymphocytes (Th1 lymphocytes), NK cells and monocytes highly depends on the presence of another chemotactic agent, interferon-γ (IFN-γ) (Ruffilli *et al.* 2014). The presence of the CXCL10 chemokine in extracellular fluids such as blood, urine and the extracellular matrix (ECM) in tissues indicates a strong immune response and thus an ongoing inflammatory process in these tissues; therefore, CXCL10 is determined in studies addressing autoimmune diseases like the Grave's disease for instance (Ruffilli *et al.* 2014). In this doctoral thesis, the expression of CXCL10 in the livers from obese diabetic NAFLD patients is analyzed in order to map the grade of hepatic inflammation in NAFLD, NASH, fibrosis and cirrhosis compared to controls and by this investigate the inflammatory nature of NAFLD pathogenesis in obesity in humans.

Tumor necrosis factor α (TNF-α)

The proinflammatory cytokine tumor necrosis factor α (TNF-α) gene is the founding member of the TNF superfamily and is of high interest, as it seems to play pivotal roles especially in autoimmune diseases, the pathogenesis of insulin resistance, tumorigenesis and many more (Cazanave *et al.* 2011, Hirsova and Gores 2015, van Furth *et al.* 1972). TNF-α is mainly secreted by activated macrophages and unfolds its various effects on cell differentiation, apoptosis, lipid metabolism and so forth by binding to its two receptors on the cell surface. These are the tumor necrosis factor receptor superfamily member 1A (TNFRSF1A), also known as TNFR1, and tumor necrosis factor receptor superfamily member 1B (TNFRSF1B), also known as TNFR2 (Marchler-Bauer *et al.* 2017, van Furth *et al.* 1972). *Via* a death domain, TNFR1 induces the NF-κB signaling pathway and hereby leads to downstream pro-inflammatory and stress responses, which are pathogen-clearing cell activities. In contrast, TNFR2 directs rather anti-inflammatory pathways and thus cell responses, which suppress inflammation (Marchler-Bauer *et al.* 2017).

Triggering receptor expressed on myeloid cells 2 (TREM-2)

The triggering receptor expressed on myeloid cells 2 (TREM-2) operates as a surface membrane receptor and binds ligands, which conduct immune responses from extracellular into the cell and therefore play a pivotal role in chronic inflammation (Bouchon *et al.* 2000), Turnbull *et al*. 2006). A very rare disease associated with gene defects in the TREM-2 gene is the polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL), the Nasu-Hakola disease (Bianchin *et al.* 2004).

The TREM molecules, including TREM-1 and TREM-2, were discovered in 2000 as novel receptors of the immunoglobulin superfamily (IgSf) expressed on neutrophils and monocytes, respectively, by Bouchon *et al.* The authors showed that TREM-1 exhibits rather pro-inflammatory characteristics, whereas TREM-2 restrains inflammatory activity, which was shown by Turnbull *et al*. in 2006. Bouchon *et al.* detected TREM-1 to induce acute inflammatory responses upon stimulation of blood neutrophils and monocytes with bacterial lipopolysaccharides (LPS). Moreover, TREM-2 has been of interest foremost in basic research about signaling pathways in acute inflammatory responses, osteoclastogenesis and diseases of the nervous system, especially pathologies of microglia and age-associated neurodegenerative diseases such as the Alzheimer's disease and Parkinson's disease (Bouchon *et al.* 2000, Kawamoto and Minato 2004). As the thesis laboratory found that TREM-2 expression in human liver is elevated in obese humans (unpublished), this work aims to elucidate the role of TREM-2 in obese patients with NAFLD for the first time.

Alpha-1-type-1-collagen (COL1A1)

Alpha-1-type-1-collagen (COL1A1) forms the pro-α-1-chain of the triple helix of type I procollagen, which is processed into type 1 collagen outside of the cell (Kuivaniemi *et al.* 1997 and 1991). Collagen itself exists in 19 different types of which two third have a fibrillary structure such as the type I collagen. Type I collagen is abundant in many different cell types of the body but mainly in connective tissue producing cell types such as fibroblasts, osteoblasts, chondroblasts and endothelial cells. In this thesis the hepatic expression of the COL1A1-gene is analyzed to define the progressed grade of inflammation, which is marked by fibrosis of the liver parenchyma, and seen in NASH. COL1A1 in the liver is expressed by myofibroblasts in the perisinusoidal space, which differentiate upon tissue damage to hepatic stellate cells (Asselah *et al.* 2008, Brenner *et al.* 2013).

1.4 Fatty acids and *de novo* lipogenesis

Fatty acids contain hydrocarbon chains that vary in chain length and number of double bonds and carry a carboxyl group at one end (Young and Zechner 2013). They carry out four main tasks in human metabolism. First, they serve as high-density storage for metabolic energy as part of TG. Second, as part of phospholipids und glycolipids they serve as cell membrane elements providing the fluid and dynamic character of these two-dimensional solutions with polarized lipids and proteins, and in addition transmitting signals over the in most eukaryotes 6 to 10nm thick membrane from extracellular to intracellular in the form of chemical messengers in signal transduction pathways. Third, fatty acids may be used in posttranslational modification in order to label proteins as 'membrane proteins', so that they are directed to their designated places. Fourth and last as known to date, processed fatty acids function as extracellular, meaning systemcial, chemical hormone-like messengers, for example prostaglandins (Peng and Chen 2017).

1.4.1 The metabolic unit of the WAT and the liver

White adipocytes and the liver work closely together in order to maintain whole body energy homeostasis according to constantly changing energetic states in the body. The unit of the WAT and liver supplies the whole body with FFAs in the catabolic state and store surplus energy from sugar, proteins and fat in the anabolic postprandial state (Scheja and Heeren 2016). In catabolic conditions, between meals, WAT releases FFAs into the blood, which the hepatic parenchymal cells, hepatocytes, absorb and process into VLDLs. VLDLs in turn are secreted into the blood stream, so that peripheral cells, mainly muscle cells, absorb them and supply their mitochondria with VLDLs for the production of energy in form of ATP (Young and Zechner 2013).

1.4.2 *De novo* lipogenesis

DNL is the synthesis of fatty acids from non-lipid precursors like glucose and amino acids (Ameer *et al.* 2014, Postic and Girard 2008). DNL is catalyzed by the fatty acid synthase (FAS or FASN), a multidimensional homodimer enzyme complex, in the cytoplasm of DNL competent cells. In two active centers supplied with acyl groups it generates palmitoyl-CoA, $NADP^+$, CO^2 and H_2O from acetyl-CoA, malonyl-CoA and NADPH from the glycolysis and proteolysis (Ameer *et al.* 2014, Jayakumar *et al.* 1997, 1995 and 1994 and Postic and Girard 2008). Malonyl-CoA is synthesized from acetyl-CoA by a carboxylation reaction catalyzed

by the acetyl-CoA-carboxylase (ACACA) (Ameer *et al.* 2014, Postic and Girard 2008). This reaction is the committed step and serves as the DNL initiating reaction (Postic and Girard 2008). The C2-chain is hereafter prolonged by adding one C2-unit after another from carboxylated, meaning activated, acetyl-CoAs under the emission of carbon dioxide and consumption of NADPH until the chain terminates in a 16 C-atom-chain, called palmitate (Ameer *et al.* 2014, Postic and Girard 2008).

DNL is performed by a variety of enzymatic activities. In mammals, they cooperate in the cytosol and are included in a single FASN polypeptide, whereas in the mammalian mitochondria and in bacteria, there are several polypeptides, which assemble into an active protein multimer (Ameer *et al.* 2014). DNL is predominantly performed by major lipogenic tissues, which are responsible for the whole body energy homeostasis – the adipose tissue and the liver (Ameer *et al.* 2014, Scheja and Heeren 2016).

1.4.3 Altered *de novo* lipogenesis in the liver under obese conditions

In obesity, the natural metabolic balance between the liver and WAT is impaired and the balance of DNL shifted to the hepatic side (Eissing *et al.* 2013, Scheja and Heeren 2016). Previous data indicate that DNL enzymes are up-regulated in the liver and down-regulated in WAT, which finally supports the development of non-alcoholic fatty liver disease (Diraison *et al*. 2002, Eissing *et al.* 2013, Scheja and Heeren 2016). The exact underlying molecular mechanisms need to be investigated and single molecules and messengers to be identified. Obesity is characterized by hypertrophic white adipocytes that secrete not only more FFAs than in a lean body but also pro-inflammatory cytokines such as TNF-α and Interleukin-6 (Eissing *et al.* 2013, Scheja and Heeren 2016). Pro-inflammatory cytokines and a high supply with FFAs in turn affect the liver resulting in a greater hepatic fat content (fatty degeneration) and eventually also subclinical inflammation (Scheja and Heeren 2016). This in turn stimulates the liver to secrete regulatory molecules such as fetuin-A and apolipoprotein C3, which signal back to the WAT. These, in turn, aggravate the subclinical inflammation, which further induces insulin resistance in the WAT, the liver and the peripheral tissues and manifests in clinical symptoms of the metabolic syndrome. Altogether, this can be seen as self-fuelling and therefore vicious metabolic cycle (Scheja and Heeren 2016).

1.4.4 Expression and regulation of DNL genes in the liver

Glucose affects hepatic lipogenic gene profile through both direct and indirect mechanisms. Directly, they are conducted through the lipogenic transcription factor carbohydrate responsive element binding protein (ChREBP), identified in 2001 (Abdul-Wahed *et al*. 2017, Ameer *et al.* 2014, Herman *et al.* 2012, Vijayakumar *et al.* 2017). Upon insulin-dependent or -independent glucose flux into the cell, dephosphorylations and posttranslational modifications, such as acetylation and O- linked N-acetylglucosaminylation of ChREBP-α, are induced (Abdul-Wahed *et al.* 2017 and Eissing *et al.* 2013). Once dephosphorylated and hereby activated, CHREBP- α is translocated into the nucleus and activates transcription of lipogenic genes and also of a slightly different isoform of itself: ChREBP-β. ChREBP-β in turn further enhances the expression of lipogenic enzymes (Eissing *et al.* 2013 and Ferré and Foufelle 2010). In a conjunctional manner with ChREBP, the lipogenic pathway is also regulated by insulin as one of the most potent activators of the sterol responsive element binding protein- 1c (SREBP-1c). SREBP-1c is bound to the inner membranes of the ER, where it matures *via* proteolytic cleavage upon insulin stimulation and in turn stimulates the expression of glycolytic and lipogenic enzymes (Ferré and Foufelle 2010). The stimulation of SREBP-1c by insulin is mediated by the phosphoinositide 3-kinase (PI3K)-dependent pathway. The transcription of SREBP-1c was shown to be enhanced by insulin, various nuclear hormone receptors such as liver receptor alpha (LXRα), nutritional factors and inhibited by glucagon, shown in cultured hepatocytes. It is subject of current research, how *de novo* lipogenic genes and their regulatory elements in the livers from obese individuals diagnosed with NAFLD are altered and which are the underlying mechanisms of these alterations. So far, data from diet-induced obese murine models have revealed an inversely regulated expression of the DNL gene FASN in the liver and the WAT, hence an up-regulation of DNL genes in the liver and a down-regulation in the WAT (Eissing *et al.* 2013).

The fibroblast growth factor 21 (FGF21) gene encodes a 181-amino-acid endocrine hormone member of the FGF family. It is of interest in the context of this study as a target gene and thus a marker of ChREBP activity in the liver, as shown in rodent studies (Fagerberg *et al.* 2014, Iizuka *et al.* 2009, Iroz *et al.* 2017), which is most likely to be the case in humans, too (Dushay *et al*. 2015, Lundsgaard *et al*. 2017). FGF21 expression is induced by various stimuli such as glucose flux into the cell, fasting states and protein deficiency and regulated by PPAR-α (stimulation) and the protease responsible for inactivation by protein cleavage (FAP) (inhibition), demonstrated in human primary hepatocytes, animal models and in humans (Laeger *et al*. 2014, Lundåsen *et al.* 2007, Sonoda *et al.* 2017). FGF21 seems to have

multiple beneficial effects on the carbohydrate and lipid metabolism and improve insulin sensitivity as observed in obese mice (Sonoda *et al.* 2017). FGF21 is therefore described to provide anti-diabetic and anti-obesity properties (Laeger *et al*. 2014, Sonoda *et al.* 2017). In the project of global analysis and classification of human tissue-specific expression patterns of proteins across organs by Fagerberg and colleagues in 2014, FGF21 RNA was highly expressed in the liver and in low quantities also in the thyroid gland, skeletal muscle and the adipose tissue (Fagerberg *et al.* 2014). The expression of the FGF21 gene in liver biopsy specimen of obese NAFLD patients and obese controls is investigated as a novel approach to explore its contradictory role in the pathogenesis of NAFLD and obesity, to picture its DNL negatively regulating function in humans and to explore its role as a novel drug opportunity in the context of the pharmacological treatment of T2DM, obesity and NASH (Sonoda *et al.* 2017).

1.5. Bile acids in liver physiology and pathophysiology

BAs conduct the following functions in mammals: They serve as disposal for excess cholesterol, emulsifiers of cholesterol, dietary lipids and fat-soluble vitamins and as signaling molecules (Russell 2003 and 1999, Thomas *et al.* 2008).

1.5.1 Primary bile acids

BA biosynthesis is made of two main parts taking place in different organs permitting hereby also spatial separation (Russell 2003). The first part is performed in the liver, involves 17 individual enzymes and produces so called primary BAs from cholesterol, mostly cholic acid (CA) and chenodeoxycholic acid (CDCA), the former catalyzed by the 12αHydroxylase (CYP8B1) (Ding *et al.* 2015, Tu *et al.* 2000, Russell 2003). CA and CDCA are then conjugated to one of the two amino acids, glycine and taurine, in the peroxisomes of the hepatocytes (Russell 2003). This conjugation step is catalyzed by the bile acid-CoA:amino acid N-acyltransferase (BAAT) (Ding *et al.* 2015 and Russell 2003).

The mixture of bile salts, phospholipids and free cholesterol is secreted into the intrahepatic located *canaliculi lumen*, where rhythmic smooth muscle contractions of the hollow muscular organs transport the gall into the gallbladder for storage, from where it may be secreted into the duodenum, which is part of the small intestine, upon an appropriate stimulus induced by food ingestion. Here, they unfold their function as emulsifiers and dissolve cholesterol,

dietary lipids and fat soluble vitamins and thus make them accessible for intestinal resorption and the liver (Ding *et al.* 2015, Russell 2003 and 1999).

1.5.2 Secondary bile acids

The second part generates the so-called secondary BAs from CA and CDCA, and is conducted in the colon by enzymes secreted from the anaerobic gut microbiota, which cut the glycol- and tauroconjugational amide bonds and catalyze the production of deoxycholic acid (DCA), ursodeoxycholic acid (UDCA) and lithocholic acid (LCA) (Alnouti 2009, Ding *et al.* 2015, Jiao *et al*. 2018, Russell 2003 and 1999, Yunpeng *et al.* 2015). BA synthesis is the major disposal mechanism for the body to excrete and by this counterbalance excess cholesterol. In addition, BAs are also recycled in the enterohepatic circulation, where they circulate 5 to 6 times in one single day (Russell 2003 and 1999) In this process 95% of BAs are reabsorbed by a group of various membranous transporter located in the distal ileum and 5% are finally excreted *via* the faeces (Russell 2003 and 1999).

1.5.3 Cytoxicity of bile acids

BAs may also exhibit cytotoxic properties, especially in the conjugated state in form of secondary BAs (Alnouti 2009). Yet, findings differ between species (animal models and humans) and within one species. Recently researchers question critically, whether liver injuries and especially the pathogenesis of cholangiopathies derive from toxic BAs as studies present opposite effects (e.g. fibrotic and antifibrotic) of BAs. Until now, no convincing evidence could be found for BAs to be toxic *per se* at physiological concentrations (Fickert and Wagner 2017).

1.5.4 Expression of pivotal bile acid metabolism regulating genes in the liver

Cytochrome P450 genes

The cytochrome P450 gene family encodes for proteins, which are functionally peroxidases, peroxygenases and monooxygenases and involved in a tremendously amount of metabolic reactions in the human body, especially in drug metabolism and infections (Hrycay and Bandiera 2012, Munro *et al.* 2018, Stavropoulou *et al.* 2018). They all use heme as a cofactor and therefore are called hemoproteins; P450 stands for their characteristic spectrophotomet-

ric peak at 450 nm in the reduced state (Stavropoulou *et al.* 2018). In humans, the cytochrome P450 family contains about 30 CYP enzymes and again is classified into the subfamilies 1 to 8, of which CYP3A4 and CYP2D6 are the most abundant in terms of drug metabolism (Stavropoulou *et al.* 2018). In BA biosynthesis and cholesterol metabolism, various CYP enzymes of the subfamilies seven and eight are key players, which will be elaborated in the following.

Cytochrome P450 family 7 subfamily A member 1 (CYP7A1)

The cytochrome P450 family 7 subfamily A member 1 (CYP7A1) gene encodes for the gene product cholesterol 7α-hydroxylase (CYP7A1). It is produced exclusively in the liver and catalyzes the rate limiting of the dominant, classical BA synthesis pathway, which is why it is tightly regulated *via* negative regulatory feedback mechanism (Russell 2003 and Thomas *et al.* 2008). The protein is located in the endoplasmic reticulum (ER) and bound to the inner site of the plasma membrane (Russell 2003 and Thomas *et al.* 2008). CYP7A1 catalyzes the hydroxylation of cholesterol into 7α-hydroxycholesterol, which in the following is oxidized by the 3β-hydroxy-Δ5-C27-steroid dehydrogenase (HSD3B7). 7α-hydroxy-4-cholesten-3-on is the product of HSD3B7 and hydroxylated by CYP8B1, see below (Šarenac and Mikov 2018).

Cytochrome P450 family 7 subfamily B member 1 (CYP7B1)

The cytochrome P450 family 7 subfamily B member 1 (CYP7B1) gene encodes for the gene product oxysterol 7α-hydroxylase, which is produced across many organs with highest expression in the thyroid gland, the liver and the brain. It is also located at the ER inner membrane side and catalyzes the rate-limiting step in the alternative pathway of BA synthesis (Russell 2003 and Thomas *et al.* 2008). Due to the high expressions in the thyroid gland, liver and the brain, it is suggested to be involved in the pathogenesis of atherosclerosis, neurosteroid and sex hormone metabolism (Russell 2003 and Thomas *et al.* 2008).

Cytochrome P450 family 8 subfamily A member 1 (CYP8B1)

The cytochrome P450 family 8 subfamily B member 1 (CYP8B1) gene encodes for the gene product sterol 12α-hydroxylase (CYP8B1). It plays a key role in terms of the final solubility of cholesterol as it catalyzes the conversion of 7α-hydroxy-4-cholesten-3-on into 7α, 12α-dihydroxy-4-cholesten-3-on and ultimately determines the CA: CDCA ratio, which is under

physiological healthy conditions 1.5:1 (Russell 2003, Šarenac and Mikov 2018, Thomas *et al.* 2008).

1.5.5 Regulation of bile acid metabolism regulating genes in the liver

The small heterodimer partner 1 (SHP1) gene, also known as the nuclear receptor subfamily 0 group B member 2 (NR0B2) belongs to the superfamily of nuclear receptors (NRs) and to the NR-subfamily of so called orphan receptors, which means that the endogenous ligands for these receptors have not been identified yet. The NRs might be novel therapeutic targets in order to develop drugs for the treatment of epidemic diseases such as diabetes mellitus type 2, breast cancer and neurodegenerative diseases (Shi 2007). Nevertheless, some ligands for the orphan receptors in general could be identified during the last 10 to 15 years and transfer the very ones into the subfamily of so called adopted orphan receptors upon successful identification (Shi 2007). Adopted orphan receptors are for example FXR, HNF4 and the peroxisome proliferator-activated receptor family (PPARs) of which the peroxisome proliferator-activated receptor α (PPARα), peroxisome proliferator-activated receptor β or δ (PPARβ or PPARδ) and peroxisome proliferator-activated receptor γ (PPARγ) play breakthrough roles in T2DM treatment (Hauer 2002, Shi 2007, Yki-Järvinen 2004).

