Diploma thesis

Fibroblast Growth Factor 21 in connection to alcohol consumption and alcoholic liver cirrhosis

submitted by

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Graz, am 20.05.2018 *Philipp Grande eh*

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Abstract

Introduction: FGF21 is a hepatokine regulating nutritional intake, especially of simple sugars. Its endocrine function relies on FGF-receptors and βKlotho, which are both present in the hypothalamus and liver. In addition, sugar and alcohol intake enhances the synthesis of FGF21 and highly increased levels of FGF21 decrease the alcohol intake in mice. Therefore, FGF21 might facilitate a liver-brain endocrine axis, by which liver tissue can decrease harmful alcohol intake. The aim of this study was to measure FGF21 levels in patients with alcoholic liver cirrhosis (ALC) and correlate them with their present alcohol consumption. Furthermore, a comparison of these results with non-alcoholic liver cirrhosis (NALC) and healthy test persons was made.

Methods: Detailed information about eating and drinking behaviour of our cohort (ALC: n=41; NALC n=34; Healthy: n=21) was acquired by a food frequency questionnaire. Declared alcohol intake was verified by urine ethyl glucuronide (ETG) levels. FGF21 plasma levels were determined by ELISA. In addition, a cell culture model with human carcinoma hepatocytes was used to test their FGF21 expression in response to alcohol and glucose.

Results: Patients with positive ETG levels (≥0.5 µg/ml; equivalent to positive alcohol consumption in the last 12 to 72 hours) showed significantly higher FGF21 plasma levels in comparison to patients with negative ETG levels. This applied to patients with ALC and NALC. The results did not show statistical significance in FGF21 plasma levels according to eating behaviour. The incubation of human hepatocytes with alcohol and glucose did not result in an increase of FGF21 synthesis.

Discussion: Increased FGF21 levels in patients with recent alcohol consumption (verified by ETG) confirmed the first part of the liver-brain endocrine axis: Alcohol consumption seemed to enhance FGF21 synthesis. However, the second part of the liver-brain endocrine axis, a reduction of alcohol intake in case of increased FGF21 levels was not observed. That points towards a pathology in this pathway, which might be caused by a malfunction of βKlotho or FGF-receptors according to other studies. Further research is required to clarify these pathologies, which may open new pharmacological treatment for patients with alcohol use disorder and alcohol dependence.

Zusammenfassung

Einleitung: Das Hepatokin FGF21 beeinflusst die Aufnahme von Nährstoffen, besonders von einfachen Zuckern. Die hormonellen Eigenschaften sind auf die Gegenwart von Cofaktor βKlotho und FGF-Rezeptoren angewiesen, welche u.a. im Hypothalamus und in der Leber zu finden sind. In Mäusen wird dieses Molekül nach Zucker- und Alkoholzufuhr verstärkt gebildet und zeigt neben dem Einfluss auf den Energiestoffwechsel eine Verringerung des Alkoholverlangens. Somit unterstützt FGF21 eine hormonelle Leber-Hirn Achse, durch die möglicherweise die Leber selbst den schädlichen Alkoholkonsum einschränken kann. Ziel dieser Studie ist es die FGF21 Konzentration bei Patienten mit alkoholischer Leberzirrhose (ALC) zu messen und diese mit dem gegenwärtigen Alkoholkonsum zu korrelieren. Zusätzlich soll dies mit der nicht-alkoholischer Leberzirrhose (NALC) und mit gesunden Testpersonen verglichen werden.

Methoden: Mithilfe eines Fragebogens wurden detaillierte Informationen über die Ess- und Trinkgewohnheiten der Kohorte erhalten (ALC: n=41; NALC: n=34; Gesund: n=21). Die Angaben zum Alkoholkonsum wurden mithilfe der Ethylglucuronide (ETG) Spiegel im Urin verifiziert. Des Weiteren wurde die FGF21 Konzentration im Plasma mittels ELISA bestimmt. Zudem wurden humane Hepatozyten kultiviert und mit Alkohol und Glukose inkubiert, um deren FGF21 Expression zu prüfen.

Ergebnisse: Patientinnen und Patienten mit positiven ETG-Spiegeln (≥0,5µg/ml; entspricht Alkoholkonsum in den letzten 12 bis 24 h) zeigten signifikant höhere FGF21 Spiegel als solche mit ETG Konzentrationen unter 0,5 µg/ml. Dies gilt für Betroffene von ALC und NALC. Bei der Inkubation von Hepatozyten mit Alkohol und Glukose konnte kein Anstieg der FGF21 Synthese beobachtet werden.

Diskussion: Erhöhte FGF21 Spiegel bei Patientinnen und Patienten mit rezentem Alkoholkonsum (geprüft durch ETG) bestätigt den ersten Teil der hormonellen Leber-Hirn Achse: Alkoholkonsum verstärkt die FGF21 Synthese. Jedoch bleibt der zweite Teil der hormonellen Leber-Hirn Achse, also die Reduktion des Alkoholkonsums in der Regel aus. Dies deutet auf eine Pathologie dieser Hormonachse hin, welche durch Störungen von βKlotho oder den FGF-Rezeptoren ausgehen kann. Weitere Forschung ist erforderlich, um dies zu klären und damit neue Behandlungsmöglichkeiten für Betroffene mit Alkoholabhängigkeit zu schaffen.

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Abbreviations

A

ALC *Alcoholic liver cirrhosis*
ALD *Alcoholic liver disease*
AML12 *Alpha mouse liver 12*

B

BAT *Brown adipose tissue*
BMI *Body-Mass-Index*

C

CDT *Carbohydrate deficient transferrin*
CNS *Central nervous system*

D

DIC *Disseminated intravascular coagulation*

E

ELISA *Enzyme linked immunosorbent assay*
ETG Ethyl glucuronide
ETS Ethyl sulfate

F

FGF Fibroblast growth factor
FGFR *Fibroblast growth factor receptor*
FHF *FGF homologues factor*

G

GWAS *Genome-wide association study*

K

KO *knockout*

M

MCV Mean corpuscular volume
MELD Model of end-stage liver disease

N

NAD oxidized nicotinamide adenine dinucleotide
NADH reduced nicotinamide adenine dinucleotide
NALC *non-alcoholic liver cirrhosis*

P

PNPLA3 *patatin-like phospholipase domain-containing 3*
PPARα *P*eroxisome proliferator-activated receptor alpha
PPARγ *peroxisome proliferator-activated receptor gamma*

S

SNP Single nucleotide polymorphism

T

TIPS *Transjugular intrahepatic portosystemic shunt*

TM6SF2 *Transmembrane 6 superfamily member 2*

TNF-alpha *Tumor necrosis factor-alpha*

W

WAT *White adipose tissue*

Γ

γ-GT *gamma-glutamyltransferase*

1 Introduction

1.1 FGF family

The family of fibroblast growth factors (FGF) consists of 18 different FGF proteins (FGF1 – 10 and FGF16 – 23) and four FGF homologous factors (FGF11 – 14)(1). These proteins can be classified into endocrine and paracrine/autocrine FGFs. Endocrine FGF, also called the FGF15/19 subfamily, regulated energy and mineral metabolism (2-5). Paracrine/autocrine FGFs are subdivided in five subfamilies, called FGF1-, FGF4-, FGF7-, FGF8- and FGF9- subfamily, and influence tissue repair and organogenesis during embryogenesis. The last not mentioned member of the FGF family is FGF 15, representing the mouse ortholog of human FGF19 (6).

Additionally, there are FGF homologous factors (FGF11 – 14), showing a high sequence identity to FGFs but do not activate FGF-Receptors (FGFR) (7). The expression of FGF homologous factors (FHF) is mainly restricted to neuronal cells, where they bind to intracellular domains of sodium channels. Depending on the type of FHF, this binding leads to activation or inactivation of sodium channels, which increases or decreases sodium currents. Genetic alteration of these proteins can lead to cerebellar ataxia in mice. Furthermore, FHFs represent cofactors for the brain specific mitogen-activated protein kinase (MAP kinase), whereby the function of these interaction is not clear so far (8).

To achieve the diverse functions of FGFs specific receptors are required. FGF-receptors (FGFR) are tyrosine kinase receptors encoded by four different genes (*FGFR1 – FGFR4*). Alternative splicing of *FGFR1 – FGFR3* leads to two different isoforms of each receptor, denominated with “b” or “c” (FGFR1b – FGFR3b and FGFR1c – FGFR3c). The b-isoforms are specific for epithelial tissue and the c-isoform for mesenchymal tissue (9). Accordingly, there are seven FGFRs with different FGF binding specificities.

In addition to these receptors, cofactors are needed. In case of the FGF family there are two different types of cofactors. The first one is heparan sulfate. Paracrine and autocrine signalling is based on the high affinity for heparan sulfate in the extracellular matrix (1, 10), which leads to the inability of FGFs to enter the blood circulation (11). Heparan sulfate is also essential for promoting and stabilizing the

contact between FGF and FGFR (11). Furthermore, it protects FGF molecules against degradation (1). Endocrine FGFs however have a very low affinity to heparan sulfate. To achieve their function at the target organs, an alternative cofactor is necessary: klotho proteins, subdivided in αKlotho proteins specific for FGF23 and βKlotho proteins specific for FGF15/19 and FGF21 (5, 11). Table 1 shows the FGF subfamilies according to their appropriate cofactors and receptors.

FGF subfamily	FGF	Cofactors	Receptor specifity
FGF1 subfamily	FGF1	Heparin or Heparan sulfate	All FGFRs
	FGF2		FGFR 1c, 3c > 2c, 1b, 4
FGF4 subfamily	FGF4 FGF5 FGF6		FGFR 1c, 2c > 3c, 4
FGF7 subfamily	FGF 3 FGF7 FGF10 FGF22		FGFR 2b > 1b
FGF8 subfamily	FGF8 FGF17 FGF18		FGFR 3c > 4 > 2c > 1c >> 3b
FGF9 subfamily	FGF 9 FGF16 FGF20		FGFR 3c > 2c > 1c, 3b >> 4
FGF15/19 subfamily	FGF 15/19	βKlotho	FGFR 1c, 2c, 3c, 4
	FGF21		FGFR 1c, 3c
	FGF23	αKlotho	FGFR1c, 3c, 4

Table 1: FGF subfamilies with receptor specificities and cofactors [from (10)].

1.2 FGF21

FGF21 with a half-life period of 0.7 – 1.1 hours is expressed in various tissues, more precisely in liver, white adipose tissue (WAT), brown adipose tissue (BAT) and pancreas (3, 12). However, the main part is expressed by the liver (13). Multiple factors can influence the increased expression of FGF21, facilitating different effects on the organism. These effects are restricted on tissues, expressing βKlotho and FGFR 1c or 3c (10, 14). While FGFRs are widespread in several tissues, βKlotho seems to be the limiting factor for the endocrine effects of FGF21. Tissues expressing βKlotho are liver, WAT, BAT, endocrine and exocrine pancreas and hypothalamus (15, 16). In addition to the expression of βKlotho in the central nervous system, FGF21 can cross the blood-brain barrier (3, 17).

1.2.1 FGF21 in relation to the energy metabolism

The energy metabolism is a very sensitive system, acting and reacting dependent on the nutritional status of the organism. This flexibility requires various hormones coordinating this process. FGF21 has been shown as an important player of this system.

Fasting for more than 12 hours strongly increases FGF21 synthesis in mice, which requires glucagon receptors and the peroxisome proliferator-activated receptor α (PPARα) (18). The same effects are seen in human cell lines, in HepG2 hepatoma cells (19). PPARα is a nuclear receptor inducing the transcription of the *fgf21* gene and is activated by fatty acids and fibrates. Thus, the FGF21 levels increase and bind to the FGFR of hepatocytes, which in turn increases gluconeogenesis and ketogenesis. The activation of these metabolism pathways by FGF21 leads to obligatory energy winning, which is necessary for an organism while fasting (18). PPARα-KO mice are FGF21 deficient and have an insufficient catabolism of fatty acids in the liver and becoming hypoketonemic and steatotic. These effects are reversible by the application of recombinant FGF21 (20). Besides these effects on the metabolism, FGF21 does induce torpor, presenting an effective mechanism for mammalians to save energy in fasted state (20) (Figure 1).

Remarkably, an induction of FGF21 expression in fed state by WAT in rodents, especially in refeeding situations after fasted state can be observed (21). This means that there are two opposite situations, fasting and feeding, that lead to an increase of FGF21. In a fed state, peroxisome proliferator-activated receptor γ (PPARγ) induces the expression of FGF21. PPARγ is activated by fatty acids and thiazolidinedione (insulin sensitizer) and is highly expressed in WAT (18, 21). Additionally, the induction of FGF21 expression in WAT does not lead to an increase of protein concentration in the blood circulation. In this case, FGF21 only has paracrine and autocrine effects. It facilitates glucose uptake in adipose tissue, enhances adipocyte differentiation and regulates PPARγ activity. The cause for the paracrine/autocrine manner of FGF21 in WAT could be an interaction between FGF21 and the extracellular matrix of WAT (21, 22) (Figure 1).

In summary FGF21 is expressed in fasted and fed state, which seems to be a contradiction at first. However, the expression of FGF21 in each nutritional state is

limited to different tissues. In fed state FGF21 is expressed by WAT and operates in an autocrine manner, whereby in fasted state FGF21 is expressed in hepatocytes and operates in an endocrine manner. Therefore, these two processes are happening in different regions of the organism, independent of each other.

1.2.2 Pharmacological effects of FGF21 on metabolic disease

FGF21 is directly and indirectly involved in the pharmacological treatment of metabolic disease.

Pharmacological studies have shown that FGF21 increases insulin sensitivity (Figure 1). It could be shown that one hour after a single injection of FGF21, the blood glucose levels decreased and the glucose tolerance and insulin sensitivity improved in insulin resistant mice (23). Diabetic rhesus monkeys showed a decline of plasma glucose, triglycerides, insulin and glucagon during a daily administration of FGF21 for 6 weeks. There were also significant improvements of cardiovascular risk factors, like a decrease of LDL cholesterol levels and an increase of HDL cholesterol. , a modest weight loss in the period of FGF21 administration was observed (24). However, no improvement of glucose uptake in peripheral tissues by FGF21 has been shown in mice, which leads to the hypothesis that all pharmacological effects were achieved by the regulation of hepatic metabolism (18).

Furthermore, fibrates and thiazolidinedione can induce the synthesis of FGF21 (1.2.1 FGF21 in relation to the energy metabolism). Fibrates represent a potent therapy for dyslipidaemia, and the insulin sensitizing effect of thiazolidinedione is an effective antidiabetic treatment (25). Therefore, FGF21 already seems to be a pharmacological target and might represent a potential treatment for metabolic diseases by itself.

