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IMPACT OF ETHANOL INTAKE ON FIBROBLAST GROWTH FACTOR 21 AND BILE ACID METABOLISM

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LIST OF ABBREVIATIONS

ABCB4:	ATP Binding Cassette Subfamily B Member 4
ACLF:	Acute on Chronic Liver Failure
ACLI:	Acute on Chronic Liver Injury
ALD:	Alcoholic Liver Disease
ALI:	Acute Liver Injury
ALT:	Alanine Aminotransferase
ASBT:	Apical Sodium-dependent Bile acid Transporter
AST:	Aspartate Aminotransferase
AUD:	Alcohol Use Disorder
BA:	Bile Acid
BAAT:	Bile Acid CoA: Amino Acid N acylTransferase
BACS:	Bile Acid CoA Synthetase
BMI:	Body Mass Index
BSEP:	Bile Salt Export Pump
CB1R:	Cannabinoid Receptor Type 1
CDT:	Carbohydrate Deficient Transferrin
CLI:	Chronic Liver Injury
CREBH:	cAMP Responsive Element-Binding protein
CRTC2:	CREB Regulated Transcription Co-activator 2
CYP27A1:	Sterol-27-hydroxylase
CYP7A1:	Cholesterol 7 alpha-hydroxylase
CYP8B1:	Cytochrome P450 family 8 subfamily B member 1
DAMP:	Damage-Associated Molecular Patterns
DSM-5:	Diagnostic and Statistical Manual of mental disorders, 5th edition
ERK:	Extracellular signal-Regulated Kinase
FGF:	Fibroblast Growth Factor

FGFR: Fibroblast Growth Factor Receptor

FXR: Farnesoid X Receptor

GGT: Gamma Glutamyl Transferase

GIP: Gastric Inhibitory Polypeptide

GLP1: Glucagon Like Peptide 1

HbA1c: Glycated Haemoglobin

HCC: Hepatocellular Carcinoma

HSC: Hepatic Stellate Cell

IFN: Interferon

INR: International Normalized Ratio

KLB: beta-Klotho

MAPK: Mitogen-Activated Protein-Kinase

MBOAT7: Membrane-Bound O AcetylTransferase domain-containing protein 7

MDR: Multidrug Resistance Protein

MRP4: Multidrug Resistance Protein 4

MTOR: Mammalian Target of Rapamycin

NAD: Nicotinamide Adenine Dinucleotide

NAFLD: Non Alcoholic Fatty Liver Disease

NIAAA: National Institute on Alcohol Abuse and Alcoholism

NK: Natural Killers

NLR: NOD-like Receptors

NR0B2: Nuclear Receptor Subfamily 0, Group B, Member 2

NTCP: Sodium Taurocholate Cotransporting Polypeptide

OST: Organic Solute Transporter

PAMP: Pathogen-Associated Molecular Patterns

PNPLA3: Patatin like Phospholipase Domain-containing Protein 3

ROS: Reactive Oxygen Species

SCN: Suprachiasmatic Nucleus

SHP: Small Heterodimer Partner

SIRT1: Sirtuin 1

SREBF1: Sterol Regulatory Element Binding Transcription Factor 1

SREBP: Sterol Regulator Element Binding Protein

TAP: Antigen Peptide Transporter

TBA: Total Bile Acids

TGR5: G protein-coupled bile acid receptor 1/ G-protein coupled receptor 19

TLR: Toll-Like Receptor

TM6SF2: Transmembrane 6 Superfamily Member 2

TUDCA: Tauroursodeoxycholic Acid

UDCA: Ursodeoxycholic Acid

WHO: World Health Organization

SUMMARY

Introduction: Alcohol use disorder remains one of the major health problems worldwide, causing excess morbidity and mortality. Alcoholic liver disease constitutes one of the major components of alcohol-associated morbidity, and alcoholic cirrhosis is the leading cause of alcohol-related death. In the course of alcoholic liver disease, alterations in bile acid metabolism play a critical role, leading to impaired bile secretion and the formation of hepatotoxic mediators. Two members of the endocrine subfamily of fibroblast growth factors, FGF21 and FGF15/19, are reported to regulate bile acid biosynthesis, but only sparse data exist hitherto about their exact functions in the setting of alcohol intake and/or alcoholic liver disease.

Patients and methods: Wild-type (C57BL/6J) and *Abcb4* knockout mice (which develop a cholestatic liver disease mimicking primary sclerosing cholangitis) were fed with either ethanol containing or control diet (using the preestablished NIAAA - National Institute on Alcohol Abuse and Alcoholism - protocol). Tissue expression and circulating levels of FGF21 and FGF15 were measured in both groups and mouse types. Serum bile acids and mRNA expression of the major enzymes of bile acid metabolism were determined. Liver specimens were histologically examined for cholestatic damage. Furthermore, recombinant human FGF21 was added to primary murine hepatocytes in order to assess the direct effect of FGF21 on the expression of CYP7A1, which encodes the rate-limiting enzyme of hepatic bile acid synthesis. Subsequently, serum levels of FGF21 and FGF19 and oxysterols were determined in patients with alcoholic liver disease and ongoing ethanol consumption and compared to a cohort of healthy control subjects, in order to translate the experimental findings to humans.

Results: Ethanol consumption consistently upregulated hepatic and circulating levels of FGF21 in both humans and rodents. The observed upregulation was preserved even in the context of preexisting cholestatic liver disease (in rodents) or alcoholic liver disease (in humans). The upregulation of FGF21 elicits *in vivo* and *in vitro* a suppression of CYP7A1. The suppression took place in a dose-dependent manner and appeared to be independent of FGF15/19 signaling.

Conclusions: Ethanol intake induces increased hepatic expression and circulating levels of FGF21, which in turn represses direct bile acid biosynthesis in a dose-dependent manner.

This action is retained even in the setting of preexisting liver disease and presents a potential hepatoprotective mechanism against alcohol-associated liver damage.

ZUSAMMENFASSUNG

Einleitung: Alkoholabusus stellt unverändert ein erhebliches Gesundheitsproblem dar und führt zur exzessiven Morbidität und Mortalität. Die alkoholische Fettlebererkrankung und Steatohepatitis sind zentrale Komponenten der alkohol-assoziierten Morbidität, und die alkoholische Leberzirrhose ist die führende alkohol-assoziierte Letalitätsursache. Im Verlauf der alkoholischen Lebererkrankung spielen Veränderungen des Stoffwechsels der Gallensäuren, die zu gestörten Exkretion in die Galle und Überproduktion von hepatotoxischen Molekülen führt, eine wichtige Rolle. Zwei Mitglieder der Familie der endokrinen Fibroblast Growth Factors, FGF21 und FGF15/19, sind an der Regulation der Synthese der Gallensäuren in der Leber beteiligt. Aktuell sind nur wenige Daten über ihre Effekte im Rahmen der alkoholischen Lebererkrankung verfügbar.

Patienten und Methodik: Die Mausinzuchtlinie C57BL/6J und *Abcb4* Knockout-Mäuse (die, eine cholestatische Lebererkrankung, ähnlich zur primär sklerosierenden Cholangitis, entwickeln) wurden entweder mit Kontrolldiät oder einer alkoholischen Diät ernährt, gemäß dem vorher etablierten NIAAA Protokoll. Gewebeexpression und Serumkonzentrationen von FGF21 und FGF15 wurden in allen Gruppen bestimmt. Die mRNA Expression der wichtigsten Enyzme der biosynthetischen Kaskade der Gallensäuren wurden ebenso bestimmt. Die Lebergeweben wurden histologisch nach cholestatischer Schädigung untersucht. Primäre Hepatozyten wurden mit reombinantem humanen FGF21 inkubiert , um dessen Einfluss auf die hepatische Expression von CYP7A1, dem geschwindigkeitslimitierenden Enyzm der Gallensäurensynthese, zu untersuchen. Anschließend wurden die Serum-Spiegel von FGF21, FGF19 und Oxysterolen bei Patienten mit alkoholischer Lebererkrankung und fortgesetztem Alkoholabusus und einer Kontrollgruppe von gesunden Probanden bestimmt.

Ergebnisse: Alkohoeinnahme führte zur Erhöhung der hepatischen Produktion und des Serumspiegels von FGF21, sowohl in den Tiermodellen als auch bei den Probanden. Diese Erhöhung blieb auch im Rahmen einer vorbestehenden cholestatischen Leberschädigung (in den Mäusen) oder der alkoholischen Lebererkrankung (bei Patienten) erhalten. Die erhöhte Produktion von FGF21 führte zu einer dosisabhängigen Suppression von CYP7A1, sowohl *in vivo* als auch *in vitro*. Diese Suppression erfolgte in einer nicht FGF15/19 vermittelten Weise.

Schlussfolgerungen: Ethanol induziert die Synthese von und erhöhte die systemischen Spiegel von FGF21. Das hochregulierte FGF21 supprimiert die Biosynthese der Gallensäuren durch einen direkten Effekt auf CYP7A1. Diese Wirkung ist potenziell

hepatoprotektiv gegenüber hepatotoxischen Einflüssen wie Alkohol in unserem experimentellen Modell.

1. INTRODUCTION

Alcohol abuse remains as one of the main causes of excess morbidity and mortality worldwide and has been further associated with social, emotional and economic consequences not only for the patient himself but for his complete environment (112). The disease burden caused by excessive alcohol intake makes it attractive to reveal the underlying pathomechanisms, which induce or retain alcohol use disorder. One of the major and most prevalent pathological conditions associated with excessive alcohol ingestion is the alcoholic liver disease (ALD) that appears to be the most prevalent type of chronic liver disease worldwide (112). However, an effective treatment currently does not exist for ALD, except the application of corticosteroids for acute alcoholic steatohepatitis treatment, which has been suggested in the 1970s, or liver transplantation in the final stages of the disease (120). Therefore, there is further need to discover potential pharmacological targets, in order to restrict alcohol use disorder or unveil innovative treatments for its detrimental effects on vital organs such as liver or heart.

Alcoholic liver disease is a progressive disease, with a wide spectrum of liver disorders ranging from hepatic inflammation (called steatohepatitis) and impaired or changed bile acids synthesis to more severe liver defects such as cirrhosis and hepatocellular carcinoma (HCC) (120). The causative pathomechanism of ALD is complicated and not yet fully understood and encompasses hepatic fat accumulation, oxidative stress and cytokine and chemokine induced inflammation (120). Since the emerging evidences demonstrate a pivotal role of the altered bile acids (BA) metabolism in the pathogenesis and physical course of ALD (46,148,157,158), exposing the underlying mechanisms could lead to potential therapeutic manipulations. In this context, the emerging roles of the two members of the endocrine fibroblast growth factors (FGF) family, the FGF21 and FGF19 (the human orthologue of FGF15 in mice) have been supported as intriguing targets of acute liver damage (29). It has been supported that FGF19 is predominantly produced in ileum postprandially and suppresses bile acid synthesis as an important part of glucose and lipid homeostasis, whereas, FGF21 is a key metabolic regulator acting as a hepatokine and primarily enorchestrating the adaptative response to starvation (29). As far as the alcohol use disorder is concerned, there is evidence from previous studies that acute ethanol ingestion upregulates both the hepatic expression and serum concentration levels of FGF21 in previously healthy humans and in mice (27,128). It has been supported that this upregulation aims to ameliorate the devastating metabolic changes caused by alcohol intake and trigger the fat accumulation and inflammation in liver (27,128). Moreover, increased levels of FGF21 appear to play a role in suppressing the desire of ongoing consumption, which

comprises a part of a liver-brain axis that regulates the gustatory preference for ethanol and sugar (27,128).

The main aim of the present study is the examination of the exact role of chronic plus binge alcohol induced FGF21 on bile acid metabolism by using a recently established acute on chronic liver injury (ACLI) mouse model with a preexisting chronic cholestatic liver injury (61). In order to obtain a pattern of a cholestatic liver damage, ATP binding cassette subfamily B member 4 gene knock out ($Abcb4^{-/-}$) mice have been used. These mice lack the canalicular phospholipid flippase, which leads to decreased ability for secretion of phospholipid into bile (9,108) causing a type of spontaneous biliary fibrosis (108). Recent studies showed a novel suppressive effect of FGF21 on bile acids synthesis rate limiting enzyme, cholesterol 7 alpha-hydroxylase (CYP7A1), which was known to be inhibited through FGF19 in liver as a negative feedback of bile acid synthesis regulatory mechanism (17,74).

In the current study, the impact of alcohol induced FGF21 on bile acids metabolism has been investigated in mice and human. The importance of the study was to observe FGF21 response mechanism and its relation to hepatic bile acid homeostasis, which is described for the first time in an alcohol related acute-on-chronic liver injury model.

1.1 PREVALENCE, PATHOPHYSIOLOGY, COMPLICATIONS AND THERAPY OF ALCOHOLIC LIVER DISEASE

The alcohol use disorder is defined as an aberrant model of habitual ethanol consumption, leading to clinically recognized significant distress and impairment of health quality, identified by the diagnostic criteria set by the diagnostic and statistical manual of mental disorders, 5th edition (DSM-5) (at least two within 1 year) (Table 1) (28).

A pathological pattern of ethanol consumption has been found to be correlated with an increase in the disease burden and mortality (156). Data from the World Health Organization (WHO) indicate that ethanol consumption and especially alcohol use disorder are associated with elevated risk of infectious, cardiovascular, malignant, metabolic, neurologic, hepatic, traumatic, reproductive, maternal, newborn/child/adolescent and psychiatric diseases and are linked to a further hazardous behavioral pattern (unprotected sex, dangerous driving, aggressive and violent attitude) and a lower socioeconomic status (156).

1. Alcohol is often taken in larger amounts or over a longer period than was intended.
2. There is a persistent desire or unsuccessful efforts to cut down or control alcohol use.
3. A great deal of time is spent in activities necessary to obtain alcohol, use alcohol, or recover from its effects.
4. Craving, or a strong desire or urge to use alcohol.
5. Recurrent alcohol use resulting in a failure to fulfill major role obligations at work, school, or home.
6. Continued alcohol use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of alcohol.
7. Important social, occupational, or recreational activities are given up or reduced because of alcohol use.
8. Recurrent alcohol use in situations in which it is physically hazardous.
9. Alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by alcohol.
10. Tolerance, as defined by either of the following: (a) A need for marked increased amounts of alcohol to achieve intoxication or desired effect; (b) A markedly diminished effect with continued use of the same amount of alcohol.
11. Withdrawal, as manifested by either of the following: (a) The characteristic withdrawal syndrome for alcohol (refer to criteria A and B of the criteria set for alcohol withdrawal); (b) Alcohol is taken to relieve or avoid withdrawal symptoms.

Table 1: Diagnostic Criteria of Alcohol Use Disorder. The fifth edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-5) criteria for the framing of patients with alcohol use disorder (AUD) (28).

Alcoholic liver disease remains currently the dominant liver disease in Europe and the United States (11,104,112). In 2010, ALD represented 10.4% of all alcohol-attributable deaths worldwide and 47.9% of all deaths attributable to liver cirrhosis were because of alcohol intake (112). However, the exact prevalence of ALD might be underestimated since many patients with mild symptoms or within the earliest phase of the disease were not registered. The relationship between alcohol consumption and liver associated mortality has already been suggested in 1967 by Terris M et al., who found out an association between decreased rates of liver cirrhosis and decreased levels of alcohol intake especially during historical events, such as the world wars (140). Subsequently, this suggestion has been proved by WHO, indicating a positive correlation between amount of reported alcohol intake and prevalence of alcoholic liver disease in European areas (11,156). The ongoing chronic alcohol consumption may increase the risk of alcoholic fatty liver disease, even if the consumed amount is in the permitted threshold. A significant risk of ALD rises with alcohol consuming above this threshold, which is also very difficult to define because of the multifactorial nature and the diverse stages of this manifestation (111) (Figure 1). The exact risk estimation becomes more challenging in the context of binge abuse, where a precise quantification is practically impossible. In most ALD cases, a predictable and usually sequential cascade of

pathophysiological events with corresponding clinical manifestations could be recognized as depicted in Figure 1.

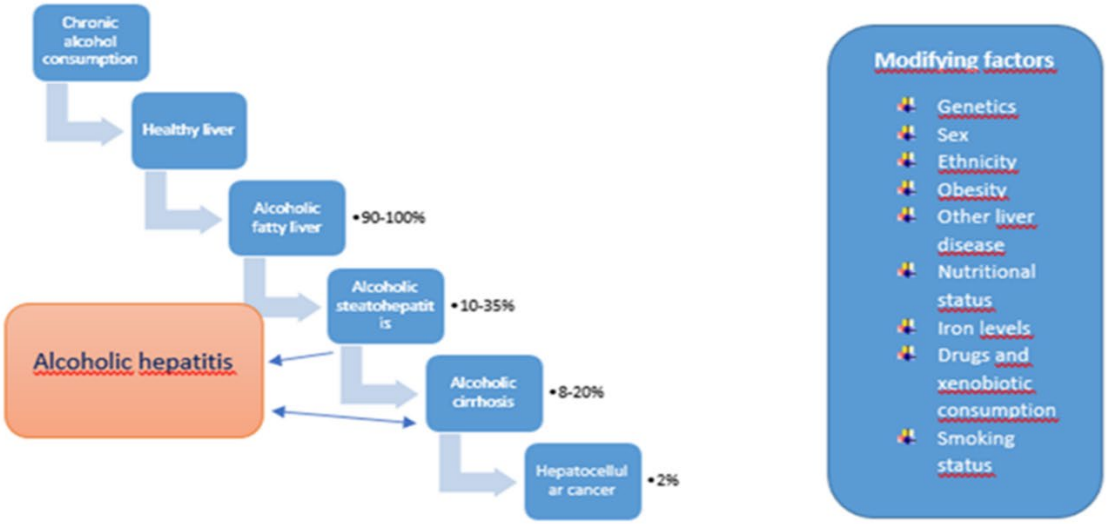

Figure 1: The typical physical course of disease progression in ALD from fatty liver disease to established liver cirrhosis with elevated risk of HCC. There are diverse factors that can unpredictably modify the time course or severity of each manifestation. Adapted from Nature Reviews/Disease Primers (120).