PPARα agonists are the fibrates, a class of drugs that enhance FFA oxidation through activity enhancement of the lipoproteinlipase and lower the concentration of serum lipoproteins HDL and LDL and TG and therefore show a positive cardioprofile with cardioprotective effects (Yki-Järvinen 2004). Favoured substances in Germany, but no longer prescribed due to side effects and unproven efficacy on disease endpoints, are bezafibrate, fenofibrate and gemfibrozil and are indicated as lipid lowering drugs of second choice after statins in the treatment of coronary artery disease (CAD). PPARγ is target of the thiazolidinediones (TZDs), also called glitazones, of which the first substance was troglitazone in 1997 and the followed by rosiglitazone and pioglitazone, which have been taken off the market due to severe drug side effects. They unfold their anti-diabetic and cardio protective effects by improved insulin sensitivity and the lowering of serum glucose and free fatty acids and therefore are also called insulin-sensitizers (Hauer 2002, Yki-Järvinen 2004). SHP1 encodes for a gene product that unfolds its functions of a NR by protein-protein binding as it is lacking the DNA-binding domain (DBD) that NRs present usually (Prestin *et al.* 2017). Through this direct protein interaction *via* the conserved ligand-binding domain (LBD) with other NRs, it negatively regulates the genetic expression of genes involved in drug and glucose metabolism and BA biosynthesis. Other than that, SHP1 is of interest for the study as it is also a good

marker for the activity of the farnesoid X receptor (FXR), which in turn is involved in the feedforward and feedbackward regulation of CYP7A1 (Goodwin *et al*. 2000, Prestin *et al.* 2017, Russell 1999). FXR binds CDCA and its glycine and taurine conjugates and seems to induce the expression of SHP-1, whereby the production of BAs *via* the SHP-1-mediated repression of CYP7A1 expression is finally repressed (Goodwin *et. al* 2000). SHP1 is regulated not only by FXR, but also by the NRs hepatocyte nuclear factor 4α (HNF4α) and the liver receptor homolog-1 (LRH1) as data suggest in several studies conducted by Prestin and colleagues in 2017 and Goodwin *et al*. in 2000.

All genes analyzed in the available dissertation are depicted in **table 2**.

Overview of Analyzed Genes

Gene	Gene Name	Function	NCBI Gene Reference
FASN	fatty acid synthase	Biosynthesis of fatty acids. Indicates DNL. Major lipogenic tissues: Liver and adipose tissue. Single polypeptide in cytosol in mammals vs. protein multimer in mammalian mitochondria and in bacteria.	NM_004104.4 (mRNA), AY451392.1 (mRNA complete cds), BC063242.1 (mRNA cDNA clone complete cds)
FGF21	fibroblast growth factor 21	Mediates the hepatocytic response upon glucose after stimulation by GLUT2 and ChREBP. Might have a DNL negative regulating function in NAFLD.	NM_019113.3 (mRNA), BC018404.1 (mRNA cDNA clone complete cds)
CXCL10	chemokine (C-X-C motif) ligand 10	Secreted by and binding to Th1 lymphocytes, NK cells and monocytes *via* a G-protein receptor (CXCR3). Indicates a strong immune response and inflammation.	NM_001565.3 (mRNA), BC010954.1 (mRNA cDNA clone complete cds)
TNF	tumor necrosis factor	Secreted by activated macrophages and binding to two cell surface receptors: TNFR1 induces pro-inflammatory and hereby pathogen-clearing cell responses *via* NFκ-B signaling pathway. TNFR2 induces anti-inflammatory cell responses.	NM_000594.3 (mRNA) AY799806.1 (partial cds)
TREM-2	triggering receptor expressed on myeloid cells 2	Membrane receptor in two isoforms: TREM-1 conducts pro-inflammatory signals into the cell, for example on blood neutrophils upon LPS stimulation. TREM-2 conducts anti-inflammatory signals. Increased expression in liver tissue of obese humans.	NM_018965.3 (mRNA), AF213457.1 (mRNA complete cds) BC032362.1 (mRNA cDNA clone complete cds)
COL1A1	collagen, type I, alpha 1 chain	Abundant in connective tissue producing cell types like fibroblasts, osteoblasts, chondroblasts and endothelial cells. In the liver expressed in myofibroblasts from the perisinusoidal space, which differentiate into hepatic stellate cells upon tissue damage. Indicates progressed stages of inflammation marked by fibrosis.	NM_000088.3 (mRNA), AF017178.2 (complete cds), BC036531.2 (mRNA cDNA clone complete cds)
CYP7A1	cytochrome P450, family 7, subfamily A, polypeptide 1	Expressed exclusively in the liver. BA- biosynthesis initiating and rate-limiting enzyme. Located at inner side of ER membrane.	NM_000780.3 (mRNA), M93133.1 (mRNA complete cds), BC112184.1 (mRNA cDNA clone complete cds)
CYP7B1	cytochrome P450, family 7, subfamily	Expressed in various organs, highest in thyroid gland, liver and brain. Rate-limiting enzyme of alternative pathway. Located at inner side of ER membrane.	NM_004820.3 (mRNA), BC136574.1 (mRNA cDNA clone complete cds),

	B, polypeptide 1		hCT1951447.2 (AppliedBiosystems Humane Genome Survey Microarray v2.0),
CYP8B1	cytochrome P450, family 8, subfamily B, polypeptide 1	Decisive for the production of cholic acid and ultimately determines the CA: CDCA ratio, which is 1.5:1 under healthy conditions..	NM_004391.2 (mRNA), hCT1640852.2 (AppliedBiosystems Human Genome Survey Microarray v2.0), BC067442.1, BC067441.1, BC067434.1 (mRNA cDNA clone complete cds),
SHP1 = NR0B2	small heterodimer partner 1 = nuclear receptor subfamily 0, group B, member 2	Belongs to the superfamily of NRs and NR- subfamily of orphan receptors. Binds other NRs *via* protein-protein interaction in the conserved LBD as it's lacking a DNA- binding domain. Regulates expression of genes involved in drug and glucose metabolism and BA biosynthesis negatively. Regulated by the NRs HNF4α, LRH1 and FXR.	NM_021969.2 (mRNA), HQ692833.1 (mRNA complete cds), BC030207.1 (mRNA cDNA clone complete cds)

Table 2 – Overview of analyzed genes. *Provides an overview of the genes that were analyzed in this study. The genes are listed in the order in which they are named in the continuous text. Species of all analyzed genes is homo sapiens (human).*

Chapter 2

2. Material and Methods

2.1 Study Cohort

Ninety-six subjects of both sexes aged 28 to 71 years with morbid obesity of an average BMI of 54 kg/m² attending to the Adiposity Center Hamburg (Adipositas-Centrum Hamburg) at the General and Visceral Surgery Department, University Medical Center Hamburg-Eppendorf (UKE) from January 2012 to June 2015 were included in the study. The study was approved by the institutional review board of the ethics committee of the Medical Association of Hamburg, and therefore guaranteed that the study was conducted in accordance with the principles described in the Amsterdam Declaration. All participants provided written informed consent. In February 2017, the Bariatric Surgery Bio Database included around 800 patients. 95% of the bariatric patients received two main surgeries: The Roux-en-Y gastric bypass (RYGB) (operation time of 90 to 150 minutes) or the Sleeve gastrectomy (SG), see **figures 5A, B** and **C** (operation time of 45 to 90 minutes). Other, less frequent procedures, are the biliopancreatic diversion and the gastric band (Wolter and Mann 2015). The patients undergoing bariatric surgery had to meet the following criteria for inclusion into the database and conduction of biopsy: First, diagnosis of obesity class II (BMI ≥ 35 to 40kg/m²) plus comorbidities or obesity class III (BMI ≥ 40kg/m²) (Klish 2018). Second, intraoperational macroscopic conspicuous liver, which means criteria of fatty degeneration and enlargement.

For the purpose of the present study, patients were divided into four groups and 12 subgroups according to 1. sex (male/ female), 2. steatosis grade (meaning percentage of fatty hepatocytes: steatosis group I < 33% of fatty hepatocytes (η = 32), steatosis group II = 33% to 66% of fatty hepatocytes (η = 32), steatosis group III > 66% of fatty hepatocytes (n = 32)), 3. fibrosis (meaning presence of fibrotic characteristics in the liver biopsy specimen assessed independently by two senior pathologists); and 4. diabetes mellitus type 2 (T2DM, yes/ no). After standardization of data gene expression profiles were compared between the three steatosis groups I, II and III and the effects of fibrotic characteristics and T2DM were measured.

2.1.1 Anthropometric parameters

Gender (female (f) and male (m)) and age (in years) were recorded. Physical examination was performed defining the patients' body mass index (BMI), which was calculated by dividing weight by height squared (kg/m²).

2.1.2 Blood parameters

Fasting serum blood glucose (FBG in mg/dl) and HbA1c (glycated hemoglobin in %) were analyzed to test for disorders of glucose metabolism. The samples were also screened for dyslipidaemia by the measurement of total cholesterol (mg/dl), fasting serum TG (mg/dl), high-density lipoprotein (HDL) (mg/dl) and low-density lipoprotein (LDL) (mg/dl). Liver damage was assessed by measuring four hepatocyte enzymes, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma glutamyl transferase (γ-GT) (all in U/l). Hepatic synthesis capacity was assessed by measuring proteins exclusively produced in the liver, namely serum albumin (g/L) as well as enzymes, that are part of the coagulation system, synthesized in phylloquinone (vitamin K)-dependency and reflected in the so called prothrombin time, mathematically adjusted into the International Normalized Ratio (INR) value which is synonymous to the Quick-value in Germany, (Barthels and Depka 2003). C-reactive protein (CRP) and leukocytes in the serum were measured as indicators of inflammation.

2.1.3 Diagnosis of non-alcoholic fatty liver disease

The diagnosis of NAFLD was performed according to the last updated guideline for NAFLD ("S2k- Leitlinie nicht alkoholische Fettlebererkrankung") of the German Society of Gastroenterology, Dyspepsia and Metabolic Disorders (Deutsche Gesellschaft für Gastroenterologie, Verdauungs- und Stoffwechselkrankheiten e.V (Roeb *et al*. 2015). All of the patients enrolled in the study received full medical evaluation, including clinical history taking and physical examination by a physician. Exclusion criteria for the diagnosis of NAFLD were a history of significant alcohol consumption greater than 20 g/day according to the recommended guidelines of the German Society of Gastroenterology, Dyspepsia and Metabolic Disorders (Deutsche Gesellschaft für Gastroenterologie, Verdauungs- und Stoffwechselkrankheiten e.V.), pre-existing liver cirrhosis, chronic renal disease, inflammatory or autoimmune disorders, acute or chronic infectious diseases, cancer, endocrine disorders, HIV-

, HCV- , and HBV-seropositivity, pregnancy and lactation and any kind of anti-inflammatory medication. Inclusion criteria for the diagnosis of NAFLD were a positive imaging diagnosis of the liver, which was performed per ultrasound in the transcutaneous B-modus of the right and left upper abdominal quadrants in order to detect eventual abnormal sonographic patterns of the liver that can occur in various liver or liver related diseases including hepatic fatty degeneration (Parekh *et al.* 2015, Roeb *et al.* 2015, Sumida *et al.* 2014). Patients not meeting these criteria were excluded from the study cohort.

2.1.4 Diagnosis of type 2 diabetes mellitus

T2DM was diagnosed *via* fasting serum blood glucose and the oral glucose tolerance test (oGTT). Therefore, blood glucose had to be higher than 125 mg/dl in fasting state and higher than 199 mg/dl after 2 hours of ingestion of 75 g glucose. Hypertension was diagnosed in patients who showed a systolic and/ or diastolic blood pressure greater than 140/90 mmHg after a minimum of 10 minutes resting.

2.2 Sample Collection

Liver tissue samples were kindly provided by the medical colleagues Dr. Stefan Wolter, Dr. Oliver Mann and Dr. Philip Busch, who are heads of the Bariatric Surgery Bio Database from the Adiposity Center Hamburg (Adipositas-Centrum Hamburg) which again is part of the General and Visceral Surgery Department at the UKE (Institut für Allgemein- und Viszeralchirurgie des UKE).

Tissue of interest was the liver and biopsies were conducted in the end of the surgical procedure with a so called wedge-biopsy from liver segment 3 and without thermo coagulation at the biopsy specimen, which were thereafter saved in 1.5 mL cryogenic tubes (Thermo Fisher Scientific, No. 50001020) and thus immediately brought to the vapour phase of liquid nitrogen in order to prevent RNA degradation. The samples were stored at -80°C.

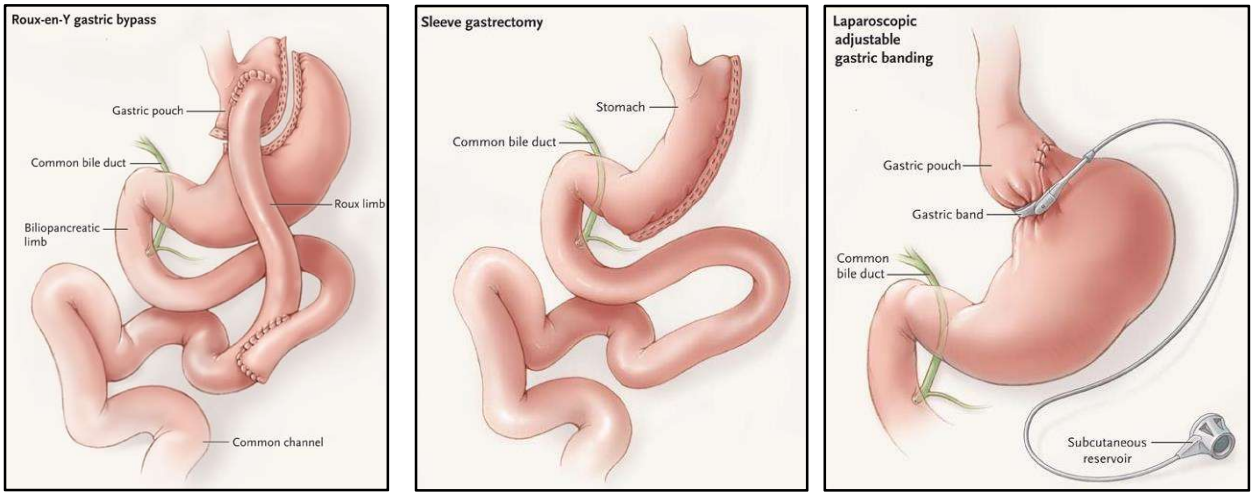

Figure 5 A, B and C – Three standard bariatric surgical procedures. *Figure 5A depicts the Roux-en-Y gastric bypass (RYGB). Figure 5B depicts the Sleeve gastrectomy (SG). Both are the most often applied bariatric surgeries today in order to treat obesity II and higher. Figure 5C depicts the method of the gastric band. Reproduced with friendly authorization from the Office of Clinical Research, University of Pennsylvania Perelman School of Medicine, 8041 Maloney Building, 3400 Spruce Street, 19104 Philadelphia.*

2.3 Statistical analysis

All statistical analyses were performed with Excel 2016. When comparing two groups, data were analyzed and significance was calculated *via* the non-paired two-tailed Student's *t*-test. All data are expressed as means ± standard error of the mean (s.e.m). Statistical significance is indicated by the *p* value (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

2.4 Method Overview

Overall aim of the conducted experiments was to detect the amount of the described enzymes of interest in liver biopsies from each of the defined three NAFLD stages on the mRNA level.

For this purpose, RNA from hepatic tissue was extracted and purified, used to generate complementary DNA (cDNA) and then determined the expression of specific mRNAs *via* real-time quantitative polymerase chain reaction (real-time qPCR).

Experiments were performed accordingly to the protocols from the vendors and supported laboratory work with technical information from Jansohn and Rothhämel 2012.

2.4.1 RNA Extraction

Total RNA was extracted 15 mg to 35 mg of frozen liver sample using the mirVana™ miRNA Isolation Kit from Ambion®, see **table 3** (Ambion®, Cat. No. AM1560).

Complete List of Reagents for RNA Extraction			
Component	**Additional information**	**Storage**	**Source**
miRNA Wash Solution 1	Add 21 Ml 100% ethanol before use	room temperature	Ambion®, Cat. No. AM1560
Wash Solution 1/3	Add 40 mL 100% ethanol before use	room temperature	Ambion®, Cat. No. AM1560
Collection Tubes	--	room temperature	Ambion®, Cat. No. AM1560
Filter Cartridges	--	room temperature	Ambion®, Cat. No. AM1560
Lysis/ Binding Buffer	--	4 °C	Ambion®, Cat. No. AM1560
miRNA Homogenate Addivtive	--	4 °C	Ambion®, Cat. No. AM1560
Acid-Phenol:Chloroform	--	4 °C	Ambion®, Cat. No. AM1561
Elution Solution	--	- 20 °C	Ambion®, Cat. No. AM1560
Nuclease-free Water	--	room temperature	Invitrogen™, Cat. No. 10977049
Hepatic Tissue Samples	Need to be kept continuously on dry ice	– 80°C	Bariatric Surgery Bio Database of the Center for General and Visceral Surgery at the UKE

Table 3 – Complete list of reagents for RNA extraction. *Lists the entire kit components and additional consumables not provided in the mirVana™ miRNA Isolation Kit from Ambion®. Ribonucleic acid (RNA), micro ribonucleic acid (miRNA).*

The kit works on the base of a so called Homogenate Additive, which binds preferably miRNA to a filter of glass-fibre and by that increases miRNA and RNA content of the final extract a lot more than classical RNA extraction methods like the TRIzol Reagent for example may provide (Ambion®, Cat. No. 15596018).

Tissues had been stored in 1.5 mL Cryogenic Tubes (Nalgene™, Cat. No. 50001020) at –80 °C according to regulations. Whole samples were then weighed with a regularly calibrated laboratory balance (PCE Instruments, Cat. No. PCE-BS 6000), tissue pieces of 15 to 35 mg cut and transferred to 2 mL Eppendorf-Safe-Lock Tubes® (Eppendorf, Cat. No. 0030120094).

Hereafter, the protocol provided by Ambion® was closely followed after being adjusted to the technical equipment of the doctoral thesis institute laboratories. Working on 12 to 24 samples similarly, 500 µl of Lysis-Binding Buffer (Ambion®, Cat. No. AM1560) containing isothiocyanate, which inactivates RNases, were added to frozen liver tissue. Immediately, stainless steel beads (QIAGEN, Cat. No. 69989) were added to each of the tubes, secured in corresponding Tissue Lyser racks (QIAGEN, Cat. No. 69982) and then homogenized in a Tissue Lyser (QIAGEN, Cat. No. 85300) at 20 hertz for three minutes two times. It was of great importance for final data quality to verify that homogenization had been complete as RNA loss threatens if pieces of tissue remain. In case of tissue pieces being still visible, affected probes were worked through at 20 hertz for three minutes per run until tissues were completely homogenized. Thereupon, 500 µl of the phenol:chloroform pre-mixed solution (Ambion®, Cat. No. AM1560) were added to each tube and thus samples shaken for 60 seconds at 20 hertz on the Tissue Lyser (QIAGEN, Cat. No. 85300). For phase separation of organic and aqueous layers samples were centrifuged for 15 minutes at speed of 13.200 rpm at + 4°C in the refrigerated centrifuge (Eppendorf, Cat. No. 5401000010). After complete phase separation, upper aqueous layers containing RNA were pipetted into new 1.5 mL Eppendorf-Safe-Lock Tubes® (Eppendorf, Cat. No. 0030120086) and novel volumes were determined by pipetting the solutions, which lay around 350 to 450 µl by mean. For precipitation of RNA, 600 µl of ice – cold Ethanol 96 % (stored at –20 °C) was added to each sample and inverted vigorously by hand. For washing and purification reasons solutions were then pipetted onto Filter Cartridges (Ambion®, Cat. No. AM1560) in novel 1.5 mL Eppendorf-Safe-Lock Tubes® (Eppendorf, Cat. No. 0030120086) and thus centrifuged for 15 seconds at speed of 10.000 rpm at + 4°C in the refrigerated centrifuge (Eppendorf, Cat. No. 5401000010).

