THESIS

NAFLD and bile acids

Non-alcoholic fatty liver disease- a new pandemic after corona?

submitted by

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Declaration of Academic Integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

Graz, 16.10.2023 Julia Fitzinger m.p.

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Abbreviations

ALD	alcoholic liver disease
12-a-OH BA	12α-hydroxylated BA (CA, DCA + their conjugated forms)
ALT	alanine transaminase
ASBT	apical sodium dependent bile acid transporter
AP	alkaline phosphatase
AST	aspartate transaminase
AT	adipose tissue
BA	bile acid
BAAT	bile acid-CoA:amino acid N-acetyltransferase
BACS	bile acid-CoA synthetase
BSEP	bile salt export pump
CA	cholic acid
CAR	constitutive androstane receptor
CCK	cholecystokinin
CDCA	chenodeoxycholic acid
ChREBP	carbohydrate-responsive element-binding protein
DAMP	damage associated molecular pattern
DCA	deoxycholic acid
ELF	ELF-Test (Enhanced Liver Fibrosis)
ER	endoplasmatic reticulum
FA	fatty acids
FFA	free fatty acids
FiB_Score	NAFLD fibrosis score
FIB-4	fibrosis-4 index
FLI	fatty liver index
FXR	farnesoid X receptor
GCA	glycocholic acid
GCDCA	glycochenodeoxycholic acid
GDCA	glycodeoxycholic acid
GLCA	glycolithocholic acid
GLP-1	glucagon-like-peptide 1
GLUT (1,2,3,4,5…)	glucose transporter
GUDCA	glycoursodeoxycholic acid
HFR diet	high fructose diet
LCA	lithocholic acid
LPC	lysophosphatidyl choline
MAFLD	metabolic dysfunction-associated fatty liver disease
MED	mediterranean diet
NAFLD	non-alcoholic fatty liver disease

NASH	non-alcoholic steatohepatitis
non-12-a-OH BA	(CDCA, LCA, UDCA + their conjugated forms)
NTCP	Na+-taurocholate-cotransporting-polypeptide
OSTα/β	organic solute transporter α/β
PAMP	pathogen associated molecular pattern
PPAR-γ	peroxisome proliferator–activated receptor gamma
PXR	pregnane X receptor
SREBP	sterol regulatory element-binding protein
SREBP-1c	sterol regulatory element–binding protein
SULT 2A1	sulfotransferase (SULT) 2A1
T2DM	type-2 diabetes mellitus
TCA	taurocholic acid
TCDCA	taurochenodeoxycholic acid
TDCA	taurodeoxycholic acid
TGR5	takeda G protein coupled receptor 5
TLCA	taurolithocholic acid
TUDCA	tauroursodeoxycholic acid
UDCA	ursodeoxycholic acid
UGT	UDP-glucuronosyltransferase
VLDL	very low density lipoprotein

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Abstract

Background/ Objectives: Worldwide, an increasing number of people is suffering from non-alcoholic-fatty liver disease (NAFLD), the most common reason for fatty liver. The main cause is metabolic syndrome, which is accompanied by cardiovascular diseases and general higher morbidity and mortality, causing higher workload and expenses in health care. Therefore, the focus of research is on finding therapeutic, but also diagnostic possibilities for the disease. Still, the ways of detecting are limited, especially for non-invasive and early diagnosis. It is important to understand the diverse affection of organs and metabolism including the role of insulin resistance, immune system, gastrointestinal microbiome as well as genetic variations and the influence of sex. Metabolomics are a way to get insight into cellular processes through creation of a profile of certain small molecules. We measured bile acid (BA) concentrations in the plasma of adult NAFLD- and alcoholic liver disease patients and controls with targeted mass spectrometry.

Hypothesis: The liver is central for BA synthesis and therefore BA may be potential markers to detect and monitor liver dysfunction. Depending on certain individual contributors and the origin of liver disease, the changes in BA concentrations could show a characteristic pattern.

Results: There are alterations, that seem to be specific for NAFLD (significant in NAFLD vs. controls and NAFLD vs. ALD) and some trends that are dependent on gender. Women presented with higher concentrations of primary BA. In male NAFLD, concentrations of secondary BA are elevated and they show a lower proportion of total free BA to total BA, but the part of free chenodeoxycholic acid (CDCA) is higher. Glycine/taurine conjugated BA and glycine conjugated CA/CDCA are higher than in controls.

Zusammenfassung

Hintergrund/Zielsetzung: Die Zahl der Diagnosen "nicht-alkoholische Fettleber Erkrankung", der häufigsten Ursache der chronischen Verfettung der Leber, steigt weltweit und ist in erster Linie durch das metabolische Syndrom bedingt. Kardiovaskulären Erkrankungen und generell höhere Morbidität und Mortalität begleiten das Krankheitsbild und steigern den Arbeitsaufwand und die Ausgaben im Gesundheitssystem. Therapieoptionen und die Ausweitung diagnostischer Möglichkeiten stehen deshalb im Fokus der Forschung, wobei bei der frühen und möglichst nicht-invasive Diagnose noch großer Aufholbedarf besteht. Es wäre wichtig, neben der Insulinresistenz, des Einflusses des Immunsystems, des Darm-Mikrobioms und unter der Berücksichtigung genetischer Variationen und des Einflusses des Geschlechts die Auswirkungen auf verschiedene Organe und metabolische Abläufe noch genauer zu verstehen. Mittels Metabolomik lässt sich ein Einblick in zelluläre Abläufe gewinnen. Durch gezielte massenspektrometrische Analyse haben wir im Plasma von erwachsenen Patientinnen und Patienten mit NAFLD, mit alkoholischer Lebererkrankung und in einer gesunden Kontrollgruppe die Konzentrationen von Gallensäuren (GS) bestimmt.

Hypothese: Die Leber ist das zentrale Organ für die Gallensäureproduktion, weshalb Gallensäuren potentielle Marker für das Erkennen von Lebererkrankungen darstellen könnten. Abhängig von individuellen Faktoren und vom Auslöser der Erkrankung könnten die Veränderungen der Gallensäurekonzentrationen charakteristische Muster aufweisen.

Ergebnisse: Es gibt Veränderungen, die speziell in NAFLD Patientinnen und Patienten (signifikant in NAFLD vs. gesunde Kontrollen und NAFLD vs. alkoholische Lebererkrankung, ALD) zu finden sind und einige, die geschlechtsabhängig waren. In weiblichen NAFLD Patientinnen wurden erhöhte Konzentrationen primärer GS gefunden. Bei männlichen NAFLD Patienten gibt es Erhöhungen sekundärer GS und der Anteil der gesamten freien GS von den gesamten GS war niedriger, der Anteil von freier Chenodeoxycholsäure (CDCA) erhöht. Das Verhältnis Glyzin/Taurin konjugierter GS und Glyzin konjugierte CA/CDCA war höher im Vergleich zu den Kontrollen.

1 Introduction

Non-alcoholic-fatty-liver disease (NAFLD) increasingly becomes a topic of interest, as around a quarter of the general world population shows increased amounts of fat in the liver, being an early sign for metabolic imbalance. Up to 70% of obese people or those with T2DM are estimated to have NAFLD, although there are big regional differences in prevalence ranging from 5 % in Ethiopia and nearly 50% in Turkey. (1)

To point out the strong metabolic component and, renaming it as “metabolic acquired fatty liver disease” (MAFLD) has been considered and would draw a clearer line between other potential non-alcoholic causes of steatosis. Abdominal obesity, type 2 diabetes mellitus, dyslipidemia and high arterial blood pressure take a central part in development of NAFLD. Furthermore, advanced age, male sex, ethnicity, environmental factors, lifestyle and genetic variations are important risk factors. All these factors contribute to dysfunctions in lipid- and sugar metabolism, disturbances in microbiome, a systemic inflammatory state and activation of immune cells, being the being central points in pathogenesis.

The earlier phases of the NAFLD include simple hepatic steatosis (NAFL, HS) and non-alcoholic steatohepatitis (NASH) and show progression, or remission over a long period. Fibrosis and lastly cirrhosis and potentially Hepatocellular Carcinoma (HCC) can develop in the course of disease. About 20% of NASH patients are rapid progressors, where advanced fibrosis occurs within a few years. Over the course of two years, about 20% of the patients with advanced fibrosis progress to cirrhosis, of which after two years 20% present hepatic decompensation.

While in fibrotic stages, incidence of cardiovascular events and extrahepatic malignancies is higher, the liver-related complications like hepatic decompensation and development of HCC become more relevant in cirrhosis. (1) Besides the known risk factors, certain comorbidities like cardiometabolic diseases, polycystic ovary syndrome, subclinical hypothyreosis, and obstructive sleep apnea can further affect progression and mortality. Higher fatal outcome, but also higher general morbidity is found in the disease, not only stemming from liver-related complications in cirrhotic stages like hepatic decompensation and development of HCC, but also from cardiovascular events and extrahepatic malignomas, that occur more often in fibrotic stages. Mortality directly from liver disease is even lower than dying from extrahepatic manifestations. (1)

The apparent increase of obesity among children in the last decades is accompanied by earlier onset and a higher risk of NAFLD, cirrhosis and liver related mortality due to cumulative effects over the years, compared to weight gain later in life.

Despite the close connection to obesity, the significance of the disease in lean patients cannot be underestimated. According to a Chinese study, the risk for metabolic syndrome and hypertension in lean NAFLD was comparable to obese NAFLD and Visceral Adipose index (VAI) was significantly higher in comparison to overweight controls. Lean NAFLD patients have a higher PNPLA3 risk allele carrier rate compared to lean controls. (2–5)

2 Pathogenesis

Central in NAFLD are hepatic steatosis (HS), overweight and insulin resistance, but in the last years, the intestine and its microbiome, diet, activation of the innate and adaptive immune system and the capacity of balancing circulating stressors have been added to considerable factors in disease progression.

Fatty acids stemming from systemic circulation, de-novo lipogenesis and diet are used in TG synthesis, oxidized (in mitochondria and peroxisomes) or are excreted out of the liver (as VLDLs). In NAFLD, these processes are disturbed, leading to high TG- plasma levels and an accumulation in the liver in the form of lipid droplets. They are not directly hepatotoxic

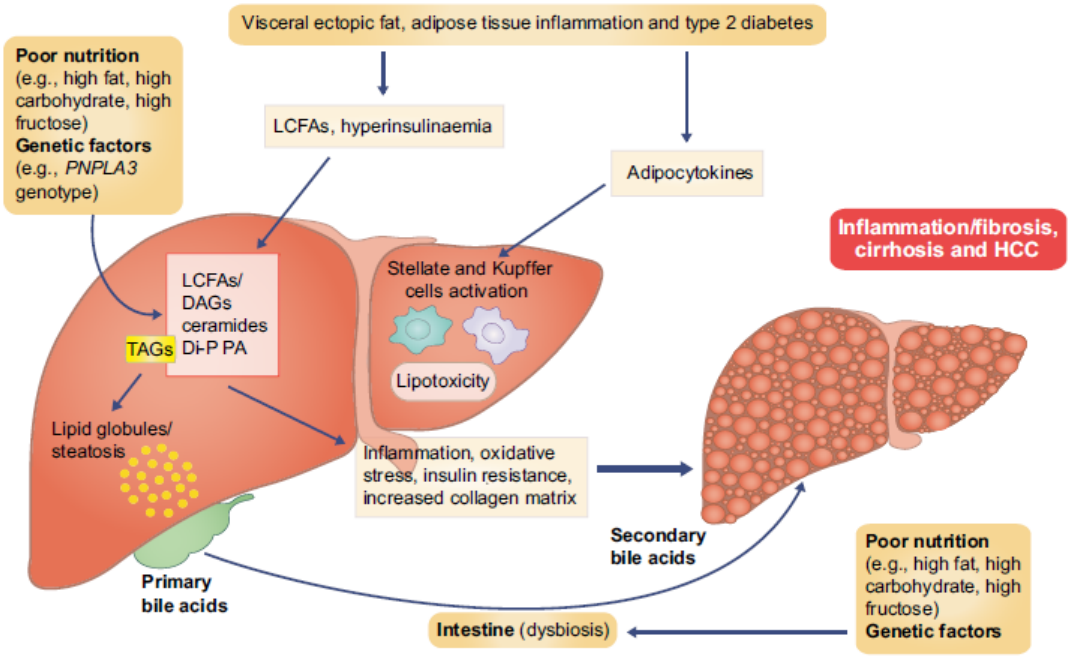

Figure 1: Pathogenesis of NAFLD (6)

and their build-up may even be a protection from more toxic lipid species like ceramides, lysophosphatidyl choline (LPC), diacylglycerol and cholesterol, which all can fuel NAFLD.

Increased amounts of fat are released out of the adipose tissue (AT) in case of energy overload or if the AT presents signs of metabolic sickness (cell hypertrophy, chronic low-grade inflammation, insulin resistance, cell death, hypoxia...). Additionally, the endocrine function of the AT includes secretion of pro-inflammatory cytokines like TNF-α, IL-6, monocyte chemoattractant protein-1, plasminogen-activator inhibitor-1 and angiotensinogen, but also leptin. In obesity, high concentrations of the latter lead to an insensitivity of leptin, diminishing its anorexigenic effect, while the profibrogenic properties persist. (6) Adiponectin is low in NASH and fibrosis and stimulates ß-oxidation, suppresses inflammation, and modulates hepatic stellate cells (HSC) as well as Kupffer cells (KC).

Free fatty acids can drive progression of NASH by elevating inflammation, ER-stress and mitochondrial stress. An overload of free fatty acids (FFA) oxidized in mitochondria can be major sources of reactive oxygen species (ROS). Reactive oxygen species harm also the function and structure of mitochondria through oxidation.

Microsomes and peroxisomes are also cellular elements that metabolize certain fatty acids. Oxidative stress has been correlated to disease severity, but in early stages, antioxidative systems may be upregulated to fight oxidative stress, subsequent become exhausted and insufficient. Besides driving inflammation and worsening insulin resistance, the peroxidation of lipids by ROS disturbs secretion of VLDL (from the liver) and plays a big role in progression, as oxidative processes in (lipid containing) membranes can lead to cell necrosis, apoptosis and induce fibrogenic responses.

Fatty acids are a source of energy through ß-oxidation. Increased ß-oxidation goes along with higher mitochondrial respiratory rates in obese without NASH, whereas patients with NASH presented with lower respiratory rates despite higher mitochondrial mass. (6)

Circulating harmful cytokines in NAFLD include TNFα that induces apoptosis and death of hepatocytes. IL-1, IL-6, IL-10 and IL-11 can for example contribute to metabolic dysfunction, impair antimicrobial defencce, and drive inflammation and fibrogenesis. (7) They have the potential, like damage-associated molecular pattern (DAMPs, stress signals) such as ATP, to induce T-effector-cell responses.

In the sterile inflammation of NAFLD, antigen-presenting cells (APCs) induce T-cell mediated destruction of hepatocytes. Moreover, anti-inflammatory APCs like specific dendritic cells (DCs) are low. Antibodies from adipose tissue may also trigger the adaptive immune system. Such antibodies bind to epitopes expressed by oxidative stress or TNF (activating also B-cells). These antibodies may correlate to NAFLD by a direct damaging effect not yet known in detail. Furthermore, metabolites like SCFA and certain bile acid derivatives affect the differentiation of T-cells.(7)

Fibrosis starts as a healing response in reaction to liver damage and after chronic inflammation. Platelets and neutrophils are quickly available and release factors that induce fibrogenesis. Kupffer cells are liver resident macrophages in the sinusoidal lumen that eliminate dead cells and pathogens, but like local lymphocytes contribute to the progression of hepatocellular damage in a paracrine way. Their pathological state, in which they release pro-inflammatory mediators, keeps sustained through the influence of damage associated molecular patterns (DAMPs). Natural killer cells can either exacerbate the situation by releasing profibrotic and proinflammatory molecules or induce cell death and be cytotoxic to hepatic stellate cells (HSC), depending on the stimulus. Stressors like inflammation, hepatic cell damage, liver sinusoidal endothelial cells (LSEC) fenestration-loss, activated KCs, activate hepatic stellate cells that can turn into myofibroblasts. They produce extracellular matrix, which contains collagen and other proteins that primarily are part of the acute regeneration process. In chronic liver injury, accumulation of these abnormal components contribute to failed tissue repair. Consecutively, the liver damage progresses by higher portal vein resistance that limits the blood supply of hepatocytes. (6)

When there is no regression, an escape out of the imbalanced, self-aggravating metabolic state gets harder and the morphological presentations slowly develop into the direction of cirrhosis. Fibrotic depletions get thicker, the tissue gets necrotic and, in the end, cirrhosis even becomes visible in macroscopic dimensions as the organ shrinks and hardens. (6)

2.1 Dietary factors

The often referred to as a “western” diet can be considered as a central starting point in the development of a metabolic imbalance. A big problem is the tendency to have a hypercaloric, carbohydrate weighted diet with high amount of industrially processed food, in worst case with unnecessary additives or lacking in certain nutrients (vitamins, minerals, with antioxidative properties, or important trace elements like zinc and copper). A diet deficient

in choline, present in western population, or dysbiosis related excessive choline degradation are leading to lower lipid transport away from the liver. Considering fat intake, it is important to estimate the type and quality of ingested fatty acids. Especially saturated FA are thought to have disease-promoting capacities. Therefore, a recommendable diet, that could be appropriate for prevention of NAFLD, may be the Mediterranean diet (MED), fulfilling the needs of being rich in unsaturated FA, micronutrients and unprocessed ingredients. Improvement of metabolic parameters and weight loss have been associated to the MED. (7,8)

Furthermore, microbiota adapt to dietary composition and as bacteria have their distinct way metabolizing available components, which can lead to release of disease driving end products. (8)

2.2 The problem with fructose

Nowadays fructose is omnipresent in industrially processed food as a favourable ingredient for mass production: It is cheap, provides long-lasting hydration and shelf life and has greater sweetening power than glucose.

It is metabolized mostly in the liver and the uptake takes place insulin-independent mainly by active transport through GLUT5 on the luminal intestinal side, but further makes its way from the intestine to portal circulation also passively through GLUT2. In patients with a high caloric diet, GLUT5 expression increases.

The conversion into glucose or lactate normally is predominant. With acute fructose overload, lipogenesis quantitatively becomes more important and long-term disruption of regulatory mechanisms of de-novo lipogenesis (DNL) may intensify the problem.