The state of steatohepatitis is characterized by the presence of an active inflammation in addition to alcoholic fatty liver with a percentage of 10-35% as it is shown in Figure 1. It has been supported that the existence of inflammation is an essential precondition for the structural alterations, which are typical for the disease hallmarks of liver fibrosis or cirrhosis (120). The evolvement of the disease from a pro-inflammatory situation to a function-relevant destruction comprises a cascade of events, which eventually starts with gut-derived pathogen-associated molecular patterns (PAMPs), suggesting that the gut microbiota and the gut-liver axis have crucial roles for the fueling of the inflammatory sequel (120). The pivotal consequence is finally the secretion of inflammatory cytokines from the Kupffer cells, which are one of the most important part of the mononuclear phagocyte system. This is ultimately leading to the release of damage-associated molecular patterns (DAMPs) (120). The induction of the inflammation

follows the stimulation of receptors, which are expressed not only by immunoactive cells, but also by the hepatocytes themselves, such as the Toll-like receptors (TLRs) and NOD-like receptors (NLRs) (57,58,103,107).

An inflammation induced hepatocellular damage is the prerequisite for fibrogenesis, which can be considered as a maladaptive healing response to liver injury that results in altered parenchymal structure and pericellular and perisinusoidal matrix accumulation (120). These pathologic changes affect only a fraction of the patients that have developed inflammatory steatohepatitis. However, the predisposing factors of inflammatory steatohepatitis and their exact impacts are not fully elucidated yet. An initial event seems to be the hyperactivation of hepatic stellate cells (HSCs) that can be induced by previously activated Kupffer cells (32,45,66,98), reactive oxygen species (ROS) produced by the changes in oxidative/reductive potential, direct activation of alcohol and its metabolites, and through the endoplasmic reticulum stress (120). Indeed, a proinflammatory state is favored by means of inhibition of anti-inflammatory agents and the fibrogenetic processes, such as activation of natural killers (NK) and production of interferon γ (IFNγ), which cause the apoptotic cell death of the activated HSCs (59,110). The main process of the fibrogenesis is the uncontrolled proliferation and activation of the HSCs and the excessive production of extracellular matrix, which destructs the parenchymal architecture of the liver. As the fibrosis progresses, the liver becomes cirrhotic and impaired blood flow in the hepatic vessels, biosynthetic dysfunction and predisposition to cholestasis take place (59,110,120).

The final stage of ALD is the progression of the disease to liver fibrosis, cirrhosis and subsequently to hepatocellular carcinoma (HCC), which is the most relevant issue in the clinical praxis. The elevated risk of HCC with the progression of advanced fibrosis or cirrhosis has been suggested to be dependent on alcohol intake and subsequently occurred functional and structural changes caused by fibrogenesis (7). Alcohol is considered to be carcinogenic because of its metabolite acetaldehyde, which is the primary carcinogenic substance (7). Acetaldehyde acts directly on DNA in a mutagenic way, inhibiting its self-repairing capacity through inactivation of the enzyme O6-methylguanine DNA methyltransferase (83,142,155). Further predisposing factors, which are associated indirectly with alcohol consumption and linked to fibrogenesis, are the release of ROS, which induce the production of carcinogenic metabolites that activate preexisting procarcinogens, such as nitrosamines, and methylation alterations causing epigenetic chromosomal instability (44,147,150). The pathophysiological cascade of ALD progression from excessive alcohol consumption to cirrhosis with increased risk of carcinogenesis is depicted concisely in Figure 2.

There are numerous risk factors, which affect the vulnerability of ALD development or its clinical complications individually. Among these risk factors, genetic susceptibility is also strongly assumed with the data indicating an association between genetic proneness and alcohol consumption and ALD development (133). This fact partly explains why only a fraction of human population has an established disease susceptibility following alcohol abuse. In this regard, patatin like phospholipase domain-containing protein 3 (*PNPLA3*), transmembrane 6 superfamily member 2 (*TM6SF2*) and membrane-bound O acetyltransferase domain-containing protein 7 (*MBOAT7*) genes have been considered to be crucial for the development and severity of ALD (12,115,132). The induction of a proinflammatory and fibrogenetic state enables the gradual progression of the disease. It is subsequently appealing to assume, that genetic alterations concerning the production of cytokines or chemokines and the inflammatory responsiveness could have a pivotal role as determinants for the severity of alcohol induced disease.

The diagnosis of ALD requires biochemical, sonographic and histopathological evidence of liver disease in addition to a significant alcohol use disorder (AUD) state. However, the detection of AUD is difficult in the clinical praxis since the amount of ethanol consumption or time of consuming is in most cases impossible to be objectified or quantified. In addition, the measurement of alcohol metabolites in plasma or urine is not common in the clinical routine, with the exception of several groups, i.e. the patients on a transplantation list. Biochemical markers, such as elevated transaminases, bilirubin, alkaline phosphatase and γ glutamyltransferase, biomarkers of reduced biosynthetic capacity, such as low albumin or elevated international normalized ratio (INR), the sonographic liver pattern (echogenicity and homogenicity of the parenchyma, tumours, cholestasis, and structural deformation) and the result from transient elastography can together be confirmed with the clinical evaluation to stage ALD. Specifically during a transient elastography, the control attenuation parameter (quantifiable in the context of attenuation of shear waves) has a high diagnostic accuracy especially for severe hepatic steatosis, although it could not appropriately distinguish its cause (30,56,143). The measured liver stiffness can diagnose cirrhosis in a non-interventional way showing sufficient correlation with the histological stage of fibrosis (89). The cutoff values for staging liver fibrosis are shown in Figure 3 in the setting of an acute liver disease. In this context, it remains disputable if the diagnosis should be histopathologically confirmed via liver biopsy, because of the associated complications, such as bleeding (36).

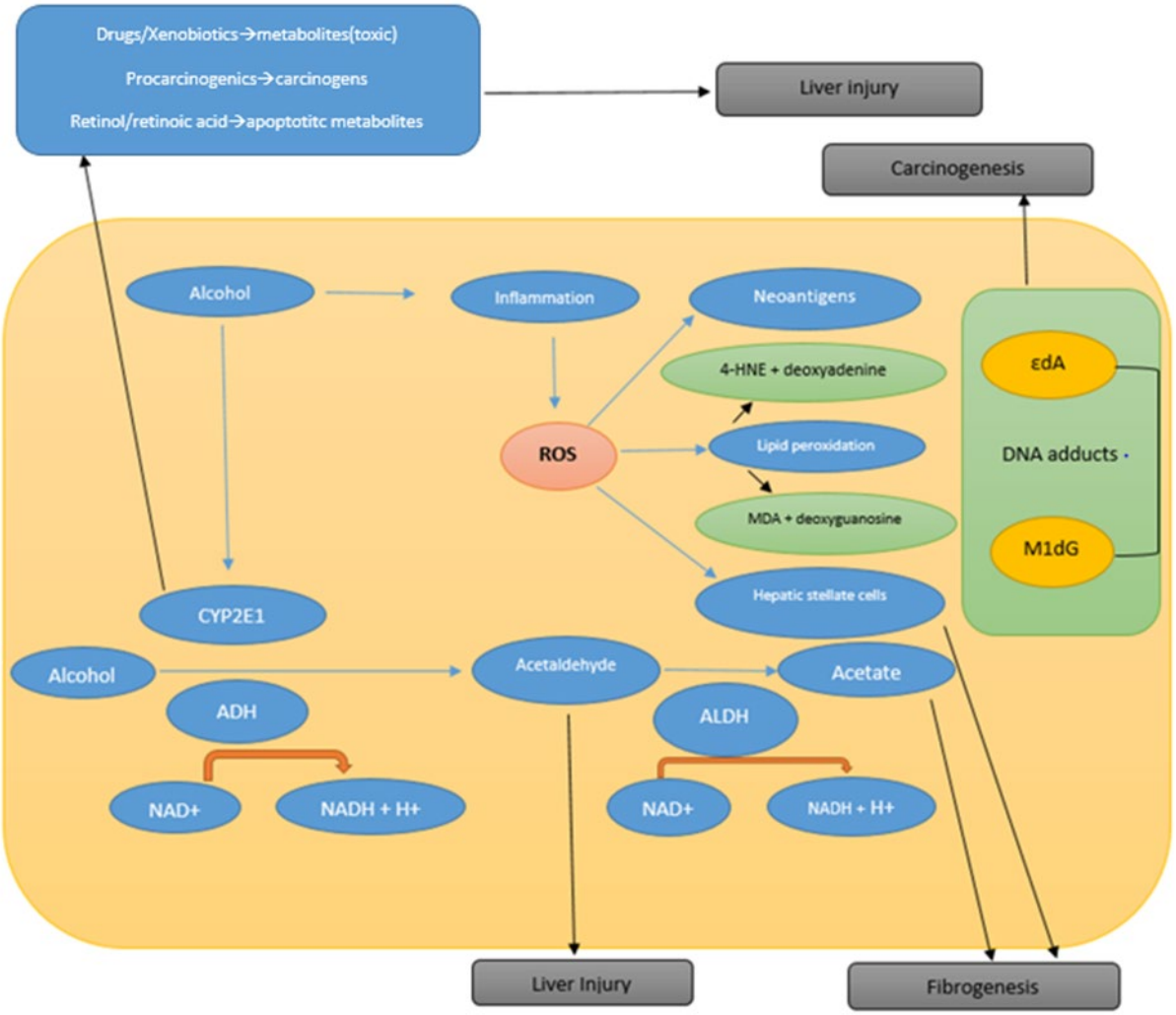

Figure 2: Pathomechanisms linked to induction of alcoholic liver diesease and progression from fatty liver to cirrhosis with elevated risk of carcinogenesis. ROS: reactive oxygen species, ADH: alcohol dehydrogenase, NAD: nicotinamide adenine dinucleotide, ALDH: aldehyde dehydrogenase, εdA: 1,N6 -etheno-2′-deoxyadenosine, M1dG: 3-(2-deoxy-β-d-erythro-pentofuranosyl)pyrimido(1,2-α)purin-10(3H)-one, 4-HNE: 4-hydroxynonenal, MDA: malondialdehyde. Adapted from Nature Reviews/Disease Primers (120)

The milestone of ALD management is alcohol cessation, which is challenging and necessitate the interdisciplinary collaboration especially between hepatologists and psychiatrists. A combination of psychological and pharmacological treatment can be proved contra-intuitively very effective with long time maintenance of the succeeded result. The abstinence therapy needs in some cases hospitalization and hepatological follow up (1,5,127).

In the state of alcoholic steatohepatitis supportive care is recommended such as albumin substitution and sufficient energy intake. In order to reduce inflammation, corticosteroids are

administered in the case of an acute alcoholic hepatitis (125,145). On the other hand, additional administration of pentoxifylline did not offer any additional advantage (82). On the contrary, N-acetyl cysteine administered with corticosteroid has been shown to be effective in improvement of liver function (94). Liver transplantation remains to be ultimate treatment modality for patients with severe cirrhosis or hepatic malignancy and should be evaluated relatively early in the course of the disease, as it is associated with better outcome in early stages (55,71,81,134).

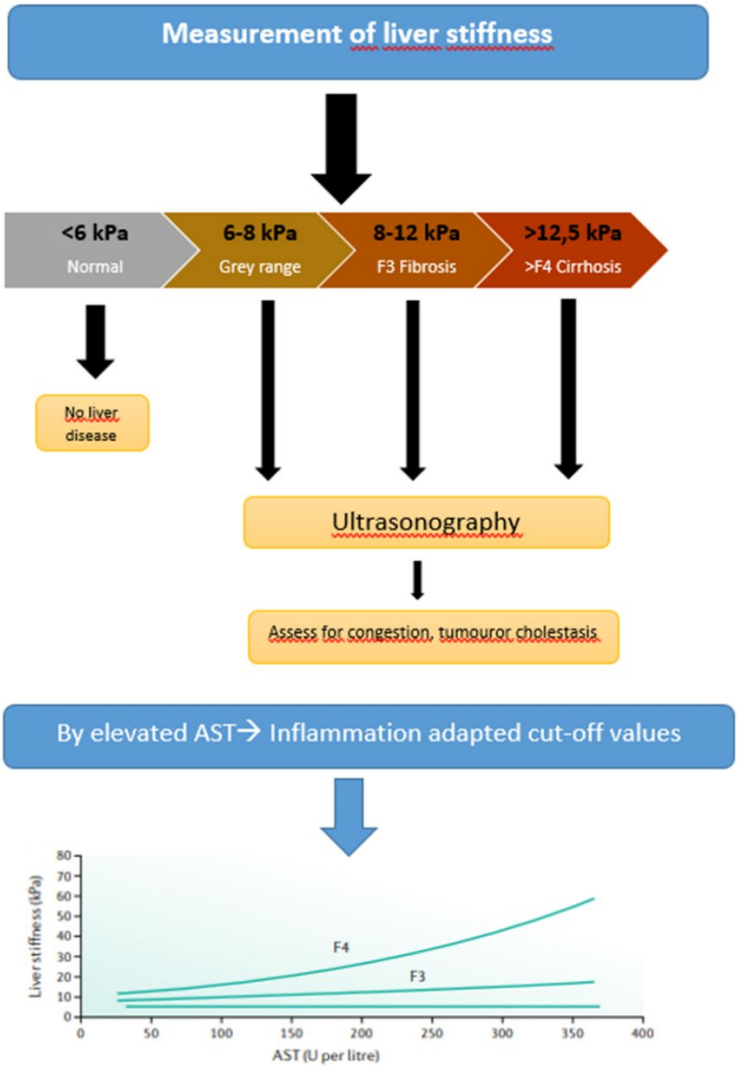

Figure 3: Algorithm for clinical interpretation of liver stiffness in ALD. AST (Aspartate Transaminase) adapted cut-off values for liver stiffness for staging of fibrosis. These values can differ in liver fibrosis of different etiology. Adapted from Nature Reviews/Disease Primers (120)

1.2 THE ROLE OF FGF21 IN ALCOHOLIC LIVER DISEASE

Fibroblast growth factor 21 was identified as a member of the subfamily of FGF19, the so-called endocrine fibroblast growth factors, and was initially considered to be a mainly in liver produced hepatokine, inducing the adaptive response to starvation (97). The fibroblast growth factors family is an evolutionarily highly preserved group of growth factors. Among the vertebrate species, between 19 and 27 FGF genes have been identified. Both mice and humans possess 22 (FGF1 to FGF23, with FGF19 being the human orthologue of FGF15 in rodents), which are furthermore classified in seven subclasses based on their phylogenetic similarity (29,97). Emerging data though indicate FGF21 as a peptide hormone, with endocrine, paracrine and autocrine function, produced and acting in diverse tissues and overtaking a pivotal role in the regulation of metabolism. The stimuli for the expression of FGF21, the precise manner of action and the downstream signaling cascade are inherently complicated and not yet fully clarified (41,131).

The signaling cascade of FGF21 is inducted through stimulation of its fibroblast growth factor receptor (FGFR) with seven isoforms having been identified hitherto in mammals, which are characterized as 1b, 1c, 2b, 2c, 3b, 3c and 4 (65). They are membrane receptors with an extracellular domain, which binds the ligand. They consist of three immunoglobulin-like subdomains, a transmembrane helix (a-helix) and an intracellular domain, which exhibits activity of tyrosine kinase. There is a very weak induction of downstream signal after the binding of FGFs to their relative receptors, so that the existence of an obligate co activator for the complete signal transduction was very early hypothesized. This co-activator is currently known to be the β-Klotho (KLB), an enzyme encoded by the *KLB* gene being the part of the three klothos subfamilies, α, β and γ (116). Different fibroblast growth factors show preference and affinity to different receptors. The preference of FGF21 has previously been reported to be FGFR1c/KLB and FGFR3/KLB (136,164). Howbeit, the 2c and 4 receptors have been suggested to be activated by FGF21 as well, even though with lower affinity (65,107). The downstream cascade is complicated and multidimensional, with the best described events being the stimulation of mitogen-activated protein-kinase (MAPK), a pathway which includes the RAF proto-oncogene serine/threonine-protein kinase (Raf-1) and the extracellular signal-regulated kinases 1 and 2 (ERK 1, 2) (38,40,131).

The first data emphasized the physiological and pathophysiological role of FGF21 in regulating mainly the glucose metabolism and energy expenditure, coordinating the response towards food deprivation (41). Further studies supposed the role of FGF21 in energy expenditure with its effect on weight loss in mice, although the food intake was not predominantly altered

(23,159). In addition to its role in energy expenditure, FGF21 was shown to have a role in improving metabolic biomarkers in diabetic monkeys and mice (64). Stepwise upregulation of FGF21 was observed after fasting and interestingly after consumption of a ketogenic diet (41). Both events include the reduction of supplied amino acids and the shift of metabolism towards catabolic processing of lipids in a common manner, which results in excessive amount of fatty acids. The key event of this upregulation of FGF21 upon fasting and high-fat low carbohydrate ketogenic diets has been shown to be the fatty acids induced activation of peroxisome proliferator activated receptor a (PPARa), which is considered to be the upstream mediator of FGF21 (8).

It is furthermore noteworthy that FGF21 can cross the brain blood barrier and pass intact to the brain in both humans and rodents, in an apparently unsaturable fashion, as there is a linear correlation between levels in plasma and cerebrospinal fluid (54, 138). Accordingly, the corresponding receptors FGFR1, FGFR2 and FGFR3 and the obligate co-receptor β-Klotho (KLB) can be detected in brain cells as well, although the ascertained amount of KLB is reported to be fairly low (83). One of the major sites of action of FGF21 intracerebrally seems to be the hypothalamic suprachiasmatic nucleus (SCN), which has been reported to be the crucial location of the FGF21 signaling in the central nervous system (41). The suprachiasmatic nucleus (SCN) is known to be a pivotal center for the circadian rhythmicity in vertebrates and overtakes an important role for the regulation of temperature, which could point to an interaction with the FGF21 induced thermogenesis in adipose tissue (113). Moreover, the crosstalk between FGF21 and SCN could create a circadian rhythmicity in diverse metabolic procedures. As it is known, the food intake in different time periods during the light and dark cycle can have dramatic effects on metabolism and a disruption in this rhythmicity could be one of the major contributors to obesity or diabetes mellitus (92). The SCN coordinates furthermore many behavioral performances and psychological processes (135). It is compelling to hypothesize that the function of FGF21 in this brain area could be a ligand between metabolism and behavior, as many metabolic disorders are accompanied with psychological distress and vice versa.