Jut of each tube was discarded and 700 µl of Wash Solution 1 (Ambion®, Cat. No. AM1560) were pipetted onto Filter Cartridges (Ambion®, Cat. No. AM1560) in novel 1.5 mL Eppendorf-Safe-Lock Tubes® (Eppendorf, Cat. No. 0030120086) and thus tubes centrifuged at 15 seconds at speed of 10.000 rpm at + 4°C in the refrigerated centrifuge (Eppendorf, Cat. No.

5401000010). Complete procedure was repeated with 500 µl of Wash Solution 2/3 (Ambion®, Cat. No. AM1560) two times in total. The last washing step was a dry centrifugation for 60 seconds at speed of 10.000 rpm at + 4°C in the refrigerated centrifuge (Eppendorf, Cat. No. 5401000010). By then, RNA had been bound to the glass fibres of the Filter Cartridges (Ambion®, Cat. No. AM1560), cleaned and free of any DNA, phenol or chloroform. 50 to 100 µl of Elution Solution (Ambion®, Cat. No. AM1560) containing 0.1 mM EDTA, which inactivates RNases, were pipetted centrally on Filter Cartridges (Ambion®, Cat. No. AM1560) in novel 1.5 mL Eppendorf-Safe-Lock Tubes® (Eppendorf, No. 0030120086) and after 60 seconds of incubation time centrifuged for 30 seconds at speed of 10.000 rpm at + 4°C in the refrigerated centrifuge (Eppendorf, No. 5401000010). Again, eluates were pipetted centrally on to the Filter Cartridges (Ambion®, Cat. No. AM1560) in novel 1.5 mL RNase-free Microfuge Tubes (Invitrogen™, No. AM12400), incubated on ice for 10 more minutes and thus samples entirely centrifuged down to tubes bottoms for 60 seconds at speed of 10.000 rpm at + 4°C in the refrigerated centrifuge (Eppendorf, Cat. No. 5401000010). RNA concentration measurements were performed with 1.4 µl of each sample per measurement on a NanoDrop™ spectrophotometer after calibration with 95 °C pre-heated DNase/ RNase-Free Distilled Water (Invitrogen™, Cat. No. 10977049) according to regulations (PEQLAB Biotechnologies GmbH, NanoDrop™ ND-1000). Hereafter, RNA was stored at – 20°C at optimal final concentration for stable RNA storage of under 400 ng/µl and therefore eventually diluted and so concentrations adjusted with 95 °C pre-heated DNase/ RNase-Free Distilled Water (Invitrogen™, No. 10977049).

2.4.2 Reverse Transcription (RT)

Complete List of Reagents for RT	
Component	**Source**
10X RT Buffer	Applied Biosystems™, Cat. No. 4368813
10X RT Random Primers	Applied Biosystems™, Cat. No. 4368813
25X dNTP Mix (100mM)	Applied Biosystems™, Cat. No. 4368813
MultiScribe™ Reverse Transcriptase (50 U/µl)	Applied Biosystems™, Cat. No. 4368813
RNase Inhibitor (20 U/µl)	Applied Biosystems™, Cat. No. 4368813
Nuclease-free water	Invitrogen™, Cat. No. 10977049
RNA samples	from storage in – 20°C

Table 4 – Complete list of reagents for reverse transcription. *Llists the entire kit components and additional consumables not provided in the kit. Catalog number (Cat. No.), ribonucleic acid (RNA), reverse transcription (RT), deoxyribose nucleoside triphosphate (dNTP).*

For the analysis, the High Capacity cDNA Reverse Transcription Kits For 1000 Reactions from Applied Biosystems™ was used (see **table 4**) (Applied Biosystems™, Cat. No. 4368813) and steps were followed according to the adjusted protocol, which was provided by vendor.

Firstly, the 2X Reverse Transcription Master Mix (Applied Biosystems™, Cat. No. 4368813) was prepared on ice according to the protocol, while RNA probes stored in – 20°C softly thawed on dry ice.

Once thawed, the appropriate volume in µl from each sample was added to the 2X RT Master Mix aiming to convert 2 µg of RNA into single-stranded cDNA; and performed RT in the thermal cycler (Biometra, Thermocycler T3) at 25°C for 10 minutes, at 37°C for 120 minutes and at 85°C for 5 seconds (see **table 5**):

Thermal Cycler Program	
10 min	25 °C
120 min	37 °C
5 sec	85 °C

Table 5 – Thermal cycler program*. Depicts the thermal cycler program according to which RT had been performed. Reverse transcription (RT).*

The cDNA concentration measurements were performed with 1.4 µl of each sample per measurement on a NanoDrop™ spectrophotometer after calibration with 95 °C pre-heated DNase/ RNase-Free Distilled Water (Invitrogen™, Cat. No. 10977049) according to regulations (PEQLAB Biotechnologies GmbH, NanoDrop™ ND-1000).
cDNA was hereafter diluted in a 1:5 ratio, which meant adding 80 µl of Nuclease-free water to the final volume of 20 µl of each cDNA sample.
cDNA was then directly processed for qPCR or stored at – 20°C for later processing in qPCR.

2.4.3 Quantitative Polymerase Chain Reaction (qPCR)

Complete List of Reagents for Quantitative Polymerase Chain Reaction	
Component	**Source**
TaqMan™ Universal PCR Master Mix plus	Applied Biosystems™, Cat. No. 4364338
Assays on Demand	see table xx
Nuclease-free water	Invitrogen™, Cat. No. 10977049
cDNA samples	from storage in – 20°C

Table 6 – Complete list of reagents for quantitative polymerase chain reaction. *Lists all components used for performance of qPCR. Quantitative polymerase chain reaction (qPCR), synonymous to real-time polymerase chain reaction (rtPCR), complementary deoxyribose nucleic acid (cDNA).*

The last step of the entire experiment works on the base of the cDNA, from which genes of interest (GOI) are amplified and thus made visible and detectable by the TaqMan Machine (Applied Biosystems™, 7900HT Sequence Detection System).
According to protocol, which is provided by the vendor, the required assays on demand (AoD) from storage in – 20 °C were used to prepare the Assay on Demand Mix (AoD Mix) according to the following formula in 1.5 mL Eppendorf-Safe-Lock Tubes®, see **tables 6, 7** and **8** (Eppendorf, Cat. No. 0030120086):

Assay on Demand Mix
285 µl Universal Mix
28.5 µl AoD
161.5 µl Nuclease-free Water

Table 7 – Assay on demand mix. *Depicts the formula which had been used to prepare the AoD Mix and thus run qPCR. Assay on demand (AoD), synonymous to gene of interest (GOI), quantitative polymerase chain reaction (qPCR).*

Assays on Demand for Quantitative Polymerase Chain Reaction		
AoD	**Gene Name**	**Assay ID**
hCOL1A1	collagen, type I, alpha 1	Hs00164004_m1
hCXCL10	chemokine (C-X-C motif) ligand 10	Hs00171042_m1
hCYP7A1	cytochrome P450, family 7, subfamily A, polypeptide 1	Hs00167982_m1
hCYP7B1	cytochrome P450, family 7, subfamily B, polypeptide 1	Hs00191385_m1
hCYP8B1	cytochrome P450, family 8, subfamily B, polypeptide 1	Hs00244754_s1
hFASN	fatty acid synthase	Hs00188012_m1
hFGF21	fibroblast growth factor 21	Hs00173927_m1
hSHP = hNR0B2	nuclear receptor subfamily 0, group B, member 2	Hs00222677_m1
hTNF	tumor necrosis factor	Hs00174128_m1
hTREM2_Exon1	triggering receptor expressed on myeloid cells 2	Hs00219132_m1
h36B4 = hRPLP0	ribosomal protein, large, P0	Hs99999902_m1
hTAF1 = DYT3	TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 250kDa	Hs00270322_m1

Table 8 – Assays on demand for quantitative polymerase chain reaction*. Lists gene assays that had been used in qPCR. The primer and probe sequence is the sequence of bases respectively nucleotides, a linear information, via which genetic information are encoded. It is equivalent to the target sequence on the gene of interest (goi), which is wished to be amplified in order to be detected and measured in its quantity in the tissue or cell of interest. Assay on demand (AoD), human (h), identification (ID), quantitative polymerase chain reaction (qPCR), synonymous to real-time polymerase chain reaction (rtPCR).*

V-Bottom Plates, Fast 96-Well Reaction Plates from MicroAmp™ (Applied Biosystems™, Cat. No. 4346907), were then loaded with 25 µl of each AoD Mix and hereafter 5 µl of each cDNA sample were pipetted into the tubes according to the previously planned pipetting schema. The plates were thus covered with optical adhesive film (Applied Biosystems™ Cat. No. 4360954) and mixed on the thermoshaker (Eppendorf, Thermomixer 5436) for 3

minutes at a speed of 600 rpm. Hereafter, v-bottom plates were centrifuged at speed of 10.000 rpm at + 4°C in the refrigerated centrifuge for 2 minutes (Eppendorf, Cat. No. 5401000010) in order to eliminate air bubbles through pipetting. In order to transfer the volumes from the 96-well plates into the TaqMan™ 384-Well Reaction Plates (Applied Biosystems™, Cat. No. 4343370) the Tecan Robot (Tecan, Genesis RSP 200 Freedom) was used, which pipetted the plates according to a pre-programmed schema. The 384-Well Reaction Plates were also covered with optical adhesive film (Applied Biosystems™ Cat. No. 4360954) and centrifuged at speed of 10.000 rpm at + 4°C in the refrigerated centrifuge for 2 minutes (Eppendorf, Cat. No. 5401000010) in order to eliminate air bubbles through pipetting. The appropriate gene expression analysis program was selected on the TaqMan machine (Applied Biosystems™, 7900HT Sequence Detection System), and the AoD array established. The cycle threshold (Ct) values of the target genes were normalized to those of the endogenous control gene (TAF1) and relative changes in the target genes were calculated using the equation $2^{-\Delta\Delta Ct}$.

Complete List of Material Used in All Experiments

Material	Additional Information	Company	Brand	Quantity	Catalog Number
Devices					
Eppendorf Centrifuge 5415 R	refrigerated, rotor FA-45-18-11, rotor lid, temperature range 0°C to 40°C, max. speed 14.000 rpm, max. capacity 18 x 1.5/2.0 mL tubes	Eppendorf	Eppendorf Quality™	1 piece	5401000010
NanoDrop™ spectrophotometer	UKE Gerätenummer 73234	PEQLAB Biotechnologies GmbH Erlangen	NanoDrop™	1 piece	ND-1000
PCE-BS 6000 Laboratory Balance	UKE Gerätenummer	PCE Instruments	--	1 piece	PCE-BS 6000
TaqMan Machine	UKE Gerätenummer 73159	Applied Biosystems™	Manufactured by ABI PRISM	1 piece	7900HT Sequence Detection System
Tecan Robot	UKE Gerätenummer 73159	Tecan	Tecan	1 piece	Genesis RSP 200 Freedom 2216
Thermo Cycler	UKE Gerätenummer 61559	Biometra	Biometra	1 piece	

Thermoshaker	UKE Gerätenummer 64191	Eppendorf	Eppendorf Quality™	1 piece	Thermomixer 5436
TissueLyser II	UKE Gerätenummer 73161	QIAGEN	Manufactured by Retsch®	1 piece	85300
Reagents					
DNase/ RNase- Free Distilled Water	500 mL	ThermoFisher Scientific™ Life Technologies	Invitrogen™	1 bottle	10977049
High Capacity cDNA Reverse Transcription Kit	for 1000 Reactions	ThermoFisher Scientific™ Life Technologies	Applied Biosystems™	5 kits	4368813
miRNA Isolation Kit	for 40 preparations	ThermoFisher Scientific™ Life Technologies	Ambion®,	5 kits	AM1560
TaqMan™ Universal PCR Master Mix plus	--	ThermoFisher Scientific™ Life Technologies	Applied Biosystems™	2 bottles	4364338
Utensils					
Adhesive Seal Applicator	--	ThermoFisher Scientific™ Life Technologies	Applied Biosystems™	1 piece	4333183
Eppendorf Safe-Lock Tubes®	1.5 mL, colorless	Eppendorf	Eppendorf Quality™	1000 tubes	0030120086
Eppendorf Safe-Lock Tubes®	2 mL, colorless	Eppendorf	Eppendorf Quality™	1000 tubes	0030120094
F1-Clip Tip™ OneChannel Pipettors with variable volume	Volume range up to 10 µl	ThermoFisher Scientific™ Life Technologies	F1-Clip Tip™	1 piece	4641170N
F1-Clip Tip™ OneChannel Pipettors with variable volume	Volume range up to 20 µl	ThermoFisher Scientific™ Life Technologies	F1-Clip Tip™	1 piece	4641180N
F1-Clip Tip™ OneChannel Pipettors with variable volume	Volume range up to 100 µ	ThermoFisher Scientific™ Life Technologies	F1-Clip Tip™	1 piece	4641200N
F1-Clip Tip™ OneChannel Pipettors with variable volume	range up to 200 µl	ThermoFisher Scientific™ Life Technologies	F1-Clip Tip™	1 piece	4641210N
F1-Clip Tip™ OneChannel Pipettors with variable volume	Volume range up to 1000 µl	ThermoFisher Scientific™ Life Technologies	F1-Clip Tip™	1 piece	4641230N
Biosphere® Filter Tip 10	neutral	Sarstedt AG & Co.	Sarstedt	Case of 480	70.1130.210
Biosphere® Filter Tip 100	neutral	Sarstedt AG & Co.	Sarstedt	Case of 480	70.760.212
Biosphere® Filter Tip 200	neutral	Sarstedt AG & Co.	Sarstedt	Case of 480	70.760.211

Biosphere® Filter Tip 1000	neutral	Sarstedt AG & Co.	Sarstedt	Case of 500	70.762.211
General Long-Term Storage Cryogenic Tubes	1.5 mL, colorless	ThermoFisher Scientific™ Life Technologies	Nalgene™	500 tubes	50001020
Optical Adehesive Film	--	ThermoFisher Scientific™ Life Technologies	Applied Biosystems™	1 box	4360954
RNase-free Microfuge Tubes	1.5 mL, colorless	ThermoFisher Scientific™ Life Technologies	Invitrogen™	500 tubes	AM12400
SemperGuard® general purpose latex gloves	Disposable Powder Free Latex Gloves, Size S	Sigma-Aldrich® Merck	SemperGuard®	100 boxes	Z560790
Stainless Steel Beads	5 mm	QIAGEN		200 pieces	69989
TaqMan™ Fast 96-Well Reaction Plates	Without barcode	ThermoFisher Scientific™ Life Technologies	Applied Biosystems™ MicroAmp®	10 plates	4346907
TaqMan™ 384-Well Reaction Plates	Without barcode	ThermoFisher Scientific™ Life Technologies	Applied Biosystems™ MicroAmp®	1000 plates	4343370
TissueLyser Adapter Set	2 x 24	QIAGEN	Manufactured by Retsch®	1 set	69982

Table 9 – Complete list of material used in all experiments.

Chapter 3

3. Results

3.1 Cohort Characterization

Around 80% of all bariatric surgery patients presented criteria of a macroscopically altered liver with regard to organ enlargement and tissue structure. **Table 10** depicts the clinical characteristics of the groups analysed, defined by steatosis grades I, II and III as well as by presence or absence of T2DM. In terms of the serum lipid profile, plasma TG showed statistically significant increases along with the grade of liver steatosis and T2DM, whereas total cholesterol, LDL cholesterol and HDL cholesterol did not exhibit any statistically significant differences between the groups (see **table 10)**. Similar to dyslipidemia, the glucose metabolism of the study cohort presented dysregulations associated with the grade of liver steatosis and the diabetic phenotype, showing statistically significant increases in fasting blood glucose and HbA1c (see **table 10)**. These findings confirm the association of a diabetic phenotype with hepatic steatosis.

The plasma activities of the liver parameters ALT, AST and γ-GT, which reflect the organ tissue integrity and tissue damage of the liver, were significantly increased along with the grade of liver steatosis, especially in the diabetic subjects (see **table 10)**. On the contrary, no statistically significant decreases or trends in the levels of serum albumin, total protein in serum, creatinine and total serum bilirubin in obese non-diabetic and diabetic NAFLD patients could be observed. The same was true for the systemic inflammation indicating blood parameters, the CRP and the leucocytes that were not found to be increased along with liver steatosis or the presence of T2DM (see **table 10)**.

