Diploma thesis

Non-invasive diagnosis of hepatic fibrosis in patients during alcohol detoxification therapy

submitted by

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Affidavit

I hereby declare that the following diploma thesis is the result of my own work. I did not receive any assistance from third parties. Furthermore, I confirm that all sources applied are listed and specified in the thesis.

Graz, 27th of August, 2020 *Alena Maria Kristof eh*

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Abbreviations

A1AT	Alpha-1 antitrypsin
A1ATD	Alpha-1 antitrypsin deficiency
AA	Acetaldehyde
AH	Alcoholic hepatitis
ALD	Alcoholic liver disease
ALT	Alanine-aminotransferase
AMPK	AMP-activated protein kinase
ASH	Alcoholic steatohepatitis
AST	Aspartate-aminotransferase
AUD	Alcohol use disorder
ATZ	alpha-1 antitrypsin Z
CAP	Controlled attenuation parameter
CDT	Carbohydrate deficient transferrin
CXCL-1 (GRO alpha)	C-X-C motif chemokine ligand 1 (growth-regulated oncogene alpha)
CYP2E1	Cytochrome P450 2E1
dB/m	Decibels per meter
e.g.	Example given
ELF	Enhanced liver fibrosis test
FIB-4	Fibrosis-4 test
IQR	Interquartile range
IQR/med	Interquartile range of the median value (%)
HCC	Hepatocellular carcinoma
HMZ	Homozygous
HTZ	Heterozygous
Hz	Hertz
kg/m^3	Kilogram per cubic metre
kPa	Kilopascals
LPS	Lipopolysaccharid
LS	Liver stiffness
LSM	Liver stiffness measurement
m/s	Metre per second
NADH	Nicotinamide adenine dinucleotide (reduced)

NAD^+	Nicotinamide adenine dinucleotide (oxidized)
NAFLD	Non-alcoholic fatty liver disease
n-WT	non-wildtype
PIIINP	Aminoterminal peptide of type III procollagen
PNPLA3	Patatinlike phospholipase domain-containing protein 3
ROS	Reactive oxygen species
SERPINA1	Serpin family A member 1
Sig.	Significance
SREBP-1c	Sterol regulatory element-binding protein 1c
TNR	True negative rate; specificity
TPR	True positive rate; sensitivity
TIMP-1	Tissue inhibitor of matrix metalloproteinases-1
TLR	Toll-like receptor
VCTE	Vibration controlled transient elastography
WT	Wildtype

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Zusammenfassung

Einleitung

Die Alkohol-assoziierte Lebererkrankung ist eine häufige Krankheit. Trotz ihrer hohen Prävalenz steht sie nicht im Fokus der Forschung. Einfache, nicht-invasive Methoden zur Detektion müssen evaluiert und optimiert werden, um Alkohol-assoziierte Leberfibrose in frühen, potentiell reversiblen Stadien detektieren zu können. Ziel der Studie war es, potentiell vorhandene Fibrose mit nicht-invasiven Verfahren in großteils asymptomatischen Alkoholkranken unter stationärer Detoxifikation festzustellen. Zusätzlich wurden die Studienteilnehmerinnen und Studienteilnehmer auf Veränderungen der Lebersteifigkeit als Folge einer Abstinenz in einem Follow-up nach drei Monaten untersucht. Letztlich wurde auch die Rolle von unterschiedlichen Genotypen von PNPLA3 und SERPINA1 exploriert.

Methoden

31 Patienten unter stationärer Detoxifikation im Zentrum für Suchtmedizin des LKH Graz-Süd-West, Standort Süd, wurden eingeschlossen. Der FIB-4 Index wurde aus klinischen und laborchemischen Routineparametern errechnet. Der ELF-Test wurde mittels Advia Centaur XP (Siemens Healthcare Diagnostics, Wien, Österreich) durchgeführt. SERPINA1 und PNPLA3 wurden in einem Routinelabortest mittels 5'-Exonuklease-Assays (TaqMan) analysiert. Die transiente Elastographie wurde nach einer Nüchternperiode von mindestens 2 Stunden mit FibroScan® 502 Touch (Echosens, Paris, Frankreich) ausgeführt. Für die statistischen Berechnungen wurden IBM SPSS Version 25 und 26 verwendet.

Ergebnisse

Etwa 16 % der initialen Studienpopulation wiesen mittels FibroScan®-Untersuchung Zeichen für fortgeschrittene Fibrose auf (≥ 15 kPa). Mit einer Lebersteifigkeit von ≥ 15 kPa als Referenzwert, detektierte ELF mit einem Cut-off von 10.5 fortgeschrittene Fibrose am besten (TPR = 80%, TNR = 100%, PPV = 100%, NPV = 96,2%). Für FIB-4 wurde 1.30 als adäquater Cut-off für fortgeschrittene Fibrose ermittelt (TPR = 60%, TNR = 80,8%, PPV = 37,5%, NPV = 91,3%). FIB-4 stieg, während ELF und Lebersteifigkeit nach dreimonatiger Abstinenz abnahmen. ALT und MCV sanken nach der Abstinenz signifikant.

Schlussfolgerung

Die Vorstellung von asymptomatischen Alkoholkranken in der Leberambulanz kann für ihre Behandlung vorteilhaft sein. ELF und FIB-4 sind adäquate Methoden für die Diagnostik von fortgeschrittener Leberfibrose anhand einer Lebersteifigkeit von ≥ 15 kPa. Die Rolle der Genotypen von PNPLA3 und SERPINA1 konnte nicht ausreichend bestimmt werden. Eine größere Kohorte sollte untersucht werden, um verlässliche Ergebnisse zu generieren.

Abstract

Introduction

Alcohol-related liver disease is a common disease. Despite its high prevalence, research has not been focused on the topic. Simple, non-invasive detection methods must be evaluated and optimized in order to detect alcohol-related fibrosis in potentially reversible stages. The aim of this study was to determine the presence of fibrosis by non-invasive means in mostly asymptomatic alcoholics undergoing detoxification. Additionally, investigation of alterations in liver stiffness due to abstinence in a follow-up visit 3 months after the respective baseline visit was conducted. Finally, the role of PNPLA3 and SERPINA1 genotypes in fibrosis development was investigated.

Methods

31 consecutive patients admitted for alcohol detoxification were enrolled. FIB-4 index was calculated using laboratory and clinical routine parameters. ELF-test was carried out on Advia Centaur XP (Siemens Healthcare Diagnostics, Vienna, Austria). SERPINA1 and PNPLA3 genotypes were analyzed in a routine laboratory test by 5'-exonuclease assays (TaqMan). VCTE was performed in patients after a fasting period of at least two hours with FibroScan® 502 Touch (Echosens, Paris, France). Statistical analyses were performed using IBM SPSS Statistics version 25 and 26.

Results

About 16% of the initial study population showed advanced fibrosis according to VCTE using a threshold of 15 kPa. Using a LS of 15 kPa as reference, for the detection of advanced fibrosis (F3, F4), ELF worked best using 10.5 as a cut-off (TPR = 80%, TNR = 100%, PPV = 100%, NPV = 96,2%). A FIB-4 cut-off value of 1.30 was found to be adequate for ruling out advanced fibrosis (TPR = 60%, TNR = 80,8%, PPV = 37,5%, NPV = 91,3%). FIB-4 increased, whilst ELF score and LS dropped after a period of 3 months. ALT (p = 0,020) and MCV (p = 0,016) significantly decreased following abstinence.

Conclusion

Introducing asymptomatic alcoholics to a liver clinic could be beneficial for their treatment. ELF and FIB-4 are adequate non-invasive tools for the detection of advanced fibrosis as defined by LS ≥ 15 kPa. The role of PNPLA3 and SERPINA1 genotypes could not adequately be determined in this study. A larger cohort must be investigated to generate reliable results on the topic.

1 Introduction

Alcohol-related liver disease is a common disease especially in the western world. Despite its high prevalence, research has not been focused on the topic. Simple, non-invasive detection methods need to be evaluated and optimized in order to detect alcohol related fibrosis in early, potentially reversible stages.

The goal of this study was to determine the presence of fibrosis by non-invasive means in mostly asymptomatic alcoholics who were admitted to the Centre of Addiction Medicine at Federal Hospital Graz II for detoxification therapy in the recruitment period between 28/02/2019 and 26/07/2019. Additionally, we investigated alterations of liver stiffness following abstinence in a follow-up visit 3 months after the respective baseline visit.