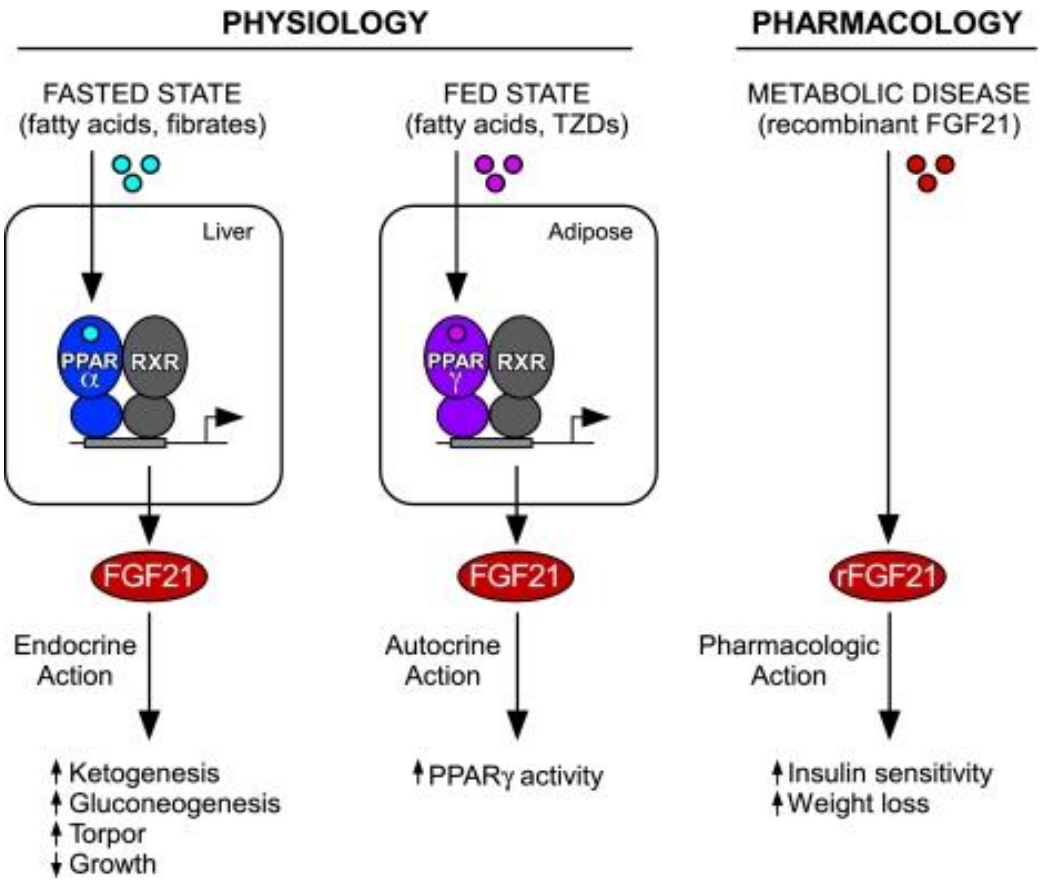


Figure 1. Endocrine, autocrine and pharmacological action of FGF21 [from (18)].

1.2.3 Eating and drinking preferences

Beside the effects on energy metabolism and the pharmacological effects, FGF21 seems to influence eating and drinking preferences. Hormones with effects on food intake are common in our organism. For example, insulin and leptin decrease the appetite by acting on the hypothalamus while the resistance against insulin and leptin leads to diseases with nutritional abnormalities, like obesity (26). This combination of effects on food intake and energy metabolism represents the foundation of a functional organism, since energy metabolism depends on nutritional habits and vice versa.

FGF21 has been shown to decrease the intake of sweets in transgenic mice with supraphysiological FGF21 levels compared to wild type mice. Both had the opportunity to drink either water or water with 3% of sucrose and the transgenic mice had an aversion to sucrose water. This effect was not observed in mice lacking βKlotho in the central nervous system (CNS) (27). βKlotho is necessary for the receptor binding of FGF21 in target organs (1.1). Thus, it seems likely that the effect of FGF21 on sweet taste preference happens in the brain. The highest levels of

βKlotho mRNA are detected in the suprachiasmatic nucleus, part of the hypothalamus. This, in turn, points to the hypothalamus as an essential region of the CNS to process the FGF21 pathway. (27). Furthermore, it has been shown that mice with an inactivated *fgf21* gene, FGF21 knockout (KO) mice, have a significant increase of mono- and disaccharide intake (28). Taken together, FGF21 has been shown to decrease the preference for sweet taste by acting on the hypothalamus in rodents (27).

Similar effects on alcohol consumption have been described. More precisely, transgenic mice with supraphysiological FGF21 levels show a significant decrease of alcohol intake in relation to wild type rodents. Since there is no difference of ethanol bioavailability between transgenic mice and wild type mice, the effects of FGF21, reducing alcohol consumption, seems to be based on alterations in the drinking behaviour and rather than alcohol metabolism (27). In addition, mice getting injections of recombinant FGF21 for a longer period, showing a reduction of alcohol intake too (29).

The mesolimbic dopamine system plays a major role in the reinforcement of alcohol consumption. Alcohol intake increases dopamine levels in the mesolimbic systems. This can lead to an alcohol-seeking behaviour, which is typical for alcoholism (30). FGF21 decreases dopamine levels in the nucleus accumbens, which in turn leads to a decrease of alcohol-seeking behaviour and confirms the effects of FGF21 on alcohol consumption (27).FGF21 reduce sweet taste and alcohol preference and vice versa alcohol or carbohydrate intake can also induce FGF21 expression.

The level of FGF21 was highly increased after 12 days of alcohol intake in mice, compared to the control group without alcohol intake, but the same amount of food. Furthermore, *in vitro* alcohol incubation of alpha mouse liver 12 (AML-12)-cells increases FGF21 expression, which in turn indicates the involvement of hepatocytes in this mechanism (31).

In addition, carbohydrates also increase the synthesis of FGF21. Twenty-four hours after ingestion of either fructose, glucose or sucrose, FGF21 mRNA and FGF21 plasma levels are elevated in mice. The group with sucrose ingestion is showing the highest levels. Mice with glucose or fructose ingestions are showing almost the

same FGF21 levels, but lower than the sucrose group (28). In humans, fructose intake has the strongest impact on FGF21 synthesis (32).

In conclusion, alcohol and carbohydrates induce the expression of FGF21 and high levels of FGF21 decrease the craving for alcohol and sugar intake. This leads to the hypothesis of a feedback mechanism, in terms of a liver-brain endocrine axis, through which components secreted by the liver are able to suppress harmful alcohol intake.

1.2.4 Overview of FGF21

Viewing our knowledge about FGF21 in contexts, leads to some open questions.

On the one side, we see metabolic situations, especially in fasted state with elevated free fatty acids leading to an increase of FGF21 expression in hepatocytes. This results in an increase of ketogenesis and gluconeogenesis, as well as an improvement of insulin sensitivity after pharmacological administration. This mechanism represents a physiological strategy to manage fasted states and FGF21 seems to play an important part in it (Figure 2).

On the other side, alcohol and carbohydrate intake induce FGF21 expression in hepatocytes, resulting in a systemic increase of FGF21 levels. By acting on the hypothalamus FGF21 can decrease alcohol and sugar preference. This hints at a liver-brain endocrine axis to prevent high alcohol and sugar intake and may in part be involved in the pathophysiology of these diseases (Figure 3).

These two pathways, involving FGF21 seem to be very useful for the organism to manage difficult situations. However, by comparing these pathways, we see some controversy. Hepatic expression of FGF21 increases in fasted state and by alcohol and sugar intake. Two opposite metabolic situations lead to the same effect: an increased synthesis of FGF21.

Furthermore, elevated FGF21 levels activate two different pathways. On the one side, FGF21 in a fasted state increase ketogenesis and gluconeogenesis to generate energy, which represents a catabolic metabolism. On the other side, FGF21 decrease the preference for sugar and alcohol, which temporarily excludes two easily accessible energy sources. In view of the energy metabolism, these two pathways are in conflict with each other.

What is the key factor regulating these different pathways of FGF21? By comparing the levels of recombinant FGF21 injected in different mouse experiments, almost the same concentration can induce either a decrease of alcohol and sugar intake or an increase of gluconeogenesis or ketogenesis (20, 23, 27, 28). Thus, the concentration of FGF21 might not be the key factor for the regulation of these different pathways. It is evident, that FGFR1c and FGFR3c show affinity for FGF21 (1.1). Different locations and different effects of these receptors could explain the differences of FGF21 pathways. Besides, the concentration or activity of co-receptor βKlotho could influence these pathways, too. Furthermore, intracellular pathways of hepatocytes or neuronal cells of the hypothalamus could process FGF21 signalling in different ways. Evidently, there are still open questions in the complexity of FGF21 that require further investigation.

However, there is an interesting feedback mechanism, which might represent a self-protection of liver tissue - the liver-brain endocrine axis. FGF21 seems to be the key player in this feedback mechanism and could represent an important role in pathophysiological pathways of diseases like alcoholism and alcoholic liver disease.

Figure 2: Pathway in fasted state

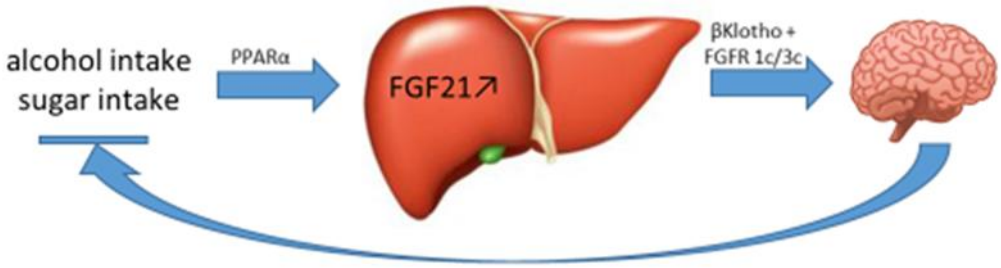


Figure 3: Pathway after alcohol or sugar intake.

1.3 Alcoholic liver disease

Alcohol consumption has a long history in humankind, starting 10,000 B.C. with the intake of fermented beverages (33). However, the knowledge of associated health problems probably remained unclear for a long time. In the 1920´s the first study was published about a higher mortality of heavy drinkers compared to moderate drinkers and abstainers. The liver gained increasing attention in regard to alcohol related health problems (34).

1.3.1 The liver

Located in the upper right quadrant of the abdominal cavity with approximately 1500g the liver is an essential organ in our organism (35). The liver represents a major player in the carbohydrate, protein and lipid metabolism, synthesizes exocrine and endocrine proteins and is necessary for the detoxification of several external and internal substances. About 80% of the liver is built up by hepatocytes, executing main functions of the organ. (36). One of these functions is the degradation of alcohol, whose excessive consumption leads to pathologic transformations of the liver tissue (25).

Macroscopically the liver is subdivided in four lobes; the right and left lobe, separated by the falciform ligament, and two smaller lobes, the caudate and quadrate lobes which are visible on the visceral surface of the liver. Based on the vascularization, there is another subdivision in eight liver segments. Each of these segments is supplied by the *Glisson-Trias*, comprising the vena portae, arteria hepatica propria and ductus hepaticus. Furthermore, there is a hepatic vein leaving each segment separate of the Glisson-Trias. All hepatic veins of each segment assemble to form one hepatic vein, anastomosing with the vena cava inferior (35).

The hepatic lobule represents the microscopic unit of the liver. The central vein is located in the centre of these hexagonal lobules and encircled by hepatocytes. Central veins of different lobules build up the hepatic vein. The Glisson-Triad (or Portal triad) is based at each corner of the hexagonal lobule, where liver sinusoids are collecting blood of the portal vein and the hepatic artery. On the way to the central vein, blood gets in contact with hepatocytes, enabling an exchange of several substances. The bile ductules are arranged in a parallel manner, carrying

bile in the opposite direction. The bile, produced by hepatocytes is entering the ductus hepaticus and later the intestine, representing an important secretion for an optimal ingestion. Beside the hepatocytes some other cell types are necessary to complete the liver. Sinusoidal endothelial cells, Kupffer cells representing the unspecific immune system of the liver, and hepatic stellate cells for fat and Vitamin A storage (36).

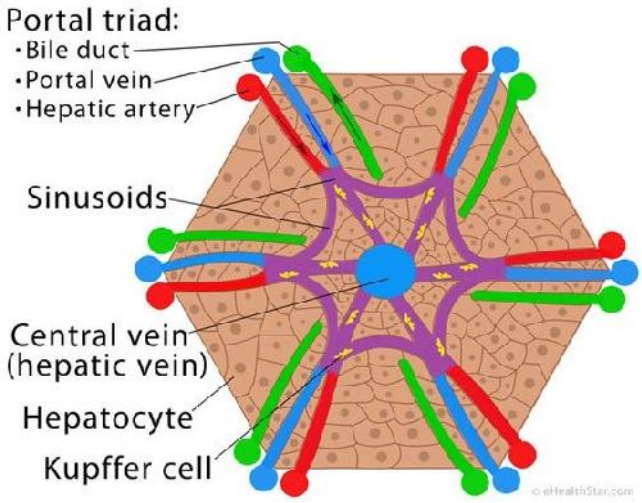


Figure 4: Hepatic lobule [from (37)]

1.3.2 Aetiology and epidemiology

The consequence of long-term alcohol intake is the alcoholic fatty liver (steatosis hepatis), representing a reversible pathology in case of reducing or stopping alcohol consumption. Without changes in drinking behaviour the pathology will proceed into alcoholic hepatitis and later to the most serious state of Alcoholic Liver Disease (ALD), alcoholic cirrhosis (34). The quantity of daily alcohol intake is a crucial factor in the pathogenesis of ALD. The toxic portion for men is specified as 40g of ethanol per day and for women with 20g of ethanol per day (25). However, duration of alcohol abuse, drinking patterns, sex, ethnicity and associated risk factors like obesity, viral hepatitis infection, iron overload and genetic factors can favour the pathogenesis of ALD (33).

In 2010 the alcohol per capita consumption was 10.9 litres of pure alcohol in Austria (European average: 11.9 litres) and the prevalence of alcohol use disorders and alcohol dependence was 14.1%, means 1,184,682 Austrians (38). This is an enormous burden for the liver and leads to more than 600 deaths per year associated with ALD in Austria (39). In 2017 Austria has the second highest rate of

liver cirrhosis in Europe (40). In general, one third of liver disease in the western world is caused by alcohol and around 5 to 10% of the western population is suffering from ALD (25). Moreover, ALD is the second most common reason for liver transplantation in Europe (41).