The effect of FGF21 on ALD is binary and seems to affect both excessive alcohol consumption and progression of the hepatic damage itself. Given the metabolic and cholestatic modifications and complications of binge and chronic alcohol intake and the suggested metabolic role of FGF21, it was reasonable to be presumed that FGF21 could someway overtake a protective action against the pathophysiological and structural alterations, which ultimately lead to liver fibrosis and cirrhosis. Therefore, it can be furthermore intriguingly postulated that the activity of FGF21 in the central nervous system, especially its hypothalamic actions, could have consequences in the behavioral attitude towards alcohol consumption.

There are emerging data indicating an upregulation of plasma FGF21 in humans after binge alcohol drinking (27,67,128). Soberg et al. showed a 3.4-fold increase in FGF21 plasma levels in healthy and fasted volunteers after consumption of approximately 25.3 grams of alcohol (128). They further observed 2.1-fold increased levels of circulated FGF21 after binge drinking during three days (by healthy volunteers at the Oktoberfest), without any relevant induction through an aggravation of hepatic biomarkers (128). These findings are supported by the study of Lang et al. who documented a 9-fold increase in plasma levels of FGF21 after both oral and intravenous administration of ethanol (0,7g/kg body weight) (67). The upregulation of FGF21 was observed approximately after 30 minutes (67). The authors could furthermore find out that this increase was accompanied with a decrease in insulin, C-peptide and glucagon with unaffected levels of gastric inhibitory polypeptide (GIP) and glucagon-like peptide 1 (GLP1), implying an incretin-independent influence of FGF21. The observed results were also independent from the route of alcohol administration. Desai et al. had previously demonstrated that upon binge alcohol intake, the peak of FGF21 elevation (in both mice and humans) occurs approximately one hour after consumption and FGF21 returns to the baseline about 6 hours afterwards (27).

It was concomitantly intriguing that FGF21 seems to have an effect on drinking habits in both rodents and humans (42,84,119,154). A negative feedback circuit enorchestrated by FGF21 has also been observed, which suppresses the appetite for alcohol consumption during the ongoing intake (42,84,119,154). Findings from rodents further supported the bidirectional correlation between alcohol intake and FGF21 levels (31,128,154). It has been recently shown that FGF21 suppresses the consumption of sugar and alcohol in mice by inhibiting their appetite for these (154). This was not surprising since alcohol is the product of alcoholic fermentation, which is the biologic process of conversion of sugars to glucose, fructose and sucrose. The latter becomes more meaningful considering that fructose intake is one of the most known factors that upregulates FGF21 (31). The intake of other macromolecules, such as lipids, proteins and complex carbohydrates seem to remain unchanged by FGF21 induction (154). Its direct effect on appetite is believed to be exerted via action in hypothalamic nuclei, as FGF21 can cross the blood-brain barrier and its main receptor FGFR1c and co-receptor KLB are expressed in many cerebral structures (154).

Another interesting study from Song et al. confirmed an FGF21-mediated stimulation of water intake in response to alcohol consumption (and ketogenic diet as well) in a vasopressin independent manner (129). During alcohol intake this could be a counteractive mechanism targeting the alcohol dilution. Another study from Turner et al. has observed an increase in water intake, urine output and blood pressure in rats, which was stimulated by FGF21

regardless of alcohol (149). These evidences, putting together, imply a new endocrine role of FGF21 in the regulation of serum and urine osmolality and balance of fluids and electrolytes.

Summarizing the existing data, alcohol induced upregulation of FGF21 in serum has been documented in both humans and rodents. This induction of FGF21 expression in liver has been proposed to be hepatoprotective when liver homeostasis is challenged by various stimuli, as in the case of alcohol (27, 39). In the course of ALD, the main metabolic alterations in liver, including fat accumulation and a shift in bile acids biosynthesis and metabolism, are therefore supposed to be affected from alcohol induced FGF21. Thus, with the sparse and controversial preexisting evidences, the revealing interactions between FGF21 and bile acids metabolism were investigated in the present study.

1.3 THE ROLE OF BILE ACIDS METABOLISM IN ALCOHOLIC LIVER DISEASE

Bile acids (BA) are hydroxylated steroids produced by hepatic cholesterol metabolism through the catalytic activation of peroxisomal enzymes. Bile acid pool comprises various amphipathic acidic steroids that have important roles in the regulation of multiple functions in liver cells. In human primary bile acids are cholic acid and chenodeoxycholic acid, which are produced directly in the hepatocytes via a cytochrome-P450 mediated oxidation of cholesterol (18,51,114). The secondary bile acids are produced via bacterial actions in colon. In humans, the most important secondary bile salts are taurocholic acid and glycocholic acid (derivatives of cholic acid) and taurochenodeoxycholic acid and glycochenodeoxycholic acid (derivatives of chenodeoxycholic acid (51).

In several studies, altered hepatic bile acid synthesis and hepatic accumulation have been found to be the potential major pathophysiological background for the induction of the structural and functional hepatocyte changes in both alcoholic steatohepatitis and non-alcoholic fatty liver disease (NAFLD) (16,157,158). Because of their amphipathic chemical structure and the distinct biochemical properties a possible detrimental effect has been presumed for the hepatocyte in case of cholestasis (43). Two main factors which influence the cytotoxic potential of bile acids are their solubility in water and their conjugation with other molecules, such as glycine or taurine (52). The cytotoxic role of bile acids in the hepatocytes initially induces acute liver injury (ALI) in a case of insufficient activation of hepatoprotective mechanisms (46,117,157). The acute liver injury is defined as objectified hepatocellular damage upon a hepatotoxic agent, biochemically ascertained with elevation of serum aminotransferase

activities, conjugated bilirubin and alkaline phosphatase levels (117). If the cellular damage becomes persistent or permanent, the disease proceeds to chronic liver injury (CLI). The influence of the same or a different hepatoxic factor can cause a deterioration of the preexisting damage, a situation characterized as an acute on chronic liver injury (ACLI). Acute on chronic liver failure (ACLF) is defined as the rapid deterioration of a preexisting liver injury due to induction of a cascade of events, including inflammation, cytotoxicity and systematic involvement where the liver fails in fulfilling its physiological metabolic role (117). The liver failure is associated with excessive morbidiy and mortality, dysfunction or failure of multiple organs and requires immediate treatment (6).

The key events affecting BA metabolism upon alcohol consumption have been reported to be their increased levels in liver, increased reabsorption in the enterohepatic circulation and increased excretion of more lipohilic and toxic bile acids (78). Therefore, the bile acid feedback loop overtakes an essential role for protection from liver damage. The major part of bile acids in liver is conjugated to glycine, taurine and sulfate (20,78). They are transported through the bile ducts in the duodenum, where they assist the emulsification of lipids, together with biliary lecithin (30,143). The BA micelles are then reabsorbed in the distal ileum, leading the way to enterohepatic circulation, in order for excessive intestinal loss to be avoided. So the negative feedback loop is constituted by the hepatic reabsorption of bile acids, which further inhibits cholesterol conversion and BA biosynthesis partly (30,141,143).

Bile acids stimulate many receptors such as the Farnesoid X Receptor (FXR) and a G protein-coupled receptor (TGR5) (141). The BA signaling via FXR seems to enorchestrate hepatic and intestinal functions, whereas the stimulation through TGR5 exerts endocrine, metabolic and neurologic actions (37). The critical rate-limiting step of BA biosynthesis is the expression of the enzyme cholesterol 7 alpha-hydroxylase (CYP7A1), which oxidizes cholesterol in the position 7 using molecular oxygen (18,114). CYP7A1 induces the classical BA synthesis pathway, in which the relative amount of primary bile acids is regulated through CYP8B1, which is a sterol 12-a-hydroxylase, located in the membrane of endoplasmic reticulum (19,20). The catalytic action of CYP8B1 leads to preferred synthesis of cholic acid, whereas chenodeoxycholic acid is produced in its absence. There is an alternative (acidic) pathway for BA synthesis that is mediated through sterol-27-hydroxylase (CYP27A1), which is a cytochrome P450 oxidase located in mitochondria (Figure 4). It is the initiating enzyme of this pathway and believed to overtake an upgraded role in the metabolism of BA in the presence of a liver disease (19).

Figure 4: Classical and alternative pathways of the bile acids biosynthesis. AKR: Aldo-Keto Reductase Family, CYP: Cytochrom P450, HSD: Hydroxysteroid Dehydrogenase. Adapted from Journal from Hepatology (20).

The feedback regulation of the CYP7A1 has been shown to be mediated mainly by a nuclear receptor signaling cascade including FXR and the small heterodimer partner (SHP) (37,78,157). Induction of intestine farnesoid X receptor/fibroblast growth factor 19 (FXR/FGF19) and activation of the farnesoid X receptor/small heterodimer partner (FXR/SHP) axis in liver by elevated bile acid levels have been suggested to be the main mechanisms for inhibition of bile acid synthesis, through suppression of *CYP7A1* transcription (37,78,157). This repression takes place, because elevated bile acid concentrations in the ileum activate FXR, which induces the secretion of the enterokine FGF19 (FGF15 in mice) into the portal circulation

and the consequential stimulation of fibroblast growth factor receptor 4 (FGFR4)/β-Klotho (KLB) complex in the hepatocyte membrane (37,78,157). Furthermore, a recently discovered protein, DIET1, has been suggested to be a regulator of FGF15/19 and its suppressive role in BA synthesis (152). DIET1 is a highly conserved protein, consisting of 2.123 amino acids, which is mainly expressed in the epithelial cells of the intestine (152). Vergnes et al. proved that DIET1 deficiency causes decreased levels of intestinal FGF15 in mice causing a negative impact on the ability of the bile acids regulation in the enterohepatic circulation (152). As a conclusion, the intact production and function of DIET1 appears to be an important factor for the integrated signaling in the FXR-FGF15/19-BA feedback loop, at least at the first part of the circuit (Figure 5).

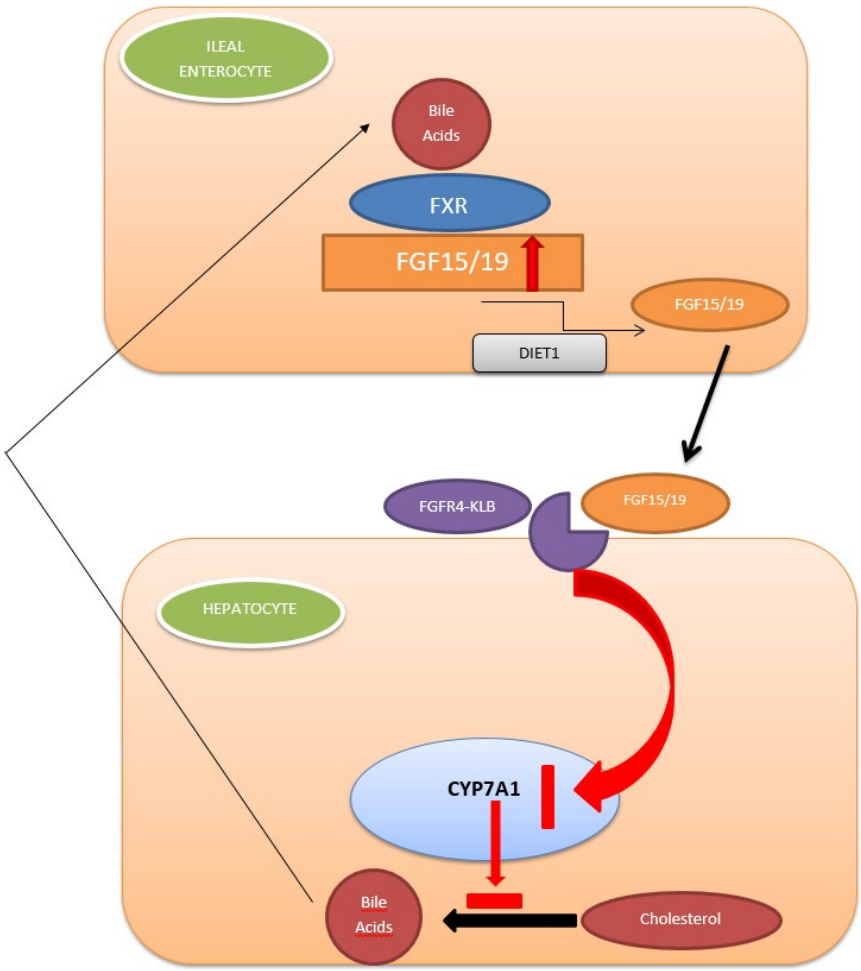

Figure 5: The negative feedback circuit of the regulation of bile acids biosynthesis through stimulation of FXR and induction of FGF15/19.CYP7A1: cholesterol 7 alpha-hydroxylase FGF15/19: Fibroblast Growth Factor 15/19, FXR: Farnesoid X Receptor, FGFR4: Fibroblast Growth Factor Receptor 4, KLB: beta Klotho. Adapted from Physiology of the Gastrointestinal Tract (Sixth Edition) (25).

A previously unidentified role of FGF21, as a negative regulator of bile acid (BA) synthesis, has been described recently (17,163). Chen et al. showed hence that FGF21 is a negative regulator of BA synthesis and supported its activation in an FGF15/19 independent manner (17). However, the underlying mechanisms of this effect remain unclear. It is howbeit reasonable to hypothesize that the signal transduction happens in one of the identified downstream signaling pathways of FGF21. FGF21 initially binds to the complex of KLB with one of its main receptors (FGFR1c, FGFR2c, FGFR3c and FGFR4) (2) and leads to activation of two main downstream pathways including the extracellular-signal-regulated kinases/ Mitogen-activated protein kinase (ERK/MAPK) and the mammalian Target of Rapamycin (mTOR) pathways (85). However, the role of FGF21 in biosynthetic regulation of bile acids has been shown to be more controversial and remains to be clarified.

The supported suppression of PPARa through SHP could lead to the initial hypothesis that FGF21 suppression is a predisposition to *CYP7A1* suppression (163). This implies an antagonistic role for FGF15/19 and FGF21, concerning the regulation of BA production. Although the signaling cascades are not well known, Minard at al. has supported that the major signaling of FGF21 occurs through mTORc1 in adipocytes (85). In hepatocytes, FGF21 has been suggested to induce the phosphoinositide 3 kinase/protein Kinase/mammalian target of Rapamycin (PI3K/AKT/mTOR) downstream signaling cascade, a fact that further implies mTOR as crucial node for FGF21 action in liver (90). Another potential signal mediation of FGF21 in the hepatocytes via the PI3K/AKT pathway suggests a possible role of a member of the sterol regulator element binding proteins (SREBPs), SREBP-1c, as a mediator of FGF21 signaling. There is a previous evidence that the PI3/AKT pathway is involved in the control of *SREB1c* mRNA expression levels (47). Moreover, SREBP1c is positively correlated with non-alcoholic fatty liver disease (NAFLD), probably activating the expression of *PNPLA3* gene and promoting fat accumulation in liver (87,109,130). In this context, there is emerging evidence for the existence of an FGF21-SREBP1c pathway, which acts as negative feedback circuit (166).

Although the concrete roles of FGF21 and FGF15/19 in ALD have not been studied in details yet, there are published evidences for their hepatoprotective role in liver damage regardless of causative background (27,39,153). An early observation was the contribution of FGF15/19 mediated reduction of bile acids synthesis to the attenuation of liver damage, by ameliorating cholestasis (86). This positive effect is howbeit to a certain degree counteracted through the mitogenic effect of FGF19, which has been suggested to promote HCC development both in a situation of marked endogenous upregulation or exogenous application (4,72). In the physical

course of an acute or chronic liver damage this ambiguous biological behavior becomes relevant. It could for instance offer an explanation for the fact that a preexisting steatosis negatively affects the liver ability to be regenerated (21,53,62). Consistent evidence has also emerged for the potential hepatoprotective role of FGF21 (27,39,160). As more detailed cited above the alcohol intake is a robust inducer of elevated plasma levels of FGF21. This upregulation could furthermore be ascertained under conditions of liver injury challenged by various stimuli in addition to alcohol challenge (160), which could be PPARa dependent or independent (122). In addition, the significance of the elevated levels of FGF21 has been proved by showing a deterioration of liver damage upon hepatotoxic agents in *Fgf21* knock out (KO) mice as compared with their wild type counterparts (122).

Moreover, the role of the BA in the physical course of liver disease poses the question about possible pharmacological effect of the lipophobic and cytoprotective ursodeoxycholic acid (UDCA) on cholestatic damage caused by alcohol. Positive evidence has indicated that UDCA and TUDCA (tauroursodeoxycholic acid, the conjugated molecule) proved a favorable effect against liver damage in human hepatoblastoma (HepG2) and hepatoma (SK-Hep-1) cells (24,50,94). Convincing data for the hepatoprotective role of UDCA in alcoholic liver disease denoted analogous treatment efforts in rodent livers (96). These data urged the beginning of clinical trials in patients with alcoholic cirrhosis, howbeit with hitherto controversial results. During clinical trials of control of UDCA versus placebo, the use of UDCA resulted in significant decrease of transaminases and bilirubin in the UDCA treated groups, but lower survival rates and more complications as well (102,105). The emerging data are auspicious but mandate further evaluation to establish a potential pharmacological value for UDCA. What furthermore remains unclear is the precise mechanism of amelioration of alcohol induced liver damage via UDCA, with many proposed pathways, such as improving impact on ATP synthesis in mitochondria, decrease of oxidative stress and oxidative damage and restoration of intracellular Prostaglandin E and linoleoyl-CoA-desaturase (75,93,137,151). All the above pathways are altered and become dysfunctional in the physical course of a liver injury.

In summary, it is compelling to clarify if and alcohol induced serum FGF21 targets not only the suppression of further intake via hypothalamic stimulation but also the early development of a defensive mechanism against liver damage. Previous studies support its hepatoprotective effect against liver damage of different etiologies. In addition to its hepatoprotective role, its recently revealed role in BA metabolism needs further investigation, in both healthy liver and during cholestasis. Therefore, in this study, the crosstalk and interactions between FGF21 and bile acids was investigated after chronic plus binge alcohol consumption in an alcohol related acute-on-chronic liver injury model.