The introduction of this thesis aims to summarize the current state of knowledge concerning alcoholism and alcohol-related liver disease.

1.1 Alcohol consumption and Alcoholism

1.1.1 Epidemiology and Public Health

Throughout many regions of the world, alcohol consumption is a common habit, even though in a global perspective the major part of adults are abstainers. Nearly every population consuming alcohol has social and health related issues. Due to the alcohol industry and globalized marketing strategies not only the consumption of alcohol but also its negative impact is rising (1). As an example, in the United States of America 80 000 deaths per year are associated with alcohol use (2).

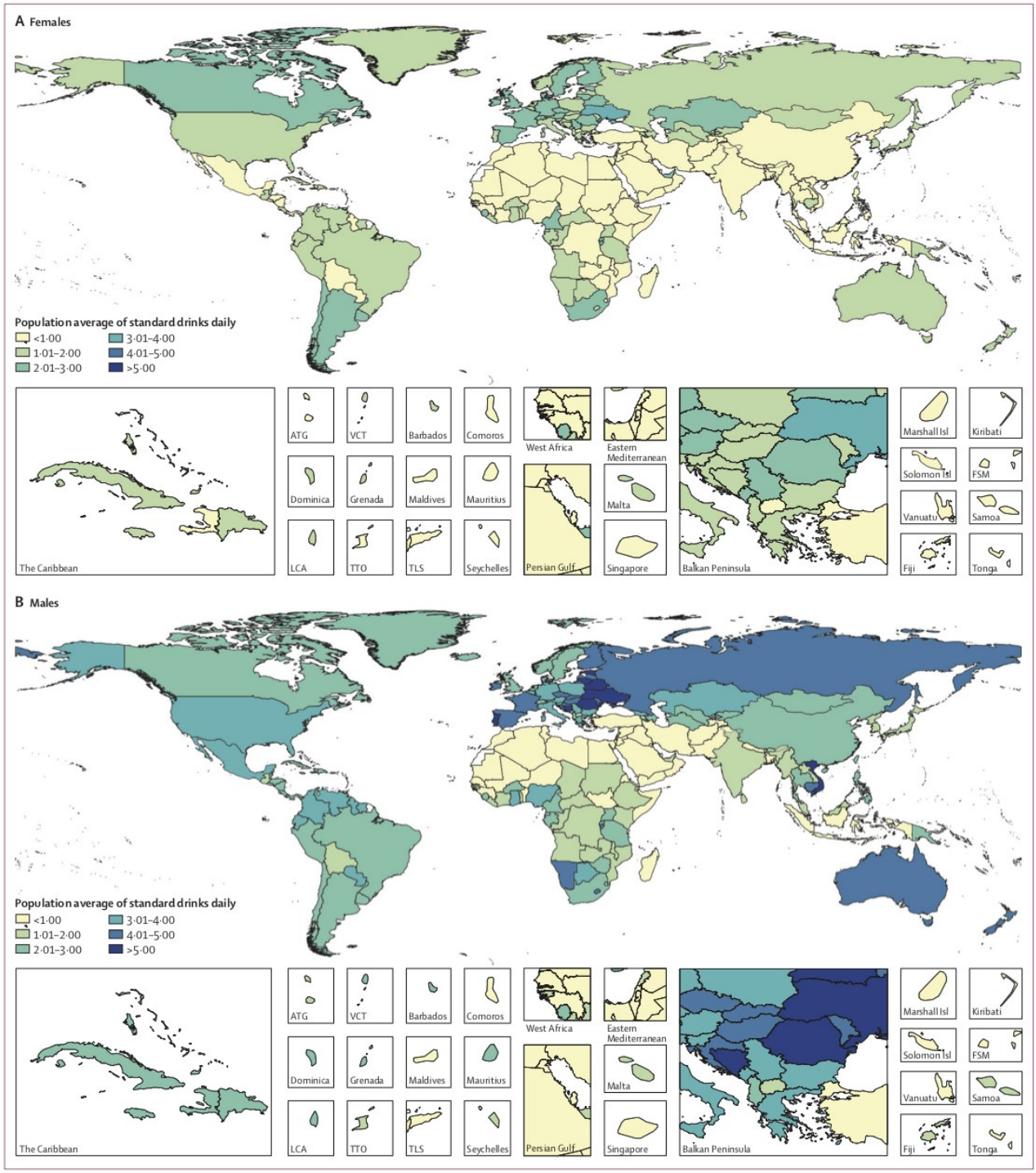
According to the first part of the *Lancet* Series *Alcohol and Global Health* published in 2009, on average, each adult consumes 6.2 liters of pure ethanol in one year. With 11.9 liters per adult, alcohol consumption in the WHO Europe region is particularly high. Throughout the world, men drink more alcohol than women. In wealthy countries women consume a bigger amount of alcohol than in low-income nations. In addition, findings unsurprisingly showed that alcohol use disorders (AUD) are more prevalent in men than in women (1).

As published in a study in *Lancet* (2018), 32.5% of the world's population consumed alcohol in 2016. A quarter of the female world population consumed alcohol at the time, as did 39%

of the male population. In absolute numbers, 2.4 billion members of the global population were active drinkers (3).

As already proposed in the mentioned *Lancet* Series *Alcohol and Global Health*, in regions with a high socio-demographic index, prevalence of alcohol users and the amount of daily alcohol intake were found to be highest (3).

Figure 1. *Average, age-standardised consumption of alcohol in 2016. Adapted from (3).*



Alcohol consumption is shown as standard drinks (1 standard drink = 10 g of pure ethanol) per day. ATG, Antigua and Barbuda; FSM, Federal States of Micronesia; Isl, Islands; LCA, St. Lucia; TLS, Timor-Leste; TTO, Trinidad and Tobago; VCT, Saint Vincent and the Grenadines.

Figure 1 displays the international differences of per capita consumption of ethanol in the male and female global population. Sex-related disparity of average ethanol intake is higher in locations with a low socio-demographic index. Nepal is considered a country with low socio-demographic index. There, only 1.5% of the female population were found to be active alcohol consumers in 2016, whereas 21% of males drank alcohol. In Sweden the sex-related difference was found to be very subtle. In this country with high socio-demographic index, 86% of females and 87 % of males consumed alcohol at the time (3).

1.1.2 Mechanisms of AUD

As penalizing addictive behavior has not been able to reduce addiction, current research indicates that it should be seen as a type of brain disease. According to the fifth edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-5) substance-use disorder is defined as repetitive consumption of alcohol or other drugs leading to considerable impairment of function. Substance-use disorder can be categorized as mild, moderate or severe. 'Addiction' describes the most serious, chronic substance-use disorder. Addicted individuals lose self-control and compulsively consume the addictive substance. They want to stop their drug consumption, but are not able to do so. 'Severe substance-use disorder' and 'addiction' are considered equivalent in DSM-5 (4).

Patients with substance-use disorder lose control over their drug use, e.g. consumption of alcohol, and develop alterations in brain regions responsible for motivation and behavior (4). With increasing severity of AUD, patients develop negative reinforcement mechanisms overweigh positive ones, which leads to negative emotions. Consumption of the substance alleviates this state. Thus, addicted individuals fail to stay abstinent (5).

As suggested in a review from 2010 by *Koob and Volkow,* there are three different stages of addiction: binge and intoxication, withdrawal and negative affect, and preoccupation and anticipation (6). This classification of stages is used in the following paragraphs.

1.1.2.1 Binge and intoxication

Surging levels of dopamine are caused by the consumption of every known addictive substance. This stimulates reward regions in the brain (7–9). With recurrent stimulation by the same substance, striatal dopamine cells quit releasing the hormone as an answer to the stimulant, but rather release it in anticipation of said reward (10). Hence, stimuli associated with the drug use, e.g. people, situations and mood, may increase dopamine and that way,

elicit longing for the substance (11) and excessive consumption (12–14). This effect may even occur after long periods of abstinence (4).

1.1.2.2 Withdrawal and negative affect

Due to the mechanisms responsible for addiction, affected individuals cannot be motivated by healthy stimulants anymore. In addicted people, reward and motivation are results of more intense dopamine release due to the respective drug (4). This process is not the only mechanism involved in addiction, though.

In contrast to a long believed hypothesis, drug use causes much lesser, not higher, dopamine releases in individuals with addiction than in those who are not addicted or even naïve to drugs (13–16). Because of this, the brain reward system loses sensitivity to the stimulating effect of rewards related to drugs and also to rewards that are not drug-related (17–19). Most of these findings about addiction were investigated in studies on cocaine-dependent individuals.