Clinical characteristic	Grade of steatosis (% of steatotic hepatocytes)					
	< 33% (group I)		33%-66% (group II)		>66% (group III)	
		T2DM		T2DM		T2DM
η	16	16	16	16	16	16
Sex (f/m)	8/8	8/8	8/8	8/8	8/8	8/8
Age (years)	47.85 ± 2		49.32 ± 1.69		47.58 ± 2.02	
BMI (kg/m²)	52.86 ± 2.29	52.98 ± 1.70	53.36 ± 2.16	53.75 ± 2.00	56.35 ± 2.52	55.12 ± 1.76
Cholesterol (mg/dl)	183.87 ± 7.95	191.00 ± 8.68	173.44 ± 5.27	193.53 ± 9.35	190.75 ± 8.23	183.56 ± 9.91
TG (mg/dl)	160.33 ±13.72	**** t2d*** **210.00 ± 19.57**	198.31 ± 23.92	**** stea ** t2d*** **308.80 ± 36.72**	204.50 ± 21.05	270.19 ± 38.39
LDL cholesterol (mg/dl)	104.80 ± 7.37	108.67 ± 8.64	99.69 ± 7.57	97.54 ± 9.18	108.56 ± 7.80	100.33 ± 8.88
HDL cholesterol (mg/dl)	48.13 ± 4.42	40.47 ± 2.07	39.88 ± 1.96	36.80 ± 1.65	41.50 ± 1.83	35.88 ± 2.28
FBG (mg/dl)	98.60 ± 2.15	***** t2d*** **128.31 ± 10.71**	103.34 ± 4.58	****** t2d*** **162.56 ± 15.42**	100.13 ± 4.08	***** stea *** t2d*** **200.00 ± 24.35**
HbA1c (%)	5.61 ± 0.12	****** t2d*** **6.93 ± 0.32**	5.64 ± 0.16	****** t2d*** **7.72 ± 0.39**	5.82 ± 0.14	***** stea *** t2d*** **8.45 ± 0.44**
ALT (U/l)	31.60 ± 1.82	26.13 ± 3.11	48.94 ± 8.52	***** stea*** **42.56 ± 5.00**	**** stea*** **51.00 ± 7.42**	****** stea*** **45.88 ± 3.76**
AST (U/l)	21.33 ± 1.82	21.88 ± 2.39	29.06 ± 4.74	***** stea*** **32.13 ± 3.85**	31.44 ± 4.60	***** stea*** **31.50 ± 2.97**
γ-GT (U/l)	34.87 ± 2.87	39.13 ± 5.36	47.25 ± 7.26	**** stea*** **66.88 ± 10.11**	51.13 ± 9.06	**** stea*** **120.50 ± 36.85**
Total protein (g/l)	77.08 ± 1.27	78.50 ± 1.23	76.58 ± 1.27	88.17 ± 9.01	80.36 ± 1.10	79.20 ± 1.10
Serum albumin (g/l)	37.42 ± 0.89	38.70 ± 0.73	37.00 ± 0.86	38.18 ± 0.93	39.36 ± 0.94	37.47 ± 0.86
Total bilirubin (mg/dl)	0.47 ± 0.04	0.49 ± 0.07	0.59 ± 0.08	0.44 ± 0.06	0.43 ± 0.08	0.45 ± 0.05
Creatinine (mg/dl)	0.92 ± 0.05	0.99 ± 0.07	0.84 ± 0.04	0.97 ± 0.06	0.91 ± 0.05	0.92 ± 0.07

CRP (mg/l)	12.27 ± 1.82	12.38 ± 1.64	12.94 ± 2.54	15.69 ± 3.32	15.50 ± 2.98	18.50 ± 4.56
Leukocytes (x 10^9/l)	10.61 ± 0.96	9.16 ± 0.50	9.91 ± 0.73	10.00 ± 0.59	10.57 ± 0.76	9.83 ± 0.63

Table 10 – Anthropometric, metabolic, hepatic and inflammatory characteristics of the study cohort. *η = 96. Data are presented as the means and standard errors of the means (S.E.M.). Statistical significance was determined via the student's t-test. Statistical significance is indicated by bold letters and by asterisks: *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001. ^{stea} compared to steatosis group I within same diabetes group, ^{t2d} compared to. non-diabetic within same steatosis group*

Analayzed Genes	**p – value**									
	vs. steatosis I		**non-diabetic vs T2DM**				**non-fibrosis vs fibrosis**			
	steat II	*steat III*	*steat I*	*steat II*	*steat III*	*all groups*	*steat I*	*steat II*	*steat III*	*all groups*
TREM2	**0.0000**	**0.0030**	0.2741	0.0873	0.1618	**0.0483**	**0.0288**	**0.0267**	0.6814	0.0781
CXCL10	0.1214	**0.0104**	0.6758	**0.0362**	0.6899	0.3328	0.0555	0.3071	0.2325	0.8009
TNFα	0.2729	**0.0382**	0.0626	**0.0384**	0.2455	**0.0200**	0.4464	0.4655	0.4406	0.2561
COL1A1	**0.0266**	**0.0017**	0.4537	**0.0315**	0.2152	**0.0396**	0.0915	0.6738	0.4072	0.2536
FASN	0.0895	0.2762	0.2895	**0.0485**	0.5181	0.3161	0.0915	0.4554	0.0687	0.2461
FGF21	0.5545	0.2899	0.1893	**0.0063**	0.7359	**0.0414**	0.3710	0.1770	0.1025	0.4078
CYP7A1	0.1768	0.3458	0.9148	**0.0398**	0.3411	0.3979	**0.0349**	0.4436	0.1967	0.5116
CYP7B1	0.2591	0.1167	0.6622	**0.0318**	0.9765	0.3233	0.0649	0.4405	0.1801	0.2921
CYP8B1	0.4021	0.6010	0.4716	0.0565	0.4686	0.5937	0.0710	0.6900	0.1410	0.5472
SHP1	0.9631	0.5679	0.7450	**0.0174**	0.6674	0.3013	0.6972	0.7676	0.2758	0.8694

Table 11 – The p-values of all analyzed genes*, which appeared to be statistically significant are presented in bold style; p < 0.05. Steatosis grade I (steat I), steatosis grade II (steat II), steatosis grade III (steat III), non-diabetic versus diabetic regardless the grade of steatosis, non-fibrosis versus fibrosis regardless the grade of steatosis.*

3.2 The expression of inflammatory genes in the liver depends on the grade of steatosis, presence of fibrosis and a diabetic phenotype

The expression of the genes involved in inflammatory and fibrotic processes, TREM-2, CXCL10, TNF-α and COL1A1 were found to be progressively increased with the degree of liver steatosis (see **figure 6**), and, in many cases, to be higher in diabetic individuals compared to the non-diabetic patients (see **figure 7**) and in the presence of fibrosis (see **figure 8**). The respective p-values are shown in **table 11**.

In detail, the hepatic expression of TREM-2 showed much higher expression in steatosis groups II and III compared to group I (see **figure 6**). Within the steatosis groups, mean expression of TREM-2 expression was higher in diabetic compared to the non-diabetic individuals (see **figure 7**). The difference was however, statistically significant only when diabetic and non-diabetic subjects of the three steatosis groups were combined (see **table 11**). Individuals with fibrosis displayed higher expression of TREM-2 in the steatosis groups I and II – but not steatosis group III – compared to those individuals without fibrosis of the same steatosis group (see **figure 8** and **table 11**). Thus, TREM-2 expression is strongly and positively associated with the degree of liver steatosis. Less pronounced positive associations are also found with fibrosis and diabetes.

Regarding the expression of CXCL10, patients with steatosis grade III showed significantly higher expressions and a trend in steatosis group II compared to group I (see **figure 6**). In addition to the differences between steatotic groups only, the patients with T2DM had higher expressions of CXCL10 in the steatosis group II compared to the non-diabetic patients of the same group (see **figure 7**). Interestingly, no elevation of CXCL10 was observed in the diabetic individuals of the group III and of the total of all groups compared to the non-diabetics (see **figure 7** and **table 11**). Now, looking at the effect of fibrosis within the steatosis groups, the group I exhibited a very strong trend in higher expression of the inflammatory gene CXCL10 compared to patients without hepatic fibrotic characteristics within the same steatosis group (see **figure 8** and **table 11**). Yet, none of the other groups (II, III and all groups combined) displayed significant differences or trends (see **table 11**).

Taken together, the expression of CXCL10 in the liver has a distinct and positive association with the degree of steatosis with less marked expression differences regarding diabetes and fibrosis. Compared to all other inflammatory genes, it shows moderate, positive associations.

TNF-α displayed statistically relevant expression differences between steatosis groups considering the parameters steatosis only and diabetes, but nothing significant regarding fibrosis (see **table 11**). Individuals from steatosis group III but not those from steatosis group II had higher expression levels of TNF-α compared to patients from group I (see **figure 6**). Looking at diabetes, the expression of TNF-α was statistically significant elevated in group II, yet not in groups I and III, and when all groups combined, compared to the non-diabetic patients (see **figure 7**). No gene expression differences at all could be detected when comparing TNF-α levels in patients with fibrosis to the non-fibrotic ones within steatosis groups (see **figure 8** and **table 11**). In short summary, TNF-α exhibits a positive correlation with the degree of liver steatosis and the presence of a diabetic phenotype, whereas fibrosis seems to have no relevant effect on the gene expression.

COL1A1, an indicator for fibrotic tissue remodeling upon inflammatory processes in the liver, showed – similar to TREM-2 – much higher expression levels in the steatosis groups III and II compared to group I (see **figure 6**). Regarding the diabetic component, these patients had statistically significant higher levels of COL1A1 in group II and when all steatosis groups combined compared to the non-diabetic individuals (see **figure 7**). Yet, again, group III didn't show expression differences compared to the non-diabetic patients of the same steatosis group (see **table 11**). Surprisingly, fibrosis didn't show much of an effect regarding this gene in general (see **table 11**). Nevertheless, individuals with fibrosis from steatosis group I exhibited a trend of higher COL1A1 expression versus the non-fibrotic patients from the same group (see **table 11**).

Taken together, the data show progressive up-regulations of the inflammatory genes TREM-2, CXCL10, TNF-α and COL1A1 along with the degree of liver steatosis. Diabetes and fibrosis display also significant, yet less consistent, positive expression differences throughout the steatosis groups, where overall fibrosis exhibits the weakest effect.

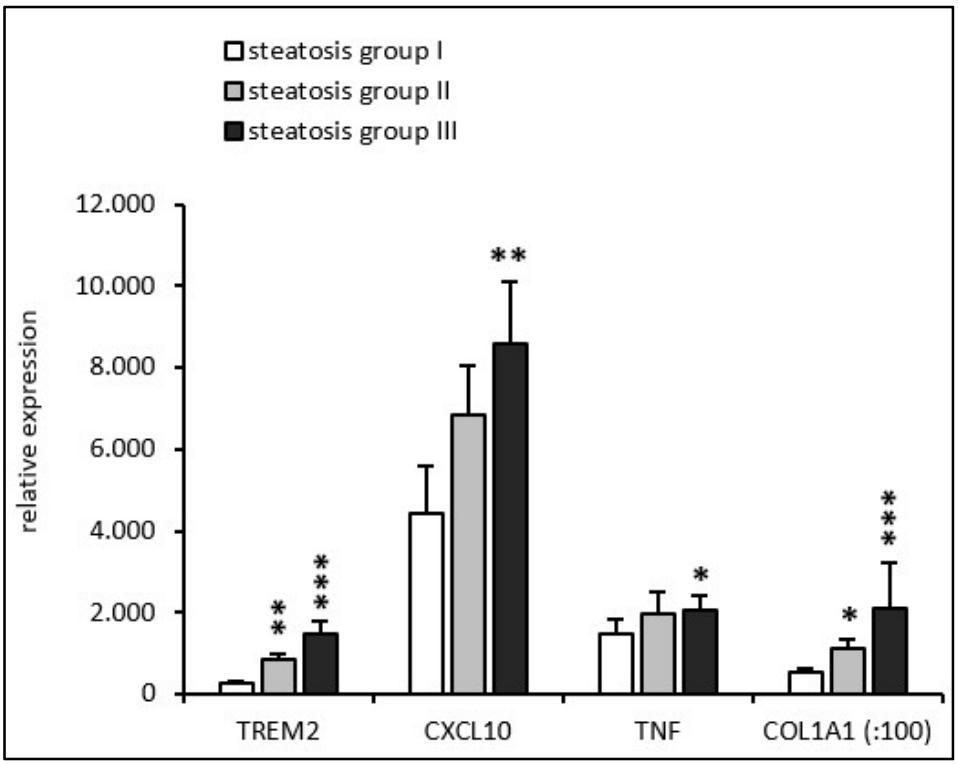

Figure 6 – Liver gene expression levels of TREM-2, CXCL10, TNF-α and COL1A1 depending on the degree of liver steatosis. *Steatosis groups I-III include all patients with steatosis grades I, II and III, respectively, including non-diabetic and diabetic individuals as well as such without and with liver fibrosis. Data are presented as the means and standard errors of the means (S.E.M.) of the normalized mRNA levels. Statistical significance was determined via the student's t-test. Statistical significance was considered if *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001.*

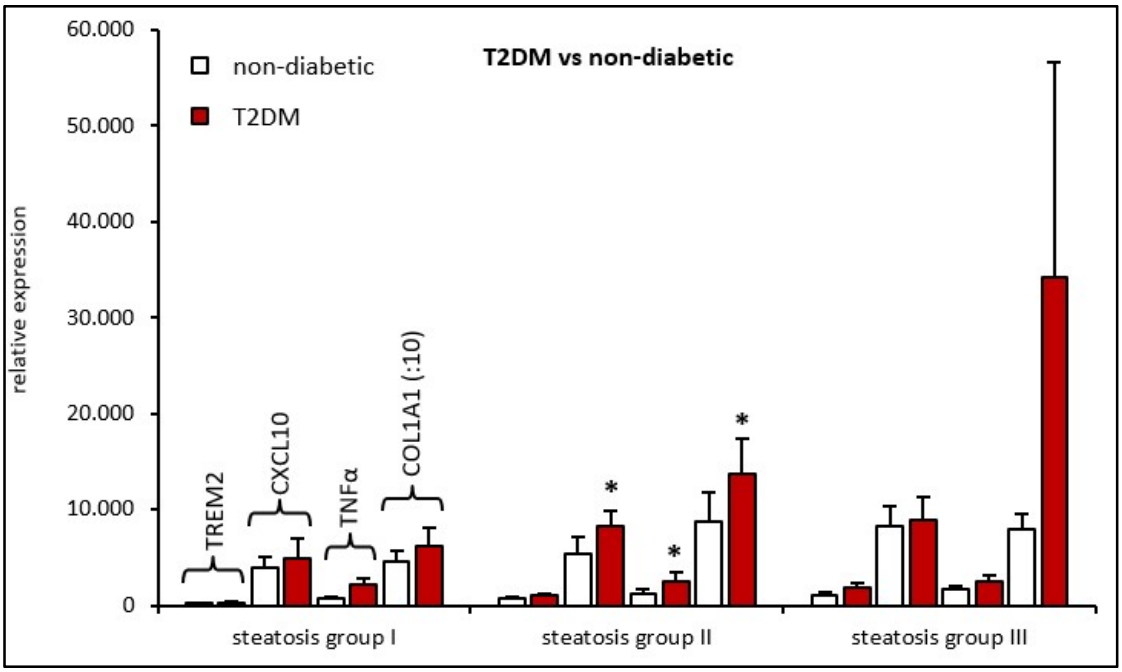

Figure 7 – Liver gene expression levels of TREM-2, CXCL10, TNF-α and COL1A1 depending on the presence of T2DM. *Each of the T2DM and non-diabetic groups with steatosis grades I, II and III, respectively, includes includes individuals without and such with liver fibrosis. Data are presented as the means and standard*

*errors of the means (S.E.M.) of the normalized mRNA levels. Statistical significance was determined via the student's t-test. Statistical significance was considered if *p-value < 0.05. Gene names are not indicated in steatosis groups II and III.*

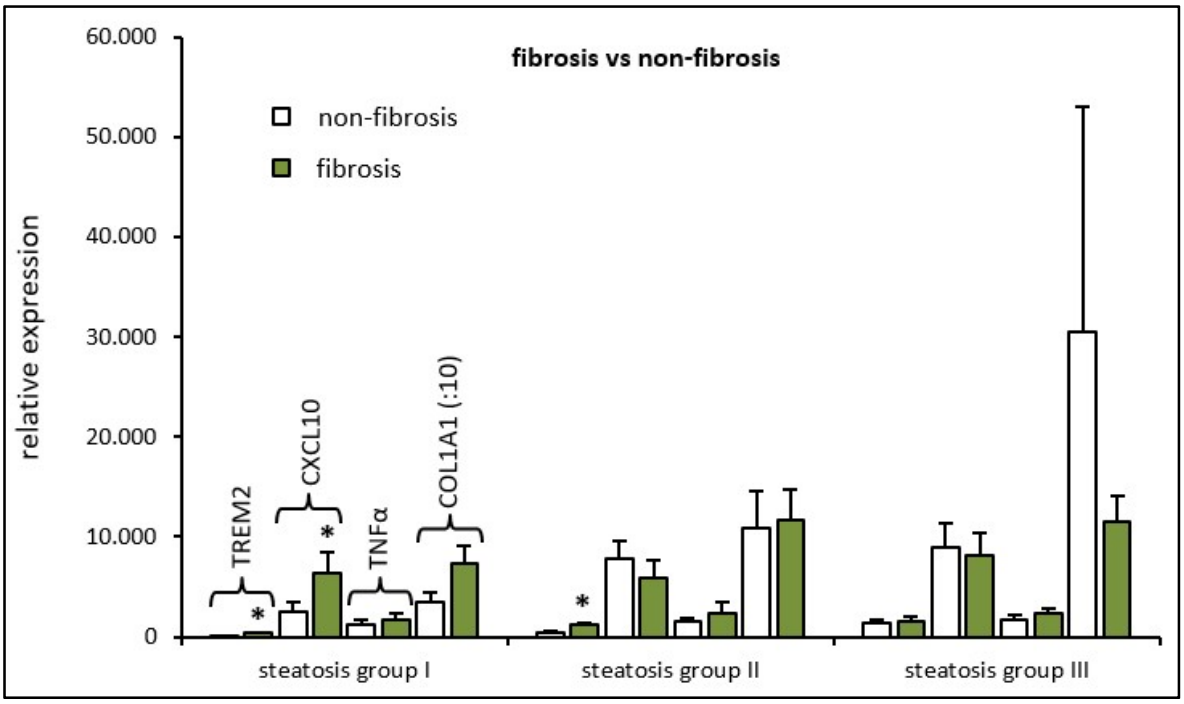

Figure 8 – Liver gene expression levels of TREM-2, CXCL10, TNF-α and COL1A1 depending on the presence of liver fibrosis*. Each of the fibrosis and non-fibrosis groups includes individuals without and such with T2DM. Data are presented as the means and standard errors of the means (S.E.M.) of the normalized mRNA levels. Statistical significance was determined via the student's t-test. Statistical significance was considered if *p-value < 0.05. Gene names are not indicated in steatosis groups II and III.*

3.3 The hepatic expression of *FASN* and *FGF21* in obese NAFLD patients is positively associated with diabetes at intermediate degree of liver steatosis

The *de novo* lipogenic genes FASN and FGF21 were found to be associated only weakly with the degree of liver steatosis and fibrosis and in some cases positive trends could be seen (see **figure 9**, **11** and **table 11**). Au contraire, within steatosis group II they displayed significantly higher expression levels in diabetic individuals compared to the non-diabetic patients (see **figure 10**). The respective p-values are shown in **table 11**.

In detail, the hepatic expression of FASN showed a positive trend in the steatosis group II versus group I, but no difference in the steatosis group III (see **figure 9** and **table 11**). Within the steatosis groups higher and statistically significant levels of FASN were found in the diabetic patients from group II compared the non-diabetic individuals from the same group (see **figure 10**). Interestingly no differences were detected in groups I and III or when diabetic patients from all groups combined were levelled against the non-diabetic patients (see **table 11**). The patients with fibrosis displayed positive trends for higher expression in the groups I and III compared to the individuals without fibrotic characteristics, and quite surprisingly no differences when all groups were taken together (see **figure 11** and **table 11**). In summary, FASN shows a positive association with diabetes, and no significant correlation with the degree of steatosis and fibrosis.

FGF21 displayed no differences between the steatosis groups (see **figure 9** and **table 11**), but higher levels in diabetic versus non-diabetic patients from group II and when all groups were combined (see **figure 10** and **table 11**). Individuals from all steatosis groups with fibrosis showed no differences compared to the ones without fibrotic characteristics (see **figure** and **table 11**). Taken together, the data reveal a strong pronounced association between FGF21 and diabetes and no association with the degree of steatosis and fibrosis.