High amounts of fructose lead to a strong induction of GLUT5 and are phosphorylated by fructokinase (Ketohexokinase, KHK) to fructose-1-phosphate (F1P). The expression of both of its isoforms increases, especially KHK-C with a higher affinity to fructose, depletes ATP and leads to an accumulation of AMP, which can turn into uric acid (UA). An overload of UA promotes systemic damages and inflammation. The inflammatory potential is explained with NFκB- activation and through greater hepatic NADPH-oxidase activity, it acts as a DAMP. The inhibition of KHK in humans presented an improvement in different stages of NAFLD and KHK-knockout mice showed a reduced activity of enzymes that link sugar and lipid metabolism, that contribute to increased fatty acid synthesis. (9)

A study comparing mice on a high fat diet (HFD) showed that both glucose and fructose influence hepatic steatosis. In mice fed a HFD in combination with fructose a significant increase in obesity, lower glucose-tolerance and impaired insulin signalling was observed, when compared to a HFD with glucose. The increase in total hepatic fat accumulation was similar, but triglyceride synthesis was especially increased by glucose and fatty acid synthesis and its intermediates like acyl-CoA by fructose.

While glucose induced higher total ChREBP (carbohydrate response element-binding protein) and ChREBP-β, fructose also affected ChREBP-β, but showed notably higher expression of transcription factor SREBP1c. Knockout-studies showed both had lipogenic effects (probably due to regulatory-gene overlap), but SREBP1c, regulating fatty acid synthesis and intracellular storage, was found to be higher in fatty liver disease related to insulin resistance. (10) Increased de-novo lipogenesis and insulin-independent glycolysis are both regulated by ChREBP.

In the large and small intestine, long term HFD in mice caused damage by reduction of proteins, that are involved in the junction of the epithelial cells. The source may be mucosal inflammation, induced by ER-stress due to F1P associated impairment of N-glycosylation. (11)

2.3 Insulin resistance and hyperinsulinemia

In insulin resistance (IR), an alteration in the insulin-signalling cascade leads to insufficient glucose uptake from the blood into fat tissue and skeletal muscle (via GLUT4). Higher blood glucose levels require increased production of insulin.

Short-term hyperinsulinemia induces an endoplasmic reticulum response protecting from cellular stress. This mechanism decreases in chronic burden of high insulin. Persistent elevated insulin levels affect the physiological structural change of its receptor through autophosphorylation after binding to it. IR in different tissues depends on accumulation of fat in the liver. With just 1.6% of intrahepatic triglyceride content (IHTG), hepatic IR showed to reach a steady state. In skeletal muscle the steady state of IR was reached, when IHTG was above 6%.

With constant energy surplus, the adipose tissues storing capacities are overwhelmed and more FA appear in systemic circulation. Insulin resistance further worsens the situation because insulin does not adequately suppress hormone-sensitive lipase (HSL) activity in

adipose tissue, an enzyme that enhances lipolysis and gluconeogenesis in the liver. Notably, only some parts of insulin signalling are dysfunctional, whereas lipogenesis stays unaffected. Hyperinsulinemia and high blood glucose levels actually upregulate transcription factor expression (SREBP-1c and PPAR-γ) of lipogenic enzymes and in NAFLD patients with high insulin levels, fasted state de-novo lipogenesis is about 3x higher than normal and does not show the physiological postprandial increase. Due to high glucose availability in the blood in IR, Glycerol-3-phosphate, necessary for reutilization of FAs in TG synthesis, decreases. (12) As lipids are diverse in their structure and functions, they have also shown a different potency in inducing IR. TG seem to be less potent in inducing IR compared to saturated FA, diacylglycerol and ceramides.

Inflammatory cytokines impair insulin signalling as they can prevent activation of insulin receptor substrate (IRS) over NF-κB, which is high in patients with NASH. Insulin resistance per se also provokes the release of cytokines and therefore inflammation. The anti-apoptotic, proliferative and activating effect of insulin on HSC contributes to progress of NAFLD/ fibrogenesis. (6,12,13)

Despite pathological insulin signalling, the mTORC1 activation, leading to impaired autophagy, may not be altered in NAFLD. (14)

2.4 Microbiome

Even if the influence of the intestinal microbiome in several (chronic) diseases is still under investigation as an essential factor in development and progression of diseases, it has increasingly become of interest during the last years. Besides a general decrease of diversity of the microbiome in NAFLD, constellations of certain species may associate with different stages of this hepatic disease, and thus may gain diagnostic and/or therapeutic impact in the future.

When the intestinal barrier with its both mechanical and immunological function is impaired, Pathogen-Associated Molecular Patterns (PAMPs) can reach the liver through the portal vein blood. They can be recognized by toll like receptors (TLR) and activate the innate immune system. Activation of TLR-4 on macrophages in the liver subsequently increases expression of pro-inflammatory cytokines like IL-6 and IL-1β or TNFα. For example, Lipopolysaccharides (LPS), associated to the cell membrane of gram-negative bacteria, act in this way.

Microbial metabolites can also enter systemic circulation over a dysfunctional gut barrier. They reach areas that are inaccessible under normal circumstances and have diverse impact like Imidazole propionate that is affecting the insulin signalling SCFAs (to which count butyrate, acetate and propionate). Such metabolites can contribute to NAFLD as promotors of fat accumulation, and have immunomodulatory effects on certain subsets of T-cells. Specifically, in NASH, the bacterial product endotoxin, inducing cytokine release by affecting the Toll-like and NOD- like signalling, was found increased.

Endogenous ethanol produced by bacteria and fungi is likely to be another important metabolite contributing to NAFLD. Though, as well as for N,N,N-trimethyl-5-aminovaleric (TMAVA) acid and phenylacetate, there are more studies needed to confirm and describe the negative effects on hepatic steatosis in human in detail.

Microbes may not be the only source of endogenous alcohol. In IR the alcohol dehydrogenase function may also be impaired and therefore (fasting plasma) alcohol levels be higher in NAFLD patients.

Dysbiosis in obese people has shown to lead to higher choline degradation and in consequence a lower capacity of lipid removal from the liver via VLDL. (6,7,15)

2.5 Genetic factors

The influence of genetics could explain ethnical differences in prevalence. Variations of PNPLA3 are not only associated with a higher hepatic fat content, but also with a greater risk for HCC and progression in NAFLD and other liver diseases, even independently from other factors like high lipid concentration and insulin resistance. (16,17) Alterations in other genes regulating the lipid- and glucose metabolism like MBOAT7 or GCKR, in genes that influence immunological response or mitochondrial metabolism also contribute to the course of this disease. (18) It is hypothesised, that the genetic variation of TM6SF2 could determine, whether cardiovascular disease and dyslipidaemia or progression of hepatic dysfunction, NASH, fibrosis, cirrhosis are predominant. (6)

2.6 Environmental factors

To highlight the non-neglectable impact of pollution of eco-systems on our health, in the following we will give an example relevant for NAFLD and other metabolic diseases, as it is hepatotoxic and can disrupt hormonal and immunological processes.

Perfluorinated alkyl substances (PFAS), industrially produced organic compounds, used as impregnating agents, in production of polytetrafluoroethylene (PTFE), which is known be used in non-stick coated cooking wear or water-repellent clothing among many other applications. They do not occur naturally in the environment, tend to be persisting and accumulating in groundwater, soil and in animal- and human tissue. (19)

PFAS could drive hepatic steatosis by increased lipogenesis and lipid flow to and decreased lipid flow away from the liver. Furthermore, PFAS interact with lipid metabolism over certain receptors (e.g. peroxisome proliferator-activated receptors (PPARs), pregnane X receptor (PXR)), and impact also bile acid synthesis and enterohepatic circulation. (20)

3 Pathway to diagnosis

Detection of NAFLD is frequently only in late stages, as it often stays asymptomatic for a long time and can occur without increase of liver transaminases. The typical symptoms of liver dysfunction normally appear in more advanced stages.

Various causes of hepatic steatosis like excessive alcohol consumption, viral hepatitis, hereditary liver dysfunction or dyslipidemias, drugs or toxins must be excluded before continuing with diagnostical and therapeutical procedures.

Presence of certain risk factors like overweight, T2DM, metabolic syndrome or arterial hypertension can justify screening and can be done in primary care by transabdominal ultrasound to detect of high grades of steatosis, but it is not possible to exclude presence of HS or differentiate between NAFL or NASH. An alteration of blood markers like the liver enzymes (ALT, AST, GGT, AP) also indicates problems in the hepatobiliary tract. In combination with other values, there are some common scores used for risk stratification in NAFLD. A high FLI (fatty liver index) can indicate liver steatosis, if ultrasound is not available. When FIB-4 score or NFS (NAFLD Fibrosis score) are high, or serum ALT levels are repeatedly increased, transfer to a specialist and further diagnostic clarification is necessary. In primary diagnosis, FIB-4, NFS and elastography serve to evaluate fibrosis stage. Measuring liver stiffness is used for exclusion of advanced fibrosis and in screening. It can be performed ultrasound or MRT-based and with Controlled Attenuation Parameter (CAP), a software that is used with vibration-controlled transient elastography (VCTE) extent of steatosis can be estimated. (2)

3.1 Diagnosis

Liver biopsy is still the gold standard for diagnosing NAFLD and is superior to non-invasive methods in detecting early stages of fibrosis. Furthermore, it is necessary for an exact staging and a definite distinction between NAFL and NASH. (2)

3.2 Biopsy results

Steatosis

The accumulation of lipids in hepatocytes, called steatosis, is considered pathological when it exceeds 5% of total liver tissue and is required for NAFL, that is further subdivided into three stages (≥5-33%, 33-66%, >66%).

A mixed pattern of micro- and macrovesicular steatosis is present in NAFLD, and it is unlikely to be just metabolic induced steatosis, if the pattern is microvesicular.

NASH

For histological definition of NASH, signs of hepatic damage in form of hepatocyte ballooning (that likely comes from oxidative stress damaged microtubule and cytoskeleton intermediate filament loss) and an infiltrate of inflammatory cells is required. Additionally, the biopsies can contain hepatocytes with Mallory-Denk bodies or glycogenated nuclei and megamitochondria.

Fibrosis

Fibrosis begins perisinusoidal, but with progression builds septa, also referred as “bridging”, and forms pseudo-lobule. The most severe stage of fibrosis is cirrhosis, which presents in a micronodular pattern in NAFLD. With decreasing steatosis levels and increasing cirrhosis, characteristics of NASH can vanish. The underlying metabolic origin in advanced stages of liver disease is even thought to be underdiagnosed as distinguishing between other causes for similar fibrosis patterns becomes more difficult. In histological results, fibrosis is divided into four stages (0 = no fibrosis, 4 = cirrhosis). (6,21)

3.3 MAFLD vs. NAFLD

In the last years, the term „Metabolic dysfunction-associated fatty liver disease“ has been considered to take the place of „non-alcoholic fatty liver disease“ as the latter may be inferior in some points. Compared to NAFLD diagnosis, a higher prevalence could be reached, applying MAFLD criteria. They have also shown to be superior in terms of identifying

individuals with higher risk and including the association to other diseases and extra-hepatic mortality. For example, MAFLD, but not NAFLD associates with asymptomatic arteriosclerosis indicating the necessity of assessment and intervention of other diseases when MAFLD diagnostic criteria are fulfilled. Another favourable aspect is, that MAFLD does not exclude alcohol as a factor of hepatic steatosis. Alcohol consumption is just a vague anamnestic parameter as it depends on the accuracy of the patients report. Furthermore, endogenous alcohol is a considerable player in the development of fatty liver. In preexisting NAFLD, alcohol consumption even below the maximum tolerable alcohol consumption per day is thought to be worsening the disease.

Apart from required steatosis ≥ 5% like in NAFLD, for diagnosis of MAFLD metabolic risk determinants (≥2/7: high waist circumference, blood pressure, plasma triglycerides, CRP, glucose levels, low HDL-cholesterol, insulin-resistance-score) are required. The diagnosis of MAFLD has been shown to be superior in identifying patients who are most at risk for clinical disease progression compared to NAFLD. (22)

4 Alcoholic fatty liver disease

Like NAFLD, pathophysiology in AFLD is also influenced by multiple factors, but the focus is on alcohol and its metabolization. Further contributors to the disease are genetics, gender and hormonal imbalances. Dietary composition, respectively malnutrition is another important factor, as in heavy drinkers, up to 50% of calorie intake comes from alcohol and diet is often lacking in unsaturated fatty acids, certain amino acids and micronutrients. Alcohol induced hepatic steatosis is found in about 90% of heavy drinkers, progression to steatohepatitis in about 20- 40% and cirrhosis in 15-20%.

The diagnosis of AFLD requires long term heavy alcohol consumption (for ASH at least until 3 weeks of the onset of the symptoms), assessment of potential other inducers of steatosis, infections and blood markers (bilirubin, ALT, ATL/AST ratio), sonographic abnormalities and indication of liver biopsy. For stratification of severity and as an orientation for therapy, scoring systems can be used. To differentiate between AFLD and NAFLD, the threshold of daily alcohol consumption is 10g in women and 20g in men, but individual susceptibility to hepatotoxic effects of alcohol needs to be considered.

Alcohol dehydrogenase (ADH) oxidizes ethanol to acetaldehyde and the enzyme in the following step, ALDH, cannot cope with the overload of acetaldehyde going along with high

alcohol consumption. In consequence leading to a higher burden of ROS, disruption of tight junctions in the gut and damage of hepatocytes. In hepatocytes, lipogenic enzymes (e.g. SREBP1c) and production of fatty acids are increased, ß-oxidation and VLDL export decreased leading to higher TG production in the liver. In the gastrointestinal tract, ethanol can directly be harming the mucosa and the motility. Mitochondrial dysfunction, ER-stress, increased gut permeability, endotoxemia and the correlated release of pro-inflammatory cytokines and activated local immune response and T- and B-cell immigration are like in NAFLD central in pathogenesis. (2,6,23)

5 Bile Acids

The synthesis of bile acids (BA) utilizes cholesterol and starts in hepatocytes following two main pathways: In the classical, side-chain cleavage follows sterol ring modification and it is reverse in the alternative way. Sufficient functionality of these pathways prevents a build-up of cholesterol in the liver. Among mammals, all BA have a C_{24}- construct, but their structures vary between different species. The circulating BA-pool is a mixture of primary and secondary BA. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are human primary BA and get conjugated with the amino acids glycine or taurine by bile acid–CoA synthase (BACS) and bile acid–CoA:amino acid N-acetyltransferase (BAAT) in the liver cells. The conjugated primary BA, glycocholic acid (GCA), taurocholic acid (TCA), glycochenodeoxycholic acid (GCDCA), and taurochenodeoxycholic acid (TCDCA), show an increase of solubility in physiological pH, ionisation and a less passive absorption.

After storage in the gall bladder, ingested fats signal production of cholecystokinin (CCK) that induces the release of BA into the bowel, where bile salt hydrolase (BSH) in bacteria deconjugates them. In the distal intestine, bacterial 7α-dehydroxylase transforms the unabsorbed fraction of primary BA to the secondary BA, deoxycholic acid (DCA) and lithocholic acid (LCA). LCA is also the product of 7β-dehydroxylation from ursodeoxycholic acid (UDCA).

Bile acids are recycled efficiently (95%) by reabsorption through the brush border membrane in the terminal ileum. Ileal bile acid binding protein (IBABP) transports them through the enterocytes, from where organic solute transporter (OSTα/β) lets them pass back over portal vein blood and liver sinusoids to the hepatocytes. Small amounts can be reabsorbed by the

renal tubes, if they had passed to the systemic circulation, or even by cholangiocytes. Fecal excretion is responsible for some loss of BA, which is balanced by de-novo-synthesis.

Apart from their direct role in lipid emulsification and solubilization BA work as nuclear receptor ligands on farnesoid X receptor (FXR), vitamin D receptor (VDR) and pregnane X receptor (PXR), but also on membranous receptors like the Takeda G-protein-receptor-5 (TGR5). Bile acids have different affinities to the BA receptors and a distinct potential of being beneficial or harmful. Therefore, a change in their concentrations can alter the signalling cascade and disturb the physiological equilibrium. In the following, some of their functions that may be influenced in metabolic diseases are described. (24,25)

FXR is involved in regulating BA synthesis, secretion and distribution. DNL and hepatic VLDL secretion are supressed by activation of FXR. Furthermore, the intestinal absorption of cholesterol is reduced due to the FXR mediated change in BA pool towards a higher hydrophilicity. When BA activate ileal FXR, fibroblast growth factor 19 (FGF19) gets released and leads to suppression of rate limiting enzyme in the classical pathway (CYP7A1). It also induces conjugation through BACS and BAAT, regulates enterohepatic BA circulation through activating bile salt export pump (BSEP), IBABP and OSTα/β and lowers expression of NTCP and ASBT. Studies suggest, that FXR regulates inflammation through FGF19 and by inhibiting NF-κB, suppresses migration of monocytes and macrophages and even work against fibrosis over HSC – gene expression. Generally, FXR activation is thought to improve metabolic syndrome by lowering blood glucose, improving IR and decreasing levels of FFA.

Takeda G protein-coupled receptor 5 (TGR5) is present in cells that are part of the biliary tract, supporting bile flow, bile bladder filling and blood flow. In intestinal enteroendocrine cells like GLP-1, activation of TGR5 induces incretine secretion and therefore effects like insulin secretion, suppression of gastric emptying, inhibition of glucagon and regulate satiety. Its anti-inflammatory properties are supported by its activation of predominantly M1-activated Kupffer cells, decreasing in NF-κB signalling and inhibition of ROS production.

Bile acid mediated PXR signalling has been linked to induction of drug metabolism and lipogenesis. Synthesis of bile acids, breakdown of glycogen, gluconeogenesis and β-oxidation showed to be decreased due to PXR signalling.

Activation of the VDR triggers expression of an enzyme (CYP3A), which metabolizes toxic LCA, prevents intestinal barrier degradation and LCA re-entry into enterohepatic circulation. (25,26)

There also is an interplay between the bile acid pool and microbiota. A changing presence in certain species in NAFLD goes along with a variation in bacterial enzymes and therefore also a change in conjugation and conversion of BA. On the other side, the physiological detergent function of BA can explain why they are capable of destabilizing bacterial membranes. (26)

With an excess of bile acids, reactive oxygen species (ROS) are produced and exceed the antioxidative capacities of glutathione. Furthermore, BA overloads can lead to ER-stress, general cellular stress accompanied by cytokine release all leading to stellate cell activation and hepatocyte damage. Accumulation of pro-inflammatory bile acids can drive liver damage. It is thought that the inflammation may impair the beneficial effect of some BA, that for example work as FXR agonists, if they are found in physiological concentrations. In NAFLD patients, studies found, that expression of FXR and signalling were decreased. (24,27,28)

6 Metabolomics

For early diagnosis and better monitoring of disease progression and efficacy of treatment, research is aiming to find an alternative to histological evaluation and less invasive possibilities than performing a biopsy to identify NAFLD in its earliest possible stages.