The joy following drug consumption is smaller in the state of addiction compared to the first time taking the substance. Additionally, this mechanism causes a lack of motivation and stimulation by components of peoples' everyday lives. For example, relationships and interests are less stimulating for addicted individuals. These alterations are deeply implanted and are not turned backwards right away solely by refraining from drug abuse (4).

Furthermore, repeatedly enhanced dopamine levels lead to alterations in the extended amygdala. Thus, the affected person becomes more reactive to stress and as a consequence, negative feelings emerge (20,21). This 'anti-reward' system is hyperactive in addicted brains, causing the dysphoria experienced during withdrawal (22). Negative affective state is also caused by lesser activity of dopamine cells in the reward system (23). So, additionally to being attracted towards the rewarding effect due to the drug consumption, addicts feel the need to seek relief from the discomfort caused by withdrawal. In many cases, addicts are not able to comprehend why they keep up their substance consumption even though they do not get pleasure from it. Many of the affected people justify their ongoing drug use by the need to escape the dysphoria they experience when they are not under drug influence (4).

1.1.2.3 Preoccupation and anticipation

Alterations do not only occur in the reward system and circuitry responsible for emotions. There are also functional changes in the prefrontal cortex. Decrease of dopamine signaling causes impairment of executional functions, such as taking decisions, self-regulation, and induction of action (24). While changing of dopamine signaling is an essential factor in addiction, it is not the only relevant neurotransmitter. Reward and emotional systems in the prefrontal brain are also disturbed by alterations of glutamatergic signaling (25). This lowers the ability to oppose intense desires or to successfully execute plans to refrain from drug abuse. Thus, addicted people can be honest and serious about aiming to stop using their drug and at the same time they are incapable of following their intentions (4).

1.1.3 Social aspects of addiction

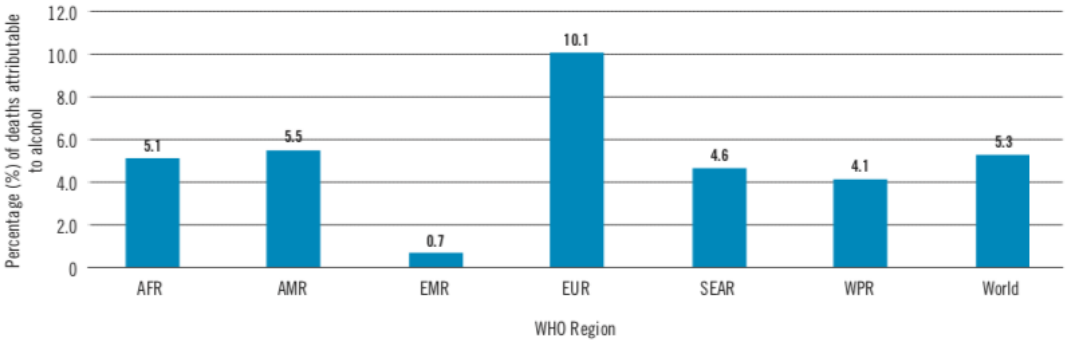
Not every drug consumer will ultimately develop addiction, because some people are less vulnerable to genetic, developmental or environmental circumstances than others (4). The susceptibility to initiating drug use, sustaining it and developing alterations in the brain is determined by many genetic factors, social situations and environmental impact (26,27). Substance use in the family, being exposed to drugs at young age, social distress, an environment where drugs are easily accessible and several mental illnesses, e.g. mood disorders or anxiety disorders, are predisposing factors for developing addiction (28,29).

1.2 Alcohol-related liver disease

As one of the main causes for liver related morbidity and mortality, alcohol-related liver disease (ALD) represents a globally relevant issue. In many regions throughout the world alcohol intake continues to be high, even though it has long been found to be a risk factor for liver disease (30). From a global perspective, 3.8 % (1) to 5.3 % (31,32) of all deaths can be referred to alcohol consumption. Particularly in Europe, where alcohol consumption leads to 10.1 % of all deaths (31) and alcohol use disorders are the main cause of liver cirrhosis (33), harmful alcohol intake seems to be a considerable problem.

AUD not only causes morbidity and mortality by inducing ALD, but also as an additional harming factor in patients with other etiologies of liver disease, such as chronic viral hepatitis or non-alcoholic fatty liver disease (NAFLD) (34).

Figure 2. *Percentage of deaths (in %) attributable to the consumption of alcohol, by WHO region, 2016. Adapted from the WHO Global status report on alcohol and health 2018 (31).*



AFR, WHO African region; AMR, WHO Region of the Americas; EMR, WHO Eastern Mediterranean region; EUR, WHO European Region; SEAR, WHO South-East Asia Region; WPR, WHO Western Pacific Region;

In most cases, ALD does not cause clinical symptoms in early stages and is therefore usually diagnosed when already advanced. Therapeutic options are limited in those cases, whilst early stages of ALD (e.g. alcoholic steatosis, fibrosis without cirrhosis) are potentially reversible (34).

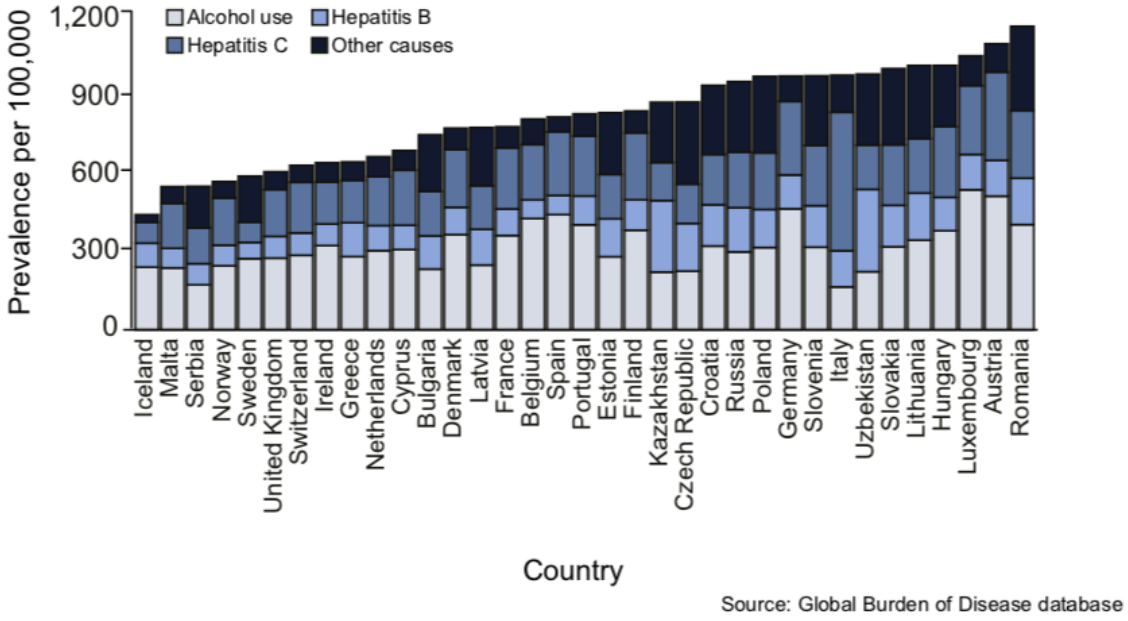
1.2.1 Epidemiology of ALD

Alcohol-related liver disease is a condition with globally growing prevalence. Especially in Europe ALD is a substantial burden, since Europeans have the highest average consumption of alcohol throughout the planet (35).

According to the *Global Burden of Disease Study* of 2010, liver cirrhosis accounted for over one million deaths and was found responsible for over thirty millions of Disability Adjusted Life Years. Almost half (47.9%) of all deaths due to liver cirrhosis were alcohol-related and more than 80 000 death cases could be led back to alcohol-related liver malignancy (33).

The exact percentage of alcohol-related liver disease is not known, since most countries do not document etiologies precisely and data vary a lot. The most exact data in Europe are offered by Finland, where 87% of liver-related deaths seem to be attributable to alcohol consumption (34). In the European Union, approximately 60% to 80% of mortality due to liver disease is caused by massive alcohol intake (36).