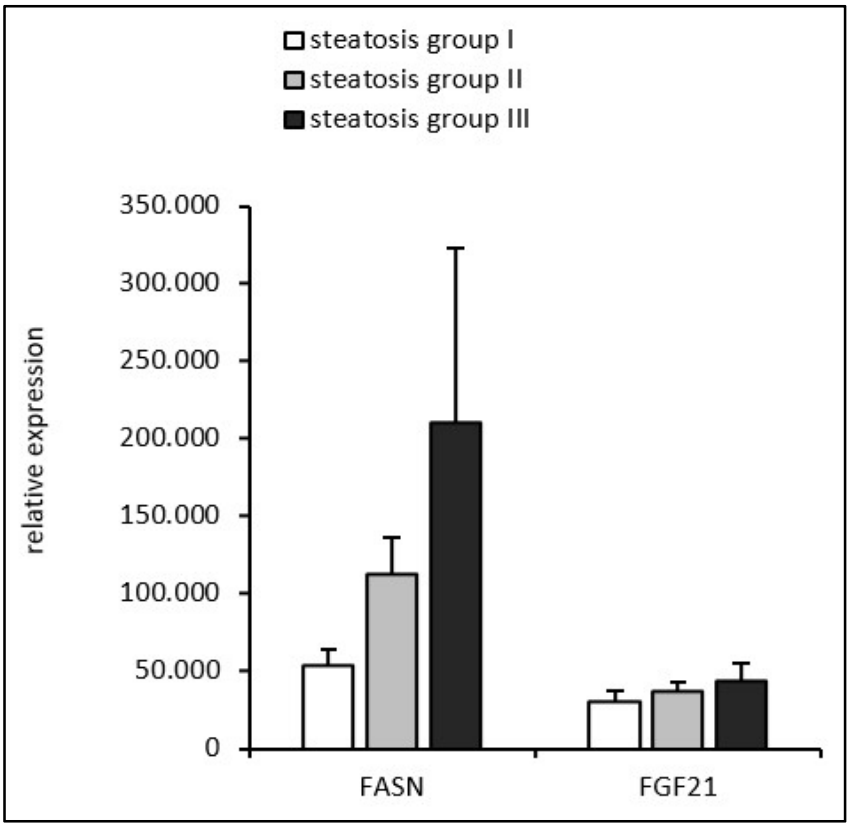

Figure 9 – Liver gene expression levels of FASN and FGF21 depending on the degree of liver steatosis*. Steatosis groups I-III include all patients with steatosis grades I, II and III, respectively, including non-diabetic and diabetic individuals as well as such without and with liver fibrosis. Data are presented as the means and standard errors of the means (S.E.M.) of the normalized mRNA levels. Statistical significance was determined via the student's t-test. Statistical significance was considered if *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001.*

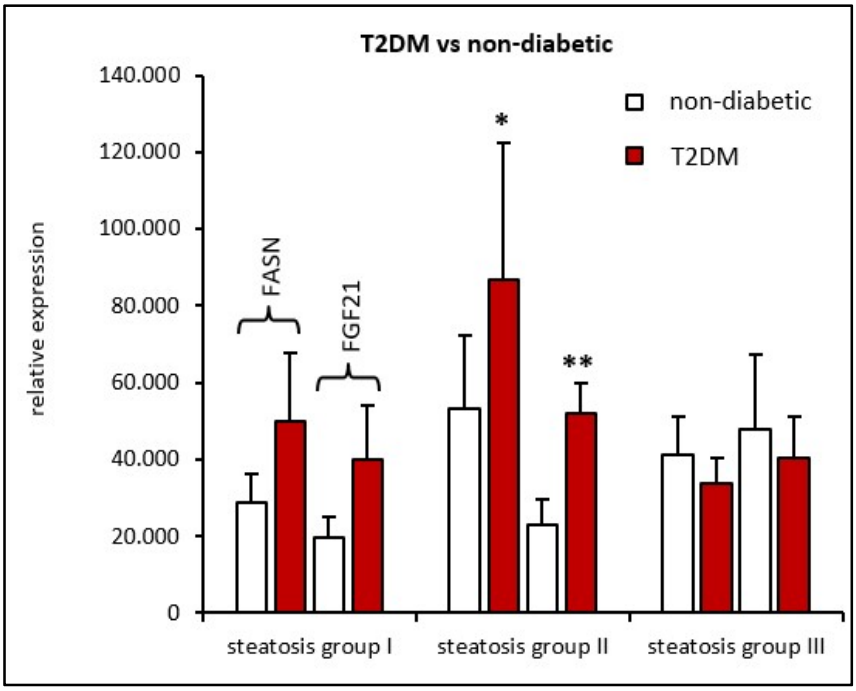

***Figure 10** – **Liver gene expression levels of FASN and FGF21 depending on the presence of T2DM.** Each of the T2DM and non-diabetic groups with steatosis grades I, II and III, respectively, includes individuals without and such with liver fibrosis. Data are presented as the means and standard errors of the means (S.E.M.) of the normalized mRNA levels. Statistical significance was determined via the student's t-test. Statistical significance was considered if *p-value < 0.05, **p-value < 0.01. Gene names are not indicated in steatosis groups II and III.*

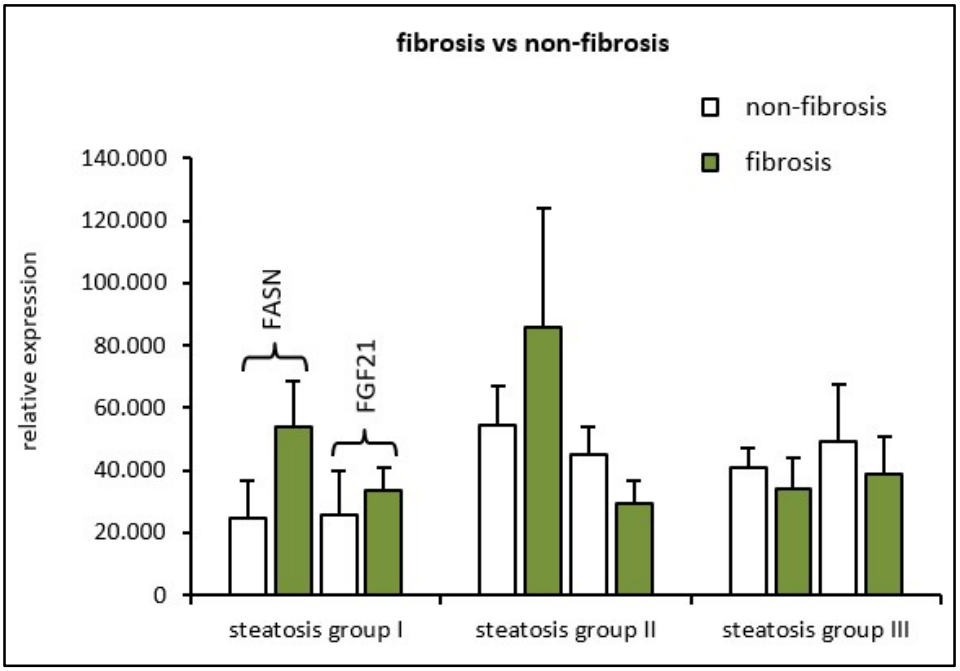

***Figure 11** – **Liver gene expression levels of FASN and FGF21 depending on the presence of liver fibrosis**. Each of the fibrosis and non-fibrosis groups includes individuals without and such with T2DM. Data are presented as the means and standard errors of the means (S.E.M.) of the normalized mRNA levels. Statistical significance was determined via the student's t-test. Statistical significance was considered if *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001. Gene names are not indicated in steatosis groups II and III.*

3.4 The hepatic expression of bile acid synthesis and regulation genes in obese NAFLD patients is positively associated with diabetes at intermediate degree of steatosis

The expression of the genes encoding BA synthesis enzymes CYP7A1, CYP7B1 and CYP8B1, and expression of the BA synthesis regulator SHP displayed no differences associated with the degree of steatosis (see **figure 12, table 11**). Contrary to this, all of these genes – except CYP8B1 – showed statistically significant higher levels in the diabetic versus the non-diabetic patients in steatosis group II but not the other steatosis groups (see **figure 13, table 11**). The expression of CYP8B1 displayed a very strong trend in the diabetic versus the non- diabetic patients in steatosis group II (see **table 11**). Patients with fibrosis exhibited a statistically significant higher level or trends for the BA synthesis genes compared to those without fibrotic characteristics in steatosis group I but not in the groups with higher degree of liver steatosis. SHP expression was independent of fibrosis (see **figure 14, table 11**). Taken together, hepatic expression of the BA genes investigated here is independent of liver steatosis, and shows moderate positive association with fibrosis only in patients with little liver fat, and clear positive association with diabetes at intermediate liver steatosis.

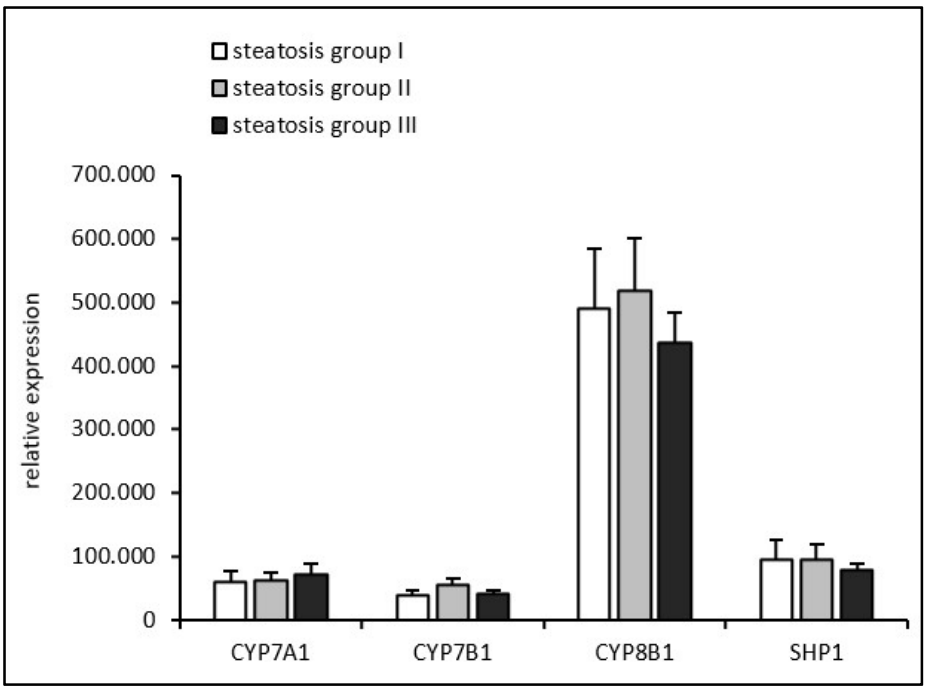

Figure 12 – Liver gene expression levels of CYP7A1, CYP7B1, CYP8B1 and SHP1 depending on the degree of liver steatosis. *Steatosis groups I-III include all patients with steatosis grades I, II and III, respectively, including non-diabetic and diabetic individuals as well as such without and with liver fibrosis. Data are presented as the means and standard errors of the means (S.E.M.) of the normalized mRNA levels. Statistical significance was determined via the student's t-test. Statistical significance was considered if *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001.*

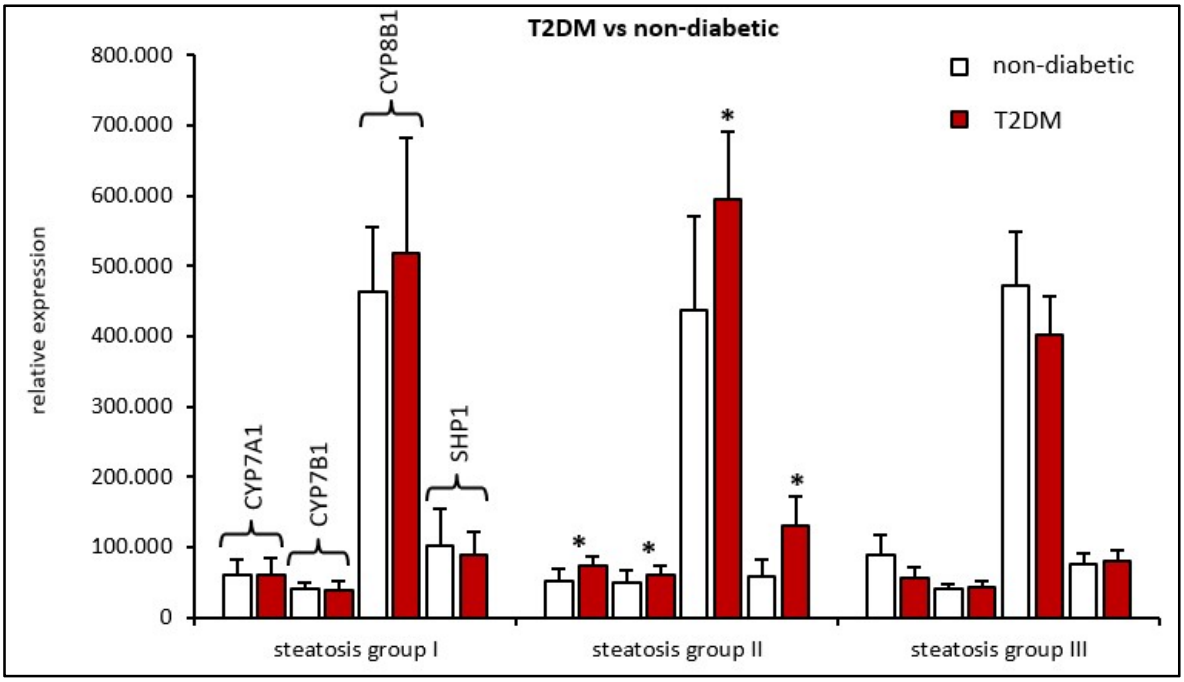

Figure 13 – Liver gene expression levels of CYP7A1, CYP7B1 and CYP8B1 and its regulatory element SHP1 depending on the presence of T2DM*. Each of the T2DM and non-diabetic groups with steatosis grades I, II and III, respectively, includes individuals without and such with liver fibrosis. Data are presented as the means and standard errors of the means (S.E.M.) of the normalized mRNA levels. Statistical significance was determined via the student's s-test. Statistical significance was considered if *p-value < 0.05. Gene names are not indicated in steatosis groups II and III.*

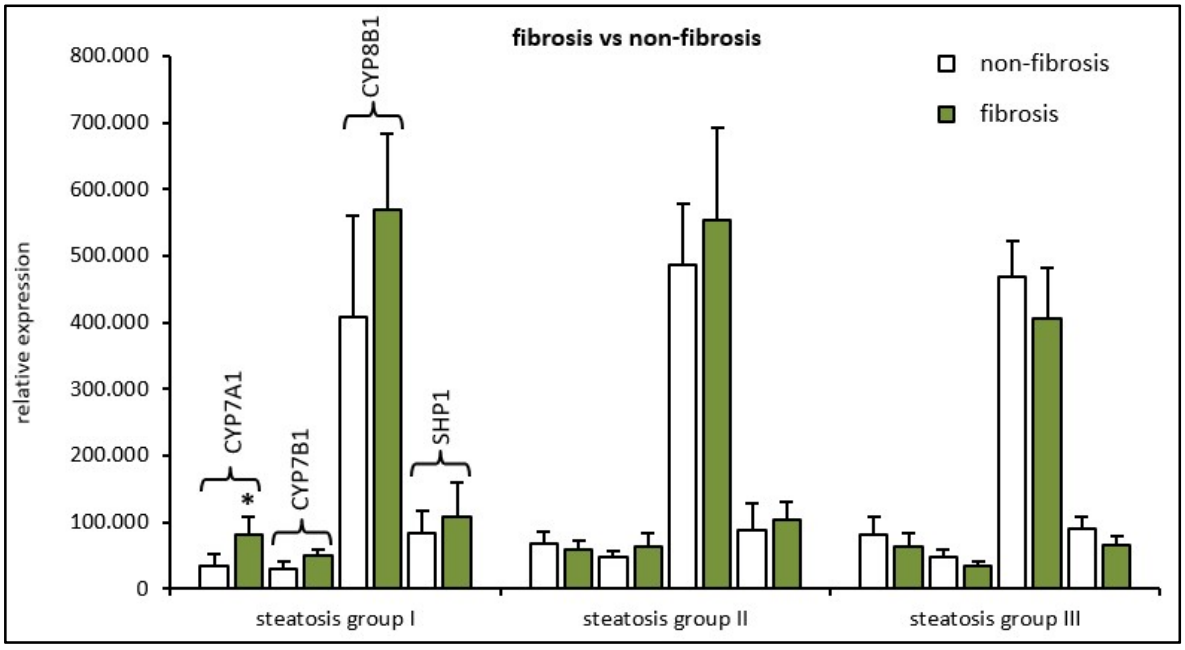

Figure 14 – Liver gene expression levels of CYP7A1, CYP7B1 and CYP8B1 and its regulatory element SHP1 depending on the presence of liver fibrosis. *Each of the fibrosis and non-fibrosis groups includes individuals without and such with T2DM. Data are presented as the means and standard errors of the means*

*(S.E.M.) of the normalized mRNA levels. Statistical significance was determined via the student's t-test. Statistical significance was considered if *p-value < 0.05. Gene names are not indicated in steatosis groups II and III.*

Chapter 4

4. Discussion

All 96 individuals in the current study were morbidly obese with a BMI of 53 to 56 kg/ m². For most of the genes under investigation, statistically significant associations were observed for at least one of the disease parameters addressed, namely degree of liver steatosis, diagnosis of type 2 diabetes or presence of liver fibrosis. Together, this general finding of the present study confirms the concept of healthy obesity claiming that the despite equal adiposity, metabolic health can vary substantially among obese patients (Smith *et al.* 2019). In the following, the associations of the selected gene expressions with the disease parameters will be discussed in detail.

4.1 Association with degree of liver steatosis

The results show a strong association between a progressive inflammation in the liver with gradually increasing accumulation of liver fat along the grades of liver steatosis. The findings support the notion that obesity is tightly associated with hepatic inflammation and plays a pivotal role in the pathogenesis of NAFLD and its further conditions such as NASH, liver fibrosis and cirrhosis, which has been demonstrated in previous findings from cell lines (Tomita *et al*. 2017 and Zhang *et al.* 2014), knockout mouse models (CXCL10 knockout mice, Zhang *et al.* 2014) and humans (Bertola *et al*. 2010 and Zhang *et al.* 2014).

The origin of the inflammatory drive is explained by the multiple-hits hypothesis, which comes from the initial two-hits hypothesis from 2010 (Buzzetti *et al*. 2016, Caluguri *et al.* 2016 and Ferro *et al.* 2020). According to this most recent concept, inflammatory stimuli in the liver are the accumulation and oxidation of FFAs in the liver due to an impaired elimination *via* the VLDL pathway in a predisposed genotype (Buzzetti *et al.* 2016). The sequence of these events promotes the progression of a simple steatosis (‘fatty liver’) into a steatohepatitis (NASH) (Buzzetti *et al.* 2016). The oxidation of FFAs leads to lipotoxic metabolites which produce mitochondrial stress and a higher amount of ROS, which in turn induce local tissue damage and stimulate pro inflammatory immune cells (Buzzetti *et al.* 2016). In addition, insulin resistance with subsequent adipocyte dysregulation and the secretion of pro-

inflammatory adipokines such as TNF-α contribute to the inflammatory pathogenesis (Buzzetti *et al*. 2016).

The hepatic TREM-2 expression is strongly and positively associated with the degree and therefore severity of liver steatosis in obese patients as the presented data illustrate. The results of the study cohort emphasize an outstanding role of TREM-2 amongst all analyzed inflammatory genes in obesity related pathogenesis of NAFLD in humans.

TREM-2 has been predominantly investigated in other inflammatory diseases such as the inflammatory bowel disease (Genua *et al.* 2014), neurodegenerative diseases like the Alzheimer's disease and various forms of dementia (Jay *et al.* 2015, Jiang *et al*. 2015) and in multiple sclerosis (Takahashi *et al*. 2007), yet the role of TREM-2 in NAFLD remains obscure. Correspondingly, the genetic expression has been detected in various cells types like the mircoglia (Sharif and Knapp 2008, Takahashi *et al.* 2007 and 2005), in endothelial cells (Chen *et al.* 2007) and in hepatic non- parenchymal cells, which are liver macrophages, a group of cells called Kupffer cells (KC) and monocyte- derived cells (Krenkel and Tacke 2017).

Recent literature strongly suggests anti-inflammatory effects of TREM-2 (Chen *et al.* 2007, Perugorria *et al.* 2019, Sharif and Knapp 2008, Subramanian *et al.* 2017, Turnbull *et al.* 2006). Findings from these studies attribute a protective role to TREM-2 in the complex process of inflammation as shown in blood neutrophils and tissue macrophages from mice (Turnbull *et al.*2006), hepatocytes and hepatic macrophages from mice (Chen *et al.* 2007) and most recently, in patients with liver injuries of diverse aetiologies, knockout mouse models and *in vitro* designs with primary hepatic macrophages from mice (Perugorria *et al.* 2019, Sharif and Knapp 2008).