Markers are usually found in alterations in pathways involved into pathophysiology of the disease. Though, right now, there are not many available for measurement in routine as their specificity and sensitivity may not be high enough and cost-benefit analysis may not be worth it yet. (29)

In the medical field, metabolomics can give an insight in the alterations of metabolic pathways, helping to understand pathophysiology of diseases by measuring metabolites that are part of biological processes in the body. As samples, body fluids and secretes, urine, stool and tissue are used and go through different preparation processes before they are measured in an instrument like a mass spectrometer. Measurement can be untargeted, producing big amounts of data, complicating quantification and the overall workup, but

enabling the search for unknown components. With a targeted method, concentrations of a selection of molecules can be measured. There are numerous endogenous, but also exogenous substances like amino acids, lipids, vitamins, co-factors and drugs or toxins, that could be measured. Having a healthy control group enables to define potentially disease related changes in samples of patients and can open new pathophysiological aspects of a disease and could also serve to develop markers for different extents of disease. (30,31)

7 Material and Methods

7.1 Study groups

In total, we analysed the samples of 205 patients. The 45 (28 male, 17 female) NAFLD patients from the hepatology department of Medical University of Graz were our base, to which we got matched 103 (51 male, 52 female) healthy controls from the Paracelsus-1000 cohort from Paracelsus Medical University of Salzburg and 57 (45 male, 12 female) patients from the ALD-Detox cohort from Medical University of Graz. Cases with severe fibrosis were excluded the ALD-Samples.

7.2 Laboratory work

After introducing internal standards (d4-DCA, d4-LCA, d4-GLCA, d4-GCDCA, and d4-TDCA, each at a concentration of 0.2 nmol), samples (10 µl) were vigorously mixed for 1 minute. To facilitate removal of proteins, 400 µl of acetonitrile were added. The mixture was vigorously mixed again, and then subjected to centrifugation at 3200g for 12 minutes at room temperature. The supernatant was then carefully removed and subsequently evaporated under a flow of nitrogen. The dried samples were reconstituted using 100 µl of mobile phase B and subsequently transferred into vials suitable for autosampling.

7.3 Mass spectrometry analyses

Bile acids were analysed by liquid chromatography-high resolution-mass spectrometry (LC-HR-MS). Chromatography of 10 uL of each sample was performed using a Nucleoshell C18 reversed phase column (Macherey-Nagel, Düren, Germany) for human bile acids. Separation was performed using aqua dest with 1.2% v/v formic acid and 0.38% w/v ammonium acetate, and elution was carried out using acetonitrile with 1.3% v/v formic acid and 0.38% ammonium acetate. Analysis was performed on Triple Quadrupole mass spectrometer 6500

(Sciex) with an ESI ion source in negative ionisation mode. The limit of quantitation of the mass spectrometer was 0.001 µmol /L for all bile acid species. Any value below this threshold was not quantitated and thus excluded from statistical analyses.

7.4 Statistics

The categorical data was summarized as relative and absolute frequencies. Metric data were presented as median and interquartile range (IQR). Percentages, that are reported, belong to the amount of non-missing answers. Mann-Whitney U or t tests were used to do comparisons between NAFLD, ALD and controls and Bonferroni correction was used for adjustment for multiple testing. Spearman's correlation coefficient was used to determine the correlations. Statistical significance was set by a two-sided p-value of 0.05. R version 4.3.1. was used to conduct all statistical analyses.

7.5 Limitations

The number of patients was quite low, especially after separation into female and male. Female vs. Male were not statistically analysed. There were only some trends visible, but the number of individuals was too little to compare both genders. Furthermore, circadian rhythms were not considered as there were no samples for a comparison between postprandial and the fasted state.

8 Results

8.1 Patient characteristics

The percentage of male patients was higher in the NAFLD group (62%, 28/45) and ALD group (79%, 45/57). The NAFLD group presented with the highest BMI, followed by the ALD, both were significantly higher (NAFLD vs. controls $p<0.0001$; ALD vs. controls $p<0.0001$) than controls. The m BMI was above the limit of obesity (BMI >30) in female NAFLD. Median levels of fasting glucose were below 100mg/dl in alcoholic and control group and significantly higher in NAFLD (NAFLD vs. controls $p<0.0001$; ALD vs. controls $p<0.0001$). Female NAFLD had higher median fasting glucose levels than male. The liver transaminases, AST and ALT, were significantly higher in NAFLD ($p <0.0001$) than in ALD and controls. GGT was significantly elevated in NALFD ($p <0.0001$) and ALD ($p <0.0001$) compared to controls. In male ALD, GGT reached higher values than in females.

Total cholesterol and LDL showed no relevant differences between the groups. HDL in NAFLD ($p<0.001$) and ALD ($p<0.001$) was significantly lower compared to the controls, with the lowest values in NAFLD. Triglyceride concentrations were inverse to the HDL levels with the highest values in the NAFLD group.

Alkaline phosphatase was elevated in NAFLD compared to controls ($p<0.001$). AP values were outside the normal AP range (30–104 U/L) only in female NAFLD and are significantly higher than ALD ($p=0.01$). Mean cholinesterase (CHE) levels were highest in NAFLD, but differences were more significant in male NAFLD (vs. controls: $p=0.004$, vs. ALD: $p=0.009$). Creatinine was highest in male NAFLD (vs. controls: $p=0.061$, vs. ALD: $p=0.002$). Urea nitrogen presented significant differences ($p<0.001$) between all groups in male patients. In NAFLD, Urea nitrogen was strongest elevated; the values of the controls were in the middle and the lowest values in ALD. Uric acid was significantly higher in NAFLD ($p<0.001$) than in controls in both genders. The highest values were found in NAFLD, the lowest in the control group and were higher in male than female. The platelet count was low in male NAFLD ($p<0.001$) compared to ALD and controls. C-reactive protein levels in NALFD ($p<0.001$) and ALD ($p<0.001$) were significantly higher than the controls. Females had highest CRP values, where mean values for CRP in NAFLD were higher than in ALD. Differences between NAFLD and ALD were only significant in male patients ($p<0.0001$).

Patient characteristics for females

Characteristic	**NAFLD**, N = 17[1]	**alcoholic**, N = 12[1]	**controls**, N = 52[1]	**NAFLD vs. alcoholic**	**NAFLD vs. controls**	**alcoholic vs. controls**
Age	53 (13)	48 (8)	55 (8)	0.703	>0.999	0.067
BMI	32.7 (27.3, 38.4)	28.3 (22.4, 31.7)	21.6 (20.5, 23.1)	0.252	<0.001	0.006
Waist (cm)	111 (100, 118)	NA (NA, NA)	78 (73, 82)		<0.001	
AST (U/l)	50 (38, 76)	26 (23, 37)	21 (18, 23)	0.016	<0.001	0.004
ALT (U/l)	71 (33, 95)	24 (20, 28)	17 (14, 20)	0.002	<0.001	0.003
GGT (U/l)	122 (72, 195)	55 (37, 77)	16 (11, 20)	0.033	<0.001	<0.001
Cholinesterase (U/l)	8,984 (6,936, 9,624)	5,959 (5,037, 7,320)	7,500 (6,588, 8,636)	0.042	0.288	0.074
Alkaline phosphatase (U/l)	111 (78, 121)	69 (53, 81)	60 (50, 70)	0.030	<0.001	0.609
Total cholesterin (mg/dl)	217 (190, 254)	209 (197, 230)	210 (189, 228)	>0.999	>0.999	>0.999
HDL (mg/dl)	45 (39, 53)	60 (48, 74)	84 (69, 93)	0.257	<0.001	0.016
LDL (mg/dl)	136 (97, 150)	128 (100, 151)	135 (106, 152)	>0.999	>0.999	>0.999
TG (mg/dl)	122 (100, 170)	102 (60, 123)	32 (28, 40)	0.219	<0.001	<0.001
Fasting Glucose (mg/dl)	110 (95, 143)	89 (85, 91)	87 (84, 90)	0.004	<0.001	>0.999
Creatinine (mg/dl)	0.78 (0.71, 0.91)	0.73 (0.60, 0.87)	0.75 (0.69, 0.81)	0.920	0.397	>0.999
Urea nitrogen (mg/dl)	31 (26, 36)	26 (18, 30)	28 (24, 32)	0.219	0.321	0.666
Uric acid (mg/dl)	5.50 (4.80, 6.00)	4.40 (3.68, 5.10)	3.98 (3.61, 4.50)	0.106	<0.001	0.906
Platelet count/ Thrombocytes (G/l)	222 (191, 272)	240 (208, 286)	253 (230, 290)	>0.999	0.198	>0.999
C-reactive protein (mg/dl)	4.8 (2.1, 8.0)	3.8 (1.6, 9.5)	0.1 (0.1, 0.1)	>0.999	<0.001	<0.001

[1] Mean (SD); Median (IQR)

Table 1: Patient characteristics for females

Patient characteristics for males

Characteristic	**NAFLD**, N = 28[1]	**alcoholic**, N = 45[1]	**controls**, N = 51[1]	**NAFLD vs. alcoholic**	**NAFLD vs. controls**	**alcoholic vs. controls**
Age	50 (14)	48 (9)	53 (7)	>0.999	>0.999	0.020
BMI	28.9 (25.9, 30.9)	26.2 (24.2, 29.4)	22.7 (21.8, 24.0)	0.073	<0.001	<0.001
Waist (cm)	103 (99, 110)	NA (NA, NA)	86 (82, 91)		<0.001	
AST (U/l)	52 (41, 72)	31 (24, 50)	23 (20, 27)	0.003	<0.001	<0.001
ALT (U/l)	82 (57, 143)	37 (25, 58)	20 (18, 23)	<0.001	<0.001	<0.001
GGT (U/l)	115 (77, 282)	121 (47, 292)	20 (16, 25)	>0.999	<0.001	<0.001
Cholinesterase (U/l)	8,335 (7,690, 9,596)	7,629 (6,374, 8,272)	7,398 (6,602, 8,253)	0.028	0.011	>0.999
Alkaline phosphatase (U/l)	70 (62, 81)	67 (60, 87)	54 (47, 68)	>0.999	<0.001	<0.001
Total cholesterin (mg/dl)	186 (175, 222)	188 (150, 214)	193 (173, 218)	>0.999	>0.999	>0.999
HDL (mg/dl)	40 (33, 46)	45 (34, 56)	64 (59, 73)	0.661	<0.001	<0.001
LDL (mg/dl)	112 (85, 142)	116 (95, 140)	130 (113, 151)	>0.999	0.145	0.152
TG (mg/dl)	139 (99, 178)	97 (75, 133)	68 (50, 80)	0.054	<0.001	<0.001
Fasting Glucose (mg/dl)	99 (92, 113)	88 (83, 94)	89 (84, 94)	0.001	<0.001	>0.999
Creatinin (mg/dl)	0.99 (0.91, 1.04)	0.83 (0.76, 0.96)	0.91 (0.82, 1.01)	0.007	0.184	0.071
Urea nitrogen (mg/dl)	33 (30, 40)	22 (18, 28)	29 (25, 33)	<0.001	0.004	<0.001
Uric acid (mg/dl)	6.40 (5.33, 7.30)	5.60 (4.90, 6.30)	5.22 (4.58, 5.77)	0.193	0.003	0.126
Platelet count/ Thrombocytes (G/l)	198 (152, 225)	257 (214, 283)	262 (223, 282)	<0.001	<0.001	>0.999
C-reactive protein (mg/dl)	1.2 (0.7, 3.2)	2.6 (1.3, 4.4)	0.1 (0.0, 0.1)	0.024	<0.001	<0.001

[1] Mean (SD); Median (IQR)

Table 2: Patient characteristics for males

8.2 Metabolic Syndrome

Diagnostic criteria of metabolic syndrome

Parameter	Threshold
Increased waist circumference	Definition specific for certain populations and countries
Increased triglycerides (or medical treatment of hypertriglyceridemia)	≥150 mg/dL (1.7 mmol/L)
Reduced HDL-C (or medical treatment of low HDL-C)	<40 mg/dL (1.0 mmol/L) in men <50 mg/dL (1.3 mmol/L) in women
Elevated blood pressure	systolic ≥130 mmHg and/or diastolic ≥85 mmHg
Elevated fasting glucose (or medical treatment of hyperglycaemia)	≥100 mg/dL or prior diagnosis of type 2 Diabetes Mellitus. If value lies above 5.6 mmol/L or 100 mg/dL, an OGTT is highly recommended, but not necessary for the definition of metabolic syndrome

Table 3: Diagnostical criteria of metabolic syndrome

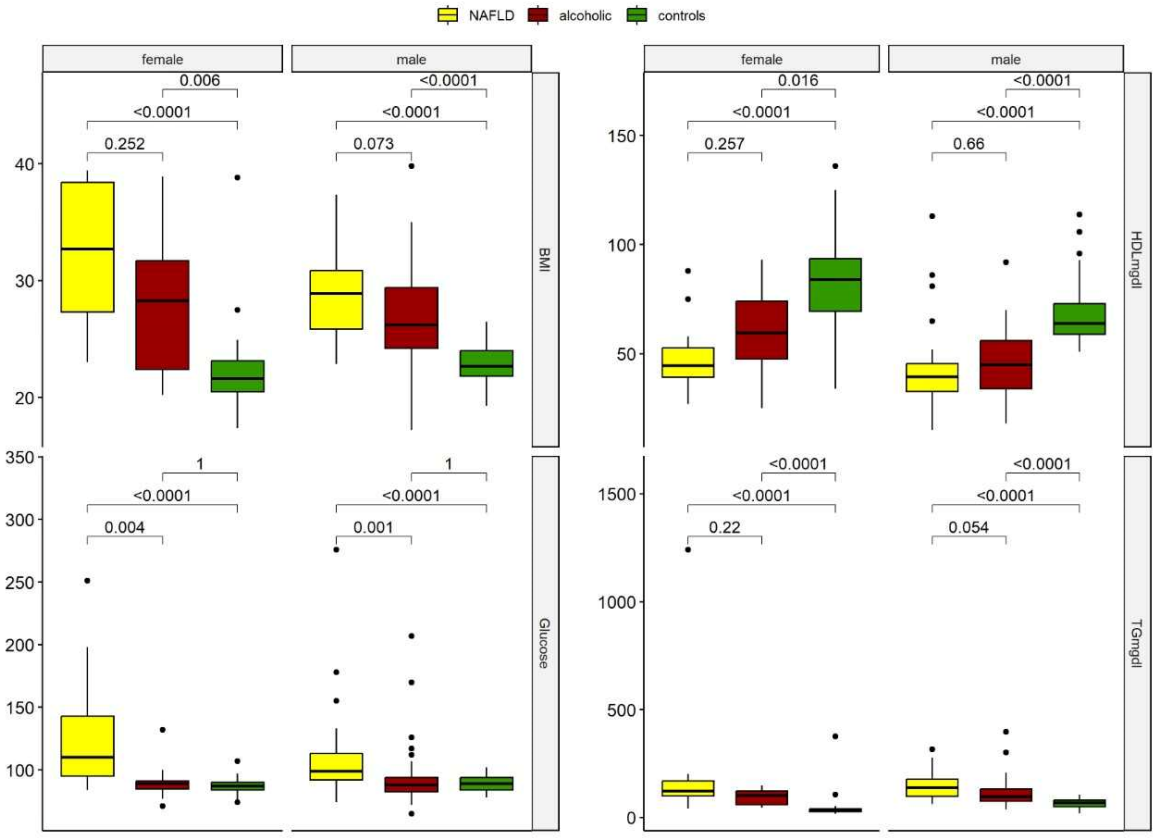

Figure 2: Diagnostical criteria of metabolic syndrome

The charts are supposed to visualize that the NAFLD cohort matches best out of all groups with the available variables that are included in the criteria for the clinical diagnosis of the metabolic syndrome. We did not receive any information about the blood pressure and as the waist circumference measurements were missing in the ALD patients, the BMI was included to show the degree of obesity.

8.3 Scores

Scores for non-invasive

detection of hepatic steatosis	FLI (Fatty liver index)	BMI, waist circumference, GGT, TG
risk evaluation for fibrosis	FiB_Score (NAFLD Fibrosis Score/NFS)	BMI, age, diabetes yes/no, AST, ALT, platelets, albumin
	Fib-4 Index (Fibrosis 4 Index)	AST, ALT, platelets, age
advanced fibrosis	ELF (Enhanced liver fibrosis)	type III prokollagen Peptide (PIIINP), hyaluronic acid (HA), tissue inhibitor of metalloproteinase-1 (TIMP1)

Table 4: Scores for non-invasive detection (2)

Fibrosis scores for females

Characteristic	**NAFLD**, N = 17[1]	**alcoholic**, N = 12[1]	**controls**, N = 52[1]	**NAFLD vs. alcoholic**	**NAFLD vs. controls**	**alcoholic vs. controls**
Fib-4 index	1.87 (1.06, 2.42)	1.13 (0.72, 1.43)	1.14 (0.92, 1.30)	0.264	0.048	>0.999
FLI score	93 (82, 98)	NA (NA, NA)	3 (2, 5)		<0.001	
ELF Score	9.24 (8.48, 10.37)	8.57 (8.25, 9.31)	NA (NA, NA)	0.438		
FiB_Score	-0.57 (-1.48, -0.09)	-2.23 (-2.31, -1.15)	-2.71 (-3.00, -2.06)	0.265	<0.001	0.327

[1] Median (IQR)

Table 5: Fibrosis scores for females

Fibrosis scores for males

Characteristic	**NAFLD**, N = 28[1]	**alcoholic**, N = 45[1]	**controls**, N = 51[1]	**NAFLD vs. alcoholic**	**NAFLD vs. controls**	**alcoholic vs. controls**
Fib-4 index	1.41 (0.89, 2.87)	1.02 (0.74, 1.36)	1.16 (0.93, 1.38)	0.136	0.264	0.823
FLI score	88 (77, 92)	NA (NA, NA)	15 (9, 20)		<0.001	
ELF Score	8.35 (8.20, 9.77)	9.45 (8.51, 10.03)	NA (NA, NA)	0.077		
FiB_Score	-1.11 (-2.63, 0.10)	-2.73 (-3.32, -1.94)	-2.16 (-3.20, -1.60)	0.044	0.098	>0.999

[1] Median (IQR)

Table 6: Fibrosis scores for males

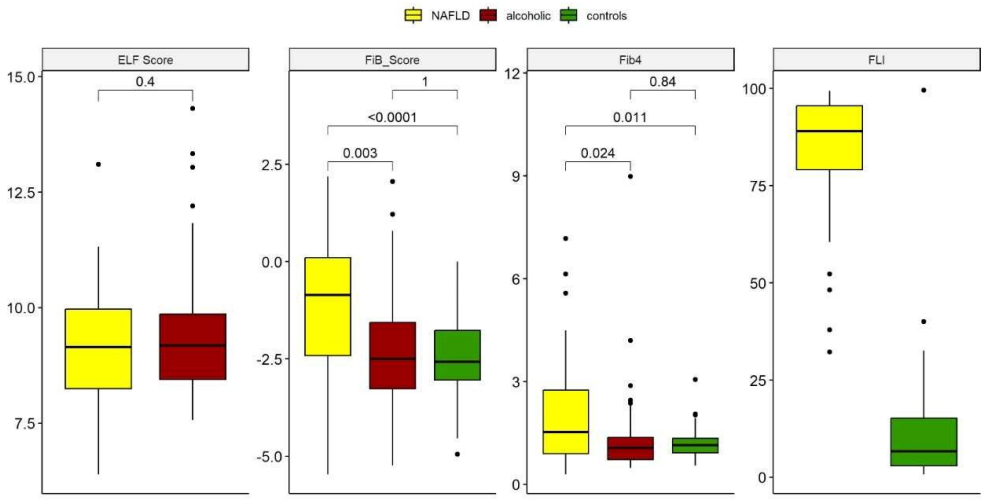

Figure 3: Fibrosis scores

8.4 Bile acids

Total bile acid concentrations were higher in NAFLD compared to the alcoholic (p=0.001) and to the control group (p=0.004). Total free/total BA were higher in male NAFLD (0.08 (0.02, 0.22)) than ALD (0.01 (0.00, 0.11), p<0.003) and lower than controls (0.16 (0.09, 0.24), p=0.027). Cholic acid (CA, free CA) only presented significantly lower (p<0.001) values in male NAFLD (0.04 (0.01, 0.12)) compared to controls (0.20 (0.17, 0.28)), whereas mean levels in female NAFLD (0.06 (0.02, 0.24)) were higher than controls (0.03 (0.02, 0.11)), but not significantly. Mean chenodeoxycholic acid (CDCA, free CDCA) was higher in NAFLD (p=0.047). In male controls (1.68 (0.88, 3.22)), free CA/free CDCA was highest (p<0.001) with no difference between NAFLD (0.18 (0.08, 0.47)) and ALD (0.17 (0.01, 1.00)). Total CA in female NAFLD (0.46 (0.21, 0.94)) exceeded female controls (0.16 (0.08, 0.33), p=0.009) and female ALD (0.07 (0.00, 0.21), p=0.025). Total CDCA was also highest in female NAFLD (0.07 (0.00, 0.21)), but only significantly compared to controls (0.46 (0.33, 0.77), p=0.011). Total primary BA (female NAFLD (1.62 (1.25, 3.45)) vs. controls (0.65 (0.45, 1.11), p=0.01) allowed the same observations. Total CA/total CDCA in male NAFLD (0.33 (0.24, 0.40)) was significantly lower than controls (0.64 (0.43, 0.86), p<0.001) and higher than in ALD (0.14 (0.08, 0.28), p<0.001).