Figure 3. *Age-standardized prevalence of liver disease by cause in 2016 - modelled data. Adapted from (37)*



As displayed in *Figure 3*, Austria was found to be the European country with the second highest prevalence of liver disease right after Romania. In terms of liver damage attributable to alcohol use, only in Luxembourg there is a higher prevalence than in Austria (37).

1.2.2 Pathophysiology

The mechanisms causing ALD are not entirely clear. Multiple factors such as behavior, environment and genetics are assumed to interact in complex ways, which ultimately can lead to a spectrum of ALD. When constantly exposed to alcohol, liver tissue develops steatosis, inflammation and fibrosis (34).

1.2.2.1 Alcohol degradation

There are two molecular systems that degrade ethanol to acetaldehyde (AA). Alcohol-dehydrogenase in the cytosol mainly processes small amounts of ethanol, as it cannot be upregulated if needed. The second enzyme system is cytochrome P450 2E1 (CYP2E1), which resides in microsomes. CYP2E1 can be upregulated and in severe alcoholics its presence is 10 to 20 times higher than normal (38). It is assumed that the intake of 40 g of ethanol per day for one week already results in an upregulation of hepatic CYP2E1 (39).

AA is the first metabolite of ethanol metabolism and it plays a crucial role in the development of ALD, since it is directly toxic to liver tissue (40). Additionally, it has been shown in animals as well as in humans that AA is strongly carcinogenic and therefore seen as a

significant factor in the connection between alcohol consumption and specific types of cancer (41). Not only AA, but also reactive oxygen species (ROS) are generated by CYP2E1, which leads to harmful oxidative stress (34).

1.2.2.2 Pathogenesis and forms of alcohol-related liver disease

Heavy chronic alcohol abuse leads to alcoholic fatty liver in 90% to 100%. Patients with alcoholic fatty liver, who keep up their high ethanol intake, will develop alcoholic steatohepatitis (ASH) in 10% to 35%. Cirrhosis only occurs in 8% to 20% of heavy alcoholics (34,42). In 70% of survivors of ASH, cirrhosis will develop and 40% of cirrhotics may develop acute-on-chronic disease (ASH in patients with cirrhosis) (42,43).

When defining thresholds for the definition of heavy drinking, biological sex needs to be taken into account. While in men, 60 g or more alcohol per day is considered heavy chronic alcohol abuse, for women the threshold is set at 40 g per day (44,45)

The International Classification of Diseases (ICD-10) acknowledges several forms of ALD (ICD-10, K70) (33). As summarized in a *Nature Rewiews Disease Primer* by *Seitz et al*. from 2018, several mechanisms are involved in the development of the different entities of ALD. The structure of the following paragraphs about is adapted from their review (42).

1.2.2.2.1 Alcoholic hepatic steatosis (K70.0)

Steatosis is the initial tissue alteration following alcoholism. It is defined as an aggregation of triglycerides, phospholipids and cholesterol esters in liver cells (46). This alcohol-related accumulation of fat in hepatocytes is caused by a disruption of beta-oxidation of fatty acids in mitochondria (47). It has also been indicated that due to ethanol ingestion more lipids are transported from the small intestines to hepatic tissue, more fatty acids are mobilized from fat tissue and also, their hepatic uptake is increased (47).

It has been suggested that alcohol influences transcription factors of the lipid metabolism. As a result, lipogenesis is induced, while fatty acid oxidation is inhibited. In liver cells, alcohol causes an increase of sterol regulatory element-binding protein 1c (SREBP-1c), which is responsible for an increase in the synthesis of fatty acids by upregulating lipogenic genes (46).

The ethanol-induced suppression of hepatic fatty acid oxidation is primarily caused by the inhibition of the peroxisome proliferator-activated receptor α (PPAR-α), the receptor inducing the gene transcription responsible for the transport and oxidation of free fatty acids (48).

Additionally, ethanol suppresses the activity of AMP-activated protein kinase (AMPK), a serin-threonin-kinase involved in fat metabolism. Ethanol can stop AMPK activity and thus, reduce the synthesis of fatty acids. However, via several pathways in which AMPK is included, alcohol leads to increasing oxidation of fatty acids. (49). By affecting AMPK, ethanol also inhibits the suppression of SREBP, which is normally carried out by AMPK. This way, alcohol causes a lack of AMPKs steatosis weakening effect (50).

Ultimately, autophagy plays a crucial role in the elimination of lipids in liver cells (51,52). Although chronic ingestion of alcohol reduces autophagy (53,54), a 2010 study indicates that short-term ethanol intake stimulates autophagy by generating ROS, which could be a protecting factor in initial stages of ALD (55).

1.2.2.2.2 Alcoholic hepatitis (K70.1)

Alcoholic hepatitis (AH) is an inflammation of the liver tissue accompanied by liver cell damage. It is assumed that 10% to 35% of hospitalized alcoholic individuals with ALD have AH (46). There are various forms of AH, ranging from discrete alterations to serious, lethal damage (56). In patients with underlying chronic liver disease, AH frequently appears acutely (57,58).

The pathophysiology of AH is characterized by multiple factors. In hepatic alcohol metabolism, alcohol dehydrogenase, CYP450 and catalase generate AA and ROS. Thus, amongst other things, lipids are oxidized and glutathione supply in the mitochondria is exhausted (46). AA is converted to acetate, which is removed from the liver quickly. Acetate itself is not directly toxic to liver tissue, but it is assumed to play a regulatory role in AH, as it up-regulates proinflammatory cytokines in macrophages (59,60).

Furthermore, alcohol activates innate immunity. Ethanol ingestion leads to alterations in the gut microbiome (61), increased enteric permeability and also causes bacterial lipopolysaccharides (LPS) to be translocated from the intestines to the liver (62–64). It has

been observed that in patients with ALD there are higher levels of LPS (46). Via TLR signaling (TLR4) in hepatic Kupffer cells, LPS leads to hepatocellular damage by causing oxidative stress and induction of proinflammatory cytokines such as TNF-α (65–68). Additionally, alcohol intake stimulates Kupffer cells by activation of complement factors C3 and C5, which results in generating TNF-α and ultimately, liver cell damage (69–71). These findings were shown in studies conducted with mice.

Contrastingly, further rodent models showed that TLR4 activation and induction of complement factors also trigger the production of protective and anti-inflammatory cytokines in hepatocytes. These mediators reduce liver injury by inducing the activity of transcription factor STAT3 in hepatocytes and Kupffer cells (72–74).

Additionally, neutrophils are recruited to infiltrate liver tissue. By production of IL-8, CXCL1 (GRO-α) and IL-17, neutrophils are attracted (46,75–77). In patients with AH, IL-17 levels are elevated. IL-17 recruits neutrophils and also induces secretion of IL-8 and CXCL1 in hepatic stellate cells (HSC) (77). Not only HSC, but also stimulated Kupffer cells produce these mediators, which was shown in a study on rat cells in primary culture (78).

Rodent studies dealing with other etiologies of liver disease indicate that neutrophils infiltrate the liver tissue and destroy liver cells by producing ROS and proteases (79). Thus, it is likely that neutrophil infiltration is partly responsible for alcohol-related liver disease as well. Not only innate immunity, but also adaptive immunity is activated in AH (46). This is indicated by elevated circulatory antibodies targeting products of lipid peroxidation and a high amount of T-cells in the hepatic tissue (80–83).

Ultimately, in a rat model it was shown that hepatic regeneration is impaired by chronic ethanol exposure (84). This leads to the assumption that chronic ethanol abuse causes liver cell destruction and additionally impairs regeneration by means of liver cell proliferation (46).

1.2.2.2.3 Alcoholic fibrosis and sclerosis of the liver (K70.2)

Fibrosis, a build-up of collagen and several other proteins (85,86), occurs as a repair mechanism responding to every type of long-term liver damage (46). This accumulation of proteins is caused by an intensified production of various chemokines, cytokines,

neuroendocrine factors and by activating the innate immune system. Subsequently, hepatic stellate cells (HSC) are stimulated and as a consequence, fibrogenesis is initiated (85,86).

As mentioned above, alcoholics have elevated serum levels of LPS. This indirectly leads to stimulation of HSC by activating Kupffer cells (87,88) and also directly via TLR4 signaling (89). Furthermore, LPS is able to initiate TLR4 signaling in cells of the sinusoidal endothelium, which has an influence on angiogenesis and finally stimulates fibrogenesis (90).