1.3.3 Pathogenesis and transformation of liver architecture

There are three different ways to catabolise absorbed alcohol in the liver. The main part is degraded by alcohol dehydrogenase, oxidising alcohol to acetaldehyde, which, in turn, is oxidised by acetaldehyde dehydrogenase to acetate. Besides, there are microsomal ethanol oxidation system (cytochrome P450 2E1) and catalase, converting alcohol in hepatocytes. Accrued acetate will be either catabolized in citrate acid cycle or converted to other biological compounds like fatty acids. These fatty acids represent one of several ways that lead to an increased level of fat in alcohol challenged hepatocytes (42). Furthermore, these two reactions of alcohol- and acetaldehyde dehydrogenase are dependent on oxidized nicotinamide adenine dinucleotide (NAD) and in case of high concentrations of incoming alcohol we see an imbalance of NAD and reduced nicotinamide adenine dinucleotide (NADH). This decrease of NAD/NADH redox ratio inhibits several reactions in metabolism, for example the fatty acid oxidation, which in turn leads to further accumulation of fatty acids (43, 44). Alcohol by itself influences fat metabolism, because it decreases the protein levels of PPARα and increase the activity of adenyl cyclase, both results in continuous increase in fatty acids (42, 45). These changes in lipid metabolism are leading to an aggregation of fat in liver tissue, so called alcoholic fatty liver. In case of abstinence it would be a reversible stadium of ALD, otherwise the progress will continue.

Especially the effects of acetaldehyde on liver tissue are crucial factors in the progression of ALD. It leads to a development of alcoholic hepatitis and later to irreversible liver cirrhosis. Furthermore, the toxicity of acetaldehyde initiates immune responses: an increase of proinflammatory cytokines, especially tumor necrosis factor-alpha (TNF-α), and a decrease of anti-inflammatory cytokines (46). This in turn leads to an infiltration of neutrophils and macrophages in the liver tissue and results in hepatocyte damage. In addition, the inflammatory process activates the synthesis of collagen in stellate cells and leads to fibrosis. The alteration of the redox state exceed the capacity of antioxidant defence mechanisms and leads to further

liver injuries (43). This alteration of the redox state arises by the changes of NAD/NADH ratio, induced by the forced activation of alcohol- and acetaldehyde dehydrogenase as mentioned above, and by an activated mitochondrial ethanol oxidation due to chronic alcohol intake. Free radicals produced by the activated mitochondrial cytochrome P450 in combination with an alteration of NAD/NADH redox ratio causes lipid peroxidation and further necrosis and liver damage, which in turn intensifies the immune responses, already started by acetaldehyde, and increases the collagen syntheses. This cascade leads to changes in liver architecture, functional and well vascularised liver tissue is replaced by fibrotic and cirrhotic tissue without function and with less vascularisation and results in a high risk of fatal consequences for the patient.(25, 42).

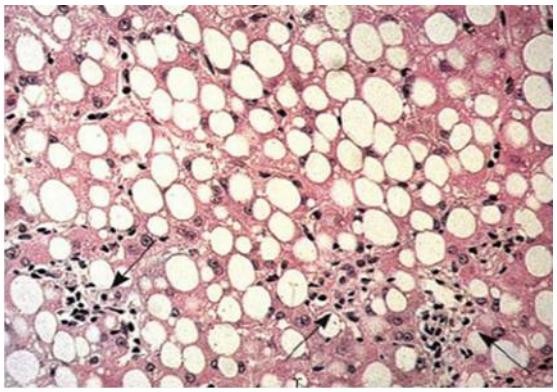


Figure 8: Microscopy of Steatosis Hepatis, black arrow: lymphocytes and Kupffer cells [from (47)]

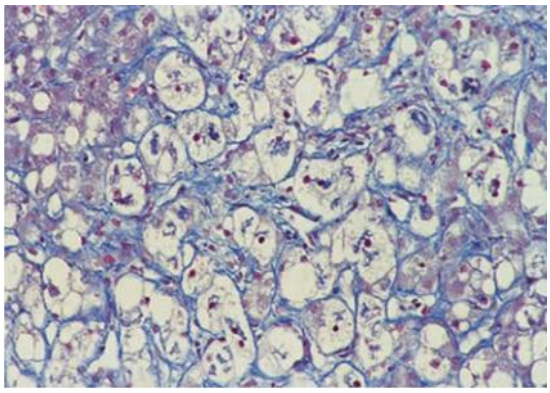


Figure 7: Microscopy of Alcoholic Hepatitis [from (47)]

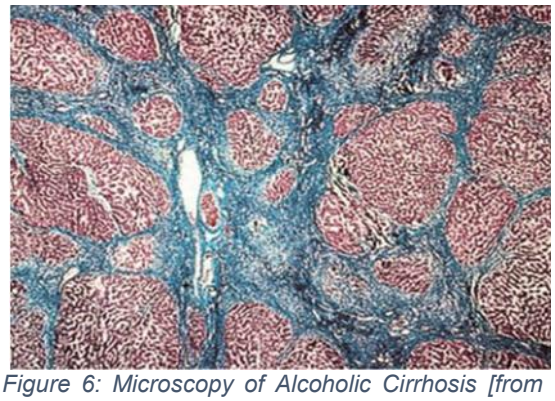


Figure 6: Microscopy of Alcoholic Cirrhosis [from (47)]

Figure 5: Macroscopy of Liver Cirrhosis [from (47)]

Steatosis hepatis shows morphological characteristics, such as an enlarged yellow liver. Furthermore, microscopic changes with lipid vacuoles and nodules can be observed, consisting of lymphocyte and Kupffer cell accumulation. In the stage of alcoholic hepatitis enlarged hepatocytes with intracellular Mallory-Denk-bodies are noticeable. Parenchyma nodules and septa of connective tissue are observable in alcoholic liver cirrhosis (47). Even though the pathologic mechanisms of ALD are

quite clear, very different developments of this disease in people with almost the same drinking practice are observed. So, why is the same amount of alcohol leading to a severe case of ALD in some cases and to marginal liver transformations in other cases? Several Genome-wide association studies (GWAS) were conducted to answer this question. In these studies, an association between variants of different genes and ALD are shown. PNPLA3 (patatin-like phospholipase domain–containing 3) is a protein expressed in adipocytes and liver with apparently lipogenic transacetylase activity and other roles in energy metabolism. It looks like a variation in this gene is an independent risk factor for liver injury (48). Furthermore, there is another protein, whose gene variation is associated with ALD, called transmembrane 6 superfamily member 2 (TM6SF2), required for very-low-density lipoprotein secretion lipid transport. A variation of this protein influences lipid displacement to the disadvantage of the hepatic lipid metabolism, leading to an increased fat accumulation in liver tissue (49). So far, several gene variations associated with ALD were identified. And of course, the most famous predisposition in context with alcohol consumption – the individual alcohol sensitivity – also influences this pathology. The crucial factor in this case is a genotype, decreasing the activity of acetaldehyde dehydrogenase, which in turn leads to an accumulation of toxic acetaldehyde. A rapid increase of acetaldehyde triggers an acute flash symptomatic in affected people (50). It remains unclear, if people with this genotype, especially represented in Asia, have a higher risk for suffering from ALD. One reason for the lack of reliable data could be the low frequency of alcohol consumption in this cohort. Furthermore, a remarkable increase of acetaldehyde in women after alcohol intake was observed. The female gender has a higher risk for ALD (42). Regardless of individual risk factors, chronic alcohol consumption represents a global health burden undoubtedly linked to the genesis and progression of alcoholic liver disease.

1.3.4 Clinical presentation

The first stage of ALD is clinically inconspicuous, in contrast to alcoholic hepatitis with more distinctive symptoms, like malnourishment, fever, jaundice and cutaneous features of chronic liver diseases. Further progress of ALD leads to liver cirrhosis, with severe symptoms and fatal complications for affected patients.

1.3.4.1 Alcoholic fatty liver

Besides an enlarged liver, the condition of patients with alcoholic fatty liver is mostly well-nurtured without any complaints. This leads to just a few coincidental diagnoses during medical check-ups through laboratory and sonographic examinations. Frequently present is an elevation of γ-glutamyltransferase (γ-GT) in combination with the sonographic characteristics of steatosis, meaning a homogenous and hyperechoic liver parenchyma with a soft edge (25, 43). Elevated levels of γ-GT linked with an increase of mean corpuscular volume (MCV) represents a parameter for chronic alcohol consumption as well as serum level of carbohydrate deficient transferrin (CDT). Both parameters γ-GT plus MCV and CDT are decreasing within 4 weeks of abstinence. However, the utility of the last-mentioned parameter is seen critically and therefore not a routine parameter (51, 52). Another indicator for alcohol intake is ethyl glucuronide (ETG), which is detectable in urine 12 to 72 hours after alcohol intake (53). Furthermore, ethyl sulfate (ETS) represents another marker for alcohol intake with a similar manner as ETG (54). In addition to these laboratory parameters, the CAGE-questionnaire is a helpful tool to identify alcoholism by anamnesis (55, 56). Certainly, a relation of trust between the patient and his/her physician is a necessity to get valid information and to achieve a commitment to abstinence, which would be the very first step to stop the progression of ALD.

1.3.4.2 Alcoholic hepatitis

The health status changes a lot in case of further progress to alcoholic hepatitis. Noticeable are increasing complaints, like loss of appetite, nausea, malnourishment, pain in the right upper quadrant of the abdomen, jaundice and fever. Moreover, we see hepatomegaly and further laboratory changes with increasing transaminase-levels and hyperbilirubinemia additional to recent γ-GT and MCV alterations. Furthermore, leucocytosis and thrombocytopenia are apparent, the first one caused by immune responses in liver tissue and the second one during the toxic effect of alcohol and a possibly existing splenomegaly (25, 43).

1.3.4.3 Alcoholic liver cirrhosis

Patients with alcoholic liver cirrhosis are severely diseased, due to the loss of function of the liver tissue, changes in portal circulation and malnourishment. The general complaints, like fatigue and nausea show further progression, accompanied by cutaneous features, both caused by the continuation of alcohol consumption.

These cutaneous manifestations are common in several liver diseases; they are non-specific, but useful in pointing toward the disease. Often seen in patients with liver cirrhosis are palmar erythema, spider angiomas, white nails, paper money skin, Dupuytren´s contracture and pruritus. Causative for palmar erythema and spider angiomas are prostacyclin, nitric oxide and androgen imbalance by triggering local vasodilatation (57). Increased peripheral conversion of androgen to oestrogen induces low testosterone and high oestrogen plasma levels (58). This leads to, the before mentioned, palmar erythema and spider angiomas, but also to gynecomastia, potency disorders and changes of body hair in men or menstrual disorders and amenorrhoea in women. The niggling pruritus may be a result of bilirubin enrichment, whose conjugation and elimination are strongly decreased (25).

The decline of the synthetic function of hepatocytes can generate further severe problems. More precisely, the decrease of vitamin K dependent factors, like factor II, VII, IX, X, protein C and S, in combination with thrombocytopenia, caused by splenomegaly due to portal hypertension, could lead to an imbalance of the coagulation system, measurable by a decrease of prothrombin time and an increase of partial thromboplastin time. Thereby we see a higher risk for bleeding as well as a higher risk for thrombosis, which is caused by an imbalance of pro- and anticoagulation factors. In some cases, a genesis of disseminated intravascular coagulation (DIC) is seen (59). These alterations of the coagulation system in combination with oesophagus varices could cause life-threatening internal bleeding, representing a common cause of death for patients with alcoholic liver cirrhosis. Furthermore, cirrhotic patients suffer from ascites and oedema, caused by portal hypertension due to increased intrahepatic vascular resistance, and enhanced by decreased albumin synthesis. Besides, ascites leads to further severe complications like spontaneous bacterial peritonitis, hepatorenal and hepatopulmonary syndrome (25).

Malnourishment is a frequent distinguishing feature in cirrhotic patients. Partly caused by decreased food intake due to alcoholism, but also based on liver dysfunction, shown throw decreased hepatic vitamin storage and inability to convert vitamins to their active forms. This leads to Wernicke´s encephalopathy and dermatological changes, like withe nails, dermatitis and erosions, intensified by iron and zinc deficiency (57).

A further consequence of hepatic insufficiency is the accumulation of ammoniac and other neurotoxic substances like mercaptan, phenols or γ-aminobutyric acid, leading to hepatic encephalopathy. This is intensified by porto-systemic shunts, generated by increased intrahepatic vascular resistance, representing a liver bypass of these toxic agents. Moreover, high protein nutrition and infections could lead to an enhanced genesis of ammoniac. Symptoms of hepatic encephalopathy are confusion, sleepiness, apathy, flapping tremor, foetor hepaticus and finally coma hepaticum (25). Furthermore, patients with liver cirrhosis are showing an increased risk for hepatocellular carcinoma (60).

The Child-Pugh´s classification is used to categorize disease severity based on clinical criteria like severity of ascites and hepatic encephalopathy, as well as synthetic performance, measured by albumin and prothrombin time, and excretion capacity obtained by bilirubin. This scoring system is helpful to predict prognosis and mortality and to define the strength of therapy (61).

Criteria	1 Point	2 Points	3 Points
Total bilirubin (mg/dL)	<2	2-3	>3
Serum albumin (g/dL)	>3.5	2.8-3.5	<2.8
Prothrombin time (%)	<70	70-40	>40
Ascites	None	Mild	Moderate or severe
Hepatic encephalopathy	None	Grade I-II	Grade III-IV

Table 2: Child-Pugh criteria [from (61)].

A patient with 5 or 6 points will be classified as Child-Pugh A and has a one year survival of 100%. Child-Pugh B (7 to 9 points) means a one year survival of 81%, and patients with 10 to 15 points are in stadium Child-Pugh C and have a one year survival of 45% (61).

Another classification to categorise the severity of liver disease, especially in the end stage, is the model of end-stage liver disease (MELD score). This score is a good predictor for 3-month mortality and therefore is a crucial factor in awarding liver transplantations. The laboratory parameters International normalized ratio (INR),

creatinine and bilirubin are the foundation of this score, starting with values of 6 up to 40, whereby 40 represents the highest mortality without liver transplantation (62).

1.3.5. Therapy

General measures like alcohol abstinence and adequate nutrition maybe accompanied by vitamin substitution is the foundation of ALD therapy. Based on the stage of ALD and the severity of complications further treatment is needed, including the evaluation of a liver transplantation in end-stage liver injury (33).