Glycocholic acid (GCA) in NALFD (f: 0.19 (0.12, 0.68), m: 0.20 (0.09, 0.35)) was higher in both genders than in controls (f: 0.07 (0.04, 0.12), p<0.002, m: 0.08 (0.05, 0.16) p<0.003) and ALD (f: 0.07 (0.00, 0.17), p=0.075, m: 0.14 (0.05, 0.25), p=0.11). The ratio GCA/total BA was significantly higher in NAFLD (f: 0.10 (0.07, 0.13), m: 0.09 (0.06, 0.11)) than

controls (f: 0.06 (0.04, 0.09), p=0.045, m: 0.04 (0.03, 0.08), p=0.001), but not compared to ALD. TCA/total BA in male NAFLD (0.008 (0.004, 0.021) was higher than male ALD (0.001 (0.001, 0.005), p<0.001), but lower than controls (0.035 (0.010, 0.049), p=0.019). In female NAFLD, TCA/total BA (0.009 (0.005, 0.020)) was higher than in controls (0.005 (0.002, 0.015)), but not significant (p=0.654). Levels of glycine conjugated BA (p=0.021) and taurine conjugated BA (p=0.097) presented significantly higher values in female NAFLD (1.89 (1.04, 5.15)) compared to female controls (0.65 (0.40, 1.10)). Only in female NAFLD, glycochenodeoxycholic acid (GCDCA (0.84 (0.61, 2.27)) increased compared to female controls (GCDCA (0.25 (0.17, 0.44), p=0.016). GDCA was higher in male NAFLD (0.30 (0.24, 1.11)) than in controls (0.18 (0.10, 0.41), p =0.009) and ALD (0.08 (0.00, 0.23), p<0.0001). The ratio GDCA/DCA was also higher in male NAFLD (1.8 (1.0, 4.6)) vs. controls (0.7 (0.4, 1.5), p=0.003) and ALD (1.0 (0.5, 1.6), p=0.022). The ratio of TCA/TCDCA in female ALD (1.00 (0.49, 1.00) was higher than NAFLD (0.26 (0.21, 0.40), p=0.004) and controls (0.17 (0.09, 0.29), p<0.001). This ratio in male controls (1.08 (0.56, 1.58) exceeded the levels in NAFLD (0.30 (0.20, 0.40), p<0.001) and ALD (0.31 (0.03, 1.00), p<0.001), which were on a similar level. GCA/GCDCA in male NAFLD (0.37 (0.30, 0.60) was higher than in controls (0.24 (0.16, 0.32), p<0.001) and ALD (0.16 (0.10, 0.27), p<0.001).

Ratios of Glycine/taurine were lower in male NAFLD (10 (5, 15)) than ALD (18 (6, 62), p<0.025) and higher than controls (5 (3, 10), p=0.09). In male NAFLD, TCA/GCA (0.11 (0.07, 0.25) p<0.001), TCDCA/GCDCA (0.18 (0.11, 0.29), p=0.04) and TDCA/GDCA (0.06 (0.02, 0.11), p<0.001) were lower than male controls (TCA/GCA: 0.62 (0.20, 1.19), TCDCA/GCDCA: 0.13 (0.06, 0.17), TDCA/GDCA: 0.31 (0.12, 0.46)).

NAFLD presented with highest median values of non-12-a-OH, significantly higher than controls in female (p=0.031) and not significantly in male (p=0.085).

ALD

Total primary BA were not significantly elevated, but in male ALD, total primary/total BA (0.68 (0.53, 0.85)) was higher than in NAFLD (0.52 (0.47, 0.59) p=0.008) and controls (0.57 (0.50, 0.63), p=0.01). ALD showed lower total free primary BA (f: 0.00 (0.00, 0.11), m: 0.05 (0.00, 0.24)) levels than NAFLD (f: 0.33 (0.13, 0.58), p=0.006, m: 0.32 (0.18, 0.54), p<0.001) and controls (f: 0.21 (0.11, 0.33), p=0.001, m: 0.32 (0.24, 0.54), p<0.001). The ratio total free/total BA was lower in ALD (Significantly only in male ALD (0.04 (0.00,

0.10)) vs. NAFLD (0.13 (0.07, 0.20), p=0.003) and male ALD vs. controls (0.20 (0.14, 0.30), p<0.001).

Conjugated CA/conjugated CDCA was significantly lower in *male* ALD (0.15 (0.10, 0.28) compared to NAFLD (0.39 (0.29, 0.57), p<0.001) and controls (0.35 (0.25, 0.52), p<0.001. In ALD, both genders showed lowest taurine conjugation (*female*: ALD (0.01 (0.01, 0.24)) vs. NAFLD (0.17 (0.10, 0.36) p=0.088), ALD vs. controls (0.05 (0.03, 0.15), p=0.084); *male*: ALD (0.05 (0.01, 0.17)) vs. NAFLD (0.10 (0.05, 0.50), p=0.02), ALD vs. controls (0.16 (0.10, 0.22), p=0.004)). TCA/total BA was only significant decreased in *male* ALD (0.001 (0.001, 0.005)) compared to both NAFLD (0.008 (0.004, 0.021), p<0.001) and controls (0.035 (0.010, 0.049), p<0.001). *Female* ALD had the highest TCA/TCDCA ratios (ALD (1.00 (0.49, 1.00)) vs. NAFLD (0.26 (0.21, 0.40), p=0.004; ALD vs. controls (0.17 (0.09, 0.29), p<0.001). Highest GCDCA/total BA were found both in *female* ALD (ALD (0.45 (0.31, 0.54)) vs. NAFLD (0.29 (0.23, 0.34), p=0.033); ALD vs. controls (0.24 (0.16, 0.29)), p<0.001) and *male* ALD (ALD (0.43 (0.32, 0.54)) vs. NAFLD (0.21 (0.17, 0.27), p<0.001), ALD vs. controls (0.23 (0.17, 0.28), p<0.001)). GCA/GCDCA was lowest in ALD (Significant only in *male* ALD (0.16 (0.10, 0.27)) vs NAFLD (0.37 (0.30, 0.60), p<0.001) and controls (0.24 (0.16, 0.32), p=0.028).

Furthermore, 12-a-OH was lowest in ALD (Significantly only in male ALD (0.49 (0.26, 0.81) compared to NAFLD (1.15 (0.64, 2.47), p<0.001) and controls (0.93 (0.63, 1.34), p<0.001). The same findings were made in 12-a-OH/non-12-a-OH (male ALD (0.53 (0.20, 1.02)) was lowest compared to NAFLD (0.94 (0.75, 1.20), p=0.03) and controls (0.98 (0.85, 1.22), p<0.001)).

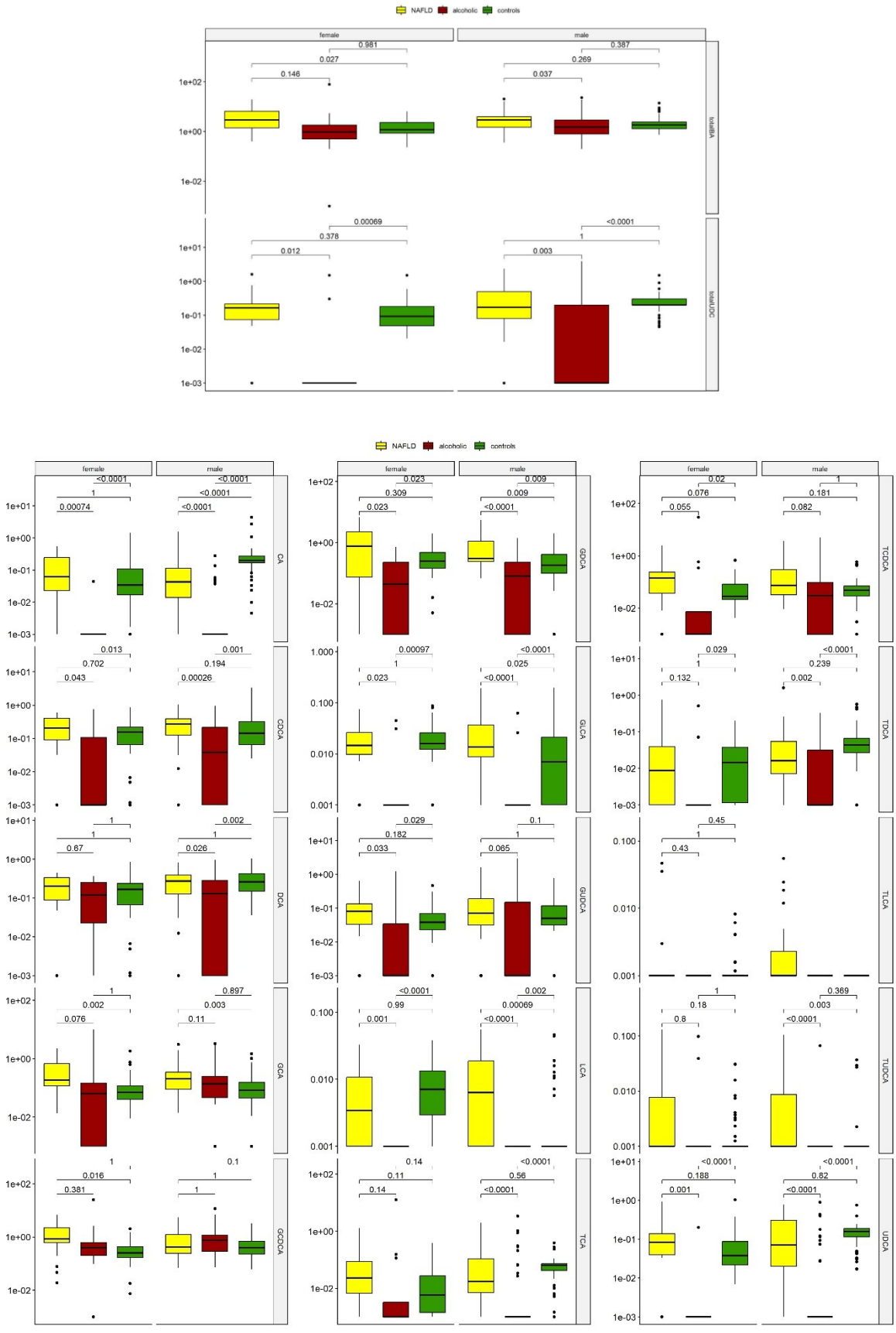

Figure 4: bile acid concentrations

8.5 NAFLD fibrosis score (FiB_Score/NFS)

Total BA only showed significant correlation to FiB_Score in NAFLD (especially male: $p<0.01$, $r=0.47$). Total free/total BA were only in NAFLD negatively correlated to FiB_Score ($p<0.01$, $r= -0.45$)

Significant correlations with FiB_Score were found in male NAFLD with total CA ($p<0.05$, $r=0.39$), total CDCA ($p<0.01$, $r=0.58$), total primary ($p<0.01$, $r=0.52$), glycine ($p<0.01$, $r=0.55$) and taurine conjugated ($p<0.001$, $r=0.62$) BA.

In female, correlations were higher in ALD for total free primary ($p<0.01$, $r=0.72$), total primary ($p<0.05$, $r=0.59$), CDCA ($p<0.01$, $r=0.72$), GCDCA ($p<0.05$, $r=0.58$), TCA ($p<0.05$, $r=0.64$), TUDCA ($p<0.05$, $r=0.65$).

BA ratios

Ratios that were stronger associated to FiB_Score in male NAFLD, were TCA/total ($p<0.01$, $r=0.54$), GCDCA/total ($p<0.01$, $r=0.52$) and TCDCA/total ($p<0.01$, $r=0.58$). Female controls showed a significant negative correlation in TCA/total ($p<0.01$, $r=0.39$).

TCA/CA and GCA/CA correlated to FiB_Score in NAFLD and alcoholic, stronger in male NAFLD (TCA/CA: <0.0001, $r=0.76$, GCA/CA: $p<0.001$, $r=0.65$) and ALD (female: TCA/CA: $p<0.05$, $r=0.64$; male: TCA/CA: <0.05, $r=0.35$, GCA/CA: $p<0.05$, $r=0.35$). Free CA/free CDCA negatively correlated to FiB_Score in female ALD ($p<0.05$, $r=-61$) and positive, but not significant in female NAFLD ($r=0.43$). 12-a-OH and non-12-a-OH correlated to FiB_Score in NAFLD (male: $p<0.05$, $r=0.47$) and in female ALD only non-12-a-OH ($p<0.05$, $r=0.59$).

8.6 Fib-4-score

Exclusively in male NAFLD, total BAs showed a significant correlation ($p<0.01$, $r=0.049$) to the Fib4-score.

Glycine conjugated primary bile acids (GCA, GCDCA) presented with the strongest association ($p<0.001$, $r =0.51$) to Fib4 in the NAFLD group. There was a correlation between these BA and Fib4 in male controls (GCA: $p<0.05$, $r=0.31$; GCDCA: $p<0.01$, $r=0.41$), but no in females.

Taurine conjugated primary BAs (TCA, TCDCA) showed a correlation in NAFLD and ALD, but not in the control group. The highest correlation was in male NAFLD (TCA:

p<0.01, r=0.5, TCDCA: p<0.001, r=0.6) and male ALD (TCA: p<0.01, r=0.45, TCDCA: p<0.001, r=0.53).

BA Ratios

TCA/CA and GCA/CA showed correlations to Fib4-score in NAFLD and ALD that were stronger in male individuals (NAFLD: TCA/CA: p<0.001, r=0.53, GCA/CA: p<0.001, r=0.52; ALD: TCA/CA: p<0.01, r=0.37, GCA/CA: p<0.01, r=0.35).

TCDCA/CDCA showed a significant correlation in NAFLD (p<0.01 r=0.47) and ALD (p<0.05, r=0.33). This was also viable in male individuals, but only in female NALFD. GCDCA/CDCA was significantly associated to Fib4 exclusively in NAFLD (p<0.001, r=0.49). In NAFLD, a correlation to Fib4 was found for TUDCA/UDCA (p<0.001, r=0.49) and GUDCA/ UDCA (p<0.01, r=0.48), whereas the latter showed better correlation in female (p<0.01, r=0.67).

8.7 AP

Total BA were correlated to AP in NAFLD (p <0.01, r =0.46) and ALD (p <0.01, r =0.36), but showed the strongest correlation in male NAFLD (p <0.001, r =0.66). Total primary (p<0.001, r=0.63), glycine (p<0.0001, r=0.7) and taurine (p<0.01, r=0.58) conjugated BA showed strongest correlation in male NAFLD.

Glycocholic acid (GCA) showed the strongest correlation to AP in NAFLD (p <0.001, r =0.52), and a weaker one in ALD (p <0.05, r =0.3) and controls (p <0.05, r =0.22). The correlation coefficient was higher in male NAFLD and in female controls.

TCA and TCDCA were correlated to NAFLD (TCA: p<0.01, r=0.43, TCDCA: p<0.01, r=0.43) and ALD (TCA: p<0.05, r=0.34, TCDCA: p<0.01, r=0.35). The correlation coefficient was highest in male NAFLD, negative for TCA in the control group and higher in female than male ALD.

BA Ratios

In NAFLD, AP was correlated to TCDCA/CDCA (p<0.01, r=0.46), GCDCA/CDCA (p<0.01, r=0.49) and GDCA/DCA (p<0.01, r=0.49). CA/CDCA was positively correlated to AP in NAFLD (p<0.05, r =0.35), but showed a negative correlation in controls (p<0.05, r= -0.22).

Female NAFLD, but also controls, had significant correlations to GCA/GCDCA (NAFLD: $p<0.05$, $r=0.54$, controls: $p<0.05$, $r=0.35$) and GCA/total BA (NAFLD: $p<0.01$, $r=0.7$, controls: $p<0.05$, $r=0.29$)

8.8 FLI

The fatty liver index is solely available for NAFLD and the control group.

Total BA were correlated to NAFLD ($p<0.01$, $r=0.47$) and controls ($p<0.01$, $r=0.32$). The correlation in female NAFLD was strongest ($p<0.05$, $r=0.66$). Total Ursodeoxycholic acid was significantly correlated in the control group ($p<0.0001$, $r=0.55$).