Acetaldehyde is predominantly synthetized by liver cells during ethanol breakdown and stimulates the production of collagen I in HSC. Not only does this reactive substance influence HSC directly, but its reaction products contribute to the effect as well (91).

Recent data show that the prognosis of early ALD is mainly predicted by fibrosis stage. Severe fibrosis (F3, septal fibrosis and F4, cirrhosis) entails a 10-year mortality of 45% (92).

1.2.2.2.4 Alcoholic liver cirrhosis (K70.3)

As fibrotic tissue alteration continues, the liver develops a cirrhotic appearance. Cirrhosis is characterized by the predomination of fibrotic tissue. This impairs blood flow and blood vessels including sinusoids get narrowed. Thus, patients with liver cirrhosis are likely to develop portal hypertension and subsequently ascites and esophageal varices. Due to the decrease of hepatocytes the liver function is impaired (42).

Ultimately, alcohol abuse can lead to liver failure (*K70.4* in ICD-10) and hepatocellular carcinoma (*C22.0* in ICD-10).

1.2.2.3 Genetics

As mentioned above, only a minority of heavy drinkers develop liver cirrhosis. This indicates that genetic factors are be relevant for the course of the disease (93).

1.2.2.3.1 PNPLA3

As a relevant protein involved in the metabolism of lipids, a specific variant of adiponutrin (patatinlike phospholipase domain-containing protein 3, PNPLA3) was identified as a risk factor for developing NAFLD and hepatocellular carcinoma (94). This indicates that adiponutrin itself is important for liver health. The gene variant rs738409 of PNPLA3 has been found to increase the risk of alcoholic liver cirrhosis and consequently, HCC (95).

A meta-analysis including most of the studies on the topic was published in 2014. It presented evidence that rs738409 plays a crucial role in ALD progression (96). In conclusion, carriers of rs738409 are at higher risk of alcoholic liver cirrhosis and hepatocellular carcinoma (93). They also develop cirrhosis after a shorter history of alcohol abuse (97), present with decompensation earlier (98) and also have a higher risk of dying of their hepatopathy (99).

Adiponectin is mainly expressed in fat tissue. In mammals, patatin-like phospholipases (PNPLAs) are responsible for maintaining the integrity of cell membranes and are involved in lipid metabolism and signaling (93). Futhermore, they balance energy homeostasis (93). Although the exact configuration of adiponectin has not yet been identified, it is assumed that in rs738409, the substitution of isoleucine with methionine on position 148 (I148M) might lead to lesser hydrolytic activity and consequently, fat accumulation (100–102).

Not only fat tissue, but also primary human HSC express PNPLA3, which is responsible for the catalyzation of retinyl ester hydrolysis (103).

The variant rs738409 shows lower activity, thus leading to an accumulation of retinyl palmitate. This was found in a mouse model, where researchers observed that reduced activity of said variant causes stress in the endoplasmatic reticulum (104). Therefore, there is a potential connection between the I148M-mutation of adiponectin and alcohol-related fibrogenesis and liver damage (93,105,106).

1.2.2.3.2 SERPINA1

SERPINA1 on chromosome 14 is the gene coding for alpha-1 antitrypsin (A1AT) (107,108). Up until now, over 100 variants of SERPINA1 have been found (109), but only some of them are connected with hepatic disease (110).

Null variants or variants leading to a defect in gene expression, translation or protein biosynthesis can cause disease manifestation (alpha-1 antitrypsin deficiency, A1ATD) (107). This metabolic disorder is inherited as an autosomal recessive trait with codominant expression, since each allele is responsible for half of the amount of the organism's total A1AT (110).

To classify SERPINA1 gene products, they have been divided by their migration speed in gel electrophoresis, hence named Fast (F), Medium (M), Slow (S) and Very Slow (Z) (111). These phenotypes define the genotypes of the alleles. The physiological phenotype is PiM and the most frequent mutations are PiS and PiZ. Whereas in PiS 50% to 60% of alpha-1 antitrypsin are expressed, PiZ only expresses 10% to 20% (110). PiMS and PiMZ are the most common carrier phenotypes, while the phenotypes PiSS, PiSZ and PIZZ cause deficiency (110). Additionally, there are other, very rare alleles causing deficiency, which are responsible for less than 5% of the A1ATD cases (110).

A1AT belongs to the serpin family. Serpins are serine proteinase inhibitors, which are mainly generated by the liver. As a proteinopathy, A1ATD results in a disturbance of cellular function via accumulation of misfolded A1AT. Patients with A1ATD have ≤ 15% of physiological serum protein levels (108).

In the lungs, A1ATD leads to emphysema by a loss-of-function mutation of A1AT. Damaged or not present A1AT fails to inhibit the serin proteinase in neutrophils, which results in the destruction of lung tissue (112).

In the liver, however, there is a different pathological mechanism that leads to damage. The mutant alpha-1 antitrypsin Z (ATZ) is a misfolded protein, which is not soluble. Thus, ATZ proteins are stored in the endoplasmatic reticulum. Ultimately, this gain-of-toxic mechanism may lead to hepatic fibrosis and even HCC (113).

1.2.2.4 Obesity, NAFLD and ALD

Non-alcoholic fatty liver disease (NAFLD) as well as ALD both have steatosis as a precondition. Additionally, non-alcoholic steatohepatitis (NASH) and alcoholic steatohepatitis (ASH) share similar microscopic aspects, indicating that they might have analogous pathomechanisms. But despite those similarities, the pathogeneses of non-alcoholic and alcoholic fatty liver (AFL) are different by some means (42).

Naturally, consumption of ethanol and excessive calories resulting from an unhealthy diet may occur simultaneously. Thus, the impact of alcohol intake on obese individuals or NAFLD patients is particularly interesting (42).

It has been shown in several epidemiological studies that heavy (> 40g/day) and moderate (20 to 40 g/day) ethanol intake can aggravate fatty liver, hepatitis, fibrosis and cirrhotic liver in overweight or obese individuals (114–120). Obesity is, without doubt, a predisposing factor for ALD (42). In conclusion there is a synergistic effect between obesity and alcohol consumption concerning liver disease.

Contrastingly, Japanese and European epidemiological research has suggested that moderate alcohol intake ameliorates fatty liver in comparison to the absence of alcohol consumption. This leads back to an improved insulin resistance in peripheral tissue (121).

Additionally, it has been suggested that regular alcohol intake may be protective concerning the histological severity of NAFLD (122). Several smaller NAFLD studies show conflicting results. In some studies, moderate ethanol consumption led to a more rapid worsening of fibrosis (120) or to higher levels of serum transaminases (119). Other research, however, did not support this discovery (123). To this day, the effect of moderate drinking on the progression of non-alcoholic fatty liver disease cannot be determined correctly.

In contrast, research on ethanol and HCC development in overweight or obese individuals delivers clearer results. Any amount of alcohol was identified as a risk factor for HCC in almost every retrospective study in NASH patients (121,123–125).

1.3 Diagnosis and assessment of alcohol-related liver disease

Generally, the clinical diagnosis of ALD is difficult. Due to a lack of symptoms in earlier stages, patients often only seek attention when they already show symptoms of severe disease (126).

Even though there is a high prevalence of alcohol use disorders (AUD), those who are affected are insufficiently diagnosed (127). When ALD is suspected in a patient, the identification of excessive alcohol abuse is crucial, since it can aggravate the development of ALD (42). Diagnosis of ALD itself in early stages is fundamental as well. This way, patients with genetic risk factors can be identified, patients without signs of addiction can be motivated to stay abstinent and by establishing screening programs, lethal complications could potentially be avoided (126). The gradual development of ALD to cirrhosis can remain unseen for several years, which causes poor outcome (126).

Due to its variability of symptoms, lacking self-reports of alcohol abuse by affected patients and missing adequate biomarkers, clinicians as well as health statistics often undervalue ALD (43,128). For that reason, the diagnosis of ALD requires clinical exams, laboratory testing, imaging and VCTE (126).

1.3.1 Clinical signs and symptoms of ALD

Whereas patients with early stages of ALD are often asymptomatic, patients suffering severe forms of ALD may show signs of decompensated hepatic cirrhosis. Patients who also have AUD may present with alcohol tolerance or withdrawal symptoms such as anxiety, insomnia or nausea. Elevated body temperature, jaundice, high leukocyte levels and ascites or hepatic encephalopathy as signs of decompensation are possible clinical manifestations of alcoholic hepatitis. Patients with alcoholic hepatitis are usually younger than patients with cirrhosis and typically the period of alcohol abuse is shorter, but more excessive (42).