Besides the necessity of alcohol abstinence, the adequate nutrition is an essential intervention to improve the health status and to prevent further complications. A study in 1984 examined the nutritional condition of more than 280 people with alcoholic hepatitis or even alcoholic cirrhosis. In no case a sufficient nutrition was observed (63). Alcoholics are usually showing a protein and calorie malnutrition as well as a deficit in vitamin and trace minerals, like vitamin A, vitamin D, thiamine, folate, pyridoxin and zinc (33). Especially the deficiency of folate and thiamine has serious consequences, like Wernicke´s encephalopathy, and should be prevented by oral substitution. A diet assessment is necessary to achieve an optimal supply of these vitamins and an adequate portion of calories and proteins. Furthermore, nasogastric feeding can be applied to achieve adequate nutrition in very malnourished patients (43). By applying these nutritional goals, we see an improvement of liver function and increased survival rates in patients with ALD (64, 65). Furthermore, an adequate nutritional therapy has even a better short-term survival then steroid therapy in patients with alcoholic hepatitis. However, in long term observations steroid treated patients show lower mortality rates than non-steroid treated patients. Therefore, a combination of a nutritional therapy and steroid medication should be verified (66). Anyhow, the benefits of steroids in the therapy of alcoholic hepatitis are still controversial (43).

Complications of liver cirrhosis represent a challenging therapeutic task. Thereby, acute varices bleeding is a life threating condition and requires fast medical attention, like a blood cycle stabilisation with infusions and a ligation of the ruptured vessel to avoid the patient bleeding to death (25). Transjugular intrahepatic portosystemic shunt (TIPS) is a prophylactic intervention to decrease the portal hypertension and prevent varices bleeding. The intensive blood flow in oesophageal

veins due to portal hypertension is attenuated by TIPS, which in turn leads to a decrease of blood pressure in this area. Furthermore, this intervention has beneficial effects on ascites and its consequences like increased risk for peritonitis, hepatopulmonal and hepatorenal syndrome (67). However, this intervention not only leads to a bypass of straining blood volume, it also leads to an increased liver bypass of toxic substances, which in turn increases the occurrence of hepatic encephalopathy (68). The problem of insufficient detoxification in a cirrhotic liver, representing the general cause of hepatic encephalopathy, is partly treatable by extra corporal detoxification treatments, like “Prometheus” (Fresenius, Austria) or molecular adsorbent recirculating system (MARS, Gambro, Sweden). To support the inadequate synthetic performance of hepatocytes, it is necessary to substitute albumin, representing another measure to prevent or reduce ascites and therefore may prevent severe infections or renal impairment. Another issue of insufficient liver synthesis is an inadequate coagulation system, which can be counteracted by the substitution of fresh frozen plasma and platelet transfusions (25). All treatment procedures mentioned so far represent a symptomatic therapy. In contrast to the symptomatic therapy options, liver transplantation is the only curative treatment option to date.

ALD is a common indication for liver transplantation in North America and Europe (69), but the self-inflicted organ injury due to alcohol misuse is seen critical as an indication for liver transplantation. This results in the fact that only 5% of patients with end-stage liver disease induced by alcohol consumption are considered for liver transplantation (70). However, the efficacy of this treatment is shown and leads to an improvement of health in affected patients (71), and survival rates of liver transplantation recipients with or without ALD history are comparable. However, up to 50% of these patients resume drinking alcohol, meaning any alcohol intake after transplantation, although heavy alcohol intake is only seen in a small fraction of transplanted patients (33).

The general problem of alcohol misuse seems to be hard to handle for patients and treating physicians. Alcohol abstinence represents an important step to improve health or achieve recovery. On the way to reduced alcohol intake or abstinence, patient´s family and friends, as well as psychiatric intervention and self-help groups like Alcoholics Anonymous are important to accomplish this goal. In addition, the

quest for helpful medication began to facilitate this process. In 1994 an opiate antagonist named naltrexone had been licensed in the United States to treat alcoholism. Naltrexone leads to a reduction of alcohol craving and decreased alcohol intake but does not achieve abstinence. Another drug to decrease alcohol consumption and craving is acamprosat, which interacts with glutamate receptors and calcium channels (25, 43). Unfortunately, the sweeping effect of this treatment stays out and does not lead to a satisfactory number of patients achieving alcohol abstinence.

1.4 FGF21, the liver-brain endocrine axis and ALD

The link between FGF21 and ALD is obviously given by the fact, that alcohol consumption represents the causative factor in ALD and FGF21 is shown to be an important part of liver-brain endocrine axis, influencing alcohol preference. Alterations in the pathway of the liver-brain endocrine axis are associated with changes of alcohol behaviour.

A GWAS with more than 105,000 people identified a single nucleotide polymorphism (SNP) in intron 1 of *klb* gene on chromosome 4p14 to be associated with alcohol intake. *KLB* encodes the transmembrane protein βKlotho (72), which represents an essential coreceptor for FGF21 (1.1). In βKlotho deficient mice FGF21 does not show effects on eating and drinking preferences, despite supraphysiological levels of recombinant FGF21 (1.2.3 Eating and drinking preferences). This GWAS strengthened the hypothesis that changes in liver-brain endocrine axis, particularly a gene variation of βKlotho, influences alcohol intake in humans. In other words, genetic variations may predispose for increased alcohol intake and alcoholism. Furthermore, the concrete identification of biological pathologies, leading to alcoholism, opens new pharmacological strategies to treat ALD.

Another possible pathophysiological mechanism in the liver-brain endocrine axis might be a receptor resistance, which is a frequent phenomenon in pathophysiology of diseases, for example diabetes mellitus type II, and in the pharmacology of drug resistances. A receptor resistance for FGF21 is already shown in obese mice (73). The idea of a receptor resistance was generated by the paradoxon that FGF21 has a positive effects on obesity (1.2.2 Pharmacological effects of FGF21 on metabolic

d) but obese humans and mice show increased FGF21 levels in obese humans and mice (73, 74). This points to an insufficient effect of FGF21 in obese patients, otherwise we would expect a lean physique of these patients. The hypothesis of receptor resistance is confirmed by an attenuated response of exogenous FGF21 in obese mice compared to lean mice. Obese mice have shown a reduced phosphorylation of extracellular signal–regulated kinase 1/2 (ERK1/2), which is a marker for FGF signalling. Furthermore, there is a decreased expression of proteins belonging to the FGF signalling pathway, in obese mice. Receptor resistance seems to occur early during weight gain, particularly within 4 weeks of high-fat diet in a mouse experiment (73).

It is not known yet, whether these findings are applicable to alcoholism and ALD, but it points towards a promising pathological hypothesis and represents a topic for further consideration.

The emergence of alcoholism and its consequences like ALD are multifactorial. Psychological and social factors contribute to this trend and represent hard to handle parameters. In addition, biological influences did not become completely clear so far and might represent the hope for a better understanding and a potential treatment. In this context FGF21 moves increasingly into focus and is already shown to be associated with alcoholism and possibly ALD.

A lot of these findings are related to mouse experiments, for example the *in vitro* experiment, in which alcohol induced FGF21 synthesis in mouse hepatocytes (31). This result is not shown for human cell lines so far and a comparison of the influences of alcohol and carbohydrates as a trigger for FGF21 expression is needed.

1.5 Aims and hypotheses

The aim of this study was to elucidate the role of alcohol in the expression of FGF21 in human and the role of FGF21 in alcoholic cirrhosis.

We hypothesized that alcohol induces the expression of FGF21 in the human hepatocyte cell line, HepG2. Secondly, we hypothesized that FGF21 is misregulated

in patients with alcoholic liver cirrhosis compared to patients with other types of cirrhosis.

2 Methods

2.1 Cell culture and incubation

HepG2 cells (ATCC, HB-8056), a cell line derived from a human hepatocellular carcinoma, were cultured in a minimum essential medium supplemented with 2 mmol Glutamine (Gibco, Waltham, USA), 1% non-essential amino acid (NEAA) (Lonza Group, Basel; Switzerland), 10% fetal calf serum (FCS) (PAA Laboratories, Cölbe, Germany) and 1% Penicillin-Streptomycin (Sigma-Aldrich, Saint Louis, USA). Cells were cultured in flasks at 37°C humidified atmosphere. At a confluency of 90% cells were passaged into a new flask at a density of 8×10^3 cells per cm^2. For the incubation of HepG2 cells with alcohol and glucose the cells were passaged and seeded in a six well plate (7.7×10^5 cells per well) with medium plus alcohol (0.2% and 0.4%) or glucose (150 mg/dl and 200mg/dl) at 37°C for 4 and 24 hours. A negative control without stimulus was incubated under the same culture conditions. The supernatants were aspirated and stored at -80°C. The cells were solved and lysed with trizol (Ambion Chemicals, Naugatuck, USA) and stored at -20°C.

This procedure was repeated with different concentrations of alcohol (1% and 2%) and glucose (200 mg/dl and 400mg/dl).

Cells lysed in Trizol were used to investigate the amount of FGF21 mRNA.

2.2 Quantitative PCR (qPCR)

RNA molecules were isolated from the suspension of lysed HepG2 cells. First, the cells were lysed by trizol and vortexed after that. To achieve a phase separation an addition of chloroform was used. By adding Isopropanolol the RNA molecules were precipitated. In the next step the RNA had to be washed with 75% of ethanol, accompanied by centrifugation, which was repeated 3 times. Before starting DNAse treatment a RNA quantification was done to determine the deficiency of RNA molecules. Then, DNase treatment with DNA-free Kit (CatNo: AM1906, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was applied. Afterwards unspecific RNA levels were determined by Nanotrop 2000 (Thermo Fisher Scientific,

Waltham, Massachusetts, USA). The mean RNA concentration was 1,27 µg/ml (Standard Deviation: 0,34 µg/ml). RNA molecules were transformed to cDNA by reverse transcription, by High-Capacity cDNA Reverse Transcription Kit (CatNo: 4368814, Thermo Fisher Scientific, Waltham, Massachusetts, USA). To amplify FGF21 cDNA, qPCR with specific Primers (right primer: AGGCCTCAGGGTCAAAGTG; left primer: CCTTGAAGCCGGGAGTTATT) was executed. As a Master Mix, SYBRgreen (Thermo Fisher Scientific, Waltham, Massachusetts, USA), including taq Polymerase, nucleotides, a buffer suspension and fluorescent colorant, plus RNase-free water, was used. All single steps of PCR, like melting (95°C), primer annealing (60°C) and elongation (60°C), were done automatically by the CFX69, which executed 40 cycles.

2.3 Patients and study design

In the period from July 2012 to September 2013 101 patients with liver cirrhosis of any aetiology were screened in the University Hospital of Graz, more precisely in the outpatient clinic at the Department of Gastroenterology and Hepatology or the Department of Transplantation Surgery. The inclusion to this study was executed in consideration of the following criteria:

Inclusion criteria:

- Patients age between 18-80 years
- Clinical and radiological evidence of cirrhosis, and/or biopsy proven liver cirrhosis of any cause
- Informed consent

Exclusion criteria:

- Child-Pugh score > 11
- Clinical evidence of active infection
- Antibiotic treatment within 7 days prior to enrolment
- Gastrointestinal haemorrhage within previous 2 weeks
- Use of immunomodulating agents within previous month (steroids etc.)
- Use of proton pump inhibitors for preceding two weeks

- Concomitant use of supplements (pre-, pro-, or synbiotics) likely to influence the study
- Renal failure (such as hepatorenal syndrome), creatinine >1.7 mg/dL
- Hepatic encephalopathy II to IV
- Pancreatitis
- Other organ failure
- Hepatic or extra-hepatic malignancy
- Pregnancy
- Presumed non-compliance to the study medication

Patients were subdivided by their aetiology of liver cirrhosis. Group one included patients with alcoholic cirrhosis and the second group consisted of patients with all other types of cirrhosis.

In addition, a healthy control group was formed and consisted of 21 individuals. Exclusion criteria are elevated liver values and chronic disease. The age of the healthy control group was comparable to the age of the patient's cohort.

A food frequency questionnaire was used to describe the nutritional behaviour of patients, as well as blood sampling to evaluate FGF21 and urine samples to measure ETG and ETS levels. Additional information was collected during the clinical investigation, such as Child-Pugh-Score, Meld-Score and BMI (Body-Mass-Index).

2.4 Food frequency questionnaire

All patients and healthy controls were asked to provide information about their eating and drinking preferences. In order to attain this information a food frequency questionnaire of the "Robert Koch Institut" named "Ernährungsfragebogen – Studie zur Gesundheit Erwachsener in Deutschland" from 2008 was used for healthy controls. This questionnaire consisted of 57 questions with single choice answer options about the intake frequency of a wide range of food items. All Patients received a food frequency questionnaire designed by the Division of Gastroenterology and Hepatology of the Medical University of Graz. This

questionnaire consists of 33 single choice questions about the intake frequency of a wide variety of food products.

2.5 FGF21

FGF21 levels were determined using a commercially available ready to use sandwich ELISA (enzyme linked immunosorbent assay) (BioVendor GesmbH, Vienna, Austria), according to manufacturer's instructions. Lithium-Heparin samples were used in a 1:2 dilution. Standards, controls and samples were incubated at room temperature on a shaker (300 rpm) for 60 minutes in a pre-coated plate with FGF21 antibodies. After washing, a biotin-labelled FGF21 antibody was added and incubated at room temperature on a shaker (300 rpm) for another 60 minutes, followed by next washing procedure. Streptavidin-HRP conjugate was added and incubated for 30 minutes at room temperature on a shaker (300 rpm). After a last washing step substrate solution was added and incubated to trigger a reaction of the remaining conjugates with the substrate solution. The reaction was stopped with acidic solution, followed by the measurement of the absorbance at a wavelength of 430nm. FGF21 concentration in pg/ml was then calculated according to the standard curve.

2.6 ETG and ETS

The ETG and ETS urine levels were measured by liquid-chromatography-mass spectrometry. Thereby was the MS/MS measurement method in use and Multiple Reaction Monitoring. TSQ Quantum Discovery is the mass spectrometer, which was used for this analysis and the Dionex Ultimate 3000 was applied for the spectrometry. (Dr. Meinitzer and his team executed this method).

2.7 Ethical consideration

The Ethics Committee of the Medical University of Graz approved the study protocol (23-096 ex 10/11). The study was performed in accordance to the declaration of Helsinki.

2.8 Statistical analysis

Data collection of the participants was done with Microsoft Excel 2016, whereas the statistical analysis was performed with SPSS 23 (IBM Germany GmbH, Ehningen, Germany). Chi-squared test/Fisher exact test was used to evaluate between-group differences of categorical variables. Furthermore, the assessment of the normal distribution of all continuous values was performed using the Kolmogorov-Smirnov test. The Mann-Withney U-test was used to evaluate significance, because the data were not normally distributed.