In 2017, a team in Nebraska analyzed – amongst multiple genes – the hepatic TREM-2 expressions in tissues from obese patients (liver, *omentum*, subcutaneous fat and blood neutrophils and monocytes) (Subramanian *et al.* 2017). They found the TREM-2 expression in all tissues of obese individuals to be decreased in a statistically significant manner (Subramanian *et al.* 2017). Form these findings, they suggested a probable association between the hepatic regulation of TREM-2 and the obesity related co-morbidities T2DM and NAFLD in terms of an anti- inflammatory function of this gene (Subramanian *et al.* 2017).

The study from Perugorria *et al.* from 2019 demonstrates that TREM-2 is expressed in the liver tissue biopsy specimen from humans, which exhibited liver cirrhosis of various aetiologies such as alcohol driven, diverse virus hepatitis and simple steatosis (Perugorria *et al.* 2019). The presented data agree with and support this pre-existing body of knowledge. The

Perugorria study also harvested primary liver cells for *in vitro* experiments from a TREM-2 knockout mouse model (Perugorria *et al.* 2019). These results show that TREM-2 limits liver injury like a natural brake in the inflammatory process during hepatocellular damage; which was experientially conducted with repetitive carbon tetrachloride and acetaminophen intoxication of the primary mouse liver cells (Perugorria *et al.* 2019). Mice lacking TREM-2 showed increased hepatocellular damage *via* elevated hepatic lipid peroxidation through a higher content of ROS and an elevated macrophage content upon the pharmaceutical intoxication (Perugorria *et al.* 2019). Additionally, the harvested primary hepatic macrophages and stellate cells from TREM-2 silenced mice showed augmented TLR4-mediated proinflammatory effects (Perugorria *et al.* 2019).

Most recently, a group from the Weizmann Institute of Science in Israel found a conserved macrophage response signature across the adipose and visceral adipose tissue and in the liver from obese patients and in obese TREM-2 knockout mouse-models (Jaitin *et al.* 2019). The colleagues newly discovered a lipid-associated macrophage, which they called LAM, expressing TREM-2 and seemingly being the major regulator in systemic lipid metabolism and also a potent immune regulator in the adipose tissue, the liver and neurons during a loss of adipose tissue homeostasis as such in obesity (Jaitin *et al.* 2019)

Therapeutical strategies of NAFLD might be not only the blocking of the immune response and blocking the infiltration of immune cells into the adipose tissue and the liver but also the enhancement of the body's inert protective pathways *via* TREM-2 positive LAM (Jaitin *et al.* 2019).

Based on this pre-existing body of literature the presented findings in the liver tissue from obese diabetic NAFLD patients can be interpreted strongly as consistent with the state of current literature and confirm this growing concept of a protective role and even defense mechanism of TREM-2, as colleagues in China and the U.S. demonstrated just recently *in vivo* and *in vitro* (Hou *et al.* 2021). They conducted a prospective clinical study with a total cohort of 1307 patients diagnosed with sepsis according to the Sepsis 3.0 criteria and 524 patients diagnosed with NAFLD *via* computertomography as well as *in vitro* experiments with a TREM-2 knockout NAFLD mouse model (Hou *et al.* 2021). They could show that the NAFLD cohort in the sepsis patients had a significantly higher 28-day and hospital mortality as well as increased needs for immunomodulatory drugs, ventilation and renal replacement therapy compared to the control group (Hou *et al.* 2021). T2DM showed comparable mortalities to the non-diabetic individuals within the NAFLD group (Hou *et al.* 2021). In addition, the livers of NAFLD sepsis patients and of the NAFLD mouse model exhibited an

abundant amount of TREM-2 positive macrophages (Hou *et al.* 2021). This was interpreted as an inert defense mechanism to rescue metabolic fitness, because conversely the *in vitro* experiments showed that the TREM-2 deficient NAFLD mouse macrophages had a higher susceptibility for sepsis in terms of a poor outcome (Hou *et al.* 2021). On the molecular level TREM-2 deficiency was associated with mitochondrial fragmentation upon mitofusion2 gene (Mfn2) degradation through a high amount of the microRNA miR-106b-5p, which blocks Mfn2 (Hou *et al.* 2021). Mfn2 is a protein gene coding for a mitochondrial membrane protein, which maintains mitochondrial integrity (Hou *et al.* 2021). TREM-2 seems to play a protective role in NAFLD-associated sepsis *via* an improved hepatic energy-supply *via* improved hepatic mitochondrial function (Hou *et al.* 2021).

Yet, the data presented here were taken from full liver samples and single cell types (parenchymal and non- parenchymal) were not differentiated, so no statement can be made regarding the cellular source and exact signaling pathways including key transcription factors and regulative elements of TREM-2 in the study cohort. This will be interesting to look at in future *in vitro* study designs for human NAFLD individuals.

To date, the gold standard for NAFLD diagnosis is the liver biopsy, which of course is an extremely invasive procedure for the patient (Buzzetti *et al.* 2016). Against the background of the progredient obesity and NAFLD pandemic non-invasive diagnostic possibilities are urgently needed. One possible biomarker to detect progressive states of NAFLD and catch the so called 'window of opportunity' for an early treatment could be circulating soluble TREM- 2. A first association between circulating TREM-2 and insulin resistance has been made in 2018 by Tanaka *et al*. and should to be investigated for NAFLD and obesity (Tanaka *et al.* 2018)

A moderate and positive association between the degree of liver steatosis and the expression of CXCL10 in obese NAFLD patients was observed (see **figure 6**). The statistical significant effect can be seen in steatosis group III versus group I (see **figure 6**).

CXCL10 belongs to the family of chemokines, whose major role is to act as an chemoattractants for immune cells (Bishara 2012). In autoimmune diseases, such as diabetes mellitus type 1, Grave's disease, lupus erythematosus, systemic sclerosis, autoimmune thyroiditis and many more, it could be shown that CXCL10, also called interferon γ-induced protein 10 (IP-10), is produced by a variety of immune cells, among them CD4+ lymphocytes, CD8+ lymphocytes, NK cells and neutrophils upon the combined stimulus of interferon-γ (IFN-γ) and TNF (Bishara 2012, Ruffilli *et al.* 2014). The role of IP-10 in the pathogenesis of obesity associated NAFLD remains obscure.

This study attributes CXCL10 to play an important role in obese diabetic NAFLD patients because other studies have already shown an inflammation fuelling mechanistic of CXCL10 not only in autoimmune diseases but also in human NAFLD (Zhang *et al.* 2014). The chinese colleagues found circulating and hepatic CXCL10 to be statistically significant higher in NASH patients (out of 147 NAFLD individuals) (Zhang *et al.* 2014). In addition to the results in patients, they set up a CXCL10 knockout dietary-induced NAFLD mouse model and demonstrated that CXCL10 deficient mice were refractory to dietary provoked NAFLD (Zhang *et al.* 2014). Furthermore the blocking of the CXCL10 signaling pathway attenuated NAFLD development in mice (Zhang *et al.* 2014). The group of Tomiat *et al.* reproduced these results in 2016 showing a liver protective effect of CXCL10 deletion in a murine diet-induced NAFLD model (Tomita *et al.* 2016). In addition, they detected attenuated macrophage liver infiltration upon dietary stimuli in the CXCL10 depleted mice (Tomita *et al.* 2016). This again has already been shown by Hintermann *et al.* in 2010, presenting a leukocyte recruitment to the liver parenchyma upon CXCL10 stimulation, which lines up with pre-existing studies (Hintermann *et al*. 2010).

The underlying signaling mechanisms are under investigation. So far, colleagues from Japan could elucidate in the human cell lines Huh7 and HLE, that CXCL10 was increasingly expressed *via* the melanoma differentiation-associated gene 5 (MDA5) pathway upon TNF-α stimulation in a signal transducer and activator of transcription 1 (STAT1)-dependent and independent manner (Kawaguchi *et al.* 2019). Similar results were presented in HepG2 cells by Tomita *et al.* in 2017 (Tomita *et al.* 2017).

Together, the pre-existing body of literature and the presented data show a tight association between NAFLD and the pro-inflammatory CXCL10. Yet, the lipid-induced CXCL10-dependent inflammation and development of NAFLD in the liver in obese NAFLD patients with the underlying mechanistic should be subject of future *in vitro* and *in vivo* studies

Because of this newly elucidated association in NAFLD patients, CXCL10/ IP-10 in the serum of patients could serve as a biomarker for progressive NAFLD.

Regarding the expression of TNF-α, a positive correlation between the expression of TNF-α in the liver from obese NAFLD patients and the degree of liver steatosis was observed (see **figure 6**). The presented findings agree with the current literature, confirm the inflammatory nature of NAFLD and in this context the role of TNF-α as a pro-inflammatory mediator. Hepatic lipid accumulation in obese NAFLD patients seems to induce TNF-α-mediated inflammation.

In 2016, Paredes-Turrubiarte *et al.* carried out a clinical study on 102 obese men and women diagnosed with different grades of NAFLD showing that the severity of NAFLD is associated with high serum-levels of TNF-α (Paredes-Turrubiarte *et al*. 2016). They confirmed findings from Crespo *et al.* made already in 2001 in 52 obese NAFLD patients, which showed a statistically significant positive correlation between NAFLD, NASH and the hepatic expression of TNF-α (Crespo *et al*. 2001).

In the presented work, it was assumed that the elevation of TNF-α in sera of obese patients very likely correlates with an increased expression of TNF-α in the liver itself. Yet, the systemic TNF-α levels of the study cohort need to be measured in order to correlate them to the findings in liver tissue with certainty. An alternative or additional source of systemic TNF-α could of course also be the adipose tissue. AT is known to have functions of an endocrine organ being able to secrete adiponectin, leptin and pro-inflammatory factors such as monocyte chemoattractant protein 1 (MCP-1) and TNF-α (Hotamisligil *et al.* 1993, Tamura *et al.* 2008). In addition, the AT and insulin resistance play a pivotal role in the pathogenesis of NAFLD, which has been suggested by one of the first, Hotamisligil *et al.* in 1993, in an obese T2DM rodent model with antibody TNF-α neutralization showing an increased peripheral glucose uptake upon insulin stimulation (Hotamisligil *et al.* 1993).

Another study from 2019 from colleagues in Pamplona, Spain, shows that TNF- α induces hepatocyte apoptosis and autophagy in morbidly obese patients with NAFLD and can be attenuated by the gut hormone ghrelin (Ezquerro *et al.* 2019). The study cohort of 158 morbidly obese patients showed a significant correlation between serum ghrelin levels and TNF-α-induced hepatocyte cell death examined in liver biopsies (Ezquerro *et al.* 2019). In addition, *in vitro* experiments on human HepG2 hepatocytes showed the statistically significant reduced TNF-α-induced hepatocyte cell death under ghrelin treatment (Ezquerro *et al.* 2019). These results underline the pro-inflammatory nature of TNF-α in the pathogenesis of NAFLD.

Further questions, which evolve from the presented findings and pre-existing literature are firstly, which cell types secrete TNF-α in the livers from patients with progressed liver steatosis? Hepatocytes, liver resident KC and recruited blood monocytes as well as their polarization froms, M1 (pro-inflammatory) and M2 (anti-inflammatory), may play a crucial role (Eguchi *et al.* 2014). And secondly, what are the extra- and intracellular mechanistics leading from fat to TNF-α secretion to inflammation?

The steatosis-induced hepatic inflammatory process of NAFLD is believed to be stimulated by a complex concert of metabolites, chemokines and various extra- und intracellular signaling cascades (Eguchi *et al.* 2014, Hirsova and Gores 2015). The TNFR1 signaling with the ligand TNF-α is one of them, yet its role remains controversial in NAFLD (Feldstein *et al.* 2004, Hirsova and Gores 2015). One study from colleagues in Baltimore, U.S., set up a murine dietary-induced NAFLD model and elucidated that an anti- TNF-α therapy (with anti-TNF-α-antibodies for 4 weeks) improves NAFLD in obese NAFLD mice (Li *et al.* 2003). In addition, *in vitro* studies in mouse hepatocytes from cathepsin B knockout mice and cultured HepG2 cells showed that FFAs induced the expression of TNF-α *via* a lysosomal pathway, which seemed to be NF-κB-dependent (Feldstein *et al.* 2004).

Studies in cultured Huh7 human hepatoma cells and hepatocytes from a murine NASH TRAIL receptor knockout model have elucidated more details about the corresponding signaling cascade of TNFR1 and TNF-α (Cazanave *et al.* 2011). Saturated FFAs such as palmitate bind to death receptors (DR), especially DR5 (also called tumor necrosis factor related apoptosis-inducing ligand receptor 2 (TRAIL-R2) or TNFRSF10B) (Cazanave *et al.* 2011). These receptors are seemingly expressed on hepatocytes and macrophages and induce hepatocyte lipoapoptosis and macrophage activation through the ligand-independent clustering and cytotoxic activation of DR5 and the down-stream caspase-dependent apoptosis (Cazanave *et al.* 2011). Upon binding to the cell surface, palmitate induced an inflammatory response *via* various pro-inflammatory messengers such as interleukin 1β (IL-1 β), interleukin 6 (IL-6) and TNF-α (Cazanave *et al.* 2011).

The described concept for the steatosis-induced TNF-α-dependent inflammation in NAFLD is of growing evidence, yet, needs to be elucidated and confirmed, especially in primary human tissue, of which only a few studies exist (Eguchi *et al.* 2014).

With the presented findings against the background of prexisting concepts, this study offers the idea that the source of the pro-inflammatory cytokine TNF-α in humans is the liver and thus one could correlate elevated serum levels of TNF-α in patients to the degree of liver disease in NAFLD. In this sense, the presented findings contribute an important aspect to the establishment of practicable clinical assessment utility of NAFLD progression into more severe stages of liver disease. Screenings of TNF-α serum levels in morbidly obese patients could be proposed in order to apply effective therapeutical interventions at an early stage.

In the study cohort a positive and strong correlation between severe NAFLD grades and hepatic COL1A1 expression could be observed (see **figure 6**). This can be seen especially in the steatosis groups III and II compared to group I (see **figure 6**).

Little is known about the impact of COL1A1 in human NAFLD and as fas as known no experimental studies in human livers have been carried out yet. Nevertheless, gene profiles of human NAFLD patients of different degrees (mild to severe) have been screened for differentially expressed genes (DEGs) (Huang *et al.* 2018 and Qi *et al.* 2017). The study from Changchun, China, showed a down- regulation of 24 genes and an up-regulation of 100 genes, amongst them also COL1A1 (Qi *et al.* 2017). COL1A1 showed such a high up- regulation that the colleagues defined it as candidate gene and attributed potential biomarker qualities to this pro- inflammatory fibrogenic gene (Qi *et al.* 2017).

Animal models showed a significant elevated expression of COL1A1 in dietary-induced NASH (Ipsen *et al.* 2019). The Danish colleagues implemented a guinea pig NASH model showing that high-fat diets for up to 25 weeks induced NASH and that amongst other inflammatory and fibrogenic pathways COL1A1 was increased about 3-fold (Ipsen *et al.* 2019).

Against the background of recent research, the presented results strongly suggest, that fat – FFAS and their metabolites – induce the secretion of COL1A1, which consequently leads to NAFLD. This in turn means, that fat and COL1A1 possess pro-inflammatory effects in the liver under increased occurrence of FFAs. Signaling cascades and molecular mechanistics especially in primary human tissue need to be elucidated in future *in vitro* and *in vivo* study designs.

The expression profiles of the *de novo* lipogenic gene FASN and the co-regulated hormone FGF21 show only weak up to no associations with the degree of liver steatosis (see **figure 9** and **table 11**). Based on the results, no major differences in hepatocyte glucose metabolism for FASN and FGF21 in morbidly obese NAFLD patients were found (see **figure 9** and **table 11**).

An explanation for this could be that significant gene regulation takes place between lean and obese phenotypes and can't be detected within obesity degrees (as in the study cohort of morbidly obese NAFLD patients). Preliminary data suggesting this concept were taken from a human obese NAFLD cohort by Eissing *et al.* in 2013. Their data from 165 obese NAFLD patients and their control groups revealed for the first time a coregulation of ChREBP and DNL enzymes (Eissing *et al.* 2013). Both FASN and FGF21 are CHREBP targets and ChREBP is activated by glucose uptake and catabolism (Eissing *et al.* 2013). Within obese

individuals they saw only trends between obese groups, so apparently all have a similar glucose metabolism and DNL seemed to have only a weak effect in obese phenotypes (Eissing *et al.* 2013).
Nevertheless, the Ipsen group from Copenhagen displayed results in their dietary-induced guinea pig NASH model showing an increased expression of FASN upon high-fat diets for 25 weeks, thus hepatic markers for hepatic DNL being up-regulated (Ipsen *et al.* 2019). Another group in Japan published similar results from a study cohort of 36 (NAFLD) patients (Kohjima *et al.* 2007). Their data illustrate the increased DNL, also by high hepatic FASN expression, in parallel to the progressive accumulation of fatty acids in the hepatocytes of NAFLD patients (Kohjima *et al.* 2007).
To which extent obesity-induced hepatic DNL is involved in the pro-inflammatory pathways of NAFLD, as it is in mice, is a matter of ongoing research.

The presented results show no differences in genetic expression of the BA genes CYP7A1, CYP7B1, CYP8B1 and the regulating element SHP1 associated with the degree of liver steatosis (see **figure 12** and **table 11**), which means that the BA synthesis is not regulated in a steatosis-dependet manner. This supports the notion that BAs don't play a role in steaosis-induced inflammatory NAFLD, yet, this concept can't be fully excluded as bile acids haven't been measured directly.

Taken together, the TREM2, CXCL10, TNF and COL1A1 data show a strong and positive association with progressively higher liver steatosis. These data can be interpreted as such that lipid accumulation in hepatocytes supports hepatic inflammation. This notion is strengthened by data, which show that a primary liver steatosis is associated with hepatic inflammation and tissue damage upon the loss of proper VLDL functioning on a predisposed genotype, e.g. by mutations in the transmembrane 6 superfamily 2 (TM6SF) and patatin-like phospholipase domain-containing protein 3 (PNPLA3) (Scheja and Heeren under preparation). Yet, the presented data could also be explained in a way that the factors promoting liver steatosis have pro-inflammatory qualities at the same time.

4.2 Association with diabetes

In addition to the effect of steatosis, the results from the expression profiles of TREM-2, CXCL10, TNF-α and COL1A1 partly show positive associations between T2DM and inflammation, yet less pronounced than the effect of liver steatosis, which was detected in the

study cohort (see **figure 7** and **table 11**). T2DM in obese subjects is accompanied by increased hepatic inflammation and moderate NAFLD in the study cohort. Differences in the gene expression profiles of TREM-2, CXCL10, TNF-α and COL1A1 are strongest for the T2DM individuals in liver steatosis II versus the control group (see **figure 7**).

T2DM is associated with systemic subclinical inflammation, which seems to manifest differently in various organs such as in the liver of obese diabetic patients, so some say that NAFLD is like the hepatic manifestation of the metabolic syndrome (Buzzetti *et al*. 2016). T2DM is associated with hyperinsulinemia and lowered insulin sensitivity up to insulin resistance of the peripheral tissue, which also seems to play a key role in the pathogenesis of NAFLD (Buzzetti *et al*. 2016, Caligiuri *et al.* 2016, Dharmalingam and Yamasandhi 2018). Effects of T2DM on the inflammatory nature of NAFLD have been poorly investigated until today in humans, animal models and cell lines. The association between pro- inflammatory markers, such as TNF-α, and T2DM has been shown in a tissue-dependent manner for the VAT in a study cohort of Asian Indians of a total of 227 individuals (Indulekha *et al.* 2011). Furthermore, light was shed on ectopic fat, also in the liver, which was seemingly associated with T2DM in lean individuals from a large UK-based cohort in a statistically significant manner (Thomas *et al.* 2012). Another study from colleagues in Spain showed a statistically significant association between systemic pro- inflammatory cytokines such as TNF-α and obesity but no link with T2DM in 25 female patients (Catalán *et al.* 2007).