Since patients with AUD are usually treated by psychiatrists, they often miss examinations of the liver (42). Biomarkers concerning alcohol use have low diagnostic accuracy, which makes them inadequate for screening purposes (129) but they may be helpful in managing ALD. Assessing alcohol-caused comorbidities such as hepatic and neurological issues or psychiatric diseases should always be included in the process of diagnosing AUD (42).

1.3.2 Non-invasive diagnosis of hepatic fibrosis

Fibrosis stage can now be estimated accurately without liver biopsy. Liver stiffness measurement with FibroScan® (vibration controlled transient elastography, VCTE) and enhanced liver fibrosis test (ELF-test) are non-invasive tools for detecting liver fibrosis. Compared to indirect serum indices for fibrosis (e.g. age-platelet-index, aspartate transaminase (AST)-platelet-ratio index), ELF-test was found to be more accurate (130). Provided that VCTE measurement produces reliable results, its diagnostic accuracy has been found superior to blood fibrosis tests (130).

1.3.2.1 Ultrasonography

Ultrasonography is a fast and cheap diagnostic method used for detecting fatty liver and confirming cirrhosis. However, it is not an ideal tool for the diagnosis of fibrosis. All imaging methods depend on sure cirrhosis signs when it comes to the diagnosis of liver fibrosis. For example, nodular appearance of the liver tissue and a re-canalization of the umbilical vein are such signs. In contrast, ascites and splenomegaly are unspecific alterations. Still, in case of pathological liver test results, imaging such as ultrasonography may be helpful in the exclusion of causes other than fibrosis (126).

1.3.2.2 Vibration controlled transient elastography with FibroScan®

VCTE is an ultrasound-based and non-invasive tool used for the assessment of liver stiffness. This technology uses shear waves, which were originally found in seismology. They are the slower waves that occur following the initial compressional wave in earthquakes. While sound waves are longitudinal, shear waves are transverse waves. In contrast to surface waves, shear waves are elastic waves permeating an object. Since shear waves are transverse, the material they affect moves perpendicularly to the direction in which the wave itself is travelling. Thus, shear waves propagate slowly and their speed is rapidly reduced by liver tissue (131,132). This attenuation effect is determined by the elasticity of the tissue. According to *Hooke's law* ($E = 3\phi v^2$), the speed of waves is proportional to the stiffness of the respective object and consecutively, wave velocity and elasticity of an object are inversely proportional. The lower the elasticity of the tissue is, the faster shear waves travel. In order to express liver stiffness (LS) in kPa instead of m/s, shear wave speed is converted into Young's modulus (E in Hooke's law). Young's modulus is expressed in kPa, the unit of tissue density (ϕ) is kg/m^3 and speed (v) is expressed in m/s. In LS calculation, tissue density

is equivalent to the density of water. In clinical routine, Young's modulus is referred to as LS (131,133,134)

The method of transient elastography was developed in 1995 at the Langevin institute with the original purpose of using it in quality control in the food processing sector. In 2001, transient elastography has been introduced to medicine as FibroScan®, manufactured by Echosens, Paris, France (131,132).

1.3.2.2.1 Liver stiffness (LS)

The FibroScan® device measures shear wave velocity using an ultrasonography probe with two transducers. The probe is put in an intercostal space close to the right lobe of the liver (131). The probe first sends a slow shear wave with a 50 Hz frequency. Following the shear wave, the probe emits fast ultrasound waves in a pulsatile way. This way, the device can determine the location of the shear wave front with regard to time. Speed is calculated from time and distance and, using Hooke's law, LS can be determined. Values vary between 1.5 and 75 kPa. Higher results indicate less elastic liver tissue. In order to assure reproducible assessments and receive results that can be compared inter-individually, the device's software has several features to minimize measurement errors. It adjusts the characteristics of the shear waves by altering amplitude and energy output in order to keep the wave's frequency and its shape constant. Furthermore, the device shows warnings and also renders the probe inoperative in case of inappropriate pressure. This feature prevents errors resulting from inadequate pressure of the probe (131,133,134).

VCTE was the first non-invasive method for measuring liver stiffness. Thus, most studies focusing on liver stiffness measurement were carried out using FibroScan® (126). LS is a very good parameter for assessing F3 and F4 in ALD and preferable to every serum marker (135). Values less than 6 kPa are commonly defined as normal and are able to rule out mild fibrosis stages as well. The precise differentiation between stages F1 and F2 via TE is not recommended for clinical routine. The range between 6 and 8 kPa is very slim and patient position or breathing may interfere with the measurement. Even though excessive tissue fat may lead to lower LS in concomitant fibrosis, it usually does not affect the determination of fibrosis stage by measuring liver stiffness (126).

Liver stiffness is associated with elevated portal pressure and with LS values above 20 kPa there is a higher risk for esophageal varices and hepatocellular carcinoma (136,137). LS elevation can also be caused by inflammation (138,139) and liver congestion (140). Furthermore, mechanic cholestasis (141) can lead to increased LS. Mentioned conditions can elevate LS even when fibrosis is absent (126). All of these may be present in individuals with ALD. Hence, imaging, laboratory findings and clinical signs must be considered in the interpretation of VCTE results (126). In the currently available literature on the topic, following cut-offs displayed in *Table 1* for the assessment of fibrosis with VCTE have been evaluated.

Table 1: *Proposed cut-offs for hepatic fibrosis assessment*

	Mueller et al. (142)	**Trabut et al. (143)**	**Thiele et al. (130)**	**Thiele et al. (144)**
Patients	group 1: 50 group 2: 101	137	289	562
Cut-off F3	≥ 8 kPa	≥ 11 kPa	≥ 15,5 kPa	≥ 13,2 kPa
Cut-off F4	> 12,5 kPa	≥ 19,5 kPa	≥ 19,7 kPa	-

kPa, kilopascals

1.3.2.2.2 Controlled attenuation parameter

Liver steatosis can not only be diagnosed by biopsy and regular ultrasonography, but also by measurement of controlled attenuation parameter (CAP) with the FibroScan® device. In CAP measurement, waves are sent through the liver and the device measures the attenuation caused by the organ tissue (145). Fatty liver tissue causes a stronger attenuation of ultrasound waves than a healthy liver (146). Regarding the detection of steatosis, CAP seems to be promising. According to a recent biopsy-controlled multicenter study, for diagnosing steatosis in patients with ALD, CAP measurement was superior to conventional ultrasonography (144). This finding was not statistically significant. However, the use of ultrasonography together with CAP was found to significantly ameliorate diagnostic accuracy for mild and severe fatty liver (144).

CAP is calculated at the same time as LS measurement with the FibroScan® device. As the ultrasound attenuation coefficient, CAP depends on the wave frequency and the characteristics of the object through which the emitted wave is travelling. Ultrasound waves with a frequency of 3,5 MHz are required for CAP measurement. Results for CAP are expressed in decibels per meter (dB/m) and range from 100 to 400 dB/m. More advanced steatosis causes higher CAP scores (131).

1.3.2.3 Fibrosis biomarkers

There are several commercially available serum markers and indices for estimating liver fibrosis. Amongst others, FIB-4 and ELF are suitable tests, as they are accessible and possess good diagnostic accuracy (130).

1.3.2.3.1 FIB-4

Fibrosis 4 index was established by *Sterling et al*. in a retrospective study published in 2006. They found that in HIV/HCV co-infected patients, age, AST, INR and platelet count were independent predictors of fibrosis. Furthermore, they found a significant association between fibrosis stage and age, AST, ALT, AST/ALT ratio, AP, albumin, platelet count, AST/platelet ratio, INR, PTT, CD4, cholesterol and bilirubin. They developed a model for the calculation of an index named FIB-4: $FIB-4 = \frac{age\ (years) \times AST\ (\frac{U}{L})}{platelet\ count\ (\frac{10^9}{L}) \times \sqrt{ALT}(\frac{U}{L})}$.

In their study cohort, which they divided into three groups by histological levels of fibrosis (*Ishak*), they chose cut-off values of FIB-4 < 1.45 to exclude advanced fibrosis (NPV = 90%) and FIB-4 > 3.25 to predict advanced fibrosis (PPV = 65%, specificity = 97%) (147).

In 2018, *Thiele et al.* validated FIB-4 as an accurate diagnostic tool that can be used in patients with ALD as well (130).