3 Results

3.1 FGF21 expression in hepatocyte cell culture

To evaluate the influence of alcohol and carbohydrates on FGF21 expression in hepatocytes, a cell culture model with human HepG2 cells was used. The cells were challenged with ethanol, 0.2% and 0.4%, glucose, 150mg/dl and 200mg/dl, and a negative control without any addition, for either 4 or 24 hours. After cell lysis, a sufficient amount of mRNA could be isolated in all samples. However, qPCR did not show the expected increase of FGF21 mRNA levels. All trials showed the same very low level of FGF21 mRNA. To optimize the experimental conditions, higher concentrations of alcohol and sugar (1% and 2% alcohol; 200 mg/dl and 400 mg/dl glucose) were applied. However, again very low levels of FGF21 mRNA were determined, independently of the incubation period and supplied additives.

3.2 Study cohort

In the time of July 2012 and September 2013 101 patients were screened for suitability, whereby 92 individuals were included and ultimately all required information and samples were attained from 75 patients. 41 of them were suffering from alcoholic cirrhosis and 34 had non-alcoholic cirrhosis. Besides, 21 healthy test persons were included.

3.2.1 Patients with alcoholic liver cirrhosis

The group of patients suffering from alcoholic liver cirrhosis consisted of 9 women (22%) and 32 men (78%). Twenty-nine patients of the whole group were in stadium Child-Pugh A (71%), eleven in stadium Child-Pugh B (27%) and one in stadium Child Pugh C (2%).

Further parameters of patients with alcoholic liver cirrhosis are summarized in Table 3.

Variable	N	Mean	Standard deviation	Median	Minimum	Maximum	Range
Age	41	55,4	9,2	56,0	32,0	71,0	39,0
Child-Pugh-Score	41	6,1	1,4	6,0	5,0	11,0	6,0
Meld-Score	41	12,5	4,4	12,4	6,5	25,9	19,4
BMI	41	27,0	4,0	27,0	18,5	40,2	21,7

Table 3: Parameters of Patients with alcoholic liver cirrhosis.

3.2.2 Patients with non-alcoholic cirrhosis

The non-alcoholic liver cirrhosis group was composed of 13 female patients (38%) and 21 male patients (62%). By looking to the Child-Pugh Classification, the observations showed 26 patients (76%) with Child-Pugh A, seven patients (21%) with Child-Pugh B and one patient with Child-Pugh C (3%).

Additional parameters of this group are summarized in Table 4.

Variable	N	Mean	Standard deviation	Median	Minimum	Maximum	Range
Age	34	57,9	9,7	60,0	33,0	71,0	38,0
Child-Pugh-Score	34	5,9	1,3	5,0	5,0	10,0	5,0
Meld-Score	34	10,6	3,4	9,7	6,4	17,8	11,4
BMI	34	27,2	4,1	26,3	20,2	37,1	17,0

Table 4: Parameters of Patients with non-alcoholic liver cirrhosis.

3.2.3 Healthy control group

The healthy control group consisted of 21 persons, including twelve women (57%) and nine men (43%). The mean age was 58 years with a standard deviation of 7 years. The mean Body-Mass-Indey was 25 kg/m² with a standard deviation of 3.1 kg/m²

3.2.4 Comparison of patient groups and healthy control group

By comparing these three groups, it is shown that healthy control group has the highest mean age with 58 years. The youngest group is the group of alcoholic liver cirrhosis with a mean age of 55.4 years. The biggest portion of female members has

the healthy control group with 57%. The smallest portion of females are represented in the group of alcoholic liver cirrhosis with 22%. The slimmest group is the healthy control group with a mean BMI of 25 kg/m². The group of non-alcoholic liver cirrhosis has the highest mean BMI (27.2 kg/m²).

The group of alcoholic liver cirrhosis has a higher mean Child-Pugh-Score (6.1) and a higher mean Meld-Score (12.5) then the group of non-alcoholic liver cirrhosis has (Child-Pugh-Score: 5.9; Meld Score: 10.6).

3.3 Alcohol consumption

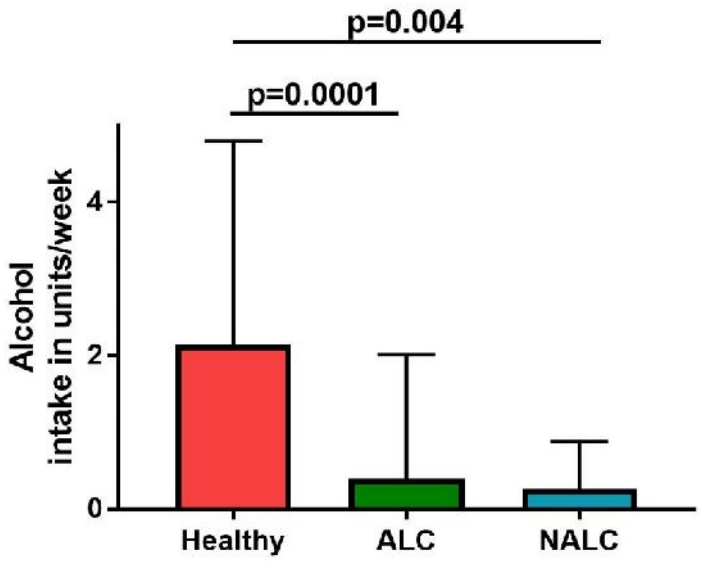
The question about alcohol intake in the food frequency questionnaire referred to beer, wine and other alcoholic beverages and asked for the frequency of intake. One person in the healthy control group withheld the information about alcohol intake.

By comparing the alcohol intake, it became evident that the healthy control group declared a higher frequency of alcohol intake then the group of alcoholic liver cirrhosis and the group of non-alcoholic liver cirrhosis.

Furthermore, 88% of patients with alcoholic liver cirrhosis indicated no alcohol intake. A total of 79% of the group of non-alcoholic liver cirrhosis declared abstinence as well as 33% in the healthy control group. Therefore, the amount of abstinent people showed the highest level in the group with alcoholic liver cirrhosis. Further information about the statements of alcohol intake of every group is presented in Table 5.

	Alcoholic liver cirrhosis (n = 41)		Non-alcoholic liver cirrhosis (n = 34)		Healthy control group (n = 21)	
Frequency of alcohol intake	N	percentage	N	percentage	N	percentage
No intake	36	88	27	79	7	33
One time a week or less	3	7	6	18	5	24
More times a week	1	2	1	3	4	19
Daily or more	1	2	0	0	4	19
N/A	0	0	0	0	1	5
Median (in units/week)	0,0		0,0		0,8	
1st quartil (in units/week)	0,0		0,0		0,0	
3rd quartil (in units/week)	0,0		0,0		3,0	

Table 5: Frequency of alcohol intake in the group of alcoholic liver cirrhosis, non-alcoholic liver cirrhosis and healthy control group.



Graph 1: Mean alcohol intake in units per week in the group of alcoholic liver cirrhosis, non-alcoholic liver cirrhosis and healthy control group.

By comparing the mean alcohol intake in units per week in every group it is noticeable, that the healthy control group shows the biggest amount of alcohol units. The amount of alcohol intake is significant higher in the healthy control group compared to the group of alcohol liver cirrhosis (p=0.0001) and to the group of non-alcoholic liver cirrhosis (p=0.004). Tough, there are no significant differences

between the group of alcoholic liver cirrhosis and non-alcoholic liver cirrhosis (p=0.386) (Graph 1).

In further analysis, the frequency of alcohol intake is described with units per week.

3.4 Ethyl glucuronide

Urine levels of ethyl glucuronide helped to get an idea about the actual alcohol intake (1.3.4 Clinical presentation). The cut-off was set at 0.5 µg/ml ethyl glucuronide. More precisely, ethyl glucuronide concentrations with at least 0.5µg/ml indicated alcohol intake in the last 12 to 72 hours.

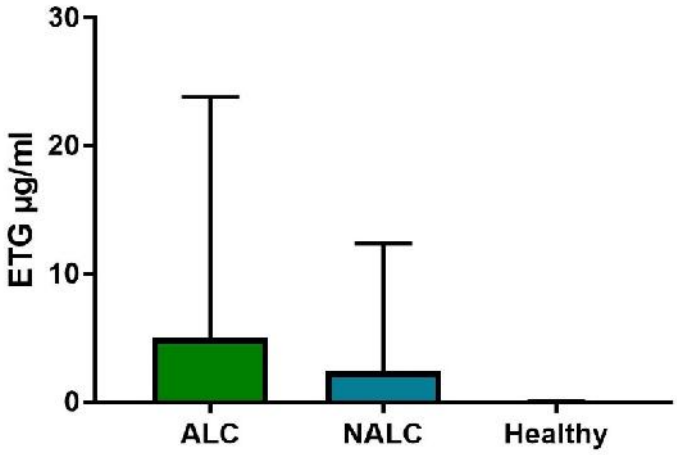
The highest number of patients with positive ETG levels was found in the group with alcoholic liver cirrhosis with 22%, followed by the group with non-alcoholic liver cirrhosis with 12%. The healthy control group showed exclusively negative ETG levels.

By comparing ETG levels with the alcohol intake given in the food frequency questionnaire some discrepancies were noticeable. In total, 95% in the alcoholic liver cirrhosis group declared not to drink alcohol or to drink on rare occasions, like one time per week or less. However, this does not compare to just 78% with negative ETG levels. In some cases, this discrepancy is also shown vice versa, so some patients declared to drink alcohol, but ETG levels were negative. In fact, all test persons in the healthy control group showing negative ETG-levels, although some declared frequent alcohol consumption.

Further information of positive and negative ETG levels in every group are shown in Table 6.

	Alcoholic liver cirrhosis (n = 41)		Non-alcoholic liver cirrhosis (n = 34)		Healthy control group (n = 21)	
ETG	N	percentage	N	percentage	N	percentage
Positive (≥0.5µg/ml)	9	22	4	12	0	0
Negative (<0.5µg/ml)	32	78	30	88	21	100
N/A	0	0	0	0	0	0
Mean (µg/ml)	6,6		3,6		0,1	
Standard deviation	21,5		12,0		0,1	
Median	0,1		0,1		0,0	
Range	114,0		54,8		0,3	

Table 6: ETG levels in the group of alcoholic liver cirrhosis, non-alcoholic liver cirrhosis and healthy control group.



Graph 2: Mean ETG levels (µg/ml) in the group of alcoholic liver cirrhosis, non-alcoholic liver cirrhosis and healthy control group.

There are no significant differences in mean ETG levels (µg/ml) between the group of alcoholic liver cirrhosis and the group of non-alcoholic liver cirrhosis (p=0.746). As well as between the group of alcoholic liver cirrhosis and the healthy control group (p=0.087) and between the group of non-alcoholic liver cirrhosis and the healthy control group (p=0.135) (Graph 2).

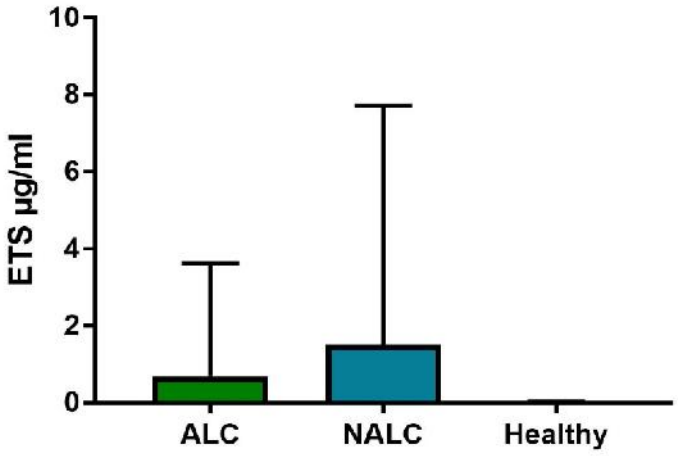
3.5 Ethyl sulfate

In addition to ETG levels, urine levels of ETS were measured and the same cut off at 0.5µg/ml for a positive ETS level was used. Unfortunately, 34 samples were not applicable due to technical reasons. Nevertheless, no positive ETS levels in the healthy control group were detected. The proportion of positive ETS levels in the group with alcoholic liver cirrhosis is similar to the group of non-alcoholic liver cirrhosis.

Additional details of ETS levels are shown in Table 7.

	Alcoholic liver cirrhosis (n = 41)		Non-alcoholic liver cirrhosis (n = 34)		Healthy control group (n = 21)	
ETS	N	percentage	N	percentage	N	percentage
Positive (≥0.5µg/ml)	2	5	2	6	0	0
Negative (<0.5µg/ml)	25	61	20	59	13	62
N/A	14	34	12	35	8	38
Mean (µg/ml)	0,7		1,5		0,0	
Standard deviation	2,9		6,2		0,0	
Median	0,0		0,0		0,0	
Range	15,1		29,1		0,1	

Table 7: ETS levels in the group of alcoholic liver cirrhosis, non-alcoholic liver cirrhosis and healthy control group.



Graph 3: Mean ETS level (µg/ml) in the group of alcoholic liver cirrhosis, non-alcoholic liver cirrhosis and healthy control group.

By comparing the mean ETS levels (µg/ml) of the group of alcoholic liver cirrhosis with the group of non-alcoholic liver cirrhosis (p=0.351) and with the healthy control group (p=0.549) there are no significant differences (Graph 3).

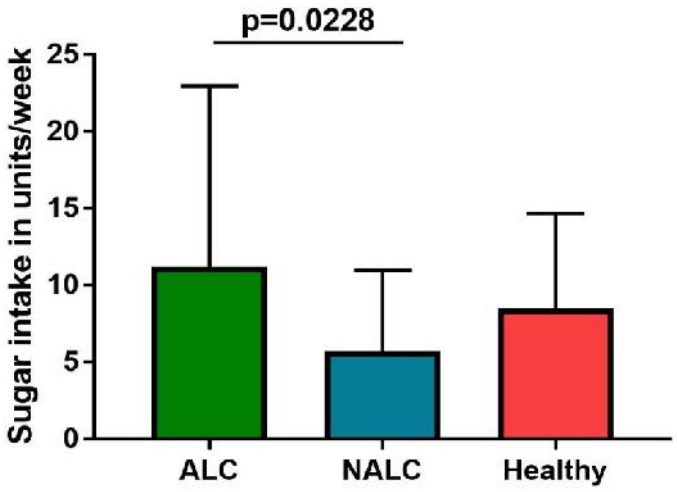
3.6 Sugar consumption

Intake of sugar containing food items included energy drinks, fruit and vegetable juices, lemonade (with and without sweetener), ice-cream, pudding, sweet dishes, cakes, cookies, tortes, chocolate and candies.

The biggest proportion of all groups declared fructose intake “several times a week” or “daily or more”. The group of non-alcoholic liver cirrhosis showed the highest percentage of the declaration “no sugar intake” and has had the lowest median. Furthermore, quite similar statements of fructose intake in the healthy control group and in the group with alcoholic liver cirrhosis were recorded. Additional data about the fructose declaration is shown in Table 8.