Some glycine conjugated BAs showed a unique correlation (especially in female) to FLI in NAFLD, namely GCDCA ($p<0.001$, $r=0.61$), GCA ($p<0.01$, $r=0.5$) and GDCA ($p<0.01$, $r=0.51$). TCA showed a significant relation to FLI in controls ($p<0.0001$, $r=0.54$) and NAFLD ($p<0.01$, $r=0.51$), whereas the correlation of the latter was stronger in female NAFLD ($p<0.01$, $r=0.74$). TCDCA showed a stronger correlation to FLI in NAFLD ($p<0.001$, $r=0.57$), particularly in female NAFLD, stronger than in controls.

Bile acids, that only correlated significantly to FLI in the controls, were CA ($p<0.0001$, $r=0.45$), DCA ($p<0.001$, $r=0.034$), TDCA ($p<0.0001$, $r=0.54$) and UDCA ($p<0.0001$, $r=0.44$). LCA was negatively correlate to FLI in controls ($p<0.0001$, $r=-0.44$), but positively in NAFLD.

Total CDCA was only correlated significantly in NAFLD ($p <0.001$, $r=0.55$) and strongest in female ($p=0.05$, $r=0.7$). Total CA was correlated to FLI in NAFLD ($p<0.01$, $r=0.45$) and controls ($p<0.0001$, $r=0.39$).

BA Ratios

GCA/CA showed a positive correlation to FLI in NAFLD ($p<0.01$, $r =0.43$), mainly in male NAFLD ($p<0.001$, $r=0.68$), and a negative correlation in the control group ($p<0.001$, $r=-0.36$). TCA/CA only showed a positive correlation to FLI in male NAFLD ($p <0.05$, $r =0.54$). TCDCA/CDCA ($p<0.01$, $r=0.46$) and GCDCA/CDCA ($p<0.01$, $r=0.47$) were associated to FLI in NAFLD. GDCA/DCA in NAFLD was positive correlated ($p<0.05$, $r=0.38$) to FLI, whereas controls showed a significant inverse result ($p<0.001$, $r=-0.35$). In male controls FLI correlated to TDCA/GDCA ($p<0.0001$, $r=0.5$) and TLCA/LCA ($p<0.0001$, $r=0.4$).

8.9 ELF score

We only calculated this score for NAFLD and ALD, where many correlations showed similar trends. In ALD, male presented with stronger correlations than female (total BA, GCA, GCDCA, TCA, TCDCA). GCA showed strongest negative correlation in female ALD ($p<0.01$, $r=-0.76$). GDCA in NAFLD correlated positively with ELF-score ($p<0.001$, $r=0.51$), but negatively in female ALD ($p<0.05$, $r=-0.62$). There was a notable difference between male ALD ($p<0.01$, $r=0.43$) and female ALD ($p<0.5$, $r=-0.66$) looking at TDCA.

8.10 ALT

Most significant correlations with ALT were found in controls. Some taurine conjugated BAs presented a significant correlation in controls (TCA: $p<0.0001$, $r=0.52$; TCDCA: $p<0.001$, $r=0.35$; TDCA: $p<0.0001$, $r=0.39$). A negative correlation to TDCA/DCA ($p<0.5$, $r=-0.38$) and LCA ($p<0.01$, $r=0.49$) was found in male NAFLD. In contrast to that, female NAFLD showed a positive, but not significant correlation to TDCA/DCA ($r=0.42$) and LCA ($r=0.45$). Ursodeoxycholic acid (UDCA) was also correlated to ALT in controls ($p<0.001$, $r=0.36$), coming from male individuals. Female NAFLD showed correlations in TCDCA ($p<0.5$, $r=0.52$), TCDCA/total BA ($p<0.01$, $r=0.61$), total CDCA ($p<0.05$, $r=0.5$), taurine conjugated ($p<0.05$, $r=0.53$), GLCA/LCA ($p<0.05$, $r=-0.51$) and glycine/taurine conjugated BA ($p<0.05$, $r=-0.56$).

8.11 TG

Except for a few significant correlations in ALD, TG were mainly correlated to the control group. CDCA ($p<0.05$, $r= 0.33$) and some linked ratios (TCDCA/CDCA, GCDCA/CDCA, total free/total BA) in male ALD were correlated to TG.

8.12 CRP

The C-reactive protein had most correlations with BAs and BA-ratios in male ALD. Only TUDCA had a significant correlation to CRP in controls ($p=0.01$, $r=0.27$).

9 Discussion

We found some specific changes of bile acid concentrations and ratios in NAFLD, but in some, NAFLD and ALD presented similar alterations compared to controls. In male NAFLD, various bile acid ratios were divergent from healthy individuals. Female NAFLD had elevated levels of bile acids in most cases, especially of some conjugated primary BA.

Total BA were significantly increased in NAFLD compared to both ALD and controls. In *female*, total BA correlated stronger to Fib4 and FLI and in *male* stronger to AP. Total primary BA were only significantly higher in *female* NAFLD. Total primary/total BA was similar between NAFLD and controls, but the part of total free BA/total BA was highest in controls and significantly lower in ALD than both groups. In NAFLD, the ratio was in between (only in male NAFLD significant), but showed a negative correlation to Fib4-score, FiB-Score, FLI, ELF-score and AP.

There was a higher ratio of free CDCA in *male* NAFLD. Cholic acid (CA), free CA/free CDCA and total free/total BA are decreased. They seemed to have more conjugated primary BA as the total primary/total BA is normal. Total CA/total CDCA in *male* NAFLD was lower than controls and higher than in ALD. Despite higher levels of total CA and total CDCA in *female* NAFLD, the ratio total CA/total CDCA was normal.

Looking at the concentrations of single BA, only GCA showed an outstanding elevation in both men and women. TCA is the only primary conjugated BA that was reduced in relation to total BA in *male* NAFLD compared to controls. A significant absolute increase in glycine conjugated BA was visible in *female* NAFLD. Both male and female NAFLD had an absolute and relative higher glycine conjugation of CA compared to CDCA. There was a higher relative taurine conjugation of CA in female and of CDCA in male. The ratio glycine/taurine conjugated BA was only significantly higher in *male* NAFLD than in controls.

Non-12-a-OH BA were increased in both sexes indicating a higher activation of the alternative BA synthesis pathway. This pathway is thought to improve lipid and glucose metabolism and help to detoxify harmful intermediates of BA. The higher activation may represent a healing response, a protection against further damage or a compensation of the overload of BA. (32)

Male NAFLD presented with a relative increase of TCDCA, but also tauroursodeoxycholic acid (TUDCA), that decreases ER-stress in vitro (26), and the secondary bile acids LCA, GLCA, TLCA and GDCA were significantly elevated in male NAFLD. In female NAFLD, the secondary BA were not significantly different compared to controls, but they had higher primary BA, which might be due to the gender-dependent disposition to cholestasis.

In accordance with a study (33) that measured BA in non-cholestatic individuals, our female controls had lower median levels of total free primary BA and total CA, total primary than male controls. In our NAFLD group, this pattern was not visible. It would be interesting to find out, whether in the course of disease, the gender-dependant differences vanish, or if there may appear other gender specific patterns compared to healthy individuals.

Interestingly, there were stronger positive correlations between bile acids and alkaline phosphatase, a marker for cholestasis, in male NAFLD (Total primary, glycine conjugated BA, 12-a-OH, non-12-a-OH), even though female NAFLD had highest levels of AP.

Alterations of physiological sexual hormone levels may contribute to fatty liver disease. Women with an estrogen deficiency, a low estrogen signalling response, that received estrogen-receptor antagonists as a breast cancer treatment or due to bisphenol A, a component of plastic that may block estrogen signalling developed steatosis. (34) Premenopausal women with age associated normal estrogen levels may be protected from severe fibrosis compared to men and postmenopausal women. (35) Other sexual hormones like progesterone or testosterone may be worth to consider in an individualized approach for NAFLD diagnosis or treatment. Nevertheless, in this study we do not have any information about the hormone levels or menopausal status of our patients.

The expression and activity of enzymes involved in bile acid metabolism is different between male and female. (34) Enzymes related to detoxification of BA (UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT) 2A1 and nuclear receptors (PXR, CAR)) were suppressed in rats fed with a high-fat-cholesterol (HFC) diet. Male HFC-fed rats showed strong and female only a slight affection. (36) However, whether these sex differences are transmittable to human needs to be confirmed.

The accumulation of BAs can be cytotoxic, and an imbalance of BA with their distinct properties can be contributing to liver disease. BA with beneficial properties could be lower and therefore cannot perform their physiological function adequately and BA with specific

harmful effects could outweigh. For example, we found a higher ratio of free CA/free CDCA. CA has less affinity to the farnesoid-X-receptor (FXR) than CDCA and the conjugated forms of CDCA. CA and TCDCA were shown to induce anti-apoptotic proteins and mRNA in hepatocytes. (37) This fact may influence the course of NAFLD by stimulation of cell proliferative processes.

The diagnostic value of bile acids analysis together with other potential markers for the individual metabolic state of a patient warrants clarification in further studies. Other factors that influence BA composition like the gut microbiome and genetic constellations may be important, but they are still not practicable outside of research. Aspects like a consistency throughout the day, the chance of short-term changes due to diet and applicability to other population groups like children may play a role. They may not only be of diagnostic value or help in exclusion of advanced stages of disease, but it could be possible to find concrete markers, that give insight in the patients' specific metabolic dysfunction to predict the development of (liver) disease or apply treatment in a very personalized way. Although there were some similar trends in men and women, our data shows that it is necessary to look separately at male and female. It would be important to understand the sex-difference in physiological (BA) metabolism more in-depth for a later use of certain metabolites as diagnostic markers.

It is likeable, that further markers will add up to existing panels that already used to increase sensitivity and specificity. A two-phase strategy, which consists of calculating Fib4- score first, followed by the ELF-Test (Enhanced Liver Fibrosis) to exclude liver damage in as many patients as possible has been found recently to increase detection of liver disease. Besides the impact on the individual suffering from liver disease, the effect on health care system and associated cost cannot be underestimated. Therefore, excluding cases in primary care through cost efficient and easy to perform diagnostical procedures like using a sequence like this would be adequate to lower expenditures. Right now, there already exist many more markers, but their transformation to routine laboratory diagnostics is missing. (38)

10 Conclusion

The main similarity in both sexes of our NAFLD patients compared with healthy controls was the higher absolute and relative value of GCA. Non-12-a-OH bile acids (BA) were elevated significantly only in female NAFLD. This represents a stimulation of the alternative bile acid pathway and may indicate a compensatory reaction of the liver cells to fat storage injury. Female NAFLD had higher primary BA and higher conjugated BA compared to controls. In male NAFLD, free CA was significantly decreased. Men also had an imbalance in conjugated BAs, some secondary BA (GDCA, LCA, GLCA, TLCA, TUDCA) were higher than in controls and the ratio of total free BA/total BA was decreased. The latter may indicate a stronger BA conjugation activity in male NAFLD cases. The causes for these gender related differences remain clarified in future studies. Apart from the underlying mechanisms, our data indicate clearly that gender specific bile acid profiles of NAFLD phenotypes are important to understand this disease. Finally, our results show that alcoholic liver disease associates with markedly different bile acid patterns compared to NAFLD.

	female NAFLD	male NAFLD
Significant in NAFLD versus controls	↑ GCA/total BA ↑ non-12-a-OH BA ↑ total BA ↑ total CDCA ↑ total primary BA ↑ glycine conjugated BA ↑ GCDCA	↑ GCA/total BA ↑ GCA ↓ free CA/free CDCA ↓ TCA/TCDCA
Significant in NAFLD versus controls and NAFLD versus ALD	↑ GCA ↑ total CA	↓ free CA ↓ total free/total BA ↓ total CA/total CDCA ↑ glycine/taurine conjugated BA ↓ TCA/total BA ↑ GDCA ↑ LCA ↑ GLCA ↑ TLCA ↑ TUDCA

Table 7: Overview of changes in BA (arrow up/down for comparison NAFLD vs. controls)

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12 Appendix

12.1 Bile acid concentrations

Bile acid concentrations in female

Bile acids for females

Characteristic	**NAFLD**, N = 17[1]	**alcoholic**, N = 12[1]	**controls**, N = 52[1]	**NAFLD vs. alcoholic**	**NAFLD vs. controls**	**alcoholic vs. controls**
Chenodeoxycholic acid (µmol/l)	0.20 (0.09, 0.41)	0.00 (0.00, 0.11)	0.15 (0.07, 0.22)	0.043	0.702	0.013
Cholic acid	0.06 (0.02, 0.24)	0.00 (0.00, 0.00)	0.03 (0.02, 0.11)	<0.001	>0.999	<0.001
Deoxycholic acid	0.20 (0.09, 0.34)	0.12 (0.05, 0.25)	0.17 (0.07, 0.24)	0.667	>0.999	>0.999
Glycocholic acid	0.19 (0.12, 0.68)	0.07 (0.00, 0.17)	0.07 (0.04, 0.12)	0.075	0.002	>0.999
Glycochenodeoxycholic acid	0.84 (0.61, 2.27)	0.40 (0.20, 0.62)	0.25 (0.17, 0.44)	0.380	0.016	>0.999
Glycodeoxycholic acid	0.77 (0.07, 2.26)	0.04 (0.00, 0.23)	0.24 (0.14, 0.48)	0.023	0.310	0.023
Glycolithocholic acid	0.015 (0.010, 0.026)	0.001 (0.001, 0.001)	0.016 (0.012, 0.026)	0.023	>0.999	<0.001
Glycoursodeoxycholic acid	0.08 (0.03, 0.13)	0.00 (0.00, 0.03)	0.04 (0.02, 0.07)	0.033	0.182	0.029
Lithocholic acid	0.003 (0.001, 0.011)	0.001 (0.001, 0.001)	0.007 (0.003, 0.013)	0.001	0.990	<0.001
Taurocholic acid	0.02 (0.01, 0.09)	0.00 (0.00, 0.03)	0.01 (0.00, 0.03)	0.145	0.106	0.143
Taurochenodeoxycholic acid	0.14 (0.04, 0.24)	0.00 (0.00, 0.09)	0.03 (0.02, 0.08)	0.055	0.076	0.020
Taurodeoxycholic acid	0.01 (0.00, 0.04)	0.00 (0.00, 0.00)	0.01 (0.00, 0.04)	0.132	>0.999	0.029

Bile acids for females

Characteristic	**NAFLD**, N = 17[1]	**alcoholic**, N = 12[1]	**controls**, N = 52[1]	**NAFLD vs. alcoholic**	**NAFLD vs. controls**	**alcoholic vs. controls**
Taurolithocholic acid	0.0010 (0.0010, 0.0010)	0.0010 (0.0010, 0.0010)	0.0010 (0.0010, 0.0010)	0.428	>0.999	0.452
Tauroursodeoxycholic acid	0.001 (0.001, 0.008)	0.001 (0.001, 0.001)	0.001 (0.001, 0.001)	0.805	0.181	>0.999
Ursodeoxycholic acid	0.08 (0.04, 0.14)	0.00 (0.00, 0.00)	0.04 (0.02, 0.09)	0.001	0.189	<0.001
total BA	2.89 (1.40, 6.50)	0.95 (0.50, 1.90)	1.18 (0.86, 2.30)	0.146	0.027	0.981
totalUDC	0.17 (0.07, 0.22)	0.00 (0.00, 0.00)	0.09 (0.05, 0.18)	0.012	0.378	<0.001

[1] Median (IQR)

Bile acid concentrations in male

Bile acids for males

Characteristic	**NAFLD**, N = 28[1]	**alcoholic**, N = 45[1]	**controls**, N = 51[1]	**NAFLD vs. alcoholic**	**NAFLD vs. controls**	**alcoholic vs. controls**
Chenodeoxycholic acid (µmol/l)	0.27 (0.13, 0.40)	0.04 (0.00, 0.22)	0.14 (0.07, 0.32)	<0.001	0.194	0.001
Cholic acid	0.04 (0.01, 0.12)	0.00 (0.00, 0.00)	0.20 (0.17, 0.28)	<0.001	<0.001	<0.001
Deoxycholic acid	0.27 (0.13, 0.40)	0.13 (0.00, 0.28)	0.26 (0.15, 0.43)	0.026	>0.999	0.002
Glycocholic acid	0.20 (0.09, 0.35)	0.14 (0.05, 0.25)	0.08 (0.05, 0.16)	0.110	0.003	0.897
Glycochenodeoxycholic acid	0.42 (0.25, 1.26)	0.75 (0.29, 1.19)	0.40 (0.23, 0.69)	>0.999	>0.999	0.102
Glycodeoxycholic acid	0.30 (0.24, 1.11)	0.08 (0.00, 0.23)	0.18 (0.10, 0.41)	<0.001	0.009	0.009
Glycolithocholic acid	0.014 (0.009, 0.037)	0.001 (0.001, 0.001)	0.007 (0.001, 0.021)	<0.001	0.025	<0.001
Glycoursodeoxycholic acid	0.07 (0.03, 0.19)	0.00 (0.00, 0.15)	0.05 (0.03, 0.12)	0.066	>0.999	0.100
Lithocholic acid	0.006 (0.001, 0.019)	0.001 (0.001, 0.001)	0.001 (0.001, 0.001)	<0.001	<0.001	0.002
Taurocholic acid	0.02 (0.01, 0.11)	0.00 (0.00, 0.00)	0.06 (0.04, 0.07)	<0.001	0.555	<0.001
Taurochenodeoxycholic acid	0.08 (0.03, 0.29)	0.03 (0.00, 0.10)	0.05 (0.03, 0.07)	0.081	0.181	>0.999

Bile acids for males

Characteristic	**NAFLD**, N = 28[1]	**alcoholic**, N = 45[1]	**controls**, N = 51[1]	**NAFLD vs. alcoholic**	**NAFLD vs. controls**	**alcoholic vs. controls**
Taurodeoxycholic acid	0.02 (0.01, 0.05)	0.00 (0.00, 0.03)	0.04 (0.03, 0.07)	0.002	0.239	<0.001
Taurolithocholic acid	0.0010 (0.0010, 0.0023)	0.0010 (0.0010, 0.0010)	0.0010 (0.0010, 0.0010)	<0.001	<0.001	
Tauroursodeoxycholic acid	0.001 (0.001, 0.009)	0.001 (0.001, 0.001)	0.001 (0.001, 0.001)	<0.001	0.003	0.368
Ursodeoxycholic acid	0.07 (0.02, 0.31)	0.00 (0.00, 0.00)	0.16 (0.12, 0.19)	<0.001	0.823	<0.001
total BA	2.88 (1.49, 3.97)	1.50 (0.80, 2.90)	1.85 (1.30, 2.43)	0.036	0.269	0.386
totalUDC	0.17 (0.08, 0.50)	0.00 (0.00, 0.20)	0.20 (0.20, 0.30)	0.003	>0.999	<0.001