1.4 CHARACTERIZATION OF THE MOUSE MODEL

In the conducted murinal experiments, mice with previously healthy liver (C57BL/6J) and a mouse model with a preexisting liver damage (*Abcb4*$^{-/-}$) have been used. C57BL/6J is a commonly used inbred mouse type and represents a good model for biological studies of several disease patterns, like atherosclerosis, obesity and diabetes (99,118). This mouse model drinks alcoholic beverages deliberately, which made it an ideal candidate for the planned studies with use of ethanol including diets. The model has been previously widely used and its characteristics and behavior are predictable (10).

The ATP-binding cassette 4 gene (*Abcb4*) knockout mice, also referred as multidrug resistance protein 2 (*Mdr2*) knockout, represent a model with a preexisting cholestatic liver injury, mimicking primary sclerosing cholangitis and biliary fibrosis (9). ABCB4 is a member of the family of ATP–binding cassette transporters and MDR/TAP subfamily. The proteins of the superfamily are translocators, enabling the intra- and extracellular transportation of various molecules (9,108). The proteins of the MDR subfamily undertake various functions, such as development of multidrug resistance (as the name of the subfamily implies), antigen presentation during the immune response and function as membrane transporters (9,108). In this frame, ABCB4 is implicated in the transportation of phosphatidylcholine from the hepatocytes into the bile, acting as member of the p-glycoprotein family of membrane proteins. In this way, it can be characterized as a floppase, namely a lipid-transporting glycoprotein, located on the cytoplasmic membrane, with transporting direction from the cytosol to the extracellular space (9,108). Its exact role has not been fully elucidated, but it is supported to have a crucial task in the excretion of phospholipids generally into the bile. Supporting for this is the fact, that the ABCB4 transporter on the hepatocytes is almost exclusively located on the canalicular side of the membrane (108). Consequently, an ABCB4 deficiency results in an unfavorable change in the proportion of phosphatidylcholine to bile salts in the bile acids pool, which precipitates the induction of cholestatic liver damage (108). In liver, phosphatidylcholine is the most abundant phospholipid and its alterations in the presence of ABCB4 deficiency are considered to occur due to changes in biosynthesis, catabolism and secretion into the bile and plasma. However, the secretory defect is suggested to bear the most crucial role for the pathogenetic cascade ultimately leading to cholestasis (9).

The cholestatic profile of *Mdr2* knockout mice has been indeed described in a previously published study, where a nearly completely lack of cholesterol and phosphatidylcholine in the homozygous deficient mice and a consequent cholestatic liver disease was ascertained (126). The lack of phosphatidylcholine in the bile increases the bile toxicity by disrupting the formation

of protective micelles with both cholesterol and bile salts (146). The *Mdr2* deficient mice develop gradually biliary fibrosis (similar to primary sclerosing cholangitis) and have elevated risk of HCC (35). A similar cholestatic pattern appears in humans with MDR3 (the human orthologue of murinal MDR2) deficiency (26). These patients have a markedly elevated potential to develop progressive familial intrahepatic cholestasis type 3. They show furthermore a predisposition for drug-induced liver damage and the females for intrahepatic cholestasis of pregnancy (26). The pathophysiological background is similarly the decreased amount of excreted phosphatidylcholine into the bile, through a defect of the canalicular transporter (26).

In the current study, the *Abcb4*−/− mouse model was combined with a standardized chronic-plus-binge ethanol feeding model (NIAAA) described previously by Bertola et al. (10). This model has been suggested to represent in a satisfactory way the pattern of alcohol abuse in patients with alcoholic liver disease and could therefore offer the preconditions to compare the findings of the murinal studies with those of the translational studies in human patients. An alternative alcohol consumption pattern of ad libitum alcohol ingestion for 4-6 weeks causes only mild hepatic fat accumulation, without virtually induction of inflammation or severe grade of steatosis (10). Thus, it has not been considered as an adequate experimental model for detection of the impact of alcohol on the biosynthesis of the bile acids and the endocrine fibroblast growth factors.

Bertola et al. have also used the C57BL/6J mouse model and combined ad libitum ethanol including diet (5% v/v ethanol liquid diet) for a time period of ten days with a following insult of binge alcohol intake (10). More recently, applying the above mentioned NIAAA feeding model, a mouse model with the *Abcb4/Mdr2*$^{-/-}$ genotype was established by Karatayli et al. Since the *Abcb4* knockout mice display the prediscussed cholestatic liver injury, additional excessive alcohol ingestion acts in this frame as a second insult, which deteriorates the preexisting pathology, leading either to a histological and biochemical worsening (ACLI, acute on chronic liver injury) or to a complete loss of the major hepatic functions (ACLF, acute on chronic liver failure). The biochemical and histopathological findings of the proposed mouse model established by Karatayli et al. has been shown to be a novel promising approach to model alcohol-related ACLI (61), which represents a reliable and convincing pattern for the physical course of alcohol abuse in human patients.

2. STUDY AIM

In the current study, we aimed to investigate the impact of chronic plus binge alcohol intake on tissue production and circulating levels of FGF21 and FGF15/19 and bile acid metabolism in a recently established ACLI mouse model with preexisting chronic cholestatic liver injury (61). It was initially hypothesized that ethanol intake will upregulate both hepatic expression of FGF21 and ileal expression of FGF19 and that the elevated production of these endocrine fibroblast growth factors will suppress the rate of bile acids synthesis, thus ameliorating ethanol-induced liver injury. It was further suggested that FGF21 would have a direct impact on regulating bile acid synthesis, acting independently of FGF15/19 in the enterohepatic circulation. It was assumed that the suggested ethanol-induced upregulation of FGF15/19 and FGF21 will be preserved even in the frame of preexisting cholestatic liver injury.

For this purpose, an animal study was conducted by using wild-type (WT, C57BL/6J) mice and the ethanol-induced acute-on-chronic liver injury (ACLI) model in *Abcb4*$^{-/-}$ mice (KO). By using different mouse groups, either fed control diet (WT/Cont and KO/Cont groups) or ethanol diet (WT/EtOH and KO/EtOH groups), the impact of chronic plus binge alcohol-induced FGF21 on bile acid metabolism should be investigated. To investigate the direct effect of FGF21 *in vitro*, recombinant human FGF21 was added to primary mouse hepatocytes. Finally, we assumed that our preclinical findings would sufficiently resemble the pathophysiological alterations of bile acid metabolism and endocrine fibroblast growth factors in human patients with alcohol-associated liver disease and ongoing alcohol use disorder. In order to translate the findings from mouse studies, human subjects with ongoing alcohol consumption and alcoholic liver disease and healthy control subjects were recruited for our study.

3. MATERIAL AND METHODS

3.1 STUDY DESIGN

3.1.1 MURINAL EXPERIMENTS

In order to clarify the impact of alcohol intake on the endocrine fibroblast growth factors and the biosynthesis of bile acids, both in a healthy liver and by preexisting cholestatic liver disease, we designed an experimental study with two mouse genotypes, the first wild type (WT, C57BL/6J) and the second with a cholestatic liver pattern, similar to primary sclerosing cholangitis (*Abcb4* KO mice). Each group consisted of 56 mice, with equal number of males and females. The two groups were furthermore divided into two subgroups, the first fed with control diet and the second with ethanol diet. The used diet models are described in detail below and rely on previously described models. We determined following parameters at the baseline and after ethanol challenge: aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl-transferase (GGT), serum ethanol, bile acids, FGF15, FGF21 and mRNA expression levels of sterol regulatory element binding transcription factor 1 (*Srebf1*), Fibroblast growth factor receptor 4 (*Fgfr4*), Fibroblast growth factor receptor 1 (*Fgfr1*), cytochrome P450 family 7 subfamily A member 1 (*Cyp7a1*), Fibroblast growth factor 21 (*Fgf21*), Farnesoid X-Activated Receptor (*Fxr/Nr1h4*), Klotho beta (*Klb*), Mechanistic target of rapamycin (*Mtor*), Peroxisome proliferator activated receptor alpha (*Ppara*), Small heterodimer partner (*Shp/Nr0b2*), Cytochrome P450 family 27 subfamily A member 1 (*Cyp27a1*), and Cytochrome P450 family 8 subfamily B member 1 (*Cyp8b1*) as well as ileal mRNA levels of MAM and LDL receptor class A domain-containing 1 (*Malrd1/Diet1*), Fibroblast growth factor 15 (*Fgf15*) and Farnesoid X-Activated Receptor (*Fxr/Nr1h4*). After the sacrifice of the mice liver tissue was obtained to perform histological evaluation. Our aim was to detect any changes to the aforementioned parameters after ethanol intake in both genotypes and any differentiations between the two genotypes.

3.1.2. TRANSLATIONAL STUDIES

We recruited 31 volunteer patients with formerly diagnosed alcoholic liver disease (24 men, 7 women) and admitted ongoing daily ethanol consumption and 27 healthy volunteers (14 men, 13 women), without preexisting liver disease, with normal body mass index and glycated

hemoglobin, who denied ethanol intake for the last 7 days prior to the conducted blood tests. The main cohort characteristics are described below. To objectify and approximately quantify the alcohol consumption (or its absence) we measured serum levels of carbohydrate deficient transferrin (CDT) in all human subjects. We determined serum levels of FGF19, FGF21, bile acids and oxysterols (7α and 27-hydroxycholesterol -7αOHC and 27OHC-, respectively). The serum oxysterols were used as biomarkers of the activity of the bile acids biosynthesis cascade. Our aim was to detect any differences in the levels of the endocrine fibroblast growth factors and the biomarkers of bile acids synthesis in the presence of alcohol consumption by preexisting liver damage.

3.2 MOUSE MODELS

For the murinal studies two mouse groups were used, consisting of fifteen week-old wild-type mice (C57BL/6J, n= 56) and *Abcb4* knock-out mice (*Abcb4*$^{-/-}$, n= 56). The accommodation took place under conditions of controlled humidity and temperature and ascertainment of a 12-hour light and 12-hour dark cycle. Based on the models described by Bertola et al (10) and Karatayli et al (61) each mouse group was divided into two subgroups, either fed control diet (WT/Cont and KO/Cont groups) or ethanol diet (5% v/v), followed by a subsequent acute ethanol intake (4 g/kg body weight) (WT/EtOH and KO/EtOH groups; n= 28 per group). The numbers of male and female mice were equal in the two groups and four subgroups. The body weight of the mice was monitored daily. In this way the recently established acute on chronic liver injury (ACLI) model was established, where *Abcb4*$^{-/-}$ was combined with a predefined chronic-plus-binge ethanol feeding model (NIAAA) (61). According to the aforementioned studies, the mice were initially fed with an ad libitum liquid diet (Rodent liquid diet, Lieber-Decarli 82; Bio-Serv, Frenchtown, NJ, USA) for five days. At the end of the 5th day, the mice diets were switched to either control liquid diet or ethanol diet (Rodent liquid diet, Lieber-Decarli 82-ethanol 4% v/v; Bio-Serv, Frenchtown, NJ, USA) for a period of 10 days, with subsequent application of isocaloric maltose dextrin gavage (9 g/kg body weight) or by an acute ethanol binge (4 g/kg body weight), respectively. All mice were sacrificed 7-9 h after gavage and plasma samples were acquired from whole blood assembled from the inferior vena cava. The harvested liver and intestine tissue samples were frozen in liquid nitrogen and stored at −80°C. Bile collected from the gallbladder was stored at -20°C. All experiments were performed in accordance with the relevant regulations of the Animal Care and Use Committee for Saarland University and were approved by Saarland University Animal Ethics Committee (TV 44/2015 and TV 06/2019).

3.3 MEASUEREMENT OF PLASMA BIOCHEMICAL MARKERS

Carbohydrate-deficient transferrin (CDT) was determined by using “The Siemens N Latex CDT kit” (BNII; Siemens Healthcare Diagnostics,Erlangen,Germany). Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and ethanol (EtOH) levels were measured in plasma (Roche Cobas 8000 c702 modular analyzer) in the Central Laboratory of Saarland University Medical Center.

3.4 HISTOLOGY

The collagen accumulation in mouse liver and the inflammation grade were evaluated by Sirius Red histochemistry and hematoxylin-eosin (H&E) staining, respectively. For histopathological analysis, paraffin-embedded, 5 μm thick formalin-fixed (4%, v/v) liver sections were used. The grading of liver fibrosis and the quantitative evaluation of inflammation were performed by means of a histomorphometric semi-automatic system for image analysis (Leica microscope, equipped with Leica application suite software; Wetzlar, Germany). An evaluation of five randomly chosen microscopic areas took place for each Sirius Red stained liver section, in order to calculate the percentage of collagenous area. H&E stained sections were also applied to find out infiltration through inflammatory cells and to calculate the dimensions of lipid droplets.

3.5 DETERMINATION OF PLASMA FGF15/19 AND FGF21 LEVELS

The plasma levels of FGF21 and FGF15 in mice were determined by means of the FGF-21 Quantikine ELISA Kit (MF2100; R&D Systems, Minneapolis, MN, USA) and mouse FGF15 ELISA kit (RD-FGF15-Mu; Reddot Biotech, Kelowna, BC, Canada). Human FGF-21 Quantikine ELISA kit (DF2100; R&D Systems) and Human FGF-19 Quantikine ELISA kit (DF1900; R&D Systems) were used for the quantification of FGF21 and FGF19 levels in human plasma samples. Each plasma sample was studied in duplicates, and average values were applied for the final analysis.

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3.6 PRIMARY HEPATOCYTE ISOLATION, CULTURE AND TREATMENT

The primary murinal hepatocytes (HC) were obtained from C57BL/6J mice (8–15 week old) using the collagenase perfusion method. The acquired HC were subsequently seeded in 6 well collagen-I-coated (collagen from rat tail, Sigma-Aldrich, St. Louis, Missuri, USA) cell culture plates at a density of 3x105 viable cells per well and incubated in 5% CO2 at 37°C for four hours. After that the cells were attached by means of incubation with medium 1 (Williams' medium E supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin/streptomycin, 100 nM dexamethasone). The medium was followingly switched to medium 2 (medium 1 without FBS) for serum starvation and cell cycle synchronisation. After 20 h, graded and no graded concentrations of recombinant human FGF21 (rhFGF21) (0.1, 0.25, 0.5, and 1 µg/ml, R&D Systems) were injected to the cells in medium 3 (Williams' medium E supplemented with 2 mM L-glutamine and 1% penicillin/streptomycin) for 24 h. Subsequently, RNA extraction and gene expression analysis were performed and PBS was included as control. All the above mentioned experiments were conducted in triplicates, and each experiment was performed three times.

3.7 RNA ISOLATION AND QUANTITATIVE REAL-TIME PCR ANALYSIS

The total RNA was acquired from frozen liver tissues by means of RNeasy Mini kit (Qiagen, Hilden, Germany). For ileum tissues and cells from cell culture peqGold Microspin total RNA kit (VWR, Erlangen, Germany) was utilized. The reverse-transcription of the obtained total RNA was conducted using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). The cDNA was amplified in 96-well plates via TaqMan™ Fast Universal PCR Master Mix (2x) (Applied Biosystems/Thermo Scientific) on a Taqman 7500 Fast Real-Time PCR system. The evaluation of hepatic steady-state mRNA expressions of Sterol regulatory element binding transcription factor 1 (*Srebf1*, Mm00550338_m1), Fibroblast growth factor receptor 4 (*Fgfr4*, Mm01341852_m1), Fibroblast growth factor receptor 1 (*Fgfr1*, Mm00438930_m1), cytochrome P450 family 7 subfamily A member 1 (*Cyp7a1*, Mm00484150_m1), Fibroblast growth factor 21 (*Fgf21*, Mm00840165_m1), Farnesoid X-Activated Receptor (*Fxr/Nr1h4,* Mm00436425_m1), β-Klotho (*Klb,* Mm00473122_m1), Mechanistic target of rapamycin (*Mtor*, Mm00444968_m1), Peroxisome proliferator activated receptor alpha (*Ppara*, Mm00440939_m1), Small heterodimer partner (*Shp/Nr0b2*, (Mm00442278_m1), Cytochrome P450 family 27 subfamily A member 1

(*Cyp27a1*, Mm00470430_m1), and Cytochrome P450 family 8 subfamily B member 1 (*Cyp8b1,* Mm00501637_s1) as well as ileal mRNA levels of MAM and LDL receptor class A domain-containing 1 (*Malrd1/Diet1*, Cg04494263_m1), Fibroblast growth factor 15 (*Fgf15*, Mm00433278_m1) and Farnesoid X-Activated Receptor (*Fxr/Nr1h4*, Mm00436425_m1) was performed via TaqMan gene expression assays (Applied Biosystems). The ΔΔCt algorithm was applied for the determination of the relative alterations of gene expressions. In the latter procedure Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, Mm99999915_g1) was used as internal control.

3.8 TOTAL BILE ACID MEASUREMENT OF MOUSE PLASMA AND GALLBLADDER

The collected bile acid samples were acquired from a total of 37 mice (11 WT/Cont, 11 WT/EtOH, 9 KO/Cont, and 6 KO/EtOH). The measurement of the total bile acid levels (TBA) was performed with the “Total Bile Acids Enzymatic Colorimetric Kit” (Cat.No. BI 3863; Randox Laboratories, Crumlin, UK) according to the manufacturer’s instructions. The taurocholic acid standard was made from powdered taurocholic acid in methanol and afterwards stored at -20°C. The prepared standard was next used in different dilution series for gallbladder (range between 5-160 µM) and plasma samples (range between 0.5-60µM). The samples were studied in duplicates and the average values were utilized for analysis. Two absorption measurements were performed in the microplate reader at 405 nm at times t0 and t1 (two minutes later than t0). The difference in absorbance E (t1) - E (t0) is regarded to be proportional to the serum concentration of bile acids. The proportionality factor was matched to the gradient of the standard dilution curve.

3.9 ANALYSIS OF SERUM TOTAL BILE ACID AND CHOLESTEROL LEVELS IN HUMANS

The “LT-SYS ® Gallensäuren Test” (LT-SYS Diagnostics, Berlin, Germany) with the Randox calibrator Cal 2351 was used to perform the measurements of the serum levels of total bile acids. Total cholesterol levels were measured using the enzymatic colorimetric determination assay using the Cholesterol Gen.2 kit (Cobas, Roche Diagnostics, Basel, Switzerland). The

measurements were conducted in the Central Laboratory of Saarland University Medical Center.