	Alcoholic liver cirrhosis (n = 41)		Non-alcoholic liver cirrhosis (n = 34)		Healthy control group (n = 21)	
Frequency of sugar intake	N	percentage	N	percentage	N	percentage
No intake	1	2	4	12	1	5
One time a week or less	3	7	5	15	2	10
More times a week	14	34	12	35	7	33
Daily or more	23	56	13	38	11	52
N/A	0	0	0	0	0	0
Median (in units/week)	7,0		4,8		8,0	
1st quartil (in units/week)	3,0		1,1		4,0	
3rd quartil (in units/week)	11,5		8,4		12,0	

Table 8: Frequency of sugar intake in the group of alcoholic liver cirrhosis, non-alcoholic liver cirrhosis and healthy control group.



Graph 4: Mean sugar intake in units per week in the group of alcoholic liver cirrhosis, non-alcoholic liver cirrhosis and healthy control group.

The comparison of mean sugar intake in units per week shows a significant higher sugar intake in the group of alcoholic liver cirrhosis compared to the group of non-alcoholic liver cirrhosis (p=0.0228). There are no significant differences between the group of alcoholic liver cirrhosis and the healthy control group (p=0.823) (Graph 4).

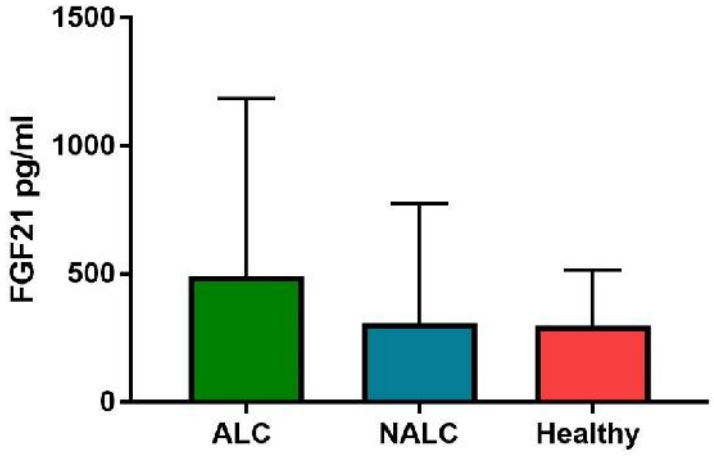
In adaption to the alcohol consumption, the frequency of sugar intake is described as units per week.

3.7 FGF21

FGF21 plasma concentrations for each group are illustrated in Table 9. The group with alcoholic liver cirrhosis has had the highest average of FGF21 concentration as well as very high maximum levels of FGF21. The healthy control group showed the lowest FGF21 level. The range of plasma FGF21 levels was especially high in the group with alcoholic liver cirrhosis and non-alcoholic liver cirrhosis while the healthy control group presented a lower range of FGF21 concentrations. The comparison of the mean FGF21 level of the group of alcoholic liver cirrhosis and the group of non-alcoholic liver cirrhosis shows no statistical difference (p=0.0818). The group of alcoholic liver cirrhosis compared to the healthy control group (p=0.5952), and the group of non-alcoholic liver cirrhosis compared to the healthy control group (p=0.3127) also showed no significant differences (Graph 5).

FGF21 (pg/ml)	Alcoholic liver cirrhosis (n = 41)	Non-alcoholic liver cirrhosis (n = 34)	Healthy control group (n = 21)
Mean	491,6	318,5	299,1
Standard deviation	693,2	470,5	216,6
Median	271,5	193,8	260,7
Minimum	31,7	30,6	52,5
Maximum	3853,8	2696,0	789,1
Range	3822,1	2665,4	736,6

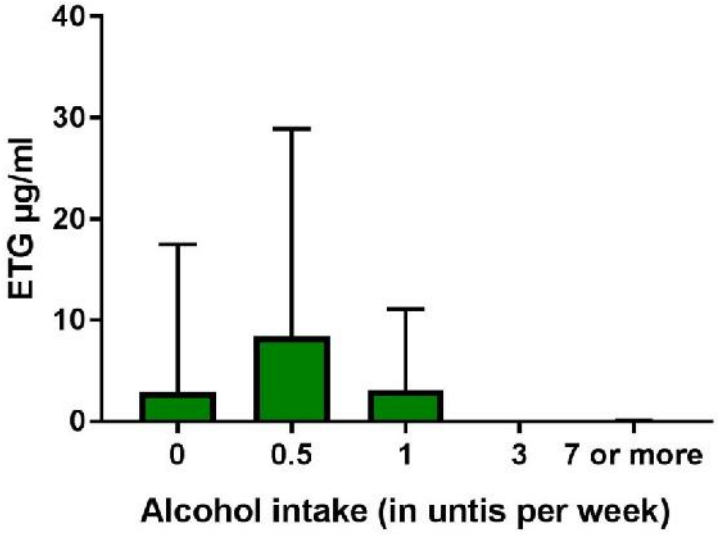
Table 9: FGF21 plasma concentration in the group of alcoholic liver cirrhosis, non-alcoholic liver cirrhosis and healthy control group.



Graph 5: Mean FGF21 level (pg/ml) in the group of alcoholic liver cirrhosis, non-alcoholic liver cirrhosis and healthy control group.

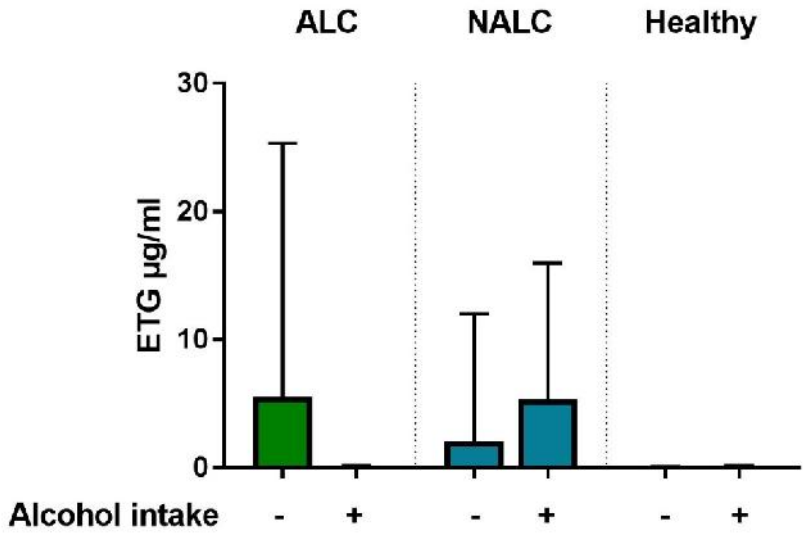
3.8 ETG, ETS and alcohol intake

By comparing the ETG levels of all patients and healthy test persons with the statements from the food frequency questionnaire some discrepancy is noticeable, especially the information about the alcohol intake. ETG as a marker for alcohol intake in the last 12 to 72 hours (1.3.4 Clinical presentation) does not confirm to the declaration of alcohol intake. More precisely, the highest ETG levels were observed in patients and healthy test persons, who declared no alcohol intake or only once a week. On the other side, very low ETG levels and a declaration of frequent alcohol intake were determined (Graph 6).



Graph 6: ETG levels according to the declaration of alcohol intake (in units per week) from food frequency questionnaire, independent of disease or health n=96.

The relationship between alcohol intake and ETG levels looks different by taking an isolated look to every group. The healthy control group showed constant low ETG levels independent of their alcohol consumption. In addition, patients with non-alcoholic liver cirrhosis and a frequent alcohol intake were showing higher ETG levels (mean ETG concentration: 5.33 µg/ml) compared to the cohort with low alcohol intake (mean ETG concentration: 2.0 µg/ml). Whereby, patients with alcoholic liver cirrhosis and low alcohol intake showed higher ETG levels (mean: 5.5 µg/ml) compared to the group with frequent alcohol intake (mean ETG: 0.1 µg/ml). In this context, alcohol consumption will be categorized in positive and negative alcohol intake to maintain clarity. The lower limit for positive alcohol intake was set at 1 unit per week (Graph 7).

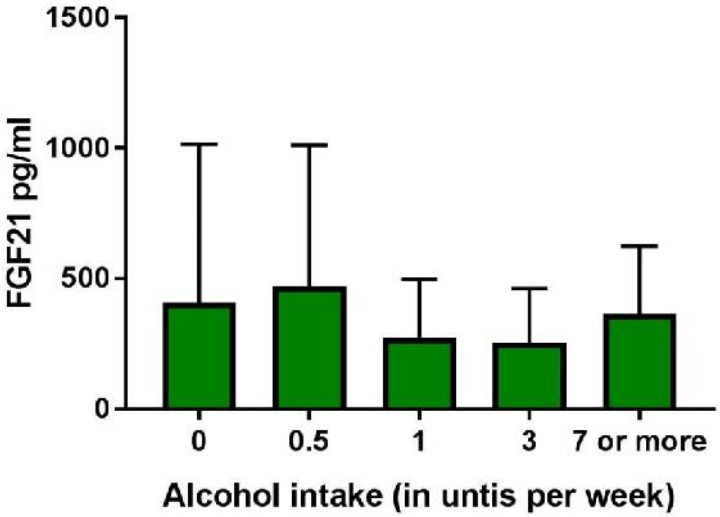


Graph 7: ETG levels according to alcohol intake, declared in the food frequency questionnaire, whereby positive alcohol intake means one unit per week or more, separated for ALC (alcoholic liver cirrhosis) n=41, NALC (non-alcoholic liver cirrhosis) n=34 and healthy control group n=21.

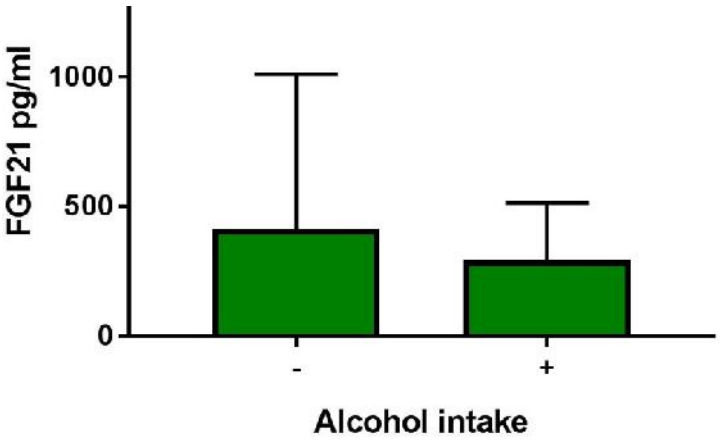
Due to the high number of not applicable ETS values there was no conclusive information according to ETS.

3.9 FGF21 and alcohol consumption

Independent of the group, high FGF21 levels in case of low declared alcohol consumption was noticeable (Graph 8). Furthermore, by categorising in positive and negative alcohol intake (limit value for positive alcohol intake: 1 unit per week) the same distribution was visible. More precisely, a mean FGF21 level of 413 pg/ml in the group with negative alcohol intake and 292 pg/ml in case of positive alcohol intake. However, the mean FGF21 levels in these two group do not show statistical significance (p=0.7812) (Graph 9).



Graph 8: FGF21 levels according to alcohol consumption (in units per week), declared of the food frequency questionnaire, independent of disease or healthiness n=96.

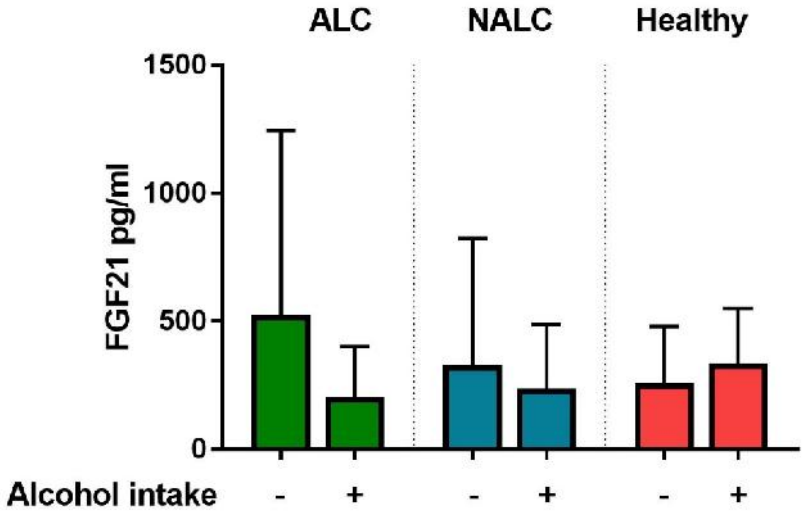


Graph 9: FGF21 levels according to negative or positive alcohol consumption, declared in the food frequency questionnaire, whereby positive alcohol intake means one unit per week or more, independent of disease or healthiness n=96.

Patients, who declared not to drink alcohol, had a non-significant trend to higher mean plasma concentration of FGF21 compared to those who admitted to alcohol use. This applies for the group of alcoholic liver cirrhosis and non-alcoholic liver cirrhosis (ALC: p=0.1589 and NALC: p=0.6992). Whereas, healthy controls, who admitted to alcohol use showed a higher mean FGF21 plasma concentration compared to those, who declared not to drink alcohol. Tough, this difference did not reach statistical significance, either (p=0.2512). (Table 10,Graph 10).

FGF 21 (pg/ml)	Mean	Median	Minimun	Maximum	Range	Standard Deviation
ALC Negative alcohol intake	523	278	45	3854	3809	721
ALC Positive alcohol intake	203	144	32	491	459	200
NALC Negative alcohol intake	329	194	31	2696	2665	494
NALC Positive alcohol intake	238	162	45	581	536	250
Healthy control group Negative alcohol intake	259	203	53	661	608	222
Healthy control group Positive alcohol intake	336	335	84	789	705	215

Table 10: FGF21 levels according to alcohol intake, declared in the food frequency questionnaire, whereby positive alcohol intake means one unit per week or more, separated for ALC (alcoholic liver cirrhosis) n=41, NALC (non-alcoholic liver cirrhosis) n=34 and healthy control group n=21.