[1] Median (IQR)

12.2 BA Ratios

BA Ratios in female

Bile acids for females

Characteristic	**NAFLD**, N = 17[1]	**alcoholic**, N = 12[1]	**controls**, N = 52[1]	**NAFLD vs. alcoholic**	**NAFLD vs. controls**	**alcoholic vs. controls**
TCA/CA	1 (0, 2)	1 (1, 30)	0 (0, 1)	0.156	0.230	<0.001
GCA/CA	5 (3, 29)	65 (1, 142)	2 (1, 6)	0.264	0.210	0.025
TCA/GCA	0.12 (0.08, 0.17)	0.36 (0.02, 1.00)	0.10 (0.04, 0.17)	>0.999	>0.999	>0.999
CA/CDCA	0.4 (0.2, 0.9)	1.0 (0.0, 1.0)	0.4 (0.1, 1.9)	>0.999	>0.999	>0.999
TCDCA/CDCA	1 (0, 2)	1 (1, 3)	0 (0, 1)	>0.999	0.434	0.403
GCDCA/CDCA	6 (2, 16)	66 (8, 324)	2 (1, 6)	0.180	0.223	0.002
TCDCA/GCDCA	0.16 (0.08, 0.35)	0.01 (0.00, 0.27)	0.15 (0.09, 0.23)	0.219	>0.999	0.114
TDCA/DCA	0.08 (0.02, 0.23)	0.01 (0.01, 1.00)	0.09 (0.04, 0.27)	>0.999	>0.999	>0.999
GDCA/DCA	3 (1, 10)	1 (1, 1)	2 (1, 6)	0.056	>0.999	0.051
TDCA/GDCA	0.03 (0.01, 0.12)	0.53 (0.02, 1.00)	0.04 (0.01, 0.12)	0.464	>0.999	0.390
TLCA/LCA	0.35 (0.18, 1.00)	1.00 (1.00, 1.00)	0.16 (0.10, 0.44)	0.056	0.078	<0.001

Bile acids for females

Characteristic	**NAFLD**, N = 17[1]	**alcoholic**, N = 12[1]	**controls**, N = 52[1]	**NAFLD vs. alcoholic**	**NAFLD vs. controls**	**alcoholic vs. controls**
GLCA/LCA	3.1 (1.2, 4.5)	1.0 (1.0, 1.0)	2.5 (1.3, 5.5)	0.077	>0.999	0.016
TLCA/GLCA	0.09 (0.06, 0.47)	1.00 (1.00, 1.00)	0.07 (0.05, 0.09)	0.146	0.230	0.002
TUDCA/UDCA	0.03 (0.02, 0.17)	1.00 (1.00, 1.00)	0.03 (0.02, 0.07)	<0.001	>0.999	<0.001
GUDCA/UDCA	0.8 (0.7, 1.3)	1.0 (1.0, 12.4)	1.0 (0.5, 1.7)	0.150	>0.999	0.234
TUDCA/GUDCA	0.03 (0.03, 0.07)	1.00 (0.35, 1.00)	0.03 (0.02, 0.05)	0.007	>0.999	0.002
Total CA	0.46 (0.21, 0.94)	0.07 (0.00, 0.21)	0.16 (0.08, 0.33)	0.025	0.009	0.210
Total CDCA	1.16 (0.68, 2.88)	0.42 (0.26, 0.89)	0.46 (0.33, 0.77)	0.265	0.011	>0.999
Total free Primary	0.33 (0.13, 0.58)	0.00 (0.00, 0.11)	0.21 (0.11, 0.33)	0.006	0.592	0.001
Total Primary	1.62 (1.25, 3.45)	0.49 (0.27, 1.05)	0.65 (0.45, 1.11)	0.146	0.010	0.994
glycine conjugated	1.89 (1.04, 5.15)	0.74 (0.32, 1.25)	0.65 (0.40, 1.10)	0.198	0.021	>0.999
Taurine conjugated	0.17 (0.10, 0.36)	0.01 (0.01, 0.24)	0.05 (0.03, 0.15)	0.088	0.097	0.084
Free CA/Free CDCA	0.4 (0.2, 0.9)	1.0 (0.0, 1.0)	0.4 (0.1, 1.9)	>0.999	>0.999	>0.999
Total CA/Total CDCA	0.38 (0.28, 0.57)	0.17 (0.06, 0.41)	0.34 (0.20, 0.50)	0.132	>0.999	0.130
T-CA/T-CDCA	0.26 (0.21, 0.40)	1.00 (0.49, 1.00)	0.17 (0.09, 0.29)	0.004	0.108	<0.001
G-CA/G-CDCA	0.36 (0.21, 0.62)	0.17 (0.06, 0.40)	0.27 (0.21, 0.39)	0.162	0.772	0.194
Conjugated CA/ Conjugated CDCA	0.33 (0.21, 0.54)	0.17 (0.06, 0.40)	0.27 (0.21, 0.39)	0.180	>0.999	0.202
Glycine/Tauro	13 (6, 15)	31 (5, 106)	10 (7, 19)	0.574	>0.999	0.328
total free/Total	0.08 (0.02, 0.22)	0.01 (0.00, 0.11)	0.16 (0.09, 0.24)	0.162	0.348	0.011
total primary / total Bas	0.59 (0.48, 0.61)	0.60 (0.52, 0.82)	0.51 (0.48, 0.56)	>0.999	0.458	0.173
G-CA/Total	0.10 (0.07, 0.13)	0.08 (0.04, 0.09)	0.06 (0.04, 0.09)	0.574	0.045	>0.999

Bile acids for females

Characteristic	**NAFLD**, N = 17[1]	**alcoholic**, N = 12[1]	**controls**, N = 52[1]	**NAFLD vs. alcoholic**	**NAFLD vs. controls**	**alcoholic vs. controls**
T-CA/Total	0.009 (0.005, 0.020)	0.002 (0.001, 0.029)	0.005 (0.002, 0.015)	0.894	0.654	>0.999
G-CDCA/Total	0.29 (0.23, 0.34)	0.45 (0.31, 0.54)	0.24 (0.16, 0.29)	0.033	0.422	<0.001
T-CDCA/Total	0.04 (0.01, 0.08)	0.00 (0.00, 0.11)	0.03 (0.02, 0.05)	0.318	>0.999	0.210
12-a-OH	1.59 (0.46, 3.27)	0.41 (0.19, 0.75)	0.59 (0.43, 1.02)	0.106	0.104	0.328
non-12-a-OH	1.45 (0.80, 3.86)	0.44 (0.28, 0.93)	0.61 (0.45, 1.07)	0.145	0.031	0.733
12-a-OH/non-12-a-OH	1.09 (0.70, 1.23)	0.90 (0.37, 1.28)	0.96 (0.87, 1.11)	>0.999	>0.999	>0.999

[1] Median (IQR)

BA Ratios in male

Bile acids for males

Characteristic	**NAFLD**, N = 28[1]	**alcoholic**, N = 45[1]	**controls**, N = 51[1]	**NAFLD vs. alcoholic**	**NAFLD vs. controls**	**alcoholic vs. controls**
TCA/CA	0 (0, 3)	1 (1, 1)	0 (0, 0)	0.495	0.088	<0.001
GCA/CA	6 (2, 16)	83 (3, 184)	0 (0, 1)	0.015	<0.001	<0.001
TCA/GCA	0.11 (0.07, 0.25)	0.02 (0.01, 1.00)	0.62 (0.20, 1.19)	0.503	<0.001	<0.001
CA/CDCA	0.18 (0.08, 0.47)	0.17 (0.01, 1.00)	1.68 (0.88, 3.22)	>0.999	<0.001	<0.001
TCDCA/CDCA	0 (0, 1)	1 (0, 12)	0 (0, 1)	0.581	>0.999	0.095
GCDCA/CDCA	2 (1, 5)	14 (5, 338)	3 (1, 7)	<0.001	>0.999	<0.001
TCDCA/GCDCA	0.18 (0.11, 0.29)	0.05 (0.01, 0.19)	0.13 (0.06, 0.17)	0.002	0.040	0.049
TDCA/DCA	0.08 (0.04, 0.28)	0.02 (0.00, 1.00)	0.18 (0.05, 0.37)	>0.999	0.572	0.585
GDCA/DCA	1.8 (1.0, 4.6)	1.0 (0.5, 1.6)	0.7 (0.4, 1.5)	0.022	0.003	>0.999
TDCA/GDCA	0.06 (0.02, 0.11)	0.08 (0.01, 1.00)	0.31 (0.12, 0.46)	0.431	<0.001	>0.999
TLCA/LCA	0.50 (0.15, 1.00)	1.00 (1.00, 1.00)	1.00 (1.00, 1.00)	<0.001	0.058	0.002
GLCA/LCA	3 (1, 8)	1 (1, 1)	1 (1, 7)	<0.001	0.107	0.005

Bile acids for males

Characteristic	**NAFLD**, N = 28[1]	**alcoholic**, N = 45[1]	**controls**, N = 51[1]	**NAFLD vs. alcoholic**	**NAFLD vs. controls**	**alcoholic vs. controls**
TLCA/GLCA	0.11 (0.07, 0.17)	1.00 (1.00, 1.00)	0.14 (0.05, 1.00)	<0.001	0.783	<0.001
TUDCA/UDCA	0.03 (0.01, 0.09)	1.00 (1.00, 1.00)	0.01 (0.01, 0.02)	<0.001	0.003	<0.001
GUDCA/UDCA	1 (1, 2)	1 (1, 37)	1 (0, 2)	0.006	0.240	<0.001
TUDCA/GUDCA	0.04 (0.02, 0.07)	1.00 (0.01, 1.00)	0.02 (0.01, 0.04)	>0.999	0.135	0.211
Total CA	0.34 (0.15, 0.67)	0.14 (0.06, 0.30)	0.38 (0.27, 0.51)	0.014	>0.999	<0.001
Total CDCA	0.83 (0.55, 2.07)	0.89 (0.36, 1.56)	0.59 (0.44, 0.98)	>0.999	0.148	>0.999
Total free Primary	0.32 (0.18, 0.54)	0.05 (0.00, 0.24)	0.32 (0.24, 0.54)	<0.001	>0.999	<0.001
Total Primary	1.23 (0.68, 2.61)	1.06 (0.43, 1.88)	0.97 (0.64, 1.50)	0.473	0.741	>0.999
glycine conjugated	1.01 (0.67, 2.72)	1.00 (0.52, 1.99)	0.88 (0.42, 1.28)	0.606	0.214	>0.999
Taurine conjugated	0.10 (0.05, 0.50)	0.05 (0.01, 0.17)	0.16 (0.10, 0.22)	0.020	>0.999	0.004
Free CA/Free CDCA	0.18 (0.08, 0.47)	0.17 (0.01, 1.00)	1.68 (0.88, 3.22)	>0.999	<0.001	<0.001
Total CA/Total CDCA	0.33 (0.24, 0.40)	0.14 (0.08, 0.28)	0.64 (0.43, 0.86)	<0.001	0.001	<0.001
T-CA/T-CDCA	0.30 (0.20, 0.40)	0.31 (0.03, 1.00)	1.08 (0.56, 1.58)	>0.999	<0.001	<0.001
G-CA/G-CDCA	0.37 (0.30, 0.60)	0.16 (0.10, 0.27)	0.24 (0.16, 0.32)	<0.001	<0.001	0.028
Conjugated CA/ Conjugated CDCA	0.39 (0.29, 0.57)	0.15 (0.10, 0.28)	0.35 (0.25, 0.52)	<0.001	>0.999	<0.001
Glycine/Tauro	10 (5, 15)	18 (6, 62)	5 (3, 10)	0.025	0.090	<0.001
total free/Total	0.13 (0.07, 0.20)	0.04 (0.00, 0.10)	0.20 (0.14, 0.30)	0.003	0.027	<0.001
total primary / total Bas	0.52 (0.47, 0.59)	0.68 (0.53, 0.85)	0.57 (0.50, 0.63)	0.008	0.629	0.010
G-CA/Total	0.09 (0.06, 0.11)	0.07 (0.04, 0.13)	0.04 (0.03, 0.08)	>0.999	0.001	0.143
T-CA/Total	0.008 (0.004, 0.021)	0.001 (0.001, 0.005)	0.035 (0.010, 0.049)	<0.001	0.019	<0.001

Bile acids for males

Characteristic	NAFLD, N = 28[1]	alcoholic, N = 45[1]	controls, N = 51[1]	NAFLD vs. alcoholic	NAFLD vs. controls	alcoholic vs. controls
G-CDCA/Total	0.21 (0.17, 0.27)	0.43 (0.32, 0.54)	0.23 (0.17, 0.28)	<0.001	>0.999	<0.001
T-CDCA/Total	0.03 (0.02, 0.06)	0.03 (0.00, 0.07)	0.03 (0.01, 0.04)	0.570	0.219	>0.999
12-a-OH	1.15 (0.64, 2.47)	0.49 (0.26, 0.81)	0.93 (0.63, 1.34)	<0.001	0.896	<0.001
non-12-a-OH	1.36 (0.81, 2.27)	0.98 (0.37, 2.07)	0.88 (0.63, 1.25)	0.373	0.085	>0.999
12-a-OH/non-12-a-OH	0.94 (0.75, 1.20)	0.53 (0.20, 1.02)	0.98 (0.85, 1.22)	0.030	0.896	0.001

[1] Median (IQR)

12.3 Correlation

Correlation for Fib4

Spearman correlation for Fib4

	all			male			female		
	NAFLD	controls	alcoholic	NAFLD	controls	alcoholic	NAFLD	controls	alcoholic
totalBA	0.44 **	0.11	0.24	0.49 **	0.25	0.22	0.39	-0.01	0.4
totalUDC	0.11	0.07	0.03	0.05	0.27	-0.09	0.2	-0.11	0.5
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Spearman correlation for Fib4

	all			male			female		
	NAFLD	controls	alcoholic	NAFLD	controls	alcoholic	NAFLD	controls	alcoholic
CDCA	-0.06	0.05	0.08	0.03	0.06	0.03	-0.1	0.06	0.34
CA	-0.07	-0.05	0.01	-0.12	-0.13	-0.05	0.05	-0.1	0.48
DCA	0.09	-0.03	-0.34 *	0.03	-0.05	-0.32 *	0.29	-0.05	-0.35
GCA	0.51 ***	0.15	0.28 *	0.56 **	0.31 *	0.25	0.52 *	-0.05	0.41
GCDCA	0.51 ***	0.18	0.33 *	0.62 ***	0.41 **	0.29	0.4	-0.04	0.53
GDCA	0.45 **	0.16	-0.09	0.49 **	0.29	-0.08	0.47	0.04	-0.11
GLCA	0.24	0.21 *	0.03	0.14	0.28	0.06	0.41	0.18	-0.05
GUDCA	0.25	0.07	0.03	0.28	0.19	-0.09	0.25	-0.06	0.4
LCA	0.35 *	0.2 *	NaN NA	0.29	0.18	NaN NA	0.48 *	0.34 *	NaN NA
TCA	0.46 **	0.04	0.4 **	0.5 **	0.22	0.45 **	0.45	-0.12	0.22
TCDCA	0.44 **	0.06	0.44 ***	0.6 ***	0.16	0.53 ***	0.32	-0.06	0.14
TDCA	0.15	-0.04	0.08	0.09	0.02	0.23	0.3	-0.1	-0.52
TLCA	0.41 **	0.12	NaN NA	0.46 *	NaN NA	NaN NA	0.35	0.22	NaN NA
TUDCA	0.42 **	-0.06	0.3 *	0.45 *	0.03	0.24	0.39	-0.1	0.53
UDCA	0	0.03	-0.11	-0.03	0.16	-0.18	0.1	-0.09	0.22
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Spearman correlation for Fib4

	all			male			female		
	NAFLD	controls	alcoholic	NAFLD	controls	alcoholic	NAFLD	controls	alcoholic
TCA/CA	0.53 ***	0.2	0.37 **	0.63 ***	0.24	0.39 **	0.26	0.15	0.22
GCA/CA	0.52 ***	0.12	0.35 **	0.6 ***	0.27	0.35 *	0.34	0.14	0.32
TCA/GCA	0.31 *	-0.04	0.16	0.4 *	-0.18	0.22	0.19	0.03	-0.08
CA/CDCA	-0.04	-0.05	-0.07	-0.11	-0.03	-0.06	0.09	-0.08	-0.16
TCDCA/CDCA	0.47 **	-0.02	0.33 *	0.51 **	0.04	0.38 *	0.41	-0.06	0.07
GCDCA/CDCA	0.49 ***	0.09	0.16	0.45 *	0.19	0.11	0.52 *	-0.02	0.22
TCDCA/GCDCA	0.16	-0.22 *	0.31 *	0.27	-0.29	0.49 ***	0.02	-0.11	-0.23
TDCA/DCA	0.13	0.02	0.25	0.33	0	0.35 *	-0.07	0.02	-0.23
GDCA/DCA	0.31 *	0.14	0.24	0.31	0.28	0.31 *	0.3	0.07	-0.13
TDCA/GDCA	-0.14	-0.15	0.15	-0.06	-0.28	0.28	-0.14	-0.13	-0.3
TLCA/LCA	0.08	-0.18	NaN NA	0.08	-0.18	NaN NA	0.06	-0.24	NaN NA
GLCA/LCA	-0.35 *	0.01	0.03	-0.36	0.09	0.06	-0.36	-0.16	-0.05
TLCA/GLCA	0.23	-0.18	-0.03	0.33	-0.28	-0.06	0.02	-0.09	0.05
TUDCA/UDCA	0.49 ***	-0.04	0.2	0.47 *	-0.13	0.22	0.48	0.05	0.18
GUDCA/UDCA	0.48 **	0.08	0.12	0.37	0.1	0.04	0.67 **	0.04	0.38
TUDCA/GUDCA	0.42 **	-0.12	0.06	0.43 *	-0.22	0.15	0.45	-0.02	-0.27
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Spearman correlation for Fib4