3.10 QUANTIFICATION OF SERUM CONCETRATIONS OF OXYSTEROLS

The quantification of the serum levels of 7α and 27-hydroxycholesterol (7αOHC and 27OHC, respectively) were quantified by means of isotope dilution GC-mass spectrometry selected ion-monitoring (GC-MS-SIM) methodology, using the corresponding deuterium labelled oxysterols as internal standards. The measurements were conducted in the laboratory of Professor Lütjohann et al., at University Medical Center of Bonn, Germany.

3.11 HUMAN SUBJECTS

Volunteer patients with preexisting alcoholic liver disease (n=31; 24 men, 7 women) and healthy controls, without any known preexisting liver disease were included in the study (n=27; 14 men, 13 women), after providing their written informed consent. The general clinical characteristics of the two groups are summarized in Table 2. The recruitment of the patients with alcoholic liver disease took place after hospital admission in the Clinic of Gastroenterology and Hepatology of the University Medical Center of Homburg, Saarland, Germany, due to decompensation of the disease and admitted ongoing daily alcohol consumption (30-100 g/day) for at least one week prior to hospitalization. The precise amount of alcohol consumption could not be measured. We therefore conducted evaluation of serum levels of carbohydrate deficient transferrin (CDT) as biochemical marker for the alcohol consumption. All the volunteers of the cohort of healthy controls reported no history of liver diseases or intake of medication. Their Body-Mass Index (BMI) and serum levels of glycated haemoglobin (HbA1c) were within the reference ranges. The study with human subjects was approved by the Ethic Committee of Saarland Medical Board (approval-number: 271/11).

	Healthy controls	ALD patients
N (women/men)	27 (14/13)	31 (7/24)
Age range (years)	36 ± 10 (24 - 70)	55 ± 11 (33 - 81)
AST (IU/l)	23 ± 7 (18 - 47)	63 ± 44 (17 - 174)
ALT (IU/l)	22 ± 11 (10 - 62)	47 ± 41 (12 - 122)
GGT (IU/l)	18 ± 6 (6 - 35)	237 ± 241 (44 - 1076)
CDT (mg/ml)	34 ± 8 (24 - 51)	51 ± 17 (24 - 79)
Total cholesterol(mg/dl)	181 ± 37 (121 - 262)	154 ± 69 (60 - 393)
Triglycerides (mg/dl)	95 ± 58 (36 - 142)	105 ± 71 (39 - 367)
BMI (kg/m²)	22.7 ± 1.2 (20.7 - 24.9)	25.8 ± 3.0 (20.4 - 31.2)
HbA1c (%)	5.2 ± 0.2 (4.8 - 5.6)	5.5 ± 0.9 (3.9 - 8.6)

Data are mean ± standard deviation (range).

Table 2: General clinical and biochemical characteristics of healthy control and ALD cohorts. AST: aspartate transaminase, ALT: alanine transaminase, GGT: gamma-glutamyl transferase, CDT: carbohydrate deficient transferrin, BMI: Body Mass Index, HbA1c: glycated haemoglobin, ALD: alcoholic liver disease

3.12 STATISTICAL ANALYSIS

Statistical analysis was conducted by means of SPSS v18.0 (IBM, Armonk, NY, USA) and GraphPad Prism (GraphPad Software, CA, USA). All data was expressed with mean and standard error (mean ± SE). A two-tailed Student's t-test was used for the comparison between two groups and one-way ANOVA followed by post hoc (Bonferroni) correction was applied for comparison of more than two groups. A p-value of <0.05 was considered to be statistically significant.

4. RESULTS

4.1 EFFECTS OF ETHANOL CONSUMPTION ON PLASMA AST, ALT AND ETHANOL LEVELS IN MICE

In order to objectify the impact of ethanol ingestion on hepatic biomarkers, we determined the serum aminotransferase activities (aspartate aminotransferase - AST and alanine aminotransferase - ALT) and serum ethanol (EtOH) levels before and after the alcohol intake in both mouse types. Ethanol could not be detected in serum before the exogenous application and could be confirmed after it. Serum aminotransferase activities showed significant elevation in both mouse types after the ethanol application ($p< 0.001$ for ALT and $p = 0.013$ for AST in wild type, $p=0.001$ for ALT and $p=0.001$ for AST in KO mice) (Figure 6). The latter observation indicates the induction of a marked hepatocellular reaction, in both previously healthy and pre-injured hepatocellular parenchyma.

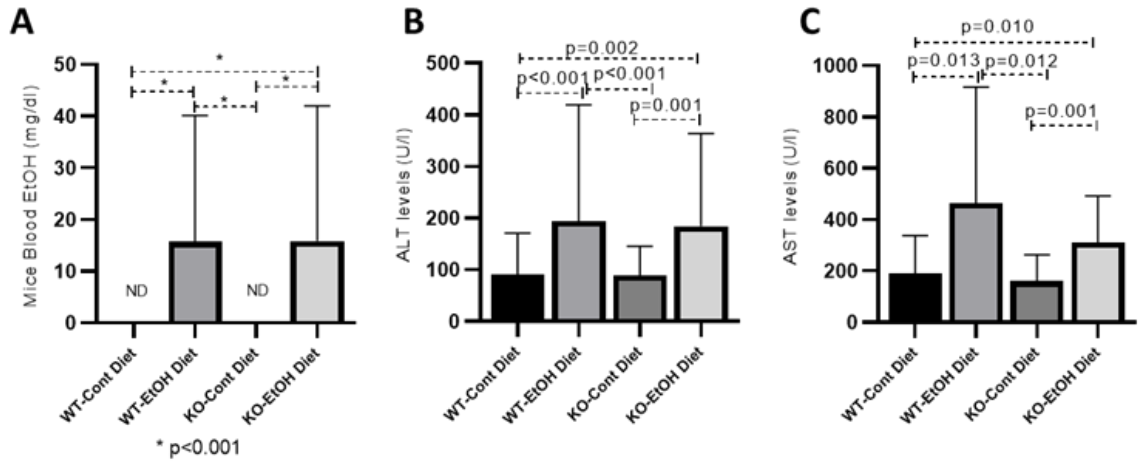

Figure 6: Plasma levels of ethanol (A) and biochemical markers ALT (B) and AST (C) in mice. ALT, alanine aminotransferase; AST, aspartate aminotransferase; EtOH, ethanol; KO, *Abcb4* knock-out mice; ND, not detected; WT, C57BL/6 J wild-type mice; $p<0.05$ was considered significant.

4.2 EFFECT OF ETHANOL CONSUMPTION ON FGF21 EXPRESSION IN HEPATOCYTES AND CIRCULATING LEVELS OF FGF21 IN MICE

Ethanol ingestion caused a significant upregulation of the expression of the *Fgf21* gene in both WT and KO mice (p=0.009 and 0.011 respectively), compared to their normal diet fed counterparts. No significant difference was observed between the two genotypes (Figure 7A). This fact indicates a preserved hepatic ability for upregulation of FGF21 upon ethanol consumption even in the setting of preexisting cholestatic damage. The upregulation of the hepatic production of FGF21 was furthermore mirrored in significantly elevated levels of circulating FGF21 in WT and KO mice after the ethanol challenge (p=0.04 and 0.048 respectively). The latter observation is consistent with the previously supported fact that FGF21 is a primarily hepatokine and the liver expression confers the major part of its circulating levels (Figure 7B).

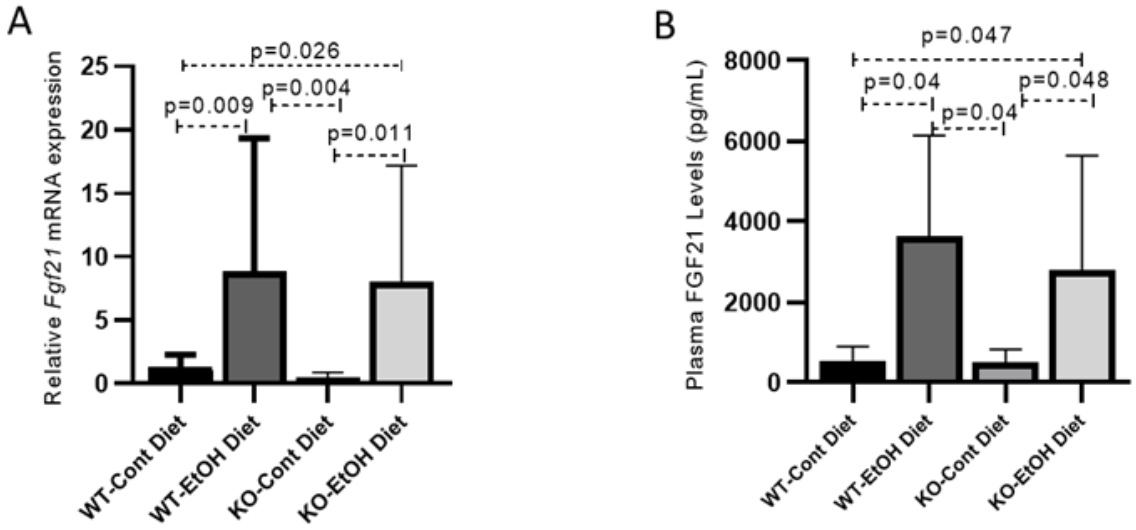

Figure 7: Effect of ethanol on FGF21 in liver and plasma. Relative quantification of *Fgf21* mRNA levels in liver (A). Plasma FGF21 levels were quantified by ELISA (B).. Cont, Control diet; EtOH, Ethanol diet; KO, Abcb4 knockout mice; WT, C57BL/6 J wild-type mice. $p<0.05$ was considered significant.

4.3 EFFECT OF ETHANOL CONSUMPTION ON HEPATIC PPARA, FGFR1, KLB, MTOR AND SREBF1 MRNA EXPRESSION LEVELS

Furthermore, the impact of alcohol on FGF21 signaling pathways was examined. In order to achieve this, hepatic mRNA levels of the *Ppara (*FGF21 upstream inducing mediator*), Mtor (*FGF21 downstream transducing signaling mediator) and *Srebf1/Srebp1c* genes (transcription regulator) were evaluated. The expression of hepatic *Ppara* showed significant upregulation after the ethanol ingestion in both WT and KO mice (p=0.01 and p=0.02, respectively), howbeit no difference was observed between the two genotypes (Figure 8A). Despite this fact, no significant difference was ascertained, as far as the hepatic mRNA expression of *Mtor* and *Srebf1/Srebp1c* between the two groups is concerned (Figure 8 B-C). More interestingly, there was noted no significant effects of ethanol intake on hepatic mRNA expression levels of FGFR1, which is the preferred receptor of FGF21, and its obligate co-receptor beta-klotho (KLB) (Figure 8 D-E). The above mentioned results in summary support the upregulating impact of alcohol on FGF21 by means of an upstream induction of PPARa but without alterations in the levels of the transcription factor SREBF1. In addition, our experiments indicate that the downstream signal transduction of FGF21 upon ethanol challenge is conceivably not mediated through the mTOR signaling pathway.

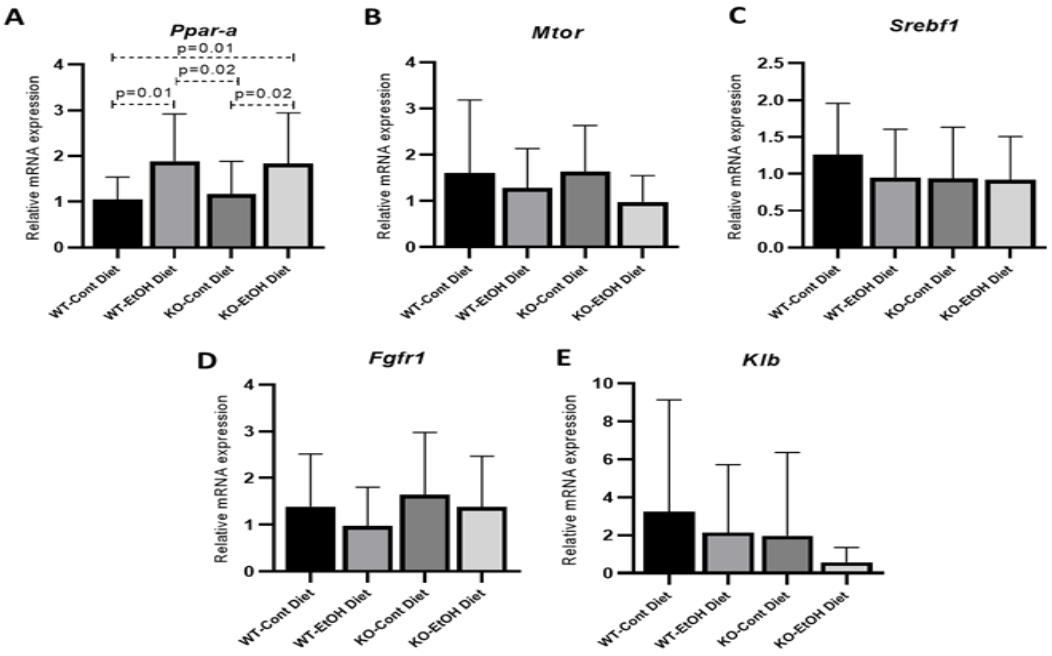

Figure 8: Relative quantification of hepatic mRNA levels. Transcriptional levels of *Ppara* (A), *Mtor* (B), *Srebf1* (C), *Fgfr1* (D) and *Klb* (E) were determined by qPCR. Cont, Control diet; EtOH, Ethanol diet; KO, Abcb4 knockout mice; WT, C57BL/6 J wild-type mice. p<0.05 was considered significant.

4.4 EFFECT OF ETHANOL INTAKE ON CYP7A1, CYP27A1, CYP8B1 AND BILE ACID SYNTHESIS

Hepatic expression of *Cyp7a1* and *Cyp27a1*, which encode cholesterol 7 alpha – hydroxylase (CYP7A1) in the classic and sterol 27-hydroxylase (CYP27A1) in the alternative BA synthesis pathway, were both significantly repressed in WT/EtOH mice ($p<0.001$ and 0.012, respectively). In the KO/EtOH mice was observed only a statistically significant suppression of *Cyp7a1* ($p<0.001$) but not *Cyp27a1* (Figure 9A-9B). Significant repression of *Cyp8b1* gene, encoding sterol 12α-hydroxylase (CYP8B1), which is required for synthesis of cholic acid, was observed only in KO/EtOH group compared with the WT/Cont ($p=0.041$), WT/EtOH ($p=0.029$) and KO/Cont groups ($p=0.027$) (Figure 9C). Although statistically not significant, chronic plus binge ethanol feeding increased TBA levels both in plasma and gallbladder bile in mice (Figure 9D-9E), showing higher plasma TBA levels in KO/Cont as compared to WT/Cont mice. These data indicate that gene expression involved in the bile acid synthesis cascade was reduced, although the bile acid pool size of gallbladder and plasma samples were increased after alcohol feeding. Together these results indicate a trend to secondary protective suppression of the cascade of bile acid synthesis, following the induction of hepatic FGF21 production after ethanol intake. It is furthermore intriguing that the suppression of *Cyp8b1* in the KO mice could suggest an altered balance between the two primary bile acids, cholic acid and chenodeoxycholic acid, after ethanol ingestion in the setting of preexisting liver damage.

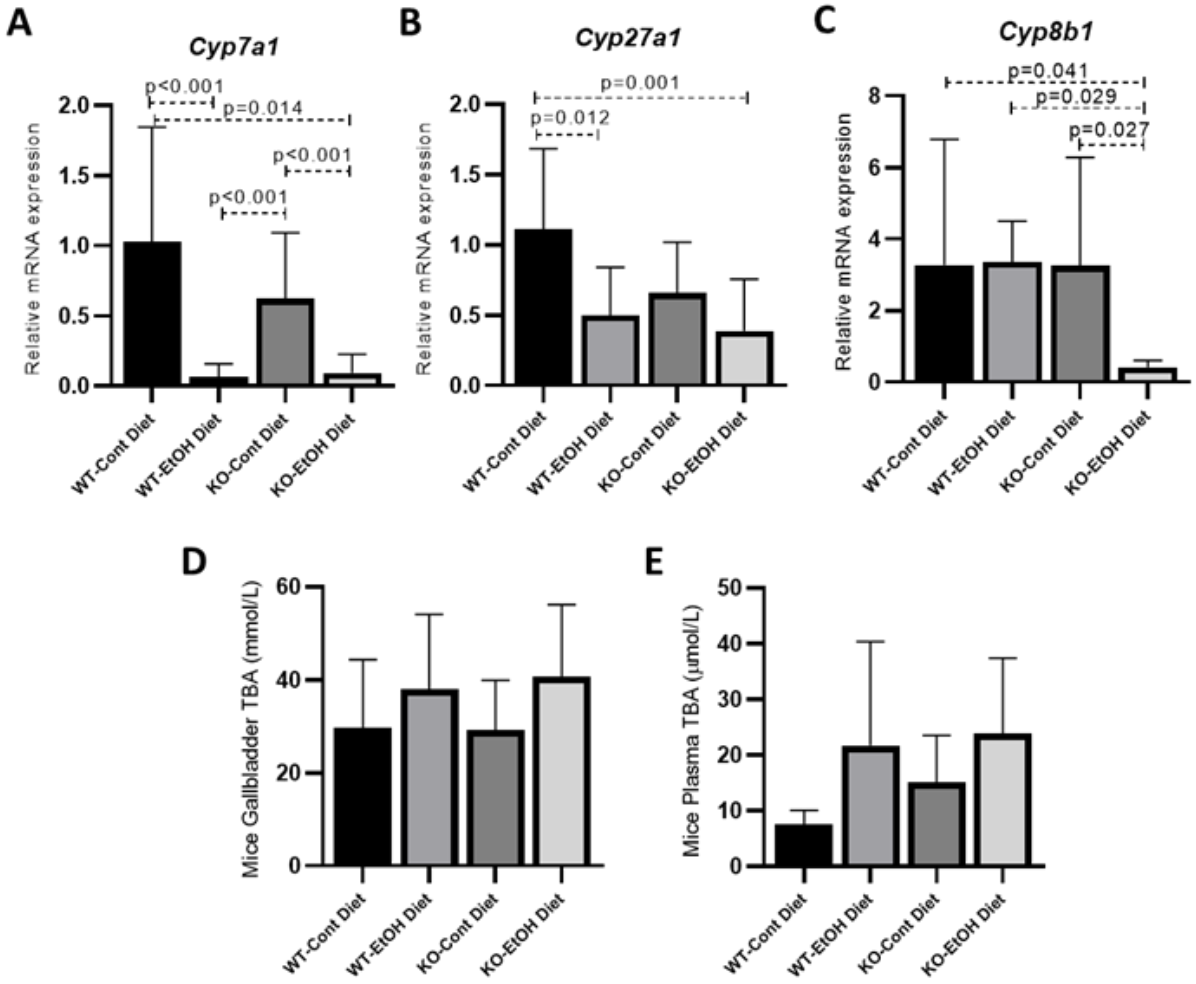

Figure 9: Relative quantification of gene expression using qPCR in liver and TBA levels in plasma and gallbladder. Hepatic mRNA levels of bile acid synthesis genes(*Cyp7a1* (A), *Cyp27a1* (B), and *Cyp8b1* (C)) were determined. Total bile acid levels in mouse gallbladder bile (D) and plasma (E). Cont, Control diet; EtOH, Ethanol diet; KO, Abcb4 knockout mice; WT, C57BL/6J wild-type mice. p<0.05 was considered significant.