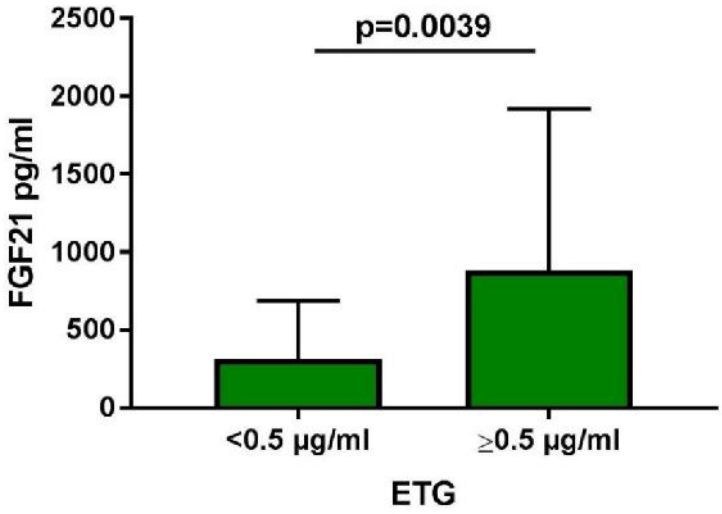


Graph 10: FGF21 levels according to alcohol intake, declared in the food frequency questionnaire, whereby positive alcohol intake means one unit per week or more, separated for ALC (alcoholic liver cirrhosis) n=41, NALC (non-alcoholic liver cirrhosis) n=34 and healthy control group n=21.

3.10 FGF21 according to ETG

Independent of the groups, an accordance between positive ETG levels (≥0.5 µg/ml) and high FGF21 plasma concentrations were determined, whereby all healthy test persons showed negative ETG levels (Table 11). The FGF21 plasma concentration

in the group with positive ETG levels was significant higher than in the group with negative ETG levels (p=0.0039) (Graph 11).

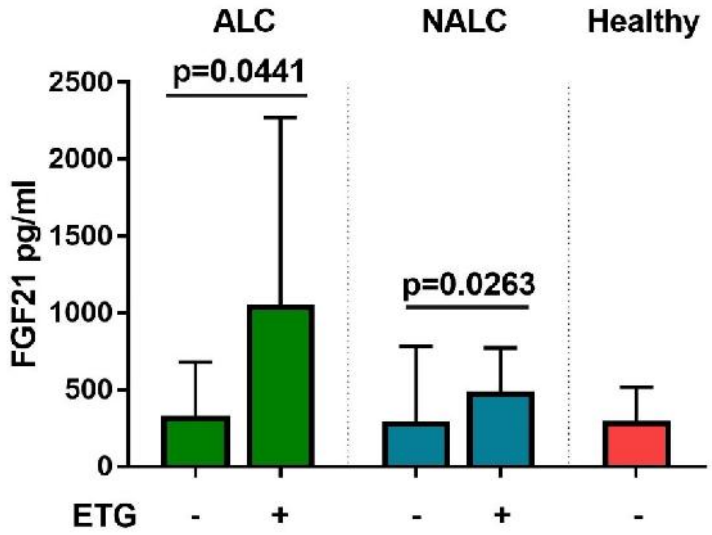


Graph 11: FGF21 levels according to negative (<0.5 µg/ml) and positive (≥0.5 µg/ml) ETG levels, independent of disease or health n=96.

A separated analysis of the different groups confirms this observation, whereby patients with positive ETG concentrations showed significantly higher FGF21 levels compared to those with negative ETG levels in the group of alcoholic liver cirrhosis (p=0.0441). The same applied for the group of non-alcoholic liver cirrhosis (p=0.0263). Furthermore, the highest mean FGF21 level was found in the group of alcoholic liver cirrhosis with positive ETG levels (maximum: 1056 pg/ml), however, the range was high (3808 pg/ml). The lowest mean FGF21 levels were present in the healthy control group and the group of non-alcoholic liver cirrhosis with negative ETG levels with 299 pg/ml and 296 pg/ml, respectively (Table 11, Graph 12).

FGF 21 (pg/ml)	Mean	Median	Minimun	Maximum	Range	Standard Deviation
ALC ETG -	333	213	32	1543	1511	347
ALC ETG +	1056	589	46	3854	3808	1216
NALC ETG -	296	182	31	2696	2665	489
NALC ETG +	490	431	247	852	605	284
Healthy control group ETG -	299	261	53	789	736	217

Table 11: FGF21 levels according to negative (<0.5 µg/ml) and positive (≥0.5 µg/ml) ETG levels, separated for ALC (alcoholic liver cirrhosis) n=41, NALC (non-alcoholic liver cirrhosis) n=34 and healthy control group n=21.



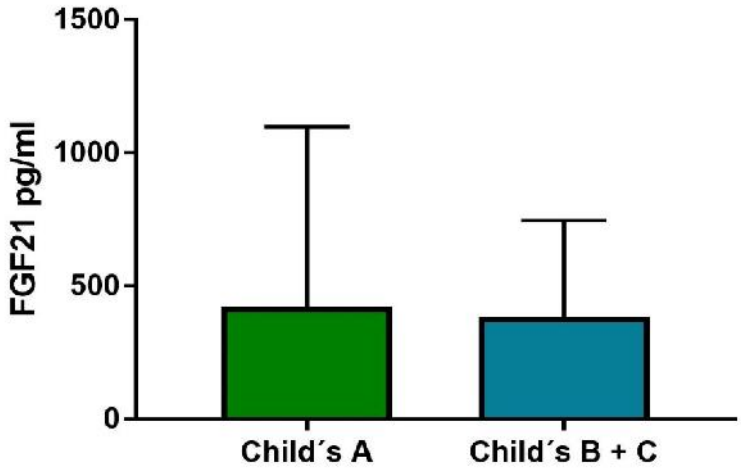
Graph 12: FGF21 levels according to negative (<0.5 µg/ml) and positive (≥0.5 µg/ml) ETG levels, separated for ALC (alcoholic liver cirrhosis) n=41, NALC (non-alcoholic liver cirrhosis) n=34 and healthy control group n=21.

3.10.1 FGF21 and ETG depending on Child-Pugh class

The Child-Pugh-Classification represents the progression and the prognosis of liver disease (1.3.4 Clinical presentation). The accordance of this classification with the corresponding FGF21 and ETG levels is shown in the following section. Caused by the small number of patients with Child´s C classification, the group of Child´s B and C were merged to one group.

The comparison of FGF21 levels and the Child-Pugh class, independently of ETG and the type of cirrhosis, did not reach differences with statistical significance. All patients with Child A showed a mean FGF21 level of 423 pg/ml and patients with

Child´s B and C a mean FGF21 level of 411 pg/ml. These differences did not reach statistical significance (p=0.4716) (Graph 13).

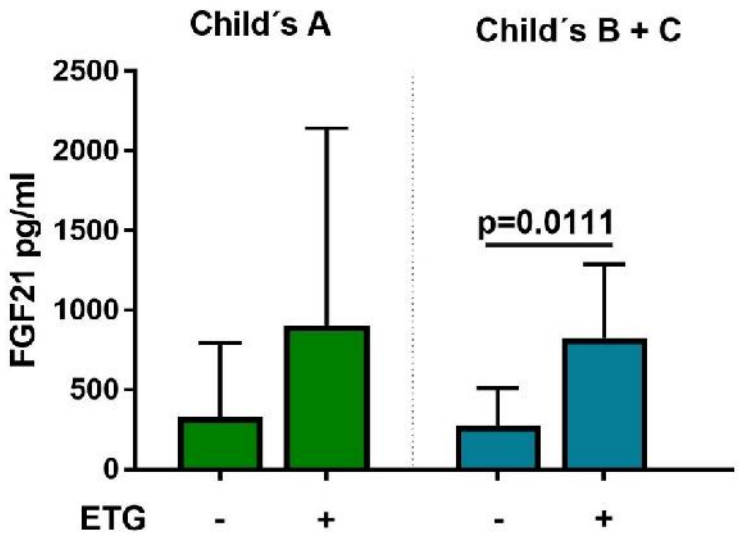


Graph 13: FGF21 levels separated for Child´s A (n=55) and B + C (n=20), independent of ETG levels and type of liver cirrhosis.

Similar results were obtained by taking a separated look at the group of alcoholic liver cirrhosis and non-alcoholic liver cirrhosis.

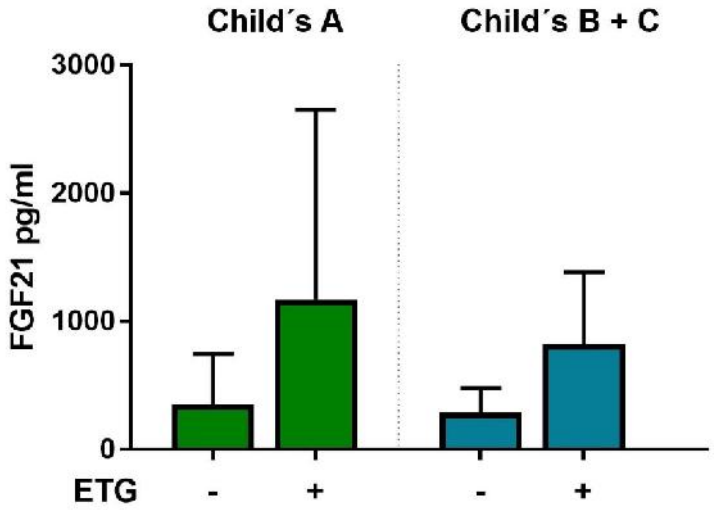
FGF21 levels in accordance to Child-Pugh class and ETG confirmed previous results, indicating higher FGF21 levels in patients with positive ETG levels compared to those with negative ETG levels. This was shown in the group of alcoholic liver cirrhosis and non-alcoholic liver cirrhosis, as well as in the group of Child's A and Child's B + C patients independent of the type of liver cirrhosis.

By analysing FGF21 according to Child-Pugh class and ETG independently of type of liver cirrhosis, a significant difference of FGF21 levels between the ETG positive and the ETG negative group in patients with Child´s B + C (p=0.0111) was observed. Patients with Child´s A and positive ETG values showed higher FGF21 levels, however these differences did not reach statistical significance (Graph 14).



Graph 14: FGF21 levels according to negative (<0.5 µg/ml) and positive (≥0.5 µg/ml) ETG levels, separated for Child´s A (n=55) and B + C (n=20), independent of the type of liver cirrhosis.

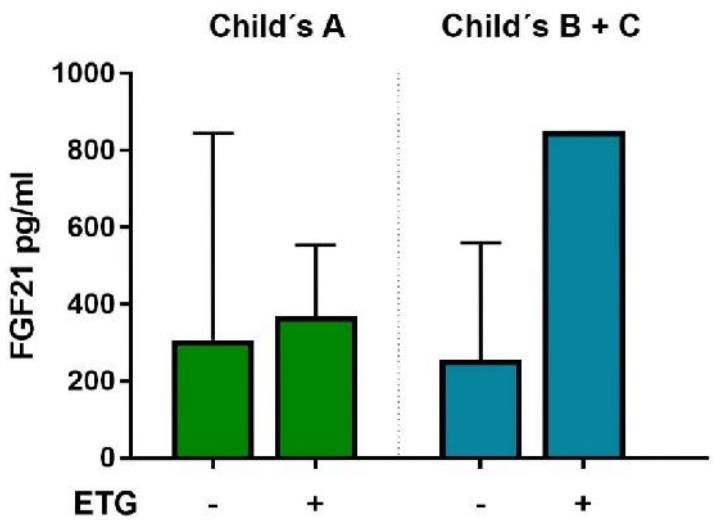
The same comparisons were made for patients with alcoholic liver cirrhosis and showed differences in the group of Child´s A and B + C, however these differences did not reach statistical significance (Graph 15).



Graph 15: FGF21 levels according to negative (<0.5 µg/ml) and positive (≥0.5 µg/ml) ETG levels, separated for Child´s A (n=29) and B + C (n=12), including only patients with alcoholic liver cirrhosis.

There was only one patient in the group of Child´s B + C with positive ETG levels and a non-alcoholic liver cirrhosis, which did not lead to a meaningful result. However, Child A patients were well-represented in this group, but showing almost the same mean FGF21 levels (Child´s A with ETG≥0.5 µg/ml: 370 pg/ml; Child´s A

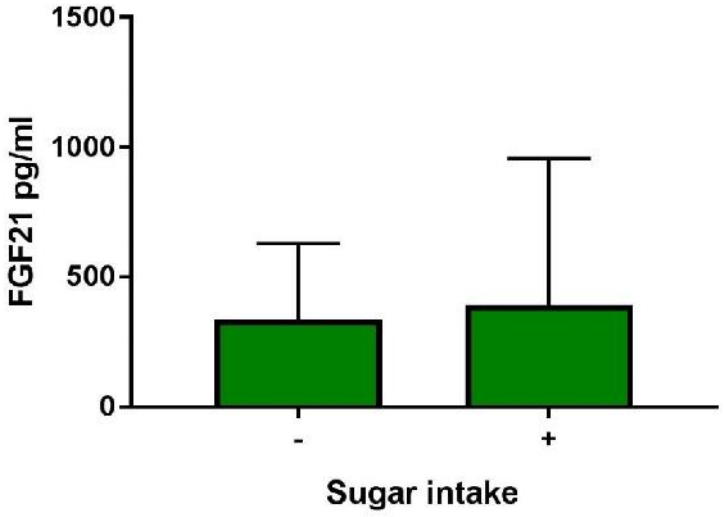
with ETG<0.5 µg/ml: 308 pg/ml), which means no group did reach statistical significance (Graph 16).



Graph 16: FGF21 levels according to negative (<0.5 µg/ml) and positive (≥0.5 µg/ml) ETG levels, separated for Child´s A (n=26) and B +C (n=8), including only patients with non-alcoholic liver cirrhosis.

3.11 FGF21 and sugar consumption

The differences of FGF21 plasma concentrations in accordance to sugar intake were low. More precisely, the mean FGF21 level was 336 pg/ml in case of low sugar intake and 393 pg/ml in case of frequent sugar intake. However, the range of FGF21 levels (3823 pg/ml) was much higher in the group with frequent sugar intake compared to the group with low sugar intake (871 pg/ml) but the differences did not reach statistical significance (p=0.9223) (Graph 17).



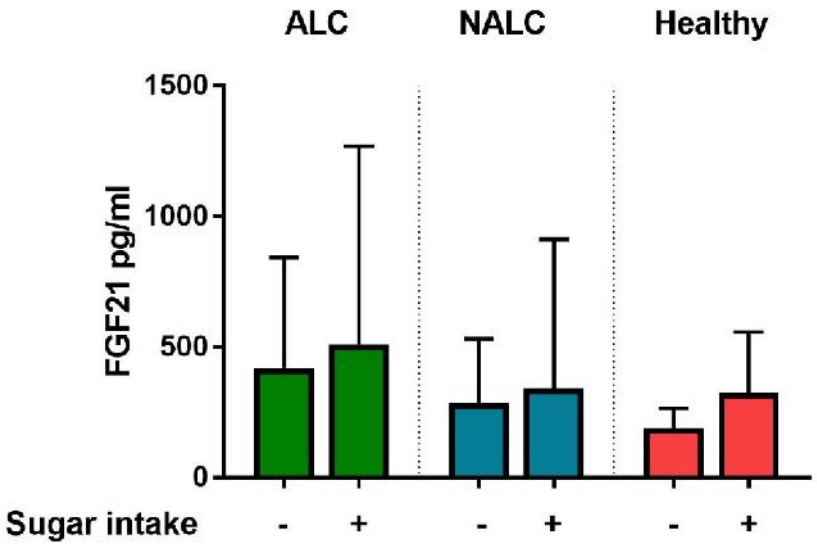
Graph 17: FGF21 levels according to negative or positive sugar consumption given in the food frequency questionnaire, whereby positive sugar intake means one unit per week or more, independent of disease n=96.