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**

Spearman correlation for Fib4

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
Total CA	0.41 **	0.02	0.34 *	0.44 *	0.05	0.33 *	0.47	-0.06	0.41
Total CDCA	0.47 **	0.18	0.37 **	0.59 ***	0.36 *	0.37 *	0.33	0	0.52
Total free Primary	-0.03	0.04	0.08	0.01	0.02	0.04	-0.04	0.01	0.34
Total Primary	0.48 ***	0.13	0.37 **	0.58 **	0.3 *	0.36 *	0.41	-0.02	0.52
glycine conjugated	0.48 ***	0.22 *	0.23	0.55 **	0.45 **	0.18	0.46	0	0.43
Taurine conjugated	0.44 **	0.03	0.4 **	0.6 ***	0.15	0.5 ***	0.34	-0.06	0.08
Free CA/Free CDCA	-0.04	-0.05	-0.07	-0.11	-0.03	-0.06	0.09	-0.09	-0.16
Total CA/Total CDCA	0.09	-0.16	0.17	-0.02	-0.29 *	0.26	0.25	-0.17	-0.13
T-CA/T-CDCA	0.24	-0.05	-0.23	0.21	-0.15	-0.27	0.15	-0.12	-0.12
G-CA/G-CDCA	0.04	-0.04	0.15	-0.13	-0.03	0.24	0.33	-0.03	-0.06
Conjugated CA/ Conjugated CDCA	0.07	-0.17	0.17	-0.07	-0.19	0.26	0.33	-0.14	-0.13
Glycine/Tauro	-0.14	0.21 *	-0.3 *	-0.31	0.36 *	-0.49 ***	0.04	0.12	0.31
total free/Total	-0.45 **	-0.13	-0.12	-0.42 *	-0.23	-0.07	-0.48	-0.06	-0.13
total primary / total Bas	0.22	0.06	0.5 ***	0.34	0.13	0.61 ****	0.08	-0.02	0.1
G-CA/Total	0.42 **	0	0.24	0.35	0.16	0.3	0.5 *	-0.12	0.09
T-CA/Total	0.38 **	-0.06	0.3 *	0.43 *	-0.06	0.38 *	0.34	-0.16	-0.15
G-CDCA/Total	0.34 *	0.17	0.22	0.52 **	0.33 *	0.22	0.07	0.04	0.1
T-CDCA/Total	0.36 *	-0.03	0.39 **	0.58 **	0.01	0.57 ****	0.15	-0.04	-0.29
12-a-OH	0.43 **	0.05	0.13	0.45 *	0.1	0.11	0.42	-0.02	0.31
non-12-a-OH	0.41 **	0.18	0.32 *	0.46 *	0.4 **	0.29	0.34	0	0.53
12-a-OH/non-12-a-OH	0.15	-0.25 *	-0.28 *	0.03	-0.37 *	-0.26	0.28	-0.08	-0.39
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Correlation for FLI

Spearman correlation for FLI

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
totalBA	0.47 **	0.32 **	NA NA	0.35	0.24	NA NA	0.66 *	-0.03	NA NA
totalUDC	0.15	0.55 ****	NA NA	0.19	0.35 *	NA NA	0.13	0.13	NA NA
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Spearman correlation for FLI

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
CDCA	0.02	0.1	NA NA	0.09	0.09	NA NA	0.08	0.02	NA NA
CA	0.02	0.45 ****	NA NA	-0.3	0.32 *	NA NA	0.23	0.1	NA NA
DCA	0.04	0.34 ***	NA NA	0.09	0.36 *	NA NA	0.1	-0.08	NA NA
GCA	0.5 **	0.11	NA NA	0.38	-0.02	NA NA	0.65 *	0.07	NA NA
GCDCA	0.61 ***	0.17	NA NA	0.54 *	0.02	NA NA	0.73 **	-0.04	NA NA
GDCA	0.51 **	-0.12	NA NA	0.41	-0.02	NA NA	0.73 **	-0.09	NA NA
GLCA	0.06	-0.26 **	NA NA	0.12	-0.24	NA NA	0.18	0.08	NA NA
GUDCA	0.24	0.27 **	NA NA	0.26	0.12	NA NA	0.28	0.08	NA NA
LCA	0.22	-0.44 ****	NA NA	0.38	-0.27	NA NA	0.15	-0.05	NA NA
TCA	0.51 **	0.54 ****	NA NA	0.31	0.32 *	NA NA	0.74 **	0.11	NA NA
TCDCA	0.57 ***	0.23 *	NA NA	0.36	0.16	NA NA	0.77 **	0.16	NA NA
TDCA	0.03	0.44 ****	NA NA	0	0.4 **	NA NA	0.3	-0.03	NA NA
TLCA	0.26	-0.15	NA NA	0.61 **	NaN NA	NA NA	-0.29	0.03	NA NA
TUDCA	0.28	0.02	NA NA	0.25	-0.04	NA NA	0.31	0.25	NA NA
UDCA	0.09	0.54 ****	NA NA	0.06	0.37 **	NA NA	0.13	0.13	NA NA
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Spearman correlation for FLI

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
TCA/CA	0.41 *	0.07	NA NA	0.54 *	0.07	NA NA	0.03	0	NA NA
GCA/CA	0.43 *	-0.36 ***	NA NA	0.68 ***	-0.26	NA NA	0.05	-0.09	NA NA
TCA/GCA	0.28	0.56 ****	NA NA	0.17	0.36 *	NA NA	0.38	0.17	NA NA
CA/CDCA	0	0.3 **	NA NA	-0.37	0.17	NA NA	0.16	0.08	NA NA
TCDCA/CDCA	0.46 **	0	NA NA	0.31	0.03	NA NA	0.5	0.08	NA NA
GCDCA/CDCA	0.47 **	0	NA NA	0.35	-0.06	NA NA	0.34	-0.01	NA NA
TCDCA/GCDCA	-0.01	0.05	NA NA	-0.05	0.22	NA NA	-0.07	0.3 *	NA NA
TDCA/DCA	0.12	0.2 *	NA NA	0.08	0.22	NA NA	0.21	-0.02	NA NA
GDCA/DCA	0.38 *	-0.35 ***	NA NA	0.23	-0.26	NA NA	0.38	-0.03	NA NA
TDCA/GDCA	-0.33	0.5 ****	NA NA	-0.1	0.31 *	NA NA	-0.43	0.06	NA NA
TLCA/LCA	0.08	0.4 ****	NA NA	0.12	0.27	NA NA	-0.15	-0.02	NA NA
GLCA/LCA	-0.38 *	-0.12	NA NA	-0.46 *	0	NA NA	-0.26	0.12	NA NA
TLCA/GLCA	0.26	0.22 *	NA NA	0.53 *	0.24	NA NA	-0.29	-0.11	NA NA
TUDCA/UDCA	0.35 *	-0.45 ****	NA NA	0.21	-0.39 **	NA NA	0.62 *	-0.03	NA NA
GUDCA/UDCA	0.2	-0.25 *	NA NA	0.15	-0.17	NA NA	0.25	-0.13	NA NA
TUDCA/GUDCA	0.24	-0.22 *	NA NA	0.21	-0.17	NA NA	0.13	0.15	NA NA
Note:									

Spearman correlation for FLI

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Spearman correlation for FLI

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
Total CA	0.45 **	0.39 ****	NA NA	0.31	0.21	NA NA	0.55	0.1	NA NA
Total CDCA	0.55 ***	0.16	NA NA	0.41	0.03	NA NA	0.7 *	-0.04	NA NA
Total free Primary	0.06	0.38 ****	NA NA	0.05	0.24	NA NA	0.18	0.07	NA NA
Total Primary	0.52 **	0.31 **	NA NA	0.41	0.15	NA NA	0.74 **	-0.01	NA NA
glycine conjugated	0.55 ***	0.09	NA NA	0.48 *	0.01	NA NA	0.66 *	-0.03	NA NA
Taurine conjugated	0.49 **	0.41 ****	NA NA	0.33	0.31 *	NA NA	0.68 *	0.08	NA NA
Free CA/Free CDCA	0	0.3 **	NA NA	-0.37	0.17	NA NA	0.16	0.08	NA NA
Total CA/Total CDCA	0.06	0.41 ****	NA NA	0.03	0.38 **	NA NA	0.04	0.22	NA NA
T-CA/T-CDCA	0.1	0.56 ****	NA NA	0.17	0.29 *	NA NA	-0.25	0.13	NA NA
G-CA/G-CDCA	-0.22	-0.01	NA NA	-0.08	0.04	NA NA	-0.26	0.22	NA NA
Conjugated CA/ Conjugated CDCA	-0.14	0.29 **	NA NA	0.02	0.26	NA NA	-0.31	0.23	NA NA
Glycine/Tauro	0.05	-0.43 ****	NA NA	0.01	-0.35 *	NA NA	0.27	-0.19	NA NA
total free/Total	-0.49 **	0.16	NA NA	-0.42	0.05	NA NA	-0.48	0.04	NA NA
total primary / total Bas	0.21	0.14	NA NA	0.09	-0.02	NA NA	0.19	0.11	NA NA
G-CA/Total	0.29	-0.11	NA NA	0.29	-0.18	NA NA	0.32	0.19	NA NA
T-CA/Total	0.37 *	0.49 ****	NA NA	0.22	0.23	NA NA	0.49	0.18	NA NA
G-CDCA/Total	0.49 **	-0.12	NA NA	0.42	-0.2	NA NA	0.6 *	-0.06	NA NA
T-CDCA/Total	0.43 *	-0.01	NA NA	0.34	0.03	NA NA	0.45	0.15	NA NA
12-a-OH	0.45 **	0.31 **	NA NA	0.3	0.29 *	NA NA	0.69 *	-0.07	NA NA
non-12-a-OH	0.53 **	0.31 **	NA NA	0.42	0.16	NA NA	0.69 *	0.01	NA NA
12-a-OH/non-12-a-OH	-0.02	-0.02	NA NA	-0.05	0.16	NA NA	0.06	-0.2	NA NA
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Correlation for ELF

Spearman correlation for ELF Score

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
totalBA	0.55 ***	NA NA	0.51 ****	0.55 **	NA NA	0.52 ***	0.5 *	NA NA	0.25
totalUDC	0.17	NA NA	0.31 *	0.18	NA NA	0.15	0.2	NA NA	0.66 *
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Spearman correlation for ELF Score

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
CDCA	-0.04	NA NA	0.25	0.14	NA NA	0.16	-0.07	NA NA	0.45
CA	-0.02	NA NA	0.06	-0.13	NA NA	-0.06	-0.01	NA NA	0.5
DCA	0.02	NA NA	-0.37 **	0.12	NA NA	-0.24	0.09	NA NA	-0.76 **
GCA	0.57 ***	NA NA	0.55 ****	0.57 **	NA NA	0.55 ***	0.57 *	NA NA	0.22
GCDCA	0.69 ****	NA NA	0.58 ****	0.72 ****	NA NA	0.52 ***	0.65 **	NA NA	0.45
GDCA	0.51 ***	NA NA	-0.04	0.54 **	NA NA	0.11	0.5 *	NA NA	-0.62 *
GLCA	0.11	NA NA	0.02	0.1	NA NA	0.27	0.19	NA NA	-0.32
GUDCA	0.36 *	NA NA	0.3 *	0.33	NA NA	0.15	0.39	NA NA	0.73 *
LCA	0.13	NA NA	NaN NA	0.1	NA NA	NaN NA	0.22	NA NA	NaN NA
TCA	0.56 ***	NA NA	0.48 ***	0.57 **	NA NA	0.5 ***	0.59 *	NA NA	0.33
TCDCA	0.68 ****	NA NA	0.57 ****	0.72 ****	NA NA	0.6 ****	0.67 **	NA NA	0.2
TDCA	0.07	NA NA	0.24	0.07	NA NA	0.43 **	0.16	NA NA	-0.66 *
TLCA	0.29	NA NA	NaN NA	0.38	NA NA	NaN NA	0.31	NA NA	NaN NA
TUDCA	0.48 **	NA NA	0.35 **	0.45 *	NA NA	0.24	0.5 *	NA NA	0.67 *
UDCA	0.11	NA NA	0.08	0.15	NA NA	-0.02	0.07	NA NA	0.4
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Spearman correlation for ELF Score

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
TCA/CA	0.55 ***	NA NA	0.4 **	0.71 ***	NA NA	0.44 **	0.31	NA NA	0.33
GCA/CA	0.48 **	NA NA	0.49 ***	0.58 **	NA NA	0.52 ***	0.42	NA NA	0.13
TCA/GCA	0.35 *	NA NA	-0.02	0.43 *	NA NA	-0.04	0.3	NA NA	0.34
CA/CDCA	0.02	NA NA	-0.2	-0.23	NA NA	-0.16	0.11	NA NA	-0.28
TCDCA/CDCA	0.61 ****	NA NA	0.31 *	0.39	NA NA	0.35 *	0.74 ***	NA NA	0.08
GCDCA/CDCA	0.62 ****	NA NA	0.04	0.34	NA NA	0.08	0.7 **	NA NA	-0.25
TCDCA/GCDCA	0.07	NA NA	0.45 ***	0.12	NA NA	0.49 ***	0.07	NA NA	0.04
TDCA/DCA	0.16	NA NA	0.42 **	0.16	NA NA	0.45 **	0.15	NA NA	0.03
GDCA/DCA	0.37 *	NA NA	0.37 **	0.19	NA NA	0.44 **	0.38	NA NA	-0.1
TDCA/GDCA	-0.26	NA NA	0.28 *	-0.18	NA NA	0.3 *	-0.15	NA NA	0.2
TLCA/LCA	0.19	NA NA	NaN NA	0.06	NA NA	NaN NA	0.38	NA NA	NaN NA
GLCA/LCA	-0.32 *	NA NA	0.02	-0.23	NA NA	0.27	-0.49 *	NA NA	-0.32
TLCA/GLCA	0.21	NA NA	-0.02	0.1	NA NA	-0.27	0.3	NA NA	0.32
TUDCA/UDCA	0.5 ***	NA NA	0.01	0.31	NA NA	0.06	0.74 **	NA NA	0.07
GUDCA/UDCA	0.39 *	NA NA	0.31 *	0.18	NA NA	0.22	0.68 **	NA NA	0.67 *
TUDCA/GUDCA	0.32 *	NA NA	-0.2	0.24	NA NA	-0.1	0.38	NA NA	-0.63 *

Spearman correlation for ELF Score

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Spearman correlation for ELF Score

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
Total CA	0.49 **	NA NA	0.54 ****	0.46 *	NA NA	0.55 ***	0.51 *	NA NA	0.22
Total CDCA	0.63 ****	NA NA	0.61 ****	0.66 ***	NA NA	0.57 ****	0.55 *	NA NA	0.41
Total free Primary	-0.03	NA NA	0.23	0.06	NA NA	0.12	-0.13	NA NA	0.45
Total Primary	0.61 ****	NA NA	0.61 ****	0.62 **	NA NA	0.58 ****	0.56 *	NA NA	0.41
glycine conjugated	0.63 ****	NA NA	0.51 ****	0.61 **	NA NA	0.49 ***	0.59 *	NA NA	0.28
Taurine conjugated	0.63 ****	NA NA	0.54 ****	0.67 ***	NA NA	0.6 ****	0.66 **	NA NA	0.12
Free CA/Free CDCA	0.02	NA NA	-0.2	-0.23	NA NA	-0.16	0.11	NA NA	-0.28
Total CA/Total CDCA	0.03	NA NA	0.29 *	-0.08	NA NA	0.33 *	0.03	NA NA	-0.04
T-CA/T-CDCA	0.18	NA NA	-0.36 **	0.29	NA NA	-0.31 *	-0.01	NA NA	-0.19
G-CA/G-CDCA	-0.22	NA NA	0.38 **	-0.29	NA NA	0.46 **	-0.1	NA NA	-0.03
Conjugated CA/ Conjugated CDCA	-0.17	NA NA	0.37 **	-0.2	NA NA	0.46 **	-0.08	NA NA	-0.11
Glycine/Tauro	-0.08	NA NA	-0.4 **	-0.18	NA NA	-0.47 **	-0.1	NA NA	0.01
total free/Total	-0.57 ***	NA NA	-0.05	-0.36	NA NA	-0.12	-0.69 **	NA NA	0.3
total primary / total Bas	0.25	NA NA	0.5 ***	0.26	NA NA	0.44 **	0.24	NA NA	0.54
G-CA/Total	0.39 *	NA NA	0.41 **	0.28	NA NA	0.42 **	0.43	NA NA	0.13
T-CA/Total	0.41 **	NA NA	0.19	0.44 *	NA NA	0.19	0.44	NA NA	0.35
G-CDCA/Total	0.54 ***	NA NA	0.15	0.49 *	NA NA	0.1	0.5 *	NA NA	0.28
T-CDCA/Total	0.47 **	NA NA	0.52 ****	0.5 *	NA NA	0.56 ****	0.51 *	NA NA	0.21
12-a-OH	0.48 **	NA NA	0.25	0.47 *	NA NA	0.28	0.45	NA NA	0.04
non-12-a-OH	0.59 ****	NA NA	0.6 ****	0.58 **	NA NA	0.57 ****	0.55 *	NA NA	0.4
12-a-OH/non-12-a-OH	-0.03	NA NA	-0.4 **	-0.1	NA NA	-0.29	-0.09	NA NA	-0.67 *
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Correlation for FiB_Score/NFS

Spearman correlation for FiB_Score

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
totalBA	0.42 **	0.13	0.23	0.47 *	0.19	0.23	0.32	0.12	0.39
totalUDC	0.11	0.1	0.06	0.14	0.24	-0.02	-0.02	0.01	0.64 *
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Spearman correlation for FiB_Score

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
CDCA	0	0.3 *	0.13	0.1	0.39	0.04	-0.14	0.26	0.72 **
CA	-0.06	-0.07	-0.04	-0.19	-0.18	-0.11	0.13	-0.06	0.48
DCA	0.13	0.11	-0.27 *	0.11	0.08	-0.25	0.23	0.12	-0.44
GCA	0.42 **	0.06	0.28 *	0.52 **	0.16	0.25	0.33	-0.03	0.33
GCDCA	0.51 ***	0.17	0.32 *	0.61 ***	0.24	0.3 *	0.32	0.1	0.58 *
GDCA	0.46 **	0.05	-0.13	0.56 **	0.04	-0.06	0.33	0.11	-0.46
GLCA	0.23	0.2	0.22	0.35	0.15	0.26	0.06	0.32 *	-0.1
GUDCA	0.19	0.12	0.1	0.3	0.15	0.01	0.06	0.09	0.64 *
LCA	0.32 *	0.24 *	NaN NA	0.47 *	0.19	NaN NA	0	0.32 *	NaN NA
TCA	0.49 ***	0.01	0.4 **	0.6 ***	0.15	0.33 *	0.42	-0.2	0.64 *
TCDCA	0.48 ***	0.01	0.35 **	0.62 ***	0.11	0.38 *	0.33	-0.1	0.45
TDCA	0.13	-0.01	0.08	0.24	0.02	0.19	0.03	-0.08	-0.22
TLCA	0.41 **	0.03	NaN NA	0.6 ***	NaN NA	NaN NA	0.06	0.07	NaN NA
TUDCA	0.39 **	-0.01	0.37 **	0.47 *	-0.01	0.26	0.25	0.04	0.65 *
UDCA	0.05	0.09	-0.09	0.06	0.22	-0.14	-0.11	0.02	0.39
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Spearman correlation for FiB_Score