4.5 IMPACT OF ETHANOL CONSUMPTION ON ILEAL EXPRESSION AND CIRCULATING LEVELS OF FGF15 (HEPATIC FXR/SHP PATHWAY AND FXR/FGF15/FGFR4 PATHWAY)

The expression of the *Fgf15, Fxr* and *Diet1* genes in ileum was subsequently controlled. Here a significant induction of the *Fgf15* gene expression in WT mice upon ethanol uptake was determined (p=0.032), but not in the KO group (Figure 10D). However the circulating FGF15 plasma levels were similar between the groups (Figure 11). The ileal *Fxr* gene expression was likewise significantly elevated after alcohol consumption only in the WT group (p=0.018)

(Figure 10E). No significant differences were observed in the expression of *Diet 1*, which has been suggested to modulate bile acid and lipid levels through the FGF15/19 enterohepatic signaling axis (Figure 10F).

To further clarify the molecular pathway of *Cyp7a1* repression upon ethanol challenge, we analyzed the hepatic expression level of the nuclear receptor FXR, which inhibits *Cyp7a1* and *Cyp8b1* gene transcription by inducing the nuclear receptor SHP. However, no significant difference in expression levels of hepatic *Fxr* and *Shp* was observed among groups (Figure 10A-10B) suggesting that the repression of *Cyp7a1* gene expression was independent of the hepatic FXR/SHP pathway in our model.

Perhaps the best known mechanism of repression of *Cyp7a1* is based on the activation of FXR in ileum, which induces the intestinal hormone FGF15. FGF15 in turn induces FGFR4 signaling in the hepatocytes. In the WT mouse group a significant upregulation of ileal *Fgf15* ($p=0.032$) and *Fxr* gene expression ($p=0.018$) could be ascertained upon ethanol intake. This effect was howbeit absent in the KO group. Interestingly we could not detect any differences in the circulating levels of FGF15 between the groups. The result seemed to be thus inconsistent. We therefore proceeded to evaluation of the expression levels of *Diet1/Malrd1* gene in ileum, which is supported to have an impact on the FGF15 secretion from its site of ileal production into the portal circulation (69). The expression levels of *Diet1* were correlated with the expression pattern of *Fgf15* indeed. The higher levels were detected in WT mice after the ethanol consumption. Despite this correlation there was absence of statistically significant differences between the mouse subgroups, as far as *Diet1*, *Fgfr4* and its co-receptor β-Klotho are concerned. Precisely this observation leads to the assumption, that the suppressive impact of FGF21 on CYP7A1 and consequently the rate limiting step of the bile acids biosynthesis could be exerted through a direct action of FGF21 and not definitely through a synergistic effect with FGF15/19. This independent action of FGF21 is presumably more marked and axial in the ACLI mouse model, where no explicit induction of the ileal FXR-FGF15 signaling could be denoted.

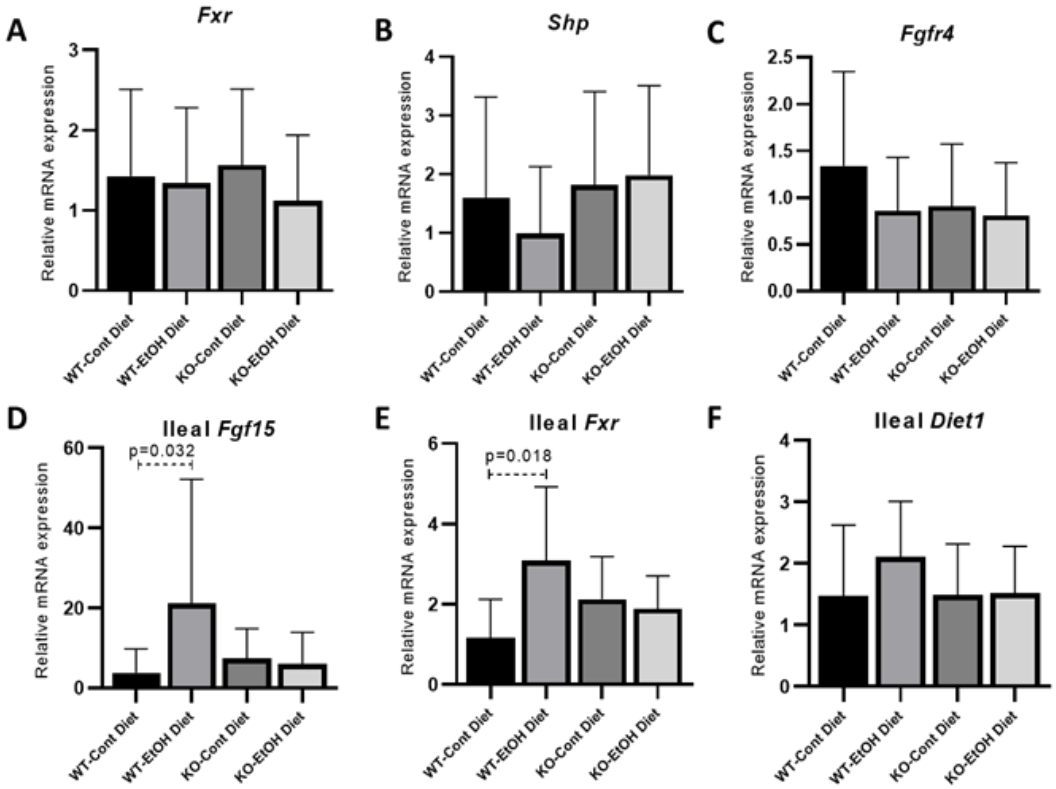

Figure 10: Relative quantification of mRNA levels of hepatic/ileal genes that play a role in transcriptional regulation of the *Cyp7a1* gene. Transcriptional levels of hepatic *Fxr* (A), *Shp* (B) and *Fgfr4* (C) and ileal *Fgf15* (D), *Fxr* (E) and *Fgfr4* (F) were determined. Cont, Control diet; EtOH, Ethanol diet; KO, Abcb4 knockout mice; WT, C57BL/6J wild-type mice. $p<0.05$ was considered significant.

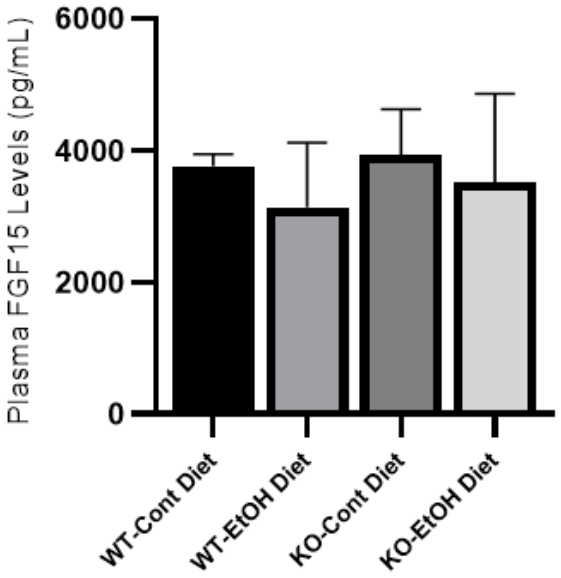

Figure 11: Plasma FGF15 levels in mice quantified by ELISA. Cont, Control diet; EtOH, Ethanol diet; KO, Abcb4 knockout mice; WT, C57BL/6 J wild-type mice. $p<0.05$ was considered significant

4.6 STUDIES IN HUMAN COHORTS

Similar pathophysiological processes concerning the endocrine fibroblast growth factors and their impact on bile acids metabolism upon ethanol consumption in humans were hypothesized. In order to verify if our findings could be translated to human cohorts, we objectified the impact of ethanol intake on FGF21 and bile acid biosynthesis by determining FGF21, FGF19 (the human orthologue of FGF15 in mice), 7α-hydroxycholesterol (7αOHC) and 27-hydroxycholesterol (27OHC) levels in blood in two cohorts of human subjects. The cohort of healthy volunteers did not have any known preexisting liver disease. The second cohort consisted of patients with previously diagnosed alcoholic liver disease and admitted ongoing ethanol consumption, which was objectified via measurement of serum CDT. For the objectification and quantification of alcohol intake and liver damage, biochemical markers in serum were determined. CDT, ALT and AST levels were found to be significantly elevated in ALD patients with ongoing alcohol ingestion ($p<0.001$, $p=0.004$ and $p<0.001$, respectively) (Figure 12).

Consistent with the results in mice, a significant ($p<0.001$) upregulation of FGF21 in the cohort with ongoing ethanol consumption was observed, furthermore supporting our conclusion that the ethanol induced elevation in circulating levels of FGF21 remains preserved, even in the frame of a preexisting liver pathology. (Figure 13A). There were no significant differences in the plasma FGF19 levels between the groups, a result which is in line with mouse plasma FGF15 results (Figure 13B).

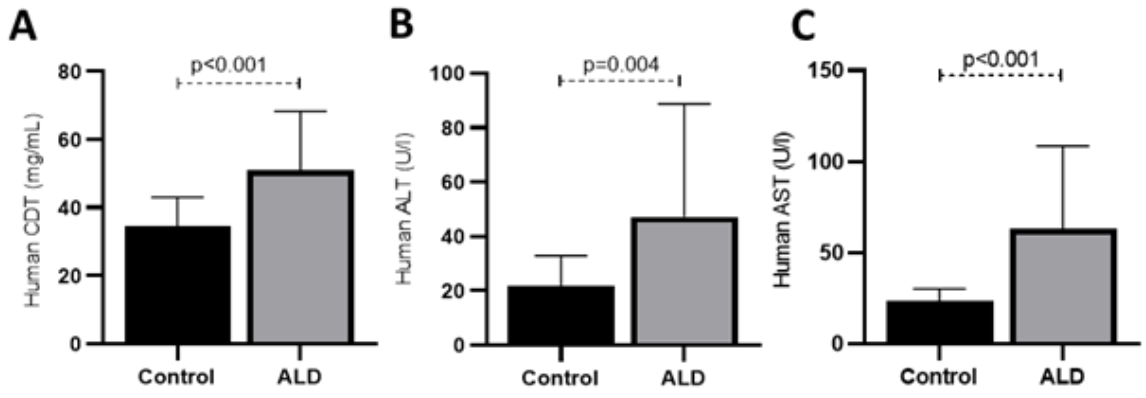

Figure 12: Plasma CDT (A), ALT (B) and AST (C) levels in humans. ALD, alcoholic liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CDT, carbohydrate-deficient transferrin. $p < 0.05$ was considered significant

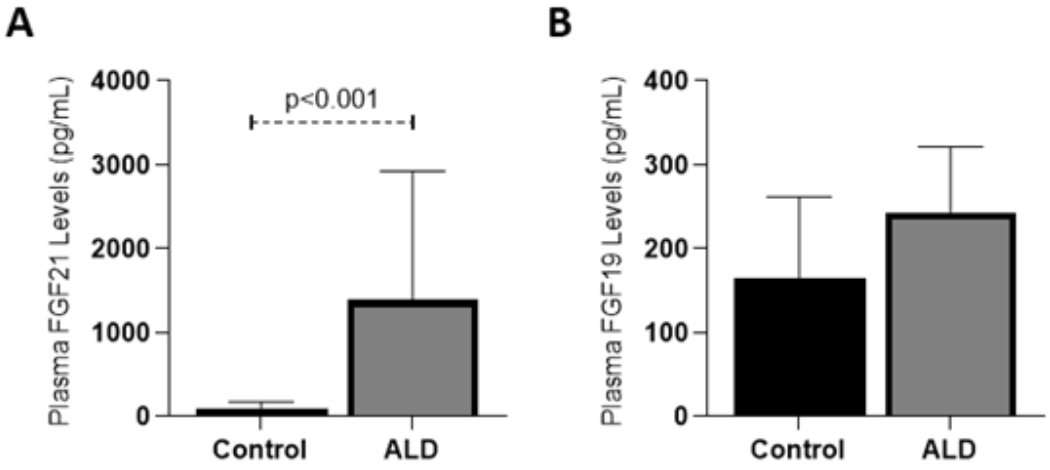

Figure 13: Plasma FGF21 (A) and FGF19 (B) levels in human plasma samples quantified by ELISA. All data are shown as means ± SE. p<0.05 was considered significant.

To further clarify a potential influence of ethanol consumption on the expression of *CYP7A1* and *CYP27A1* genes in humans, we evaluated the serum concentration of the specific metabolites 7a-hydroxycholesterol (7aOHC) and 27-hydroxycholesterol (27OHC). 7aOHC is suggested to be a biomarker for the 7a-hydroxylase activity, and 27-hydroxycholesterol a biomarker for CYP27A1 activity in humans respectively. In line with our observations in mice, a significant suppression of 7aOHC was denoted in patients with alcoholic liver disease (p= 0.02) (Figure 14A). Unexpectedly however, the levels of 27OHC were similar between groups (Figure 14B).

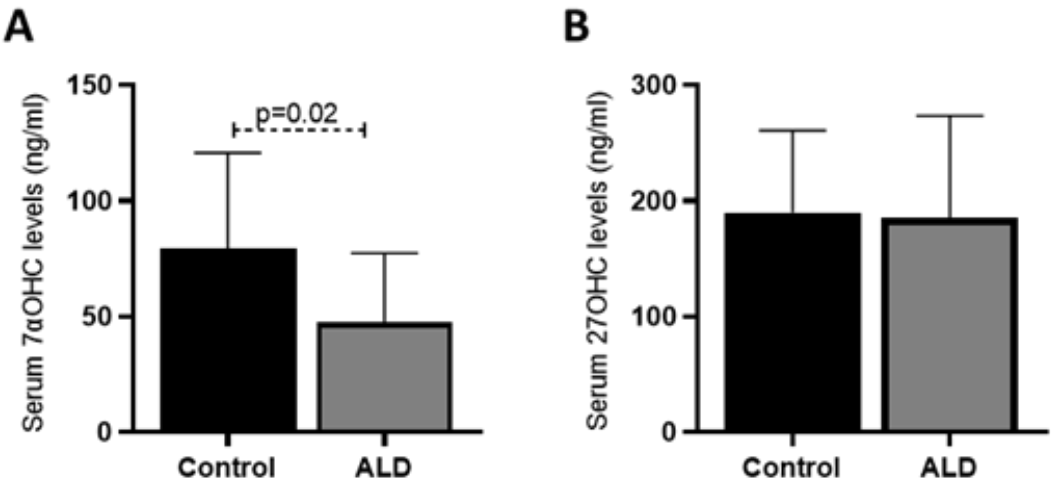

Figure 14: Serum levels of 7αOHC (A) and 27OHC (B) in humans detected by isotope dilution GC-mass spectrometry selected ion-monitoring (GC-MS-SIM). p<0.05 was considered significant.

During the basic screening of the recruited humans, serum TBA were found to be significantly elevated (p=0.01) despite a non-significant reduction of total cholesterol levels in the group of patients with alcoholic liver disease (Figure 15). In summary, these data suggest a significant elevation in TBA and FGF21 levels in plasma of ALD patients in the absence of significant changes in circulating FGF19 levels upon ongoing ethanol consumption.

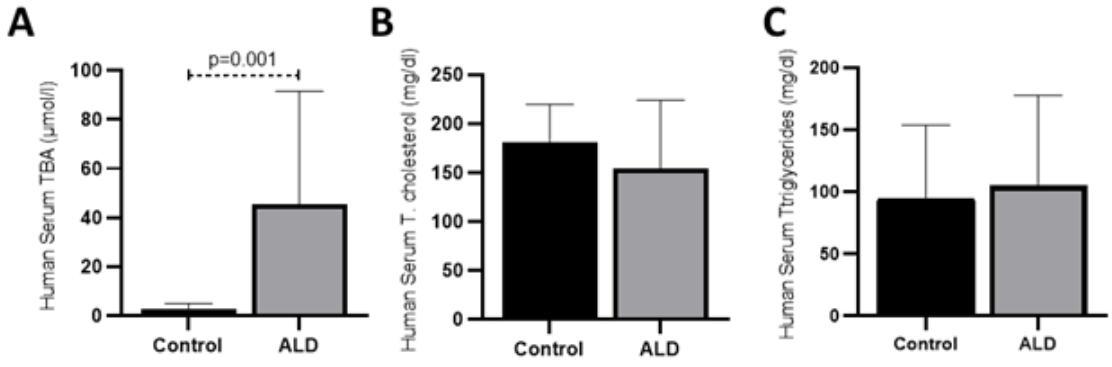

Figure 15: Human serum total bile acid (A), total cholesterol (B), and triglyceride (C) levels. $p<0.05$ was considered significant.

4.7 EFFECT OF EXOGENOUS HUMAN RECOMBINANT FGF21 ON THE EXPRESSION OF CYP7A1 IN PRIMARY MOUSE HEPATOCYTES

We extended our experiments in order to collect more data supporting a direct suppressive effect of FGF21 in the biosynthesis of the bile acids. We applied human recombinant FGF21 to murinal hepatocytes in order to dissect its effect on the rate limiting step of BA biosynthesis.