The differences in FGF21 levels depending on sugar intake did not reach statistical significance in any group. (ALC: p=0.7925; NALC: p=0.7798; Healthy: p=0.5170) (Table 12,Graph 18).

The lower limit for positive sugar intake was set on one unit per week or more.

FGF 21 (pg/ml)	Mean	Median	Minimun	Maximum	Range	Standard Deviation
ALC Negative sugar intake	326	326	156	497	341	241
ALC Positive sugar intake	500	271	32	3854	3822	709
NALC Negative sugar intake	412	329	59	930	871	394
NALC Positive sugar intake	306	194	31	2696	2665	484
Healthy control group Negative sugar intake	194	194	110	277	167	118
Healthy control group Positive sugar intake	310	261	53	789	736	224

Table 12: FGF21 levels according to sugar intake, declared in the food frequency questionnaire, whereby positive sugar intake means one unit per week or more, separated for ALC (alcoholic liver cirrhosis) n=41, NALC (non-alcoholic liver cirrhosis) n=34 and healthy control group n=21.



Graph 18: FGF21 levels according to sugar intake, declared in the food frequency questionnaire, whereby positive sugar intake means one unit per week or more, separated for ALC (alcoholic liver cirrhosis) n=41, NALC (non-alcoholic liver cirrhosis) n=34 and healthy control group n=21.

4 Discussion

4.1 Alcohol and sugar does not induce FGF21 synthesis in human hepatocytes

The basis of the liver-brain endocrine axis and the presented study, is the impact of alcohol on hepatocytes, which leads to an increased synthesis of FGF21. To get a confirmation of this hypothesis, which is not shown for human hepatocytes so far, a cell culture model with HepG2 cells, a hepatocellular carcinoma derived human hepatocyte cell line was used.

These cells were incubated with alcohol and sugar and mRNA levels of FGF21 were determined. Thereby, no elevation of FGF21 mRNA levels were observed. Furthermore, an incubation with higher concentrations of alcohol and sugar was executed, but there was no increase of mRNA levels, despite sufficient amount of unspecific RNA.

The absence of FGF21 synthesis in this cell line could be caused by an insufficient activation of transcription. However, the alcohol and sugar concentration were at a high level in the incubation trials compared to alcohol concentrations in the vena portae in vivo (75). Even an increase of alcohol and sugar concentrations in this experiment up to 5 times did not show any differences. Otherwise, the non-appearance of FGF21 mRNA could be caused by an inactivation or loss of the *fgf21* gene. Genome aberrations in malignant cells are common and could explain the results (76, 77). However, Xia *et al* have already shown the synthesis of FGF21 in HepG2 cells, whereby the induction of synthesis was achieved by starvation of the cells and not by incubation with alcohol or sugar (19). Furthermore, it could be possible that the expression of FGF21 in response to alcohol is dependent on a metabolite of alcohol that is not provided in the cell culture model.

In summary, the exact reason for the absence of FGF21 mRNA remains elusive. Accordingly, the current hypothesis regarding the induction of FGF21 synthesis by alcohol in this cell culture model could not be confirmed in this study.

4.2 No relation between FGF21 and sugar consumption in patients or healthy controls

The comparison of declared sugar intake in the food frequency question and the FGF21 plasma levels of patients and healthy test persons did not show cohesion in this observational study.

The connection between FGF21 and sugar intake is shown in mice and monkeys. More precisely, FGF21 administration decreases the preferences for sweet taste in these animals, whereby the FGF21 levels are at a supraphysiological level (27). Furthermore, genetic loss of FGF21 increases the preference for sweet taste (28).

The situation of unphysiologically high or low FGF21 levels were not simulated in this observational study. However, detailed information about food preferences in combination with FGF21 plasma levels were acquired. In this context, no significant correlation was seen between these two parameters, neither in the patient's cohort nor in the healthy control group. Therefore, the expectation of increased FGF21 levels in patients with low preferences for sugar intake does not find confirmation.

The factors influencing the demand for sweet taste are multifactorial. On one side, there is the gut microbiome with an enormous diversity, influencing our nutrient sensing, appetite and satiety-regulating system (78). On the other side, there is another big influence, the hypothalamus, synthesizing a lot of different peptides, like neuropeptide Y, ghrelin, melanocortin, cholecystokinin et cetera, influencing our eating behaviour (79). This central feeding organ is also influenced by FGF21, which points towards a non-negligible role of FGF21 (1.2). Thus, there are strong indications that FGF21 influences our appetite for sugar. Probably we could not exclude enough other factors to get valid information about FGF21 in this context.

Furthermore, obtaining information by questionnaire may lead to unspecific descriptions about the nutrition of patients and healthy controls. It represents the general eating behaviour of the last few weeks and does not give exact information about the sugar intake shortly before the blood sampling. In addition, the exact amount of sugar of the food items the individuals actually ate is not known. Therefore, further experiments with a specific focus on eating behaviour, including

standardized nourishments meals, are necessary to get an idea about FGF21 in this context in humans.

4.3 Alcohol declarations and ETG levels

Monitoring the alcohol use is an important part of the treatment of patients with ALC. Furthermore, this information represents an essential basis of our studies and is a crucial fact during the evaluation of the results. Probably it is not a rarity, that patients and even healthy people's declaration of alcohol intake is unreliable(80).

Due to this uncertainty, an objective marker is needed. We used urine ethyl glucuronide levels, a frequently used test for alcohol intake in the last 12 to 72 hours with a sensitivity of 76% and a specificity of 93%. A similar marker is ethyl sulfat with a sensitivity of 82% and a specificity of 86% (53) (1.3.4 Clinical presentation).

Caused by technical reasons, the ETS measurement revealed 35% invalid results. ETG levels provided the opportunity to compare them with the information about alcohol consumption. In this context a good match of high ETG levels and positive alcohol declaration is seen in the group of non-alcoholic liver cirrhosis. However, even in this group a few patients declared low or no alcohol intake and show positive ETG levels. In addition, the opposite combination with negative ETG levels and a declaration of frequent alcohol intake could be observed. This is a rare fact in the group of non-alcoholic liver cirrhosis, but frequent in the group of healthy controls. Everybody in this group has negative ETG levels, whereby four of them declared daily alcohol intake and ten declared to drink alcohol at least one time a week. The motif for an exaggerated alcohol declaration is not obvious so far. So, this discrepancy might be based on false negative ETG levels, which is quite unlike in almost 50% of the cases declared frequent alcohol intake in the healthy control group, even though this test has just a sensitivity of 76%. Furthermore, excessive alcohol consumption on weekends and strict abstinence in the rest of the week presents a more probable explanation. We might see negative ETG levels although the units of alcohol per week are high, if they are consumed outside the 72-hour time slot in which ETG is detectable.

Furthermore, we see inconsistencies in the group of alcoholic liver cirrhosis, too. More precisely, all nine patients with positive ETG levels declared absolute no alcohol intake. It is not ideal, but plausible not to concede to your alcohol consumption, because of shame or uncertainty or because the patients fear problems in their medical treatment in a liver clinic (e.g. access to liver transplantation) when they admit their alcohol consumption. The opposite combination represents the more dubious option; one patient declared daily alcohol intake, one patient declared 3 alcohol units per week and two patients declared weekly alcohol intake and all of them showed negative ETG levels. This could mean four false negative results of ETG levels in a group with 41 patients, which seems more likely compared to the situation in the healthy control group, but still leaves a lack of clarity. In addition, patients with usually frequent alcohol intake could have stopped their alcohol consumption just a few days before the urine sampling was done. Furthermore, the alcohol intake of these 3 patients could be fulfilled in more than 72 hours before urine sampling.

4.4 Malfunction of liver-brain endocrine axis

The basic concept of the liver-brain endocrine axis is the self-protection of liver tissue by influencing the hypothalamus, which in turn leads to a reduction or cessation of alcohol consumption. This is achieved by the synthesis of FGF21, which passes the blood-brain barrier and affects the hypothalamus by binding to FGFR1c or 3c in presence of βKlotho (1.4).

This mechanism sounds beneficial and highly promising, however the long history of alcohol consumption and abuse in humankind is showing a different reality or rather this feedback mechanism seems to be vulnerable.

To get a better idea of this feedback mechanism and its vulnerability a closer look at FGF21 levels in different diseases and alcohol consumption, is needed. Significantly higher FGF21 levels with positive ETG levels are seen compared to those with negative ETG levels. This applies to the group of alcoholic liver cirrhosis and non-alcoholic liver cirrhosis alike. This in turn indicates, that increased alcohol consumption leads to increased FGF21 levels, which would confirm the first part of the liver-brain endocrine axis. However, there is a discrepancy in the further process

of this feedback mechanism, insofar as the patients do not stop drinking alcohol. Ideally, a reduction of alcohol intake would be expected, but this does not seem to be the case.

This finding points to an abnormality in the transmission of information to the hypothalamus, or rather in the processing of this information.

Involved in the transmission of information are FGF21, βKlotho and FGFR1c and 3c and so they could represent a potential part of the pathophysiology in the liver-brain endocrine axis. FGF21 is available in sufficient concentration therefore a lack of FGF21 does not seem to be the crucial factor in the genesis of the pathological transformations of this system. Rather, increased FGF21 levels may represent the consequence of the underlying problem in these patients.

βKlotho as an essential cofactor in the signal transduction of FGF21 could be a weak point in this system. A GWAS study has already shown, that a variation in the *klb* gene (encoding βKlotho) is associated with abnormal alcohol intake with genome wide significance (72)(1.4). This could represent an explanation for the continuation of alcohol consumption in patients despite high FGF21 levels. More precisely, synthetized FGF21 attains the hypothalamus, but a signal transduction is not possible, because of a loss of function or reduced function of βKlotho. This leads to an absence of the desired cessation of alcohol intake. In addition, the persistent alcohol intake induces further FGF21 synthesis and would explain the elevated FGF21 levels.

Another flaw in the transmission of information in the liver-brain endocrine axis represents the receptor, FGFR1c and 3c. Alterations in the sensitivity of receptors are well known. The most famous example for a receptor resistance is probably diabetes mellitus type II, whereby insulin levels are high, but the hormone does not exert an effect, caused by insulin resistance (25). Parallels can be drawn to the situation of the patients in this study with elevated FGF21 levels and persistent alcohol consumption. Another example is the development of an opioid tolerance; due to long term opioid intake the responsiveness of the μ-receptors to their agonist decreases, which leads to a decreased pain-relieving effect (81). Both cases are caused by massively elevated levels of signalling substance over an extended period of time. This long-term elevation of the signalling substance may also apply

to the patients in the current study since they usually have a long drinking history and thus increased FGF21 levels for a long period. This could lead to a receptor resistance, which starts a vicious circle with the consequence of further alcohol intake and further increase of FGF21 levels and so one. Furthermore, a FGFR-resistance in obese mice is already known. These mice do not show the positive effects of administered recombinant FGF21 on metabolism and weight loss like lean mice do (1.4). In addition, a genetic variation of FGFRs is another possibility in the quest for the causing factor in this context.

The further processing of information, delivered by FGF21, in the hypothalamus could be another cause in the genesis of alcoholism. However, there are several factors influencing the hypothalamus and they are hard to distinguish from each other. Although, the hypothalamus plays an important role in every drug addiction, it will be hard to determine whether the crucial changes are actually based on the effects of FGF21 on the hypothalamus. Furthermore, the psychological and social factor predisposing to alcoholism are certainly multifactorial and impede the research for the causing factor of FGF21 miscommunication.

Therefore, a focus on the transmission of information and the involved components, like FGF21, βKlotho and FGFR 1c and 3c, is necessary. The results of this observational study, considering FGF21 levels according to ETG, confirm our hypothesis of increased FGF21 levels in patients with alcoholic liver cirrhosis and frequent alcohol intake. However, this is also present in the group with non-alcoholic liver cirrhosis. Which leads to the question, if patients with non-alcoholic liver cirrhosis, positive ETG and increased levels of FGF21 suffering from alcoholism or ALC, too? And do other types of liver cirrhosis predispose for alcoholism and ALC?

Anyway, our findings in combination with contemporary literature point towards pathologies of the cofactor βKlotho or/and the receptors of FGF21, FGFR1c and 3c. Further experiments are necessary to concretise the pathology to one of these two subjects. However, a combination of issues regarding βKlotho and FGFRs are also a plausible explanation or some patients showing pathologies in βKlotho and some showing a receptor resistance.

Based on the Child-Pugh class there is further information of this patients and their disease, which allows a comparison of values, depending on the progression of liver

cirrhosis. Child C is under represented with just 2 patients, which does not allow any conclusions about this state of liver cirrhosis. However, by comparing Child A and B independent of the type of liver cirrhosis, a more distinctively difference in FGF21 levels compared to ETG positivity or rather negativity in patients with Child B is seen. That points towards an association between the progression of liver cirrhosis and the progression of pathologies in the liver-brain endocrine axis. Furthermore, it is unclear if the cirrhosis enhances the further progression of pathologies in the liver-brain endocrine axis or is the progression of liver cirrhosis based on the advancement of pathologies in receptor resistance or βKlotho malfunction.

By taking a separated look to each group of liver cirrhosis in consideration of the Child-Pugh class, there are no obvious differences between Child A and B in the group of alcoholic liver cirrhosis. The group of non-alcoholic liver cirrhosis has only one patient with Child B and positive ETG levels, which does not allow any conclusions.

To better understand the progression of pathologies in the liver-brain endocrine axis, further studies are needed to figure out if treatment affecting this feedback mechanism is helpful in the therapy of alcoholism and ALC. In mice it is already shown, that long term FGF21 administration does decrease alcohol intake (29). But do high therapeutic concentrations of FGF21 lead to a reduction of alcohol intake, despite a potential receptor resistance in human? And is it possible to administer βKlotho in case of genetic alterations of this protein? Further research of this topic could lead to an effective treatment of patients suffering from alcoholism and ALC. In addition, this could reduce a lot of costs in the health care system, related to the long-term consequences of alcohol intake and furthermore decreases social and environmental problems, associated with frequent alcohol consumption. These issues are omnipresent in Austria and a lot of other European countries and should be targeted to reduce the burden of the disease. The liver-brain endocrine axis represents a potential mechanism for pharmacological treatment to achieve an improvement of the situation.

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