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
TCA/CA	0.54 ***	0.11	0.4 **	0.76 ****	0.26	0.35 *	0.15	0.04	0.64 *
GCA/CA	0.49 ***	0.08	0.33 *	0.65 ***	0.19	0.35 *	0.2	0.14	0.3
TCA/GCA	0.37 *	-0.03	0.23	0.54 **	0.05	0.16	0.2	-0.23	0.47
CA/CDCA	-0.01	-0.23	-0.15	-0.25	-0.26	-0.11	0.43	-0.2	-0.61 *
TCDCA/CDCA	0.47 **	-0.21	0.2	0.48 *	-0.13	0.24	0.47	-0.24	0.15
GCDCA/CDCA	0.45 **	-0.08	0.09	0.39 *	-0.08	0.08	0.55 *	-0.06	-0.2
TCDCA/GCDCA	0.11	-0.25 *	0.25	0.31	0	0.34 *	-0.17	-0.32 *	0.24
TDCA/DCA	0.19	-0.11	0.28 *	0.42 *	0.06	0.32 *	-0.08	-0.2	0.26
GDCA/DCA	0.28	-0.08	0.2	0.34	-0.11	0.32 *	0.17	0.01	-0.36
TDCA/GDCA	-0.13	-0.05	0.23	0.11	0.02	0.25	-0.31	-0.18	0.34
TLCA/LCA	0.07	-0.3 *	NaN NA	-0.04	-0.19	NaN NA	0.3	-0.4 **	NaN NA
GLCA/LCA	-0.34 *	-0.07	0.22	-0.41 *	0	0.26	-0.19	-0.01	-0.1
TLCA/GLCA	0.23	-0.21	-0.22	0.27	-0.15	-0.26	0.14	-0.33 *	0.1
TUDCA/UDCA	0.43 **	-0.09	0.19	0.4 *	-0.24	0.19	0.63 **	0.04	0.06
GUDCA/UDCA	0.28	0	0.22	0.26	-0.06	0.15	0.34	0.06	0.59 *

Spearman correlation for FiB_Score

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
TUDCA/GUDCA	0.45 **	-0.15	0.02	0.46 *	-0.24	0.06	0.48 *	-0.06	-0.52
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Spearman correlation for FiB_Score

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
Total CA	0.36 *	0.01	0.29 *	0.39 *	-0.06	0.29	0.37	0.02	0.33
Total CDCA	0.47 **	0.21	0.35 **	0.58 **	0.37	0.35 *	0.29	0.12	0.59 *
Total free Primary	0.01	0.24 *	0.1	0.06	0.2	0.02	-0.03	0.24	0.72 **
Total Primary	0.45 **	0.15	0.35 **	0.52 **	0.25	0.34 *	0.35	0.11	0.59 *
glycine conjugated	0.47 **	0.13	0.21	0.55 **	0.26	0.19	0.34	0.1	0.34
Taurine conjugated	0.46 **	0	0.32 *	0.62 ***	0.1	0.36 *	0.28	-0.12	0.42
Free CA/Free CDCA	-0.01	-0.23	-0.15	-0.25	-0.26	-0.11	0.43	-0.2	-0.61 *
Total CA/Total CDCA	0.07	-0.21	0.04	-0.05	-0.27	0.14	0.28	-0.19	-0.19
T-CA/T-CDCA	0.27	-0.03	-0.1	0.32	0.09	-0.16	0.14	-0.29	-0.44
G-CA/G-CDCA	-0.11	-0.22	0.06	-0.18	-0.14	0.17	0.04	-0.25	-0.12
Conjugated CA/ Conjugated CDCA	-0.04	-0.26 *	0.07	-0.07	-0.24	0.18	0.1	-0.31 *	-0.15
Glycine/Tauro	-0.11	0.22	-0.24	-0.36	0.11	-0.32 *	0.2	0.35 *	-0.29
total free/Total	-0.45 **	0.09	-0.09	-0.4 *	0.07	-0.1	-0.52 *	0.06	0.24
total primary / total Bas	0.09	0.04	0.43 **	0.17	0.06	0.52 ***	-0.02	0.03	0.4
G-CA/Total	0.29	-0.2	0.13	0.28	0.01	0.25	0.3	-0.27	-0.06
T-CA/Total	0.41 **	-0.14	0.33 *	0.54 **	0.02	0.27	0.33	-0.39 **	0.44
G-CDCA/Total	0.39 **	0.06	0.2	0.52 **	0.05	0.25	0.18	0.09	0.01
T-CDCA/Total	0.34 *	-0.18	0.33 *	0.58 **	-0.02	0.39 **	0.11	-0.22	0.34
12-a-OH	0.41 **	0.07	0.12	0.47 *	0.07	0.13	0.32	0.1	0.17
non-12-a-OH	0.4 **	0.2	0.31 *	0.47 *	0.38	0.28	0.23	0.11	0.59 *
12-a-OH/non-12-a-OH	0.11	-0.32 **	-0.24	0.05	-0.58 **	-0.22	0.26	-0.1	-0.48
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Correlation for AP

Spearman correlation for AP

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
totalBA	0.46 **	0.05	0.36 **	0.66 ***	0	0.32 *	0.07	0.16	0.38
totalUDC	0.16	-0.07	0.11	0.23	-0.2	0.09	0.03	0.13	0.15
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Spearman correlation for AP

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
CDCA	-0.22	0.15	0.02	-0.11	0.23	-0.08	-0.34	0.06	0.29
CA	0.35 *	-0.14	-0.22	0.29	0.03	-0.39 **	0.36	-0.18	0.48
DCA	-0.22	0.05	0.15	-0.11	0.08	0.13	-0.34	0.05	0.17
GCA	0.52 ***	0.22 *	0.3 *	0.57 **	0.14	0.28	0.39	0.34 *	0.21
GCDCA	0.51 ***	0.06	0.27 *	0.69 ****	0.05	0.25	0.09	0.11	0.29
GDCA	0.51 ***	0.16	0.26	0.69 ****	0.1	0.28	0.09	0.19	0.13
GLCA	0.16	0.14	-0.11	0.24	0.12	-0.17	-0.17	0.17	0.11
GUDCA	0.37 *	0.1	0.17	0.51 **	0.02	0.13	0.18	0.21	0.38
LCA	0.13	0.18	NaN NA	0.15	0.16	NaN NA	-0.06	0.15	NaN NA
TCA	0.43 **	-0.01	0.34 *	0.48 *	-0.13	0.31 *	0.27	0.23	0.49
TCDCA	0.43 **	0.11	0.35 **	0.55 **	0.03	0.29	0.2	0.23	0.43
TDCA	0.06	0	0.33 *	0.16	-0.02	0.3	-0.18	0.17	0.35
TLCA	0.19	0.07	NaN NA	0.49 *	NaN NA	NaN NA	-0.31	0.04	NaN NA
TUDCA	0.33 *	-0.04	-0.07	0.45 *	-0.25	-0.22	0.03	0.1	0.24
UDCA	0.05	-0.06	-0.05	0.07	-0.26	-0.02	-0.09	0.13	-0.22
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Spearman correlation for AP

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
TCA/CA	0.27	0.15	0.4 **	0.34	-0.13	0.43 **	-0.07	0.34 *	0.49
GCA/CA	0.19	0.27 **	0.4 **	0.29	0.21	0.47 **	-0.16	0.34 *	0.15
TCA/GCA	0.28	-0.12	0.08	0.38	-0.29 *	0.04	-0.02	0.12	0.44
CA/CDCA	0.35 *	-0.22 *	-0.16	0.29	-0.24	-0.19	0.36	-0.2	-0.09
TCDCA/CDCA	0.46 **	-0.02	0.21	0.48 *	-0.12	0.19	0.34	0.08	0.31
GCDCA/CDCA	0.49 **	-0.04	0.16	0.46 *	-0.12	0.13	0.24	0.03	0.18
TCDCA/GCDCA	0.22	0.08	0.28 *	0.22	0.06	0.3 *	0.13	0.08	0.3
TDCA/DCA	0.29	-0.01	0.06	0.39 *	-0.11	0.01	0.08	0.14	0.23
GDCA/DCA	0.49 **	0.11	0.1	0.46 *	0.09	0.06	0.24	0.07	0.05
TDCA/GDCA	-0.15	-0.11	-0.07	-0.09	-0.2	-0.11	-0.26	0.1	0.07
TLCA/LCA	0.08	-0.2	NaN NA	0.23	-0.16	NaN NA	-0.09	-0.22	NaN NA
GLCA/LCA	-0.14	0.02	-0.11	-0.06	0.09	-0.17	-0.13	-0.1	0.11
TLCA/GLCA	0.15	-0.17	0.11	0.43 *	-0.12	0.17	-0.07	-0.24	-0.11
TUDCA/UDCA	0.29	0.03	0.06	0.3	0.07	-0.02	0.17	-0.04	0.47

Spearman correlation for AP

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
GUDCA/UDCA	0.28	0.08	0.23	0.32	0.15	0.16	0.35	-0.01	0.53
TUDCA/GUDCA	0.17	-0.17	-0.23	0.19	-0.23	-0.19	-0.02	-0.14	-0.38
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Spearman correlation for AP

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
Total CA	0.51 ***	-0.01	0.28 *	0.56 **	-0.02	0.25	0.35	0.11	0.21
Total CDCA	0.47 **	0.12	0.29 *	0.64 ***	0.08	0.25	0.06	0.18	0.27
Total free Primary	0	-0.02	-0.04	0.05	0.14	-0.16	-0.02	-0.08	0.29
Total Primary	0.49 **	0.05	0.28 *	0.63 ***	0.02	0.26	0.17	0.13	0.27
glycine conjugated	0.52 ***	0.11	0.28 *	0.7 ****	0.03	0.25	0.15	0.21	0.32
Taurine conjugated	0.43 **	0.02	0.37 **	0.58 **	-0.06	0.31 *	0.21	0.25	0.49
Free CA/Free CDCA	0.35 *	-0.23 *	-0.16	0.29	-0.24	-0.19	0.36	-0.2	-0.09
Total CA/Total CDCA	0.26	-0.13	0.11	0.14	-0.19	0.17	0.44	0	-0.09
T-CA/T-CDCA	0.12	-0.08	-0.17	0.13	-0.21	-0.09	-0.07	0.19	-0.37
G-CA/G-CDCA	0.05	0.25 *	0.25	-0.14	0.1	0.36 *	0.54 *	0.35 *	-0.09
Conjugated CA/ Conjugated CDCA	0.09	0.07	0.26	-0.04	-0.11	0.38 *	0.51	0.29 *	-0.1
Glycine/Tauro	-0.16	0.1	-0.27 *	-0.25	0.24	-0.26	-0.02	-0.1	-0.33
total free/Total	-0.37 *	-0.09	-0.3 *	-0.42 *	0.04	-0.29	-0.14	-0.21	-0.26
total primary / total Bas	0.39 *	-0.02	-0.08	0.26	0.06	-0.07	0.3	-0.1	-0.16
G-CA/Total	0.36 *	0.22 *	0.11	0.19	0.14	0.21	0.7 **	0.29 *	-0.2
T-CA/Total	0.29	-0.07	0.1	0.3	-0.19	0.13	0.26	0.18	0.16
G-CDCA/Total	0.41 **	0.03	-0.34 *	0.43 *	0.09	-0.24	0.17	-0.04	-0.59 *
T-CDCA/Total	0.33 *	0.06	0.2	0.41 *	-0.05	0.22	0.19	0.15	0.22
12-a-OH	0.48 **	0.05	0.47 ***	0.62 ***	0.02	0.47 **	0.13	0.14	0.41
non-12-a-OH	0.43 **	0.07	0.28 *	0.64 ***	0.01	0.25	0.05	0.18	0.28
12-a-OH/non-12-a-OH	0.22	0.02	0.17	0.16	0.05	0.17	0.36	-0.02	0.31
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Correlation for CRP

Spearman correlation for CRP

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
totalBA	0.09	0.04	0.45 ***	0.14	0.19	0.45 **	-0.01	-0.1	0.45
totalUDC	-0.02	0.09	0.06	-0.02	0.13	0.01	0.03	0.04	0.37
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Spearman correlation for CRP

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
CDCA	-0.14	0.04	0.06	-0.13	0.12	0.06	-0.11	-0.05	0.07
CA	-0.02	-0.04	-0.07	-0.16	0.07	-0.06	-0.07	-0.06	-0.14
DCA	-0.2	-0.04	-0.02	-0.13	0.02	-0.01	-0.15	-0.14	0.01
GCA	0.11	0.11	0.48 ***	0.16	0.26	0.5 ***	-0.08	-0.02	0.44
GCDCA	0.14	0.07	0.38 **	0.1	0.15	0.41 **	-0.02	-0.01	0.22
GDCA	0.05	0.07	0.23	0	0.11	0.32 *	0.03	0	0.07
GLCA	-0.17	0.01	0.15	-0.21	0	0.09	-0.19	0.01	0.27
GUDCA	0.06	0.19	0.08	0.06	0.29 *	0.06	0.07	0.1	0.13
LCA	-0.1	0.01	NaN NA	-0.1	-0.01	NaN NA	-0.11	0.05	NaN NA
TCA	0.09	0.04	0.44 **	0.05	0.02	0.42 **	0.06	0.09	0.35
TCDCA	0.16	0.1	0.43 **	0.11	0.05	0.42 **	0.18	0.14	0.57
TDCA	-0.1	0.03	0.48 ***	-0.01	-0.04	0.48 **	-0.04	0.07	0.5
TLCA	-0.05	0.12	NaN NA	-0.06	NaN NA	NaN NA	0.08	0.17	NaN NA
TUDCA	0.11	0.27 **	0.2	0.21	0.22	0.13	0.05	0.29 *	0.25
UDCA	-0.05	0.06	-0.13	-0.1	0.08	-0.22	0.13	0	0.55
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Spearman correlation for CRP

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
TCA/CA	0.12	-0.01	0.4 **	0.15	0	0.36 *	0.12	0.04	0.35
GCA/CA	0.08	0.1	0.45 ***	0.18	0.1	0.44 **	0.03	0.02	0.57
TCA/GCA	0.11	-0.06	0.01	0.08	-0.27	-0.04	0.25	0.16	-0.08
CA/CDCA	0.09	-0.04	-0.06	-0.01	0	-0.05	0	-0.02	-0.16
TCDCA/CDCA	0.26	-0.02	0.27	0.23	-0.12	0.23	0.24	0.12	0.47
GCDCA/CDCA	0.26	-0.04	0.12	0.29	-0.11	0.12	-0.07	0.03	-0.03
TCDCA/GCDCA	0.12	0.08	0.34 *	-0.01	0.03	0.31 *	0.28	0.17	0.5
TDCA/DCA	0.09	0.08	0.37 **	0.03	0.06	0.31	0.23	0.13	0.69 *
GDCA/DCA	0.21	0.09	0.3 *	0.17	0.14	0.37 *	0.12	0.07	0.09
TDCA/GDCA	-0.04	-0.01	0.2	-0.11	-0.08	0.11	0.09	0.06	0.47
TLCA/LCA	0.23	0.02	NaN NA	0.16	0.01	NaN NA	0.41	0.03	NaN NA
GLCA/LCA	-0.09	0.06	0.15	-0.12	0.08	0.09	0.16	0.01	0.27
TLCA/GLCA	0.19	0.02	-0.15	0.18	0	-0.09	0.47	0.06	-0.27

Spearman correlation for CRP

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
TUDCA/UDCA	0.23	0.1	0.17	0.3	0.05	0.25	0.08	0.1	-0.46
GUDCA/UDCA	0.01	0.13	0.26	0.2	0.2	0.31 *	-0.31	0.02	-0.16
TUDCA/GUDCA	0.2	0.01	-0.01	0.28	-0.15	-0.03	0.15	0.13	0.02
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									

Spearman correlation for CRP

	all			male			female		
	NAFLD	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**	**NAFLD**	**controls**	**alcoholic**
Total CA	0.13	0.03	0.49 ***	0.09	0.19	0.51 ***	0.04	-0.06	0.44
Total CDCA	0.1	0.04	0.39 **	0.07	0.16	0.43 **	0.01	-0.07	0.22
Total free Primary	-0.11	-0.03	0.02	-0.17	0.07	0.02	-0.13	-0.15	0.07
Total Primary	0.13	0.03	0.41 **	0.09	0.16	0.45 **	0	-0.1	0.22
glycine conjugated	0.13	0.12	0.39 **	0.12	0.22	0.38 *	-0.03	0.01	0.38
Taurine conjugated	0.11	0.05	0.47 ***	0.07	0.01	0.43 **	0.1	0.09	0.53
Free CA/Free CDCA	0.09	-0.04	-0.06	-0.01	0	-0.05	0	-0.02	-0.16
Total CA/Total CDCA	0.13	0.01	0.39 **	0.05	0.11	0.39 *	0.12	-0.01	0.43
T-CA/T-CDCA	0.07	-0.04	-0.14	0.15	-0.17	-0.1	0.04	0.13	-0.66
G-CA/G-CDCA	-0.02	0.15	0.49 ***	-0.01	0.28	0.53 ***	0.08	0.02	0.42
Conjugated CA/ Conjugated CDCA	0	0.06	0.51 ***	0.01	0.07	0.54 ***	0.08	0.05	0.47
Glycine/Tauro	-0.07	0.03	-0.39 **	-0.04	0.16	-0.33 *	-0.21	-0.12	-0.62
total free/Total	-0.2	-0.01	-0.19	-0.32	0.12	-0.18	-0.05	-0.1	-0.05
total primary / total Bas	0.16	0.06	0.2	0.08	0.19	0.28	-0.08	-0.08	-0.18
G-CA/Total	0.1	0.15	0.4 **	0.13	0.2	0.45 **	0.03	0.11	0.18
T-CA/Total	0.12	0	0.2	0.12	-0.08	0.16	0.18	0.17	0.17
G-CDCA/Total	0.19	-0.03	-0.1	0.17	-0.1	-0.02	-0.22	0.04	-0.43
T-CDCA/Total	0.23	0.1	0.35 *	0.2	-0.05	0.36 *	0.22	0.26	0.33
12-a-OH	0.04	0.02	0.43 **	0.01	0.16	0.45 **	0.04	-0.13	0.33
non-12-a-OH	0.09	0.09	0.36 **	0.13	0.23	0.39 *	-0.03	-0.03	0.22
12-a-OH/non-12-a-OH	-0.05	-0.14	0.01	-0.11	-0.08	-0.01	0.01	-0.24	0.07
Note:									
(**** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05, . p<0.10)									