In murinal primary hepatocytes, the application of rhFGF21 suppressed the expression of *Cyp7a1* dose-dependently. There was initially observed that the levels of *Cyp7a1* were marginally reduced after application of lower rhFGF21 doses (0.1 and 0.25 µg/ml). After application of 0.5 µg/ml and 1 µg/ml doses a marked suppression relative to controls took place, by 25% and 70% respectively (Figure 16A). To clarify whether these alterations were induced directly or indirectly, expression levels of *Fxr* and *Shp* were determined. There were observed no significant changes for both. (Figure 16B-16C). Furthermore, there were no significant differences in gene expression of hepatic *Fgf21, Fgfr1* and *Klb* ascertained after the exogenous administration of FGF21 (Figure 16D, 16E and 16F). Consistent with the previously mentioned *in vivo* findings of the current study, these results enhance the existence of a direct impact of exogenously administered rhFGF21 on *Cyp7a1* gene suppression, which acts independently from the FXR/SHP signalling pathway.

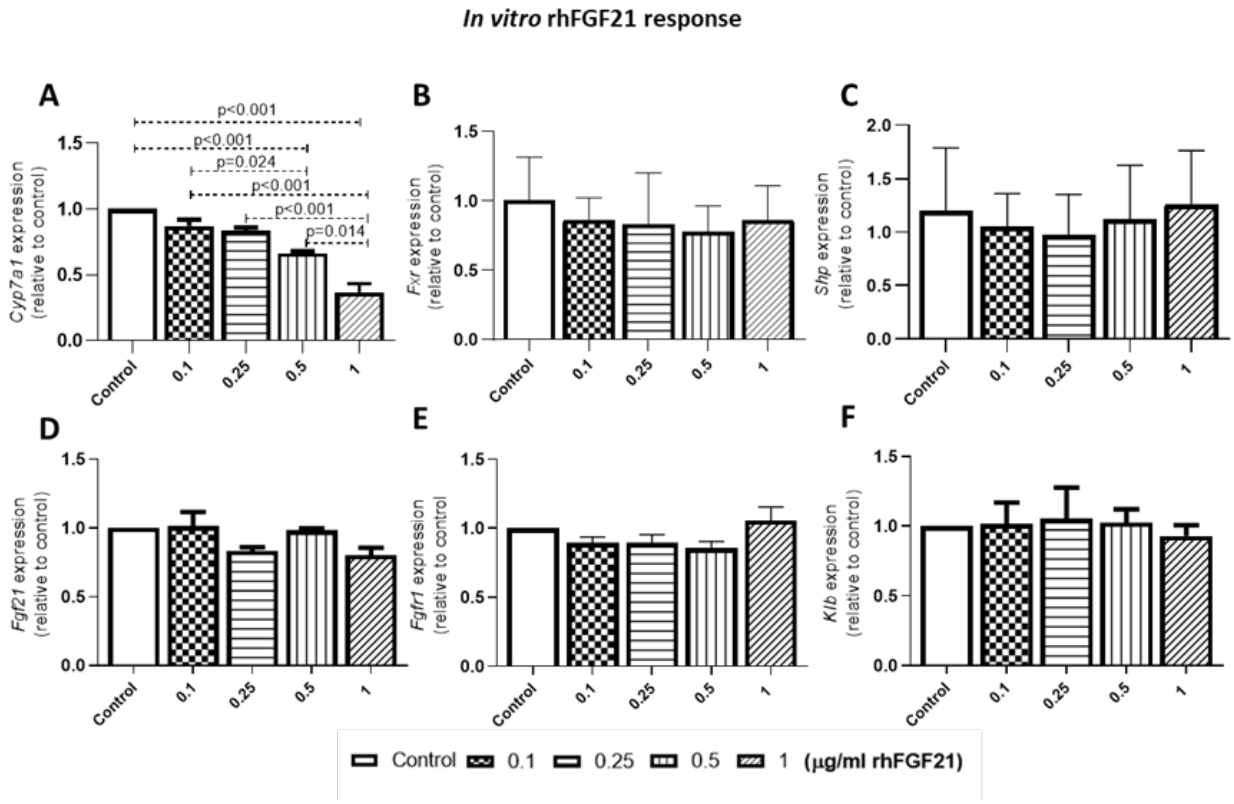

Figure 16: Effect of rhFGF21 on primary mouse hepatocytes. Transcriptional levels of the *Cyp7a1* (A), *Fxr* (B), *Shp* (C), *Fgf21* (D), *Fgfr1* (E) and *Klb* (F) genes in primary mouse hepatocytes treated with rhFGF21 (0.1-1 µg/ml) or vehicle (white) for 24 h. All data represent -fold change compared to control hepatocytes (mean ± SE, n=3). $p<0.05$ was considered significant.

4.8 HISTOLOGICAL ASSESSMENT OF MICE LIVER

The histological evaluation of the murinal liver tissues revealed signs of hepatic inflammation with infiltration with numerous inflammatory cells detected by H&E staining in mice after ethanol intake regardless of genotype (Figure 17A). The latter indicates the existence of increased hepatic steatosis. Sirius red staining of liver sections pointed to an aggravation of liver fibrosis in both WT and KO mice, induced via ethanol consumption (Figure 17B).

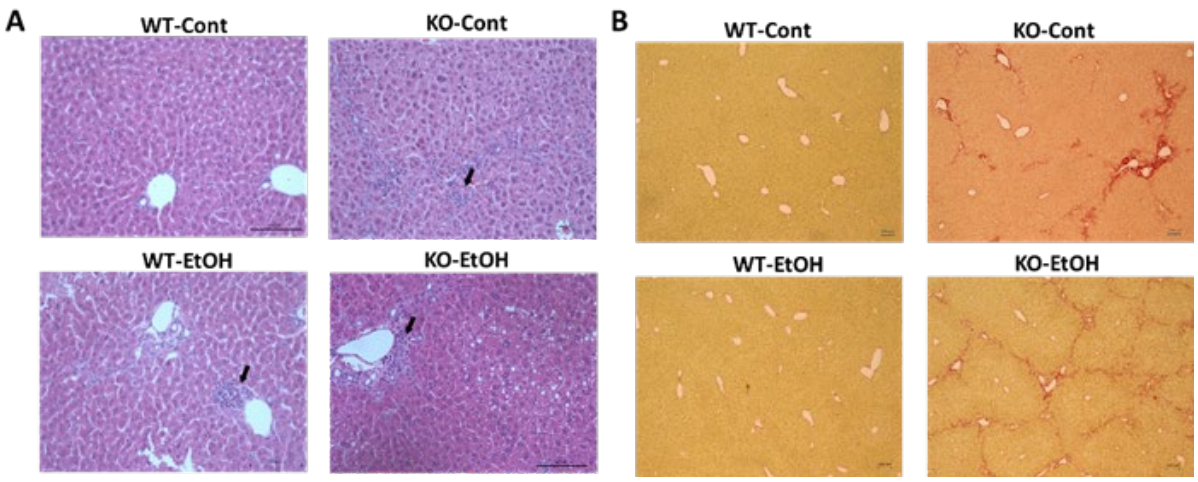

Figure 17: Assessment of liver fibrosis and inflammation. Hematoxylin and eosin staining of liver sections with 20X magnification (A) and Sirius Red stained liver sections with 5X magnification (B). Black arrows show infiltrating inflammatory cells. Each scale bar is 100 µm. EtOH, Ethanol diet; KO, *Abcb4* knock-out mice; WT, C57BL/6J wild-type mice.

5. DISCUSSION

The conducted study aimed to offer a multidimensional vision over the impact of alcohol ingestion and alcoholic liver disease on the endocrine fibroblast growth factors, FGF21 and FGF15/19, and vice versa. A further purpose was to clarify the role of FGF21 in bile acid metabolism, which could have pharmacological interest. In our knowledge there are only sparse data about the influence of alcohol on FGF15/19. Despite the previously observed intense upregulation of FGF21 after binge ethanol intake (27,128), the impact of chronic alcohol abuse or an underlying liver pathology has not yet been well studied. In this setting, we pursued to delineate the crosstalk between FGF15/19, FGF21 and bile acid metabolism, elucidate a part of their biological function in the frame of alcohol intake and figure out any alterations caused by a preexisting cholestatic liver pathology.

The results of the present study support a marked upregulation of hepatic and circulating FGF21 levels in both humans and rodents after ethanol ingestion. The significant upregulation was preserved in patients with underlying ALD as well as in *Abcb4* KO mice. This implies that ethanol induced upregulation of hepatic and serum FGF21 levels remains intact even in the context of a preexisting liver pathology. The *in vivo* studies pointed furthermore to a significant downregulation of hepatic *Cyp7a1* expression after chronic plus binge ethanol intake. The *in vitro* studies supported this suppression to take place in a dose-dependent manner. Moreover, since there were not any changes in expression levels of hepatic *Fxr, Shp* or plasma FGF15/19 levels after ethanol challenge, we propose that alcohol induced FGF21 has a direct effect on *Cyp7a1* downregulation, which is independent of FXR-SHP or FGF15/FGFR4 pathways. The upregulation of FGF21 during an emerging hepatocellular damage could be seen as a reactive mechanism for the attenuation and diminished progression of the metabolic and oxidative alterations, leading firstly to steatosis and ultimately to fibrosis and cirrhosis (60). However, the impact of FGF21 on bile acids synthesis and metabolism has been shown to be controversial. FGF21 has been suggested recently to act as a negative regulator in the biosynthesis of bile acids and interestingly also in an FGF15/19 independent manner (17). The findings of the cited study indicated a complicated mechanism of action, which is dependent on the administered doses of FGF21 and the duration of administration. There were furthermore slight differences in the biological actions of recombinant human FGF21 and endogenous FGF21. These data suggest an existing role of FGF21 in bile acids metabolism, which could be influenced through not yet fully elucidated parameters. In another recent study, a completely opposite action of FGF21 has been supported, with direct antagonization of the effect of FGF15/19 on the down regulation of CYP7A1 (163). Interestingly, upregulation of *Cyp7a1* levels has also been

observed with long-term administration of high doses of FGF21, supporting the study mentioned above (17). On the contrary, an expected downregulation of *Cyp7a1* was observed following acute FGF21 administration in rodents with unchanged FGF15 levels, which consistently supports an FGF21 induced suppression of bile acids synthesis independent of FGF15/19 (17). These data are supportive for the existence of a feedback loop between CYP7A1 and FGF21, with still unclarified regulating biologic factors, presumably FGF15/19 independent.

In both WT and KO mice, a statistically significant elevation of serum transaminases was ascertained after chronic plus binge ethanol ingestion, following the elevation of serum ethanol values, which is indicative of the biochemical impact of alcohol ingestion on liver parenchyma (induction of an acute or acute on chronic liver injury respectively). The evaluation of hepatic expression and serum levels of FGF21 revealed a statistically significant upregulation in KO mice as well as WT mice, showing preserved effect in the frame of a preexisting liver pathology. Of course the elevated serum level of an endocrine factor could mean either a reactive upregulation for boosted biological effect or presence of a resistance state. The latter has been presumed for FGF21 in obesity and also diabetes (38) and should not be underestimated, taking into account the complex interaction of FGF21 with its receptor and the necessity of an obligate co - receptor for the downstream signaling cascade. What is compelling in the present study is the fact that the FGF21 elevation was not due to the liver pathology but the alcohol itself. In addition, there was no significant difference between FGF21 levels of WT and KO mice at the baseline, indicating that the liver disease itself did not cause a change in hepatic *Fgf21* expression levels.

We have additionally examined the impact of chronic plus binge ethanol intake on FGF15, which is an ileal produced enterokine. Similar to FGF21 results we could not detect any base line differences between WT and KO mice, a fact suggesting an irrelevant impact of the preexisting liver disease itself. Upon ethanol challenge we denoted a significant upregulation of ileal expression of *Fgf15* mRNA in the WT group but not in the *Abcb4* KO mice. Given the previously supported role of FGF15 in the downregulation of bile acids synthesis, this suppression could impede the progression of fatty liver disease and steatohepatitis. Thus, the observed upregulation of FGF15 could have a hepatoprotective effect, which is presumably not preserved in the frame of a preexisting cholestatic liver pathology. This could further imply, that on the physical course of the different stages of alcoholic liver disease, a progressive deprivation of the FGF15 mediated feedback control in the bile acids synthesis takes place, an event that could in turn accelerate the aggravation of the hepatic disease. However, in this study, circulating levels of FGF15 did not show any significant differences upon ethanol

challenge between mice groups, a fact suggesting a rather local action of it in the intestine-liver axis, at least in our experimental setting.

There seems to be a bidirectional relationship between bile acids synthesis and alcoholic liver disease, as the ethanol consumption itself has an impact on BA synthesis in liver. This action has been suggested to be predominantly mediated by the farnesoid X receptor (78). The increased BA synthesis has been reported to emerge through induction of *Cyp7a1* and *Cyp8b1* transcription with inhibition of *Fgfr4* expression, which is suggested to be a transcription inhibitor of *Cyp7a1* (157,158). Since the expression levels of FGFR4 remained unchanged in our study, these steps seem to be influenced upon ethanol intake in the present experimental setting. This could albeit presumably imply a counteraction of the inhibitory effect of ethanol itself on FGFR4. The crucial factor appears to be the elevation of hepatic and circulating levels of FGF21. Following this elevation, a suppression of *Cyp7a1* and *Cyp8b1* takes place. Furthermore, it has previously been described that the ethanol-induced upregulation of *Cyp7a1* and *Cyp8b1* is enorchestrated by activation of cAMP responsive element-binding protein (CREBH) through ethanol induced stimulation of the cannabinoid receptor type 1 (CB1R). CREBH is besides considered as a central metabolic regulator and these data support the consideration of the alterations in BA synthesis as part of a metabolic shift caused by alcohol intake (16). The altered conjugation of bile acids, which directly influences their toxicity, is induced via analogous changes in the necessary metabolic enzymes. As expected, the existing evidence supports the reduction in the levels of bile acid CoA: amino acid N acyltransferase (BAAT) and the upregulation of bile acid CoA synthetase (BACS) after alcohol intake. The first catalyzes the taurine conjugation and the latter the glycine conjugation (158). The elevated reabsorption of bile acids in the enterohepatic circulation following the ethanol consumption is mirrored in the expression of liver and ileal transporters, which lead to both elevated excretion and reabsorption. The liver transporters, which are reported to be upregulated include the bile salt export pump (BSEP), organic solute transporter α/β (OST α/β) and multidrug resistance protein 4 (MRP4), all of them increasing the BA efflux. The BA reuptake transporters, such as the sodium taurocholate cotransporting polypeptide (NTCP), have been found downregulated. The upregulated ileal transporters include the apical sodium dependent bile acid transporter (ASBT) and organic solute transporter β (OST β)(157,158). The FGF21-CREBH axis could bear in the light of the above data a pivotal hepatoprotective role under conditions of chronic or binge alcohol intake.

At least a part of the effects of ethanol consumption on the bile acid synthesis was proposed to be mediated via an ethanol induced down regulation of the farnesoid X receptor, which is suggested to suppress the expression of CYP7A1 and CYP8B1 and induce the expression of BSEP and FGF15/19 (48,74,124). The key event for the decreased activation of FXR seems

to be a reduced acetylation of the receptor, mediated perhaps through downregulation of Sirtuin 1 (SIRT1), which is a histone deacetylase dependent on nicotinamide adenine dinucleotide (NAD+) and on the other side activation of acetyltransferase p300 (95,157,162). In this way, it is proved one more time that the ethanol induced alterations in the oxidative/reductive potential in the hepatocyte can trigger multiple structural and functional changes that have altogether a negative impact on its function. The indication of the negative role of the ethanol mediated downregulation of FXR leads to the testing of FXR agonists as pharmacological agents for amelioration of the alcoholic liver disease, with two of them (WAY and 6a-rthyl-chenodeoxycholic acid-6ECDCA) showing an improving effect on liver steatosis (73,157). However, there are also converse findings reported. For instance, FXR has been suggested to deconjugate cAMP response element binding Protein (CREB) from its co-activator CREB regulated transcription co-activator 2 (CRTC2), inhibiting indirectly the autophagy via decreased expression of associated genes (121). The precise mechanism has not yet been fully elucidated as other contributing factors, as the AKT, are influenced by ethanol consumption as well (79). Another receptor inducing autophagy seems to be the PPARa, whose activation occurs under different circumstances from those needed for stimulation of FXR. The latter is rather activated in the fed phase and after the return of bile acids into the liver via the enterohepatic circulation, whereas the main stimulus for PPARa is the production of free fatty acids during starvation (14,33,49,63,77,144). PPARa and FXR are claimed to have opposite effects on the transcription of autophagy related genes (70), a fact that endorses a stimulating for the autophagy role of PPARa and an inhibiting role of FXR. The above mentioned role of FXR is not endorsed by our findings, where no significant alteration of the expression of this receptor were observed between the mice genotypes neither at baseline nor after alcohol ingestion. On the contrary, the anticipated upregulation of PPARa was indeed ascertained. The notion that a procedure, such crucial for the progression of alcoholic liver disease as the autophagy, is regulated from known receptors, which could pose pharmacological targets, is compelling, but the existing data are inconsistent and further basic and clinical research is yet required in this field.

The present study ascertained a downregulation of the expression of *Cyp7a1*, which encodes the rate limiting enzyme of the biosynthetic procedure of BA in both ethanol challenged WT and *Abcb4* KO mice. The observed suppression reached the limit of statistical significance in both mouse groups, although in the group with the preexisting liver damage the expression of *Cyp7a1* was reduced as compared with the WT group at the baseline. This is a further reflection that different causes of a hepatic pathology induce a mechanism that suppresses the synthesis of bile acids, in order to avoid hepatotoxic effect. The induction of ileal FGF15 only in the WT and not in the KO group was the first indication that the observed suppression

of the rate limiting enzyme of the BA synthesis in the latter group is presumably not mediated through FGF15. Taking into account that the FGF21 upregulation was consistent in both mouse groups, we can presume that the observed downregulation tendency in the KO group occurs due to the direct action of FGF21, without the cooperative action of these two endocrine fibroblast growth factors. This leads furthermore to the assumption that, in the course of a liver disease, the capability to reduction of hepatotoxicity of the BA through quantitative and qualitative alterations in their pool can progressively be abolished. Further research is required in order to clarify if there is a causative correlation between these two processes and a linear association between stage of liver disease and reduction in feedback control of BA synthesis.