For TREM-2 a positive effect on the expression of TREM-2 when all T2DM groups combined versus the control group was found (see **figure 7**). This effect was less pronounced than in steatosis only. To date, there are no studies available which investigated TREM-2 expression, T2DM and obesity-associated NAFLD. So this study is the first to report a sort of triangular association between the progressive accumulation of fat in the liver, the dysregulation of glucose metabolism in terms of a diabetic phenotype and local (hepatic) inflammation.

A statistically significant positive association between the expression of CXCL10/ IP-10 and T2DM in moderate steatosis (group II) versus the control group was also observed, yet interestingly no differences in the severe steatosis group and when all groups combined (see **figure 7**). As far as known, the probable associations between the pro-inflammatory factor CXCL10, T2DM and NAFLD has been poorly investigated in humans, animal models or cell lines.

Colleagues in Taiwan have carried out a study in 132 NAFLD patients with and without T2DM (Chang *et al*. 2015). Their results show a positive correlation between serum

CXCL10 levels and NAFLD as well as with diabetic NAFLD (Chang *et al*. 2015).They suggested CXCL10 to be a potential non- invasive biomarker for progressive NAFLD and the development of T2DM in the following (Chang *et al*. 2015).

The presented findings support the idea of a link between T2DM and NAFLD *via* inflammatory pathways mediated by CXCL10.

The data show statistically significant up- regulations of TNF-α in patients with T2DM and moderate forms of NAFLD (steatosis group II) versus the control group (see **figure 7**). In addition, significant changes could be found when all groups with T2DM were combined and calculated against the control group (see **figure 7**).

A clincial trial in NAFLD patients with and without T2DM had been conducted in 2011 with 105 individuals showing higher plasma levels of TNF-α in T2DM NAFLD patients compared to NAFLD without T2DM (Shams *et al.* 2011).

In the adipose tissues from mice with immunohistochemical analysis (Weisberg *et al.* 2003, Xu *et al.* 2003) and in humans (Bai and Sun 2015), it could be shown that the number of adipose macrophages is increased under obese conditions and *vice versa* that the inhibition of macrophage recruitment to the adipose tissue attentuates obesity associated insulin resistance. However, the behavior and roles of TNF-α secreting liver resident macrophages and recruited blood monocytes in diabetic NAFLD remain enigmatic.

It remains also largely unknown, which cell types other than the liver resident macrophages, KC, produce the hepatic derived TNF-α and by which signaling pathways they are stimulated to do so. Liver macrophages have a probable great impact on obesity-induced insulin resistance and obesity associated co-morbidities such NAFLD as literature indicates already (Huang *et al.* 2010, Krenkel and Tacke 2017). In the diet-induced insulin resistance and NASH animal model with rats from 2010, it could be shown that the depletion of liver KC attenuates the progression of hepatic steatosis, NASH and insulin resistance (Huang *et al.* 2010). These findings indicate that KC orchestrate inflammation in the liver and in addition, are very likely crucial turning points about the direction of the inflammatory response into an either pro-inflammatory or anti-inflammatory direction, or in other words, whether the resolution or progression of the hepatic inflammatory process is supported (Adwitia *et al.* 2014, Huang *et al.* 2010, Krenkel and Tacke 2017).

Besides the clinical importance and therefore integration of the study results into clinical diagnostic algorithms more research needs to be conducted on the cellular and molecular level.

The study cohort reveals statistically significant up- regulations of COL1A1 in patients with T2DM and moderate forms of NAFLD (steatosis group II) versus the control group (see **figure 7**). In addition, significant changes are seen when all groups with T2DM were combined and calculated against the control group (see **figure 7**). Interestingly, there are no differences in severe NAFLD.

Only little is known about the interdependence of COL1A1, T2DM and NAFLD. In 2019, the colleagues from Pamplona, Spain, showed in a case- control study with 98 subject in a tissue-dependent manner the correlation between COL1A1 and AT inflammation (Unamuno *et al.* 2019). They revealed parts of the underlying signaling mechanistic by showing that blocking significant pathway proteins attenuated COL1A1 expression and reduced AT inflammation and subsequent fibrosis (Unamuno *et al.* 2019). The reproduction of these experiments in diabetic NAFLD individuals would be beneficial.

One explanation for no elevated COL1A1 expressions in severe NAFLD T2DM patients in the study cohort could be because of cell death in large numbers and replacement of hepatocytes with connective tissue in liver fibrosis. During this process no viable cells remain, which are able to transcribe DNS into proteins and enyzmes, and could produce COL1A1. Or in other words, the amount of COL1A1 correlates with the number of hepatocytes and immune cells producing collagen upon tissue damage and inflammation, which decreases along with the progression of organ damage like in NAFLD.

The expression profiles of the *de novo* lipogenic gene FASN and the DNL co-regulated endocrine factor FGF21 reveals a tight association between a diabetic phenotype and the degree of NAFLD in the study cohort in a statistically significant manner (see **figure 10**). In short summary, a positive association between FASN and diabetes and a strong pronounced association between FGF21 and diabetes was found (see **figure 10**). All differences displayed especially in moderate NAFLD (steatosis group II) (see **figure 10**).

Literature already shows a dysregulated DNL in the major lipogenic tissues of the human body – the adipose tissue and the liver – to result in a disruption of the systemic lipid homeostasis and therefore induce metabolic disorders and clinically manifest in diseases such as the most common at the moment and epidemic illnesses worldwide – obesity, T2DM and NAFLD (Ameer *et al.* 2014, Eissing *et al.* 2013). FASN is considered a marker of lipogenesis and has been categorized as housekeeping gene of the liver by Jensen-Urstad and Semenkovich in 2012 (Jensen-Urstad and Semenkovich 2012).

It could be already shown that insulin resistance, as found in T2DM, mainly regulates DNL in the liver; demonstrated *via* studies in human and micse (Eissing *et al.* 2013, Postic and Girard 2008). Most recently, a clinical trial from a group in the U.S. has shown very nicely that increased plasma glucose and insulin stimulate hepatic DNL in patients with NAFLD in a statistically significant manner (Smith *et al.* 2020). They took data from lean, obese and obese with NAFLD individuals, total number of study participants included 67 (Smith *et al.* 2020).

DNL in the liver is shown to be induced mainly by a carbohydrate rich dietary regimen, and hereby hepatic DNL is highly responsive especially to simple sugars (in contrast to complex carbohydrates) and to fructose, which could be shown in blood samples from healthy study subjects by Parks *et al.* in 2008 (Parks *et al.* 2008). In 2003, Diraison *et al.* presented data from plasma samples from 15 volunteers, which illuminate that the liver is more sensitive to a high-carbohydrate diet than the adipose tissue, shown by induced DNL in the liver (Diraison *et al.* 2002). It has been shown in obese patients, that substrates for hepatic DNL are also monosaccharides such as glucose (Eissing *et al.* 2013). Eissing *et al.* also revealed the link between glucose influx and DNL in the liver, which is covered by ChREBP-β, in obese patients (Eissing *et al.* 2013).

The underlying mechanisms between obesity, FFAs, hepatic DNL, decreased peripheral insulin- sensitivity and NAFLD are seemingly lipotoxic factors and insulin resistance promoting palmitate- ceramides, which seem to activate the innate immune system in the liver (Chavez and Summers 2012, Eissing *et al.* 2013, Fessler *et al.* 2009).

Against the background of this pre-existing knowledge the association between T2DM, hepatic DNL and NAFLD could be explained in such a way, that the hepatic DNL could be preceded by increased hepatic uptake of circulating carbohydrates in an abnormal amount due to insulin resistance of the peripheral tissues such as the skeletal muscle, as it can be found in T2DM, and promotes NAFLD. Whether in NAFLD this happens in a ChREBP-β-dependent manner needs to be confirmed in future studies.

Upon high blood glucose, increased glucose influx into the liver, DNL and accumulating lipids in the liver create an inflammatory response *via* the hepatocytes themselves and immune competent cells in the liver parenchyma (resident and recruited immune competent cells).This lipoinflammation in the liver in turn would induce hepatic and systemic insulin resistance and aggravate the T2DM phenotype – a true ping-pong-effect, which would partly explain the link between NAFLD, T2DM and obesity; of what is sometimes still called the

‘metabolic syndrome’, a fuzzy term for interdependent but most probably different diseases, which should be differentiated carefully one from another.

The DNL regulative element, FGF21, stimulates adipocytes to the postprandial uptake of glucose upon carbohydrate induced insulin secretion as shown in clamp studies from Samms *et al.* in 2017 and additionally to the uptake of free fatty acids demonstrated by Schlein *et al.* 2016 in mice (Kharitonenkov *et al.* 2005, Samms *et al.* 2017, Schlein *et al.* 2016). These inert qualities of FGF21 were revealed just recently. However, the growth hormone had come into focus of diabetes and adiposity research already in 2005, when it was found that pharmacological doses of FGF21 had anti-diabetic and weight reducing effects by lowering serum TG and controlling blood glucose in mice (Kharitonenkov *et al.* 2005).
Moreover, FGF21 seems to be able to even reverse liver steatosis in rodents and non- human primates and therefore may be a potential novel therapeutic agent for an effective treatment of T2DM, obesity, NAFLD and NASH without many of the adverse drugs reaction (Liu *et al.* 2015, Sonoda *et al.* 2017, Xu *et al.* 2009).
In dietary-induced obese mouse models FGF21 treatment revealed amelioration of peripheral and hepatic insulin resistance and suppression of hepatic DNL and reversion of hepatic steatosis (Coskun *et al.* 2008, Xu *et al.* 2009). In addition, FGF21-overexpressing mice seem to be resilient to dietary-induced obesity (Kharitonenkov *et al.* 2005). In contrast, dietary-induced obese knockout mice showed increased insulin resistance (Markan *et al.* 2014).
The underlying signaling mechanistics are subject of on-going research. Parts of the pathways could already be elucidated and show the necessity of PPARα in the ChREBP-mediated and glucose-induced FGF21 expression in the liver (Iroz *et al.* 2017). The french colleagues used a ChREBP knockout mouse model to show a markedly decrease in hepatic FGF21 expression upon glucose stimulation (Iroz *et al.* 2017).
The hepatokine FGF21 today is seen as a novel and promising approach to treat NAFLD and its co- morbidities such as T2DM and obesity effectively by enhancing the body endogenous hepatokine (Iroz *et al.* 2017, Sonoda *et al.* 2017).
In contrast to this positive aspect of FGF21, a recent wave of studies during the last 5 to 10 years sheds another light on FGF21 in energy metabolism and liver steatosis. Studies in mice show that FGF21 secretion may also be induced by intra-organ stress upon organ injury through inflammatory processes such as in NAFLD and NASH and therefore could be interpreted as a hepatic stress signal that entrains also harmful effects on other organs (Luo and

McKeehan 2013). In addition, when focusing on the metabolic unit of the liver and the adipose tissue, FGF21 seems to induce adipocytes to secrete not only so called adipokines such as adiponectin and leptin, but also free fatty acids (FFAs), which in turn could aggravate the steatotic situation in the liver (Luo and McKeehan 2013).
Like the COL1A1 expression, also the FASN expression correlates with the number of vital hepatocytes, which decreases along with the progression of organ damage as seen in fibrotic stages of NAFLD, which could explain the indifferences of FASN expression seen in severe NAFLD of the study cohort.

The data show, that T2DM increases intrahepatic BA expression in moderate NAFLD. A clear association between T2DM at intermediate liver steatosis and the regulation of the BA genes CYP7A1, CYP7B1, CYP8B1 and one of their regulatory elements SHP1 could be found (see **figure 13**).
Results from Haeusler *et al.* and Kahn *et al.* already suggested a CYP- regulating function for insulin in humans and rodent models (Biddinger *et al.* 2008, Haeusler *et al.* 2013, Saltiel and Kahn 2001). In a study cohort of 235 patients, they found shifts in the composition of plasma BAs in diabetic human patients; a twofold increase of BAs in total plasma of T2DM patients (Haeusler *et al.* 2013) and an insulin-dependent FoxO1-mediated CYP8B1 regulation in rodent models (Haeusler *et al.* 2012). In more detail, the LDL-knockout-FoxO1-knockout mice revealed that FoxO1 is required for the qualitative regulation of BA synthesis, where evidence grows that this could be one crucial link between abnormal hepatic lipid homeostasis and insulin dysregulation such as in T2DM (Haeusler *et al.* 2012). Mechanistic studies in HepG2 cells have shown that the rate-limiting enzyme CYP7A1 can be suppressed in a sort of negative feedback-loop by BAs (Lew *et al.* 2000).
This is not only crucial in order to reveal the pathogenesis of T2DM and insulin resistance but also to find novel medications. First attempts have been already made in the last 5 years or so, yet, promising results for effective pharmacological treatments of T2DM-associated NAFLD are waited for.
In the study cohort plasma BAs haven't been measured, which would be very interesting to look at as this hasn't been done yet in a human NAFLD cohort as fas as known.

Taken together, the TREM2, CXCL10, TNF and COL1A1 data show a less pronounced association with T2DM than with liver steatosis. These data can be interpreted as a pro-

inflammatory effect of T2DM, peripheral insulin-resistance and the development of an (inflammatory) liver steatosis.

FASN and FGF21 data reveal strong pronounced associations with T2DM independent of the degree of liver steatosis and illuminate the growing concept between a dysregulated glucose metabolism and liver steatosis and moreover confirm the role of FGF21 as a probable therapeutical point by enhancement strategies for this enzyme.

Data from the BA genes CYP7A1, CYP7B1, CYP8B1 and the regulating element SHP1 show interesting clear pronounced positive associations between T2DM in moderate NAFLD and seem to support the growing hope of effective hepatoprotective pharmacons by stimulating BA synthesis.

4.3. Association with fibrosis

TREM-2 shows a less pronounced positive association with fibrosis than with the degree of liver steatosis (see **figure 8** and **6**). The data reveal a statistically significant up- regulation of the genetic expression of TREM-2 in patients with moderate NAFLD and fibrotic characteristics compared to those without fibrosis of the same group (see **figure 8**). The link between TREM-2 and fibrosis hasn't been widely researched yet in NAFLD, especially not in human cohorts. As described already above, Perugorria *et al.* have looked for the role TREM-2 in the liver of humans, TREM-2-knockout mice and primary hepatic macrophages and hepatic stellate cells lacking TREM-2 (Perugorria *et al.* 2019). Their experiments in cell lines, mice and man show that liver inflammation and damage upon the application of hepatotoxic substances is increased when TREM-2 is absent and mediated by hepatic macrophages and hepatic stellate cells (Perugorria *et al.* 2019). Yet, the group didn't focus on NAFLD in their study cohort of 44 patients, which was characterized by diverse aetiologies of liver injury (Perugorria *et al.* 2019).

Most recently, in contrast to the anti- inflammatory effects of TREM-2, a group from the UK, unrevealed pro-fibrogenic characteristics for a macrophagic TREM-2 positive subpopulation in the liver which seems to inhabit the fibrotic niche as they explained (Ramachandran *et al.* 2019). Although, their study population didn't specifically include NAFLD patients but also diverse cirrhotic aetiologies (Ramachandran *et al.* 2019).

Nevertheless, with pre-existing literature in mind, the data indicate a correlation between TREM-2 expression and liver fibrosis as a consequence of hepatic inflammation upon excessive fat accumulation in obese phenotypes.

In the expression of CXCL10 a less marked expression difference regarding fibrosis in the study cohort could be found. Yet, none of the groups other than steatosis group I with fibrosis versus non- fibrotic of the same group displays any significant differences or trends (see **figure 8**).

Very freshly, data from a pilot- study with 44 NAFLD patients and their controls correlated the intrahepatic gene expression and plasma levels of CXCL10 with NAFLD degrees (Kriss *et al.* 2020). They show positive and progressive up- regulations of CXCL10 expression in the liver according to the histological degree, and a clear association between plasma CXCL10 and non-fibrotic NAFLD, which offers the usage as a non- invasive biomarker (Kriss *et al.* 2020).

Fibrosis doesn't show a relevant effect on the gene expression of TNF-α (see **figure 8** and **table 11**). And surprisingly, fibrosis doesn't show much of an effect regarding the expression of COL1A1 gene (see **figure 8** and **table 11**). Nevertheless, individuals with fibrosis from steatosis group I exhibited a trend of higher COL1A1 expression versus the non-fibrotic patients from the same group (see **table 11**).

COL1A1 expression in general depicts the association between an inflammatory processes and the scarring of tissue or in other words the replacement of vital cells with connective tissue (Sun *et al*. 2020). Collagen in the liver is produced by myofibroblasts, which originate from hepatic stellate cells, which are also called Ito cells or fat-storing cells (Asselah *et al*. 2008, Brenner *et al.* 2013, Sun et al. 2020). Hepatic stellate cells in the perisinusoidal space (space of Disse) transform into Ito cells upon stimulation with transforming growth factor β1 (TGF-β1) in case of extensive organ damage (Asselah *et al.* 2008, Brenner *et al.* 2013). Physiologically, collagen maintains the sinsusoidal tonus in the liver (Asselah *et al*. 2008, Brenner *et al.* 2013). In case of tissue damage, two rescue mechanisms for restoring organ homeostatis are avaible (Asselah *et al.* 2008, Brenner *et al.* 2013, Sun *et al.* 2020). Tissue damage is believed to happen due to hepatocyte loss upon various stimulus, and specifically in NAFLD due to lipoapoptosis upon FFAs *via* the activation of the proapoptotic p53 protein and the inhibition of the antiapoptotic protein Bcl-2 as shown in the livers from NAFLD patients by Panasiuk *et al.* in 2006 (Panasiuk *et al.* 2006). The two rescue mechanisms are the following: First is, that hepatocytes are able to divide and create an exact copy of themselves, and second is, that the liver likely contains stem cells, the hepatic oval cells in the intrahepatic bile ducts, which are able to differentiate into mature hepatocytes, if needed (Miyajima and Tanaka 2014). These self-restoring mechanisms work pretty well if tissue damage doesn't exceed an abnormal level, which can occur under mainly two circumstances:

Acute excessive liver damage, as for instance during an acute virus hepatitis or during pregnancy the HELLP syndrome; and under chronic hepatotoxic influences such as in chronic inflammation due to lipid accumulation in hepatocytes in NAFLD.
Previous findings in blood samples and liver biopsy specimen from 203 patients with hepatitis C virus infection showed the elevation of CXCL10 in plasma and were correlated with increasing stages of fibrosis and inflammation (Crisan *et al.* 2017). These findings account for the case of virological liver damage and need to be pursued for NAFLD.
So far, the data are interpreted in such a way that an induced expression of COL1A1 in the liver tissue indicates a high number of myofibroblasts, that differentiated into stellate cells, which express and transcribe the COL1A1 gene and produce excessively extracellular collagen. This process may also be called hepatic scarring and it happens only upon extensive liver damage. It can be also concluded that an up-regulation of this gene indicates progressed liver destruction and therefore a progression from simple *steatosis hepatis* into NASH and more severe stages of NAFLD. The suggested concept of an association between progressed liver steatosis and hepatic scarring in obese NAFLD patients represented by the COL1A1 gene expression was confirmed.
Future studies, especially immunohistochemial stainings of the livers from obese NAFLD patients should be performed aiming to distinguish the cell type origin of the induced COL1A1 expression and the expression of TREM-2 and CXCL10. Yet, it is also largely unknown by which signaling pathways exactly Ito cells differentiate into collagen producing myofibroblasts and in turn which downstream receptors and ligands are involved and lead to the induced expression of COL1A1 in myofibroblasts. Further research on this topic is suggested as its holds opportunities of blocking these elusive pathways and mechanisms and by this suppressing the expression of TREM-2, CXCL10 and COL1A1 and extensive fibrosis and scarring in the liver. The inhibition of these signal cascades could eventually attenuate the progression of *steatosis hepatis* and NASH into the irreversible stages of fibrosis, cirrhosis and malignant entities and therefore finally serve in the development of effective treatment strategies.