1.3.2.3.2 ELF

Enhanced liver fibrosis test was introduced for clinical use in 2010 (ADVIA Centaur CP immunochemical analyzer, Siemens Healthcare Diagnostics) (148). In contrast to fibrosis-4 index, enhanced liver fibrosis (ELF) test uses direct markers of extracellular matrix. For the calculation of ELF score, serum concentrations of hyaluronic acid (HA), aminoterminal propeptide of type III collagen (PIIINP) and tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) are needed (149,150).

For the diagnosis of fibrosis stages, the cut-off values proposed by the manufacturer are 7.7 as a threshold for significant fibrosis and 9.8 as a cut-off value for advanced fibrosis (151).

In a study published in 2018, *Jabor et al.* aimed to determine the analytical performance and evaluate the range of reference values and the biological variability of HA, PIIINP, TIMP-1

and ELF. They performed investigations on reference ranges for ELF in 40 healthy individuals. For the lower reference limit of ELF, they found a median value of 7.14. The median of the upper reference limit was found to be 9.55 (152). There are discrepancies between their findings and the manufacturer's recommendations. The producers of the Siemens Centaur System recommend three cut-offs: 7.7 as a low cut-off, a low-upper cut-off of 9.8 and a high-upper cut-off of 11.3. These values suggest no or weak fibrosis in results below 7.7, moderate fibrosis under 9.8, severe fibrosis between 9.8 and 11.3 and ultimately cirrhosis in results above 11.3 (152,153). However, in the healthy study population of said study in 2018, most patients' results were classified as moderate fibrosis, while results under 7.7 were exceptions (152).

In study published in 2019, *Staufer et al.* found that an ELF-score of 9.1 is a more adequate threshold for significant fibrosis (≥ F2) (154).

Regarding the detection of advanced alcoholic fibrosis (≥ F3), *Thiele et al*. found an ELF score threshold of 10.5 to be a more adequate cut-off than the manufacturer proposed value of 9.8 (130).

1.3.3 Invasive diagnosis: liver biopsy

As an invasive procedure, liver biopsy always includes risks such as blood loss, leading to the estimated morbidity rate of about 2%. Thus, the indication for a liver biopsy needs to be very strict and use-risk balance is required in every case (155).

Liver biopsy may be necessary in patients whose liver disease cannot be diagnosed by non-invasive methods. Furthermore, physicians may need to perform a biopsy in individuals with concomitant liver disease. This can be required in order to achieve a precise staging of alcohol-related liver disease and for the evaluation of the prognosis (156,157) as it correlates with some histological features of ALD (42).

Alcoholic hepatitis may occur in individuals with present ALD and ongoing heavy alcohol intake (155,158). Hence, in 10-50% of patients presenting with clinical features of ALD with concurrent inflammation, the diagnosis cannot be confirmed histologically (159–162).

The histological spectrum of ALD consists of alcoholic fatty liver (AFL), alcoholic steatohepatitis (ASH), alcoholic fibrosis, cirrhosis and hepatocellular carcinoma (42).

1.3.3.1 Alcoholic fatty liver

Features of AFL include extensive lipid droplets inside hepatocytes. The lipid accumulations dislocate the nucleus towards the membrane of the cell (42).

1.3.3.2 Alcoholic steatohepatitis

Hepatocellular injury, ballooning and Mallory-Denk bodies are typical alterations of ASH. Furthermore, necrosis, lobular infiltration with mononuclear cells and neutrophils and macrovesicular steatosis can be found (57,163). In serious cases of ASH, more severe alterations may occur: due to hepatocellular, canalicular and ductular cholestasis, bile pigment may be observed in respective components of the liver tissue. (42).

1.3.3.3 Alcoholic fibrosis and cirrhosis

In the majority of fibrosis cases without cirrhosis, collagen fibers begin to accumulate along sinusoids and around centrilobular hepatocytes. This pattern of fibrosis is called pericellular fibrosis. As fibrosis progresses, collagen fibers expand towards the lobular parenchyma. They often form septa, connecting central veins and portal tracts. In alcoholic liver fibrosis, perivenular fibrosis and fibro-obliterative alterations of veins are common. As fibrosis continues to intensify, cirrhosis is likely to develop (42).

About 50% of patients in early, compensated stages of ALD present with septal fibrosis or cirrhosis. In advanced ALD, the majority of the affected have it (164).

Not only in ALD, but also in NAFLD these histological patterns may occur. However, wide-ranging microvesicular steatosis, cholestasis and fibro-obliteration of veins in hepatic tissue have not been observed in NAFLD (42).

1.3.3.3.1 Fibrosis stages

In 2005*, Kleiner et al.* published a study on the histological classification of non-alcoholic fatty liver disease. Regarding fibrosis, they established stages ranging from 0 to 4. Fibrosis stage 1 was further divided into three levels (1A, 1B, 1C) (165). Their definitions of fibrosis stages are listed in *Table 2.*

Table 2. *Fibrosis stages. Adapted from (165)*

Fibrosis	Stage
None	0
Perisinusoidal or periportal	1
Mild, zone 3, perisinusoidal	1A
Moderate, zone 3, perisinusoidal	1B
Portal/periportal	1C
Perisinusoidal and portal/periportal	2
Bridging fibrosis	3
Cirrhosis	4

As a specific staging system for use in ALD is currently lacking, the Kleiner staging system developed for NAFLD is also used for ALD.

1.4 Liver stiffness in ALD

Since increased liver stiffness is not only caused by liver fibrosis, other mechanisms must be considered as well. Fibrosis can occur due to acute liver damage caused by acute inflammation (138), mild hepatitis (139,166), cholestasis (141) and liver congestion (140). Fibrosis in ALD is often accompanied by steatohepatitis, which can noticeably elevate liver stiffness (142). The correlation between histological grade of alcoholic steatohepatitis (ASH) and elevation of AST is commonly known; in patients with AST below 100 U/L, no significant inflammation has been detected in previous studies (167,168).

For identical stages of fibrosis, there seems to be a difference in liver stiffness due to the cause of damage. While in patients with alcoholic and non-alcoholic steatosis the cut-off for diagnosing cirrhosis is set at approximately 20 kPa, for cirrhosis due to viral hepatitis the threshold is approximately 13 kPa (135,169,170). It is assumed that this might be caused by the micronodular structure of alcohol-associated cirrhosis (58).

1.4.1 Decrease of LS following detoxification

Liver stiffness in ALD notably decreases when patients are consequently abstinent from alcohol. There was also found a correlation between a decrease in liver stiffness and decreasing AST during detoxification: patients with higher initial AST are more likely to show a considerable decrease in liver stiffness, whereas in patients with AST lower than 100 U/L, there were no significant alterations in VCTE results. This observation leads to the assumption that decreasing liver stiffness during alcohol detoxification is most likely caused by the resolution of hepatic inflammation (142).

In a study published in 2011, significant improvement of liver stiffness after eight days was detected in 13 out of 23 participating patients. During the course of the study, participants were divided into two groups in terms of abstinence or relapse. The longer the abstinence period lasted, the higher was the percentage of patients with a significant decrease in liver stiffness (50% on day 30, 66.7% on day 60). Contrastingly, in the cohort experiencing relapse no improvement of TE results could be observed (171).

In contrast to previous studies (142,171), *Trabut et al.* found that liver stiffness declines significantly during alcohol abstinence not only in patients with high AST, but also at AST levels lower than 100 U/L (143). However, liver stiffness decreased more rapidly in individuals with higher baseline AST, which supports the assumption that necro-inflammation in ALD aggravates liver stiffness (143).

In a retrospective study including 37 patients hospitalized for either compensated ALD or weaning therapy, results were similar to those of the studies mentioned above. TE results markedly decreased following alcohol detoxification in 85 % of the abstinent patients (172). It was also found that decrease in liver stiffness was proportional to abstinence (172), as already suggested by *Gelsi et al.* (171). Additionally, they detected a significant difference in alteration of fibrosis stage and alteration of liver stiffness between the group of relapsers and the group of abstinents (172).

The results of previous studies investigating the correlation between abstinence, liver stiffness and biochemical activity (inflammation) indicate that in patients with ALD, VCTE has to be used with caution. An ideal time for non-invasive assessment of liver fibrosis still needs to be found.

1.5 Prognosis of ALD

Most patients are diagnosed when they present with decompensation. Hence, research has been focused on predictors of short-term mortality of ALD. Unfortunately, there are only few studies on the prognosis of ALD concerning patients with early stages of this condition.