We also observed the impact of ethanol intake on *Cyp27a1,* which is a gene encoding the enzyme sterol-27-hydroxylase, which is located in diverse tissues and responsible for many reactions, but undertakes a major role in the bile acid synthesis. It exhibits a cholesterol 27-hydroxylase and vitamin D3-hydroxylase activity and its intracellular localization is in the mitochondria. Due to its role in the biosynthetic pathway of BA it can be used as a biomarker of extrahepatic cholestasis (13) and as such was entailed in our experimental study. As found in expression levels of *Cyp7a1* as well, we ascertained a statistically significant suppression of *Cyp27a1* in WT mice. A suppression of *Cyp27a1* in the KO mice challenged with ethanol was found only when compared to WT-Cont mice. In the control diet challenged KO mice, there was a trend of reduction as well, although the downregulating trend in this group was not statistically significant. These data support further the decline of the capability for controlling the BA biosynthesis in the frame of previous liver damage at one further step and could be associated with the previously mentioned importance of FGF15 in the regulation of the whole feedback loop. The latter has been already documented in the literature (60).

CYP8B1 (Cytochrome P450, family 8, subfamily B, polypeptide 1), also named sterol 12-alpha-hydroxylase, which is an endoplasmic reticulum membrane located enzyme and catalyzes the conversion of 7-alpha-hydroxy-4-cholesten-3-one into 7-alpha,12-alpha-dihydroxy-4-cholesten-3-one has also been investigated in the current study. The relevance of this catalytic reaction lies in the regulation of the relative amounts in the BA pool of the two primary bile acids, namely the cholic and chenodeoxycholic acid. This monooxygenase is further relevant in many reactions controlling drug metabolism and synthesis of cholesterol, steroids and other lipids and can influence the solubility of cholesterol. Although there are published data indicating that CYP8B1 can aggravate dyslipidemia (101), the exact pathophysiological role of this enzyme in cholestasis and lipid regulation has not yet been fully elucidated. Our findings showed an immersing suppression of *Cyp8b1* after ethanol intake only in the KO group emphasizing the importance of an established chronic liver injury in the presence of an acute insult as in the case of ACLI model.

In addition, interestingly, no significant effect of alcohol challenge on hepatic mRNA levels of *Fgfr1* and *Fgfr4* was observed. Although it needs further investigation, this fact suggests that the expression levels of these receptors could be enough for an effective binding of the endocrine fibroblast growth factors and a subsequent upregulation may not be needed. In addition, similar to *Cyp8b1*, although it was not statistically significant, a decrease in expression levels of hepatic *Klb* has been observed only in the KO-EtOH mice group, pointing to the modulating effect of pre-injured liver in ACLI. Although several studies have showed the role of KLB in tumorigenesis (106,161), these results are controversial and its exact role in ACLI needs further investigations. We could furthermore discern a significant impact on the expression of ileal *Fxr* only in the WT-EtOH mice group, in which ileal *Fgf15* expression levels were significantly upregulated. The induction of ileal *Fgf15* gene expression could be suggested to take place following the activation of FXR in the frame of an FXR-FGF15/19 enterohepatic pathway. However, the lack of significant changes of ileal *Fxr* and *Fgf15* expression levels and plasma FGF15 levels in the KO-EtOH mouse group suggest that downregulation of hepatic *Cyp7a1* is independent of FGF15 in our ACLI model.

The small heterodimer partner (SHP) or else NR0B2 (nuclear receptor subfamily 0, group B, member 2) is a member of the nuclear receptor family of intracellular transcription factors (68). Although classified as a nuclear receptor is missing a DNA binding domain and has a variety of interacting partners, a fact that indicates a multidimensional potential, regulating drug, glucose, bile acids, cholesterol and triglycerides metabolism (15). As far as the BA metabolism is concerned, SHP is considered to inhibit the conversion of cholesterol to bile acids, undertaking hence a potential hepatoprotective role preventing excessive cholestasis (15,100). Previously published data point to an essential role for SHP signaling in ethanol catabolism, as *Shp* -/- mice displayed enhanced ethanol catabolism and clearance and reduced ethanol induced hepatic inflammation compared with their wild type counterparts (100). Consequently, the suppression of SHP could be part of a favorable hepatoprotective pathomechanism. However, in this study, no significant difference in expression levels of hepatic *Fxr* and *Shp* was observed between groups suggesting that repression of *Cyp7a1* gene expression was also independent of FXR/SHP pathway in our model.

The sterol regulatory element-binding transcription factor 1 (SREBF1) or sterol regulatory element-binding protein 1 (SREBP-1) is another key regulator of lipids metabolism in liver. It is related with the signaling pathway of PPARa and circadian rhythm related genes (123). Recent data indicate its association with multiple further cellular signals, implicating SREBF1 in diabetes mellitus type 2, malignancies, immune response, neuroprotection and autophagy (123). Its emerging role in lipogenesis, autophagy and inflammation and association with fatty liver disease (87), challenged us to examine a potential alteration of this gene expression

during ethanol consumption. Since there was no significant difference in hepatic expression levels of *Srebf1* in our study, this result suggests that the transcription factor SREBF1 does not have a direct impact on FGF21 upregulation in our ACLI model. However, our results showed statistically significant upregulation of hepatic *Ppara* expression levels after ethanol challenge in both WT and KO mice showing no difference between genotypes. These results suggest that PPARa, but not the transcription factor SREBF1, may have a direct impact on FGF21 upregulation. Indeed, there is a linear positive correlation between PPARa and FGF21 demonstrated both in humans and mice (76). It is thereupon expected that we found concomitantly with the upregulation of hepatic and circulating FGF21 upon ethanol challenge, a statistically significant elevation of PPARa as well. The protective role of PPARα in alcoholic liver disease was suggested from Nakajima et al. already since 2004 (91). Later FGF21 was proposed as the mediator of the metabolic favorable function of PPARα both in alcoholic and metabolic associated liver disease and our study results support the suggested correlation. The impact of PPARa on FGF15 and the FXR-FGF15 axis is more controversial. The existence of an inverse correlation has previously been supported (148). This presumably inverse correlation between PPARa and FGF15/19 is further reinforced from a demonstrated downregulation of FGF19 after treatment with PPARa-agonists (fenofibrat) in patients with Type 2 Diabetes Mellitus (88).

To investigate the effect of alcohol on FGF21 signaling pathways, hepatic mRNA levels of the *Mtor (*downstream mediator mTOR), which is a member of the phosphatidylinositol 3 –kinase-related family of protein kinases, was also investigated in this study. There are sparse data proposing a suppression in mTOR signaling pathway in ethanol exposed hepatocytes (139). Additionally, a suppressed mTORC1 signaling has been suggested to ameliorate fat accumulation and inflammation in alcoholic liver disease and to be associated with improved prognosis (34,165). It has also been supported that the mTOR signaling is upregulated in hepatocellular carcinoma (34), a fact that has pharmacological extensions. In our study, a slight tendency of downregulation of *Mtor* in mice challenged with ethanol was observed. However, since there was no statistically significant changes between mice groups, our results denote that the downstream signal transduction of FGF21 is possibly not mediated through an mTOR pathway, which was previously reported to have a regulatory role in the FGF21 signaling network (85).

Significant upregulation of ileal *Fgf15* and *Fxr* gene expressions in the WT mice were observed upon ethanol uptake but not in the KO group. However, interestingly, the circulating FGF15 plasma levels did not differ between groups. To clarify this incompatible result, we also measured the expression levels of *Diet1/Malrd1* gene in ileum, which has been shown to play a role in the regulation of intestinal expression of *Fgf15* undertaking a potential role in the

feedback control loop of bile acid synthesis (152). DIET1 is expressed on the epithelial cells of intestine (and on cells of kidney cortex), but not in hepatocytes. DIET1 deficiency is suggested to inhibit the production of ileal FGF15/19 and thus impair the intact function of the enterohepatic feedback loop (152). Conversely, the increase in DIET1 levels enhances the FGF15/19 production and subsequently suppresses *Cyp7a1* (152). The influence of DIET1 on FGF15/19 emerges through a post-transcriptional mechanism, possibly through an intracellular mechanical interaction (152). In our knowledge there are no present data for the impact of alcohol intake on DIET1 expression. In the present experiments, although the expression levels of *Diet1* showed similarity with the expression pattern of *Fgf15*, with higher levels in the WT mice challenged with ethanol, we did not observe any significant difference in the basal levels of DIET1 between mice groups and upon ethanol challenge. Our results do not support a significant impact of alcohol intake on a DIET1 mediated regulation of FGF15 in both WT or *Abcb4* deficient mice.

We considered the detected upregulation of FGF21 upon ethanol challenge in both WT and KO mice and its potential correlation with the suppression of the biosynthesis of bile acids via direct action on the rate limiting step of their biosynthetic cascade the most compelling finding. In addition, our results did not support FXR-SHP or FGF15/FGFR4 pathways dependent downregulation of *Cyp7a1.* Therefore, to investigate the direct effect of FGF21 on hepatic *Cyp7a1* mRNA suppression, cell culture studies were performed by using mouse primary hepatocytes. The results showed that rhFGF21 decreased *Cyp7a1* expression levels in a dose dependent manner. This outcome strongly supports the direct effect of FGF21 on the BA biosynthesis, independently of FGF15/19.

Previously well-designed studies had already proved a robust upregulation of FGF21 upon alcohol ingestion in healthy volunteers and proposed a negative feedback loop in the liver brain axis that leads to suppression of excessive alcohol consumption. It is further suggested that this loop could be the evolutionary remnant of an analogous mechanism for the limitation of consumption of fermentated carbohydrates in our ancestors (154). In our study we also investigate if this potential hepatoprotective mechanism remains intact even in the presence of a preexisting liver disease. Therefore, we also examined the effect of alcohol intake on FGF21, FGF19, and bile acid regulation in human subjects with alcoholic liver cirrhosis and admitted ongoing ethanol abuse compared to healthy controls. We could not submit the patients to a quantifiable exogenous alcohol application as this would be unethical. Therefore, we objectified the admitted consumption via detection of alcohol metabolites (carbohydrate-deficient transferrin - CDT). In our enrolled ALD patients the CDT levels were elevated in a statistically significant level, as compared with a control group of healthy subjects, who denied any alcohol intake in the last 7 days. We furthermore controlled the metabolic profile of the

patients comparatively to the control cohort and we identified alterations common in ALD. The serum transaminases were significantly elevated in the ALD cohort, as were the serum total bile acids, a fact that indicates a present dysregulation of bile acids metabolism. On the contrary, serum total cholesterol was detected significantly reduced compared to the control group. This is consistent with previous findings revealing a significant reduction in levels of total cholesterol in patients with alcoholic liver cirrhosis in comparison to control groups and coherent with the expected pathophysiological impact of ethanol on cholesterol metabolism (22).

In accordance with the current *in vivo* data, an upregulation of circulating FGF21 in the cohort of patients with alcoholic liver disease was confirmed compared to the healthy control group. Although the hepatic expression levels of FGF21 has not been examined in the human subjects, previous data consistently support that the liver production of this hepatokine conferes the major part of the plasma levels. Our data support that the beforehand regarded robust upregulation of FGF21 in healthy subjects upon ethanol challenge remains preserved even in the setting of an existing liver damage and the established hepatocellular injury seems not to significantly influence the capability of upregulated FGF21 production. In our knowledge there are sparse hitherto published data for the influence of alcohol use disorder and alcoholic liver disease on FGF21 in humans. However, we cannot definitely conclude if the elevated serum FGF21 levels are attributable to the induction of a reactive protective pathomechanism or imply a resistance state, as in patients with diabetes and obesity. Independently from that, our findings suggest that exogenous FGF21 application could be studied as a potential pharmacological treatment for the amelioration of chronic and acute liver damage of alcoholic etiology.

In accordance with our findings in mice, the circulating levels of FGF19 in the patients with alcoholic liver disease and ongoing ethanol consumption did not significantly differ, although a slight trend to upregulation could be detected. This finding suggests that a considerable endocrine action of FGF19 is not induced in humans with alcoholic liver disease after ongoing ethanol abuse. At this point must howbeit be noted that the main expression site of FGF19 is the small intestine and that FGF19 does not exclusively act in an endocrine manner, but as a paracrine factor in the liver-intestine axis as well. Hereby can we though refer to the murinal findings, where an intestinal upregulation of FGF15 in the cholestatic liver after alcohol intake could not be ascertained neither. As a whole, we can reliably assume that the main endocrine fibroblast growth factor affected by alcohol intake in humans with previously established liver disease is FGF21. We can consequently suggest that the further observed alterations, especially as far as the metabolism of bile acids is concerned are mainly FGF21- and not FGF19-mediated.

After the recognition of the ethanol induced changes in the levels of FGF21 and FGF19 in the frame of ongoing alcohol consumption by preexisting alcoholic liver disease, we extended our study in order to clarify the ensuing consequences for the biosynthesis and metabolism of bile acids. To this purpose, in order to translate our findings to humans, we evaluated the effects of alcohol consumption on the oxysterols 7a-hydroxycholesterol (7aOHC) and 27-hydroxycholesterol (27OHC) levels in serum samples of both human groups. Since CYP7A1 catalyzes the conversion of cholesterol to 7a - OH cholesterol, which is a precursor of bile acids in the classic pathway, its serum levels represent an estimation of the CYP7A1 activity and consequently the saturation of the rate limiting step in the BA biosynthesis. The serum levels of 7a - OH cholesterol were significantly suppressed in the ALD group with continuing alcohol intake compared to healthy controls, which was in line with our *in vivo* findings. On the contrary, 27OH cholesterol levels, which is minor precursor of the bile acids synthesis, did not show significant differences between the groups. The latter finding is very interesting, because it correlates with the lack of statistically significant suppression of CYP27A1 in the *Abcb4* knockout mice in our murinal experiments. This could imply a deficit in the ability of control of this step of BA synthesis in the frame of a preexisting liver pathology. It could furthermore be associated with the fact, that 27-OH cholesterol has multiple functions, not only in BA metabolism, but endocrine as well. It can for instance act as a selective estrogen receptor modulator and thus is part of more than one feedback control loops (80). In summary, the findings of the present study point to a correlation between alcohol induced FGF21 and suppression of the rate limiting enzyme in BA synthesis by an unknown FGF15-independent pathway in ACLI.

6. CONCLUSIONS

The main finding of the present experimental study is the suppression of the bile acid synthesis rate limiting enzyme CYP7A1 by chronic plus binge alcohol-induced FGF21 in an FGF15/19-independent pathway, due to the lack of changes in neither expression levels of hepatic *Fxr* and *Shp* nor plasma FGF15/19 concentrations after ethanol challenge. Our results suggest a direct effect of FGF21 on *Cyp7a1* suppression, which is supported by our *in vitro* studies. Therefore, our findings are in line with studies supporting a previously unidentified role of FGF21 as a negative regulator of bile acid synthesis (79). In addition, the results support the recent notion proposing the *Cyp7a1* suppression effect of FGF21 to be mediated independently of FGF15/19 (79, Figure 18). Moreover, in accordance with the *in vivo* results, our translational human studies showed a significant FGF21 upregulation and a significant reduction of serum 7aOHC concentrations in patients with alcoholic liver disease and ongoing ethanol consumption but unchanged plasma FGF19 levels. However, in the current study, the details of the interaction mechanisms between FGF21 and CYP7A1 are not clarified and need further investigations.

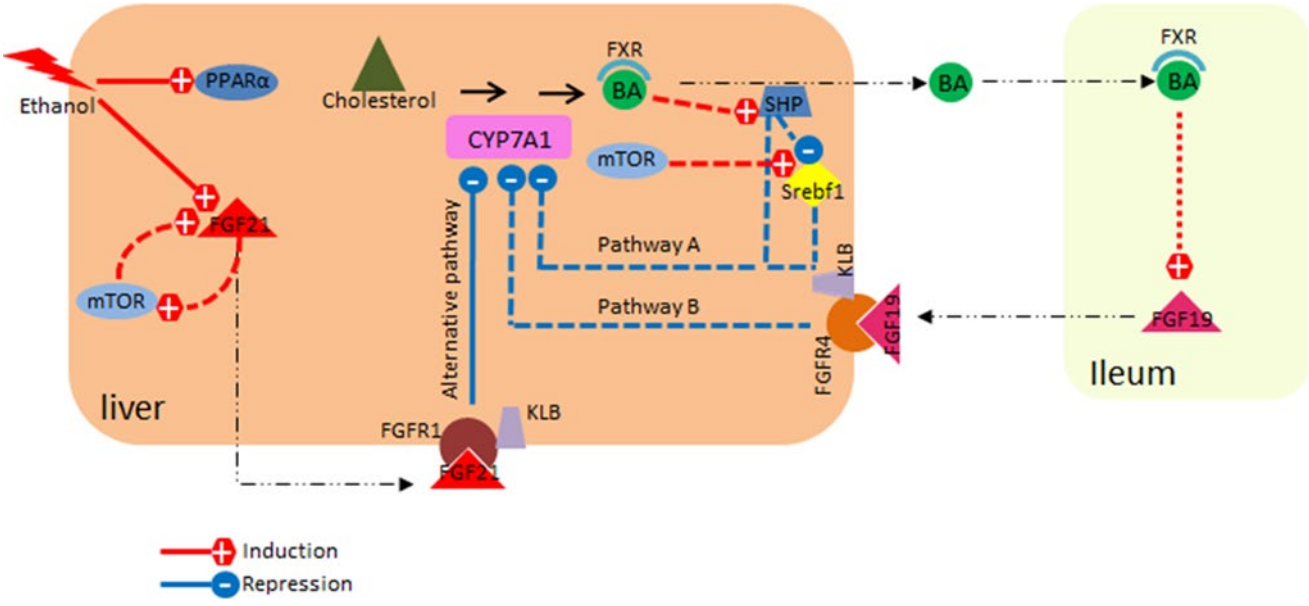

Figure 18: Suggested alternative pathway for bile acid synthesis regulation upon ethanol challenge. Dashed lines for induction and repression are based on literature data, whereas straight lines show the results of the current study. Adapted from Al-Aqil et al. 2018 (3).

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