Quite strikingly, the data reveal no statistically significant correlation between fibrosis in NAFLD patients and the expression of FGF21 and FASN (see **figure 11**). Yet, positive trends for higher expression of FASN in the NAFLD groups III and I with fibrosis versus the same groups without fibrosis are seen (see **figure** and **table 11**).

Two years ago, Lee *et al.* presented data from patients diagnosed with viral and alcoholic hepatitis, which illuminate the role and underlying pathways of FGF21 in liver inflammation and fibrosis (Lee e*t al.* 2018). Their findings indicate a positive correlation between FGF21 expression and liver fibrosis as well as liver inflammation (in AFLD and viral hepatitis) (Lee e*t al.* 2018). Furthermore, they give insight into the probable FGF21 signaling pathway *via* the IL-1β-induced NF-κB-dependent β-Klotho supression, simultaneous c-Jun N-terminal kinase (JNK) induction and by this induced expression of FGF21 (Lee e*t al.* 2018).
The low signal of genetic expression of FASN and FGF21 in the fibrosis groups compared to their control groups was interpreted as a hepatocyte loss due to lipoapoptosis and progressive hepatic scarring, which has been suggested already by other authors (Cazanave *et al.* 2011, Hirsova and Gores 2015). This process involves a loss of living cells and a simultaneous increase of extracellular dead matrix, which isn't capable of gene expression and protein production. In order to investigate the impact of T2DM on hepatic scarring further studies will be needed, which examine not only the mRNA level, but use also for example immunohistochemical staining methods.

The data reveal moderate positive associations in NAFLD patients with little liver fat and fibrosis, seen in group I with fibrosis versus group I without fibrosis (see **figure 14**). SHP1 seems to be independent of fibrosis (see **figure 14** and **table 11**).
Probable anti- cirrhotic (anti- fibrogenic) and therefore hepatoprotective effects of BAs *via* the BA-induced differentiation of hepatic stellate cells have been studied so far only in the primary biliary cirrhosis in humans and in knockout mouse models, not in NAFLD yet (Liu *et al.* 2016, Yang and Duan 2016).
In addition, the induction of BA synthesis, especially *via* the alternative pathway, possibly serves as a self-protection mechanism of the liver in order to eliminate excessive fat in forms of FFAs and cholesterol *via* the BA circulation and finally the faeces (Worthmann *et al.* 2017). This self-protection mechanism of the liver itself has been reported by Worthmann *et al.* 2017 in transgene and dietary-induced obesity mice exposed to cold (Worthmann *et al.* 2017).
As SHP1 is a negative regulator in the expression of BAs, these findings were interpreted in a way, that a still enigmatic mechanism represses SHP1 itself and hereby, the negative feedback control of SHP1 escapes and BA genes are induced in expression. The underlying mechanisms remain to be revealed.

The findings from CYP7A1, CYP7B1, CYP8B1 and SHP in the study cohort suggest that liver steatosis may induce a self-protection mechanism in the liver *via* the bile acid alternative pathway.

Taken together, the TREM2, CXCL10, TNF-α, COL1A1 and FASN and FGF21 data show that hepatic scarring is associated with inflammation and DNL.
Data from the BA genes CYP7A1, CYP7B1, CYP8B1 and the regulating element SHP1 suggest that BAs and their genetic regulation hold the potential to have hepatoprotective characteristics in NAFLD.

Chapter 5

5. Conclusion

5.1 Conclusion

DNL gene analysis reveal strong pronounced associations with T2DM independent of the degree of liver steatosis and illuminate the growing concept between a dysregulated glucose metabolism and liver steatosis. In addition, the analysis confirms the role of FGF21 as a probable therapeutical point by enhancement strategies for this enzyme.

The inflammatory gene analysis show a strong and positive association with progressively higher liver steatosis and suggest that lipid accumulation in hepatocytes supports hepatic inflammation. This notion is strengthened by data showing that a primary liver steatosis is associated with hepatic inflammation and tissue damage upon the loss of proper VLDL functioning on a predisposed genotype, e.g. by mutations in the transmembrane 6 superfamily 2 (TM6SF) and patatin-like phospholipase domain-containing protein 3 (PNPLA3) (Scheja and Heeren under preparation). Yet, the presented data could also be explained in a way that the factors promoting liver steatosis have pro-inflammatory qualities at the same time. The inflammatory genes show a less pronounced association with T2DM than with liver steatosis, which points to the fact of pro-inflammatory effects of T2DM, peripheral insulin-resistance and the development of an (inflammatory) liver steatosis. Hepatic scarring is associated with inflammation and DNL as gene analysis of the inflammatory genes depicts.

BA synthesis is not regulated in a steatosis-dependent manner, which supports the notion that BAs don't play a role in steatosis-induced inflammatory NAFLD, yet, this concept can't be fully excluded as bile acids haven't been measured directly. Interestingly, clear pronounced positive associations between T2DM in moderate NAFLD and BA genes were found and support the growing hope of effective hepatoprotective pharmacons by stimulating BA synthesis. The exposed differences between murine models and humans, especially in the regulation of BA synthesis genes, demands further research in order to be elucidated fully.

In conclusion, the work supports the growing concept that NAFLD, obesity and T2DM form a metabolic unit of mutually aggravating disorders. It can be also confirmed, that the underlying mechansims of this are alterations in the hepatic and systemic fat and glucose metabolism *via* DNL induction in the liver and hepatic inflammation. The underlying mechanisms of hepatic inflammation need to be elucidated in detail in future *in vitro* and *in vivo* studies.

These findings also offer novel therapy strategies for NAFLD *via* the inhibition of hepatic DNL, the suppression of hepatic inflammation and the induction of BA synthesis genes. This has the potential to avert long term effects of NAFLD derived malignant entities like the hepatocellular carcinoma. In addition, it might be important to have NAFLD patients under a tight monitoring in order to detect the progression from mild steatosis hepatis into NASH. Furthermore, novel biomarkers for NASH screening in obese patients *via* the serum biomarkers CXCL10 and TNF-α are offered. This may also be beneficial for clinicians, because the current diagnostic of NAFLD and NASH remains invasive and thus associated with side effects as described in the beginning of this thesis.

5.2 Zusammenfassung

Die DNL-Genanalysen zeigen stark ausgeprägte Assoziationen mit Diabetes Mellitus Typ 2 unabhängig von dem Grad der Leberverfettung. Sie stützen die zunehmende Vermutung über den Zusammenhang zwischen einem dysregulierten Zuckerstoffwechsel und der Lebersteatose. Zusätzlich legen die DNL-Genanalysen nahe, dass FGF21 eine Rolle in der medikamentösen Therapie von NAFLD spielt und als pharmakologischer Zielpunkt angesteuert und aktiviert werden könnte. Mit dieser Beobachtung reihen sich die erhobenen Daten in die bestehende Fachliteratur ein.

Die Datenanalysen der entzündlichen Gene zeigen eine starke und positive Assoziation mit den progredienten höheren Lebersteatose-Graden. Sie lassen vermuten, dass eine Lipid-Anreicherung in den Hepatozyten die Leberentzündung unterhält. Diese Vorstellung wird von den Ergebnissen gestützt, die zeigen, dass eine primäre Lebersteatose mit hepatischer Entzündung und Gewebsschädigung assoziiert ist. Dies geschieht vermutlich infolge des Verlusts von vollständig funktionierendem VLDL in einem prädisponierten Phänotyp; zum Beispiel durch Mutationen in dem Transmembran-6-Super-Familie-2 (TMSF6) und Patatin ähnlichen Phospholipase-Domän enthaltenden Protein-3 (PNPLA3) (Scheja und Heeren in Arbeit).

Gleichzeitig können die vorgestellten Daten auch so interpretiert werden, dass die Faktoren, welche die Lebersteatose fördern, gleichzeitig entzündungsfördernde Eigenschaften besitzen. Die entzündlichen Gene zeigen eine weniger stark ausgeprägte Assoziation mit T2DM als mit der Lebersteatose. Dies weist auf die entzündungsfördernden Eigenschaften von T2DM, der peripheren Insulinresistenz und der Entwicklung der entzündlichen Lebersteatose hin. Hepatische Vernarbung ist mit Entzündung und DNL assoziiert wie die Gen-Analysen der inflammatorischen Gene zeigen.

Die Gallensäuren-Synthese ist nicht Steatose abhängig reguliert. Diese Beobachtung stützt die sich entwickelnde Vorstellung, dass Gallensäuren keine Rolle in der Steatose induzierten Entzündung in NAFLD spielen. Gleichzeitig kann dieses Konzept nicht vollständig ausgeschlossen werden, da die Gallensäuren in der vorliegenden Studie nicht direkt gemessen worden sind. Interessanterweise wurden jedoch deutlich ausgeprägte positive Assoziationen zwischen T2DM mit milder NAFLD und Gallensäuren-Genen gefunden. Diese Ergebnisse nähren die Hoffung auf effektive hepatoprotektive Medikamente, welche die Gallensäuren-Synthese direkt stimulieren. Die dargestellten Unterschiede zwischen Maus-Modellen und Menschen, insbesondere in der Gallensäuren-Synthese, inspirieren zu weiteren Untersuchungen und Experimenten.

Summa summarum unterstützt die vorliegende Arbeit das sich etablierende Konzept darüber, dass NAFLD, Adipositas und T2DM eine metabolische Einheit von sich gegenseitig negativ beeinflussenden Erkrankungen bilden. Es kann ebenfalls bestätigt werden, dass die zugrunde liegenden Mechanismen Veränderungen im hepatischen und systemischen Fett- und Zuckerstoffwechsel aufgrund von DNL-Induktion und Entzündung in der Leber sind. Die Mechanismen der Leberentzündung sollten in *in vitro* und *in vivo* Experimenten im Detail weiter erforscht werden.

Die vorliegenden Beobachtungen weisen ebenfalls auf neuartige Therapiemöglichkeiten für NAFLD hin. Neue Strategien können beispielsweise die Hemmungen von DNL und Entzündungskaskaden sowie die Induktion der Gallensäuren-Synthese in der Leber sein. Diese neuen Ansätze hätten das Potenzial, die Langzeitfolgen von NAFLD induzierten malignen Erkrankungen wie beispielsweise das hepatozelluläre Karzinom zu verhindern oder zumindest zu reduzieren. Es mag zusätzlich von Bedeutung sein, NAFLD-Patienten in ein engmaschiges Monitoring aufzunehmen, um frühzeitig die Übergänge von milder Steatose in eine NASH zu erkennen. Weiterhin schlägt die vorliegende Arbeit neuartige Biomarker wie CXCL10 und TNF-α zur frühzeitigen Erkennung von NASH in adipösen Patienten vor. Auch dies mag für klinisch tätige Ärztinnen und Ärzte nützlich sein, da der gegenwärtige

diagnostische Goldstandard für NAFLD und NASH die invasive Leberbiospie und daher mit zahlreichen Risiken und Nebenwirkungen für die Patientinnen und Patienten verknüpft ist, wie eingangs beschrieben.

Chapter 6

6. References

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Chapter 7

7. Appendix

7.1 Abbreviations

A	**ACACA**	**acety-CoA-carboxylase**
	ALP	alkaline phosphatase
	AoD	assay on demand
	AST	aspartate aminotransferase
	AT	adipose tissue
	BA	bile acid
B	**BAAT**	bile acid-CoA:amino acid N- acyltransferase
	BAT	brown adipose tissue
	BMI	body mass index (kg/ m²)
C	**CA**	cholic acid
	CAD	coronary artery disease
	Cat. No.	catalog number
	CDCA	chenodeoxycholic acid
	cDNA	complementary deoxyribonucleic acid
	ChREBP-α/ -β	carbohydrate responsive element binding protein alpha/ beta
	CO^2	chemical formula of carbon dioxide
	CoA	coenzyme A
	COL1A1	alpha-1-type-1-collagen
	CRP	C-reactive protein
	CT	computertomography
	CYP7A1	cytochrome P450 family 7 subfamily A member 1
	CYP7B1	cytochrome P450 family 7 subfamily B member 1
	CYP8B1	cytochrome P450 family 8 subfamily B member 1
	CXCL10	C-X-C motif chemokine ligand 10
	CXCR3	C-X-C motif receptor 3
D	**DBD**	DNA- binding domain
	DEG	differentially expressed gene
	DNA	deoxyribonucleic acid
	DNL	*de novo* lipogenesis
	DR	death receptor

	DR5	death receptor 5, also called tumor necrosis factor (TNF-) related apoptosis-inducing ligand receptor 2 (TRAIL-R2) or TNFRSF10B
E	**ECM**	extracellular matrix
	ER	endoplasmic reticulum
	ERK1/2	extracellular-regulated kinases 1 and 2
	EUS-LB	endoscopic ultrasound-guided liver biopsy
F	**f**	female, gender
	FASN	fatty acid synthase
	FBG	fasting blood glucose
	FFA	free fatty acid
	FGF19	fibroblast growth factor 19
	FGF21	fibroblast growth factor 21
	FXR	farnesoid X receptor
G	**GOT**	glutamate oxaloacetate transferase
	GOI	gene of interest
	GPT	glutamate pyruvate transferase
	γ-GT	gamma glutamyl transferase
H	**HbA1c**	glycated hemoglobin
	HBV	hepatitis B virus
	HCV	hepatitis C virus
	HDL	high density lipoprotein
	HIV	human immunodeficiency virus
	HNF4α	hepatocyte nuclear factor 4 alpha
I	**ID**	identification
	IFN-γ	interferon-gamma
	IL-1β	interleukin 1 beta
	IL-6	interleukin 6
	INR	International Normalized Ratio
	IP-10	induced protein 10, synonym for CXCL10
	JNK	c-Jun N-terminal kinase
K	**KC**	Kupffer cell
L	**LBD**	ligand- binding domain
	LDL	low density lipoprotein
	LPS	lipopolysaccharide
	LXRα	liver receptor alpha
M	**m**	male, gender
	MDA5	melanoma differentiation-associated gene 5
	MCP-1	monocyte chemoattractant protein 1
	miRNA	micro ribonucleic acid
	mRNA	messenger ribonucleic acid.
N	**NADPH**	nicotinamide adenine dinucleotide phosphate
	NAFLD	non-alcoholic fatty liver disease

	NASH	non-alcoholic steatohepatitis
	NCBI	National Center for Biotechnology Information
	NF-κB	nuclear factor kappa-light-chain-enhancer of activated B-cells
	NK	natural killer (cells)
	NR	nuclear receptor
	NR0B2	nuclear receptor subfamily 0 group B member 2
O	**oGTT**	oral glucose tolerance test
P	**PCR**	polymerase chain reaction
	PLOSL	polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy
	PNPLA3	patatin- like phospholipase domain- containing protein 3
	PPARα/δ/γ	peroxisome proliferator-activated receptor family alpha/ delta/ gamma
Q	**qPCR**	quantitative polymerase chain reaction, synonym to rtPCR
R	**RNA**	ribonucleic acid
	ROS	reactive oxygen species
	RT	reverse transcription
	rtPCR	real-time polymerase chain reaction, synonym to qPCR
	RYGB	Roux-en-Y gastric bypass
S	**S.E.M**	standard error of the mean
	SG	sleeve gastrectomy
	SHP1	small heterodimer partner 1
	SREBP-1c	sterol responsive element binding protein 1c
	STAT1	signal transducer and activator of transcription 1
T	**T2DM**	type 2 diabetes mellitus
	TG	triglyceride
	TGF-β1	transforming growth factor beta 1
	Th1 lympho-cyte	T helper 1 lymphocyte
	TLR4	toll like receptor 4
	TM6SF2	transmembrane 6 superfamily 2
	TNF-α	tumor necrosis factor alpha/α
	TNFRSF1A	tumor necrosis factor receptor superfamily member 1A
	TNFRSF1B	tumor necrosis factor receptor superfamily member 1B
	TRAIL-R2	(TNF-) related apoptosis-inducing ligand receptor 2
	TREM-1	triggering receptor on myeloid cells 1
	TREM-2	triggering receptor on myeloid cells 2
	TZD	thiazolidinedione
U	**UKE**	Universitätsklinikum Hamburg-Eppendorf
V	**VLDL**	very low density lipoproteins

W	**WAT**	white adipose tissue
	WHO	World Health Organisation

Table 12 – List of abbrevations in alphabetic order.

7.2 Units

C	**cm**	centimeter, a metric unit of length, equal to one hundredth of a meter.
	c/nl	cells per nanoliter.
D	**dl**	deciliter, a metric unit of capacity, equal to one tenth of a liter.
G	**g**	gram, a metric unit of a mass, equal to one thousanth of a kilogram.
K	**kg**	kilogram, the SI unit of mass, equal to the international standard kept at Sèvres near Paris.
	kg/m²	kilogram per square meter.
L	**l**	liter, a metric unit of capacity, formerly defined as the volume of one kilogram of water under standard conditions, now equal to 1.000 cubic centimeters.
M	**m**	meter, the fundamental unit of length in the metric system, equal to 100 centimeters.
	mg	milligram, a unit of mass, equal to one thousandth of a gram.
	mg/dl	milligram per deciliter
	mL	milliliter, a unit of volume, equal to one thousandth of a liter.
	mol	mole, the SI unit of amout of substance, equal to the quantity containing as many elementary units as there are atoms in 0.012 kg of carbon -12
	µl	microliter, a unit of volume, equal to one million of a liter.
	µmol	micromole, one millionth of a mole.
	%	percent, by a specific amount in or for every hundred
R	**rpm**	rotations per minute.
U	**U/l**	unit per liter = µmol per min per liter = 1 enzyme unit per liter = 1 micromole substrate per minute per liter

Table 13 – List of units in alphabetic order.

7.3 Greek Characters

α	**alpha**
β	beta
δ	delta
γ	gamma
κ	kappa

Table 14 – List of greek characters in alphabetic order.

7.4.a Ethical Standard

Blood samples and liver biopsy specimens of human participants involved in this study were in accordance with the ethical standards of the institutional reserach committee, the 'Ethik-Kommission der Ärztekammer Hamburg', the German and Employement Law and the International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use (ICH GCP). Approval and permission for the study 'Einrichtung einer Bio- und Datenbank: Organschäden und Komorbiditäten bei Morbider Adipositas' were given under the number PV4889. Informed consent in writing was obtained from each patient enrolled in the study.

7.4.b Conflict of Interest Statement

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author certifies that she has NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria, educational grants, participation in speakers' bureaus, membership, employment, consultancies, stock ownership, or other equity interest, and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

7.4.c Statement in lieu of an oath

I hereby confirm that I have written this thesis on my own and that I have not used any other media or materials than the ones referred to in this thesis.

7.4.d Declaration of Consent

I agree to make both versions of my thesis (with a passing grade) accessible to the public by having them added to the library of the University Clinic of Eppendorf.

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7.6 Curriculum Vitae

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7.7 Eidesstattliche Erklärung

Ich versichere hiermit ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst habe. Ich habe keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt. Die aus den von mir benutzen Werken wörtlich oder inhaltlich entnommenen Stellen habe ich einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzen Werkes kenntlich gemacht. Ferner versichere ich, dass ich die Dissertation bisher keinem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um die Zulassung zur Promotion beworben habe. Ich erkläre mich damit einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät der Universität Hamburg mit einer gängigen Software zur Erkennung von Plagiaten überprüft wird.

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