1.5.1 Histological stage

Lackner et al. retrospectively investigated the long-term prognosis of ALD, especially focusing on the histological stages of fibrosis. Patients were classified by compensated disease vs. decompensation. The investigators found that liver-related mortality at 5 years was 13% in the subgroup with compensated ALD, whereas 43% of decompensated patients died within 5 years. They evaluated the prognostic role of fibrosis stage in their study cohort. Around half of the included patients with compensated ALD presented with severe fibrosis (stage F3, septal fibrosis) or cirrhosis (stage F4) at baseline. As evaluated in a follow-up after 10 years, no deaths had occurred in the group of patients who presented with F0-F2. In patients with severe fibrosis, 45% had deceased after 10 years (92).

1.5.2 Biological sex

Additionally, *Lackner et al*. found female sex to be an independent predictor of liver-related death in the study cohort with decompensation of ALD. Importantly, this influence was not found to be associated with higher abstinence rates (92). Previous studies suggest that females are more vulnerable to ethanol-related liver damage than males. In female individuals, smaller amounts of ethanol intake may lead to steatosis and AH (173,174). Additionally, in females the risk of fibrosis aggravating towards cirrhosis (175–177) seems to be higher, even when abstinence is obtained (177–179). A few mechanisms may be responsible for this difference in susceptibility to liver injury. A smaller percentage of body water, different drinking habits more frequently causing outbreaks of severe AH (180) and estrogen-mediated pro-inflammatory mechanisms in the liver as observed in rodent models (181–183) may be influencing factors. Furthermore, it has been found that autoantibodies are more prevalent in female alcohol abusers and that their prognosis is worse, even when they achieve abstinence. Thus, alcohol might initiate autoimmune mechanisms towards liver tissue. These autoimmune reactions might persist even when patients are abstinent. In conclusion, these hypotheses indicate that the effect of abstinence is less favorable for the prognosis in females than in males (177,178).

2 Methods

A prospective, single-center study at a tertiary referral center (Medical University of Graz) was conducted in order to assess the prevalence and severity of hepatic fibrosis in alcohol-dependent individuals with or without known ALD. Furthermore, the study aimed to investigate the effect of abstinence on liver fibrosis.

2.1 Patients

In the period from February 28th to July 26th 2019, 31 consecutive patients undergoing alcohol detoxification at the Centre of Addiction Medicine at Federal Hospital Graz II were enrolled. Informed consent was obtained prior to baseline visit.

2.1.1 Inclusion criteria

- Patients above the age of 18 undergoing alcohol detoxification therapy
- Signed informed consent

2.1.2 Exclusion criteria

- Benzodiazepine addiction
- Opioid addiction
- Polytoxicomania
- Presence of concomitant viral hepatitis B or C
- Presence of hepatic or extrahepatic malignoma

2.2 Study design

The study was conducted in collaboration between the Centre of Addiction Medicine at Federal Hospital Graz II (OA Dr. Werner Heran) and the Liver Clinic, Division of Gastroenterology and Hepatology, Medical University of Graz.

A baseline study visit at the Liver Clinic was held with in-patients of the Centre of Addiction Medicine during their short-term (3-week) or long-term (8-week) alcohol detoxification treatment. Furthermore, study participants were offered three follow-up visits at the Liver Clinic after 3, 6 and 12 months.

2.3 Baseline visit

Written informed consent was obtained at the Liver Clinic. A separate informed consent form regarding the genetic analyses was used.
Baseline visit included the following examinations:

- Patient history including detailed information about alcohol consumption
    - Years of alcohol abuse and daily alcohol intake
    - Number of underwent detoxification therapies
    - Abstinence period prior to baseline visit
- Vital signs, physical examination
- Collection of blood samples
    - Blood count
    - Liver function tests
    - ELF-Test, FIB-4 Index
    - CDT
    - PNPLA3
    - SERPINA1
- Collection of urine samples
    - Urinary EtG
- Abdominal ultrasonography
- Vibration controlled transient elastography (FibroScan® 502 Touch)

All obtained data were pseudonymized.

2.3.1 LS and CAP measurement

VCTE was performed in patients after a fasting period of at least two hours. FibroScan® 502 Touch (Echosens, Paris, France) was used. Sizes M and XL of FibroScan® probes were available at all times during the course of the study. XL probe was used, when Body mass index was 30 kg/m^2 or higher.

Following the suggestions in The Liver-FibroSTARD standards published in 2015 (184), LSM values were considered reliable if 10 successful measurements could be obtained. For values above 7.0 kPa, IQR/med < 30% was considered necessary for the results to be valid.

2.3.2 Sampling and Sample preparation

Blood and urine samples for laboratory testing were collected by the study nurses at the baseline and follow up visits. Blood samples were centrifuged at 3000 rpm in a refrigerated centrifuge. 400µl-aliquots of serum, plasma and urine were stored at -80 °C.

2.4 Follow up visit

After three months, patient history regarding alcohol abuse was re-evaluated. Blood and urinary analyses and VCTE were performed.

2.5 Analytics

ELF-test was performed on an Advia Centaur XP (Siemens Healthcare Diagnostics, Vienna, Austria).

FIB-4 index was calculated from laboratory and clinical routine parameters which were obtained during the study visits.

SERPINA1 and PNPLA3 genotypes were analyzed in a routine laboratory test by 5'-exonuclease assays (TaqMan). Primer and probe sets were designed and manufactured using Applied Biosystems 'Assay-by-Design' custom service (Applera, Austria). End-point fluorescence was measured in a Flexstation 3 plate reader (Molecular Devices).

2.6 Data collection

Patient data were collected with Microsoft ® Excel for Mac Version 15.27. In order to maintain data protection, patients were given numbers chronologically and their names were not mentioned in the table.

2.7 Statistical analyses

For statistical analyses, IBM SPSS Statistics version 25 and 26 were used.

Continuous variables are displayed as mean (± standard deviation) if they are normally distributed or median (Q1; Q2) in case they are not normally distributed. Normal distribution was tested using Kolmogorov-Smirnov test. Categorical variables are displayed as absolute and relative frequencies.

Comparison of parameters between baseline and follow up was performed by using either t-test for dependent samples for normally distributed variables or Wilcoxon-test for dependent samples in case that the variables were not distributed normally. If a variable was distributed normally at one study visit, and not distributed normally at the other visit, Wilcoxon-test was used for the comparison. This applied for bilirubin and ELF. Mentioned parameters are displayed as median (Q1; Q2) despite the normal distribution of bilirubin at baseline visit and ELF at 3 months follow up visit.

Comparison of the differences among two independent groups with non-normally distributed values was performed by Mann-Whitney U test. In order to compare differences between more than two independent groups, Kruskal-Wallis test was used. For the comparison of the differences among two independent groups with normally distributed values and unequal variances, Welch's t-test was performed.

The estimation of possible correlations between variables was performed using Spearman correlation test.

For the comparison of the frequencies for categorical outcomes between two independent groups, Chi-square or Fisher's exact test was used as appropriate.

The significance level was set at 5%.

2.8 Histological examination

For the histological diagnosis of liver fibrosis, a percutaneous liver biopsy was performed. HE stain and Berlin blue stain for the detection of iron were used.

3 Results

3.1 Initial patient collective

Table 3. *Patient characteristics at baseline visit*

	All at baseline n = 31
Age (years)	48 (±11)
Male sex (%)	64,5
BMI	27,4 (±4,9)
AST (U/L)	27 (21; 39)
ALT (U/L)	25 (18; 39)
AP (U/L)	67 (59; 78)
GGT (U/L)	53 (27; 142)
Bilirubin (mg/L)	0,50 (± 0,2)
Albumin (g/dL)	4.4 (4.1; 4.6)
PT (%)	116 (106; 120)
PT INR	0.91 (0.87; 0.96)
Creatinine (g/dL)	0.85 (± 0.2)
Sodium (mmol/L)	140 (139; 141)
CRP (mg/dL)	2.0 (0.8; 6.0)
Serum glucose (mg/dL)	86 (80; 95)
Leukocytes (G/L)	7.79 (± 2.59)
Thrombocytes (G/L)	257 (± 59)
%CDT (%)	1.51 (1.22; 1.84)
EtG path. (%) *	16.67
FIB-4	1.1 (± 0.5)
ELF	8.68 (8.24; 9.69)
LSM (kPa)	5.6 (4.1; 9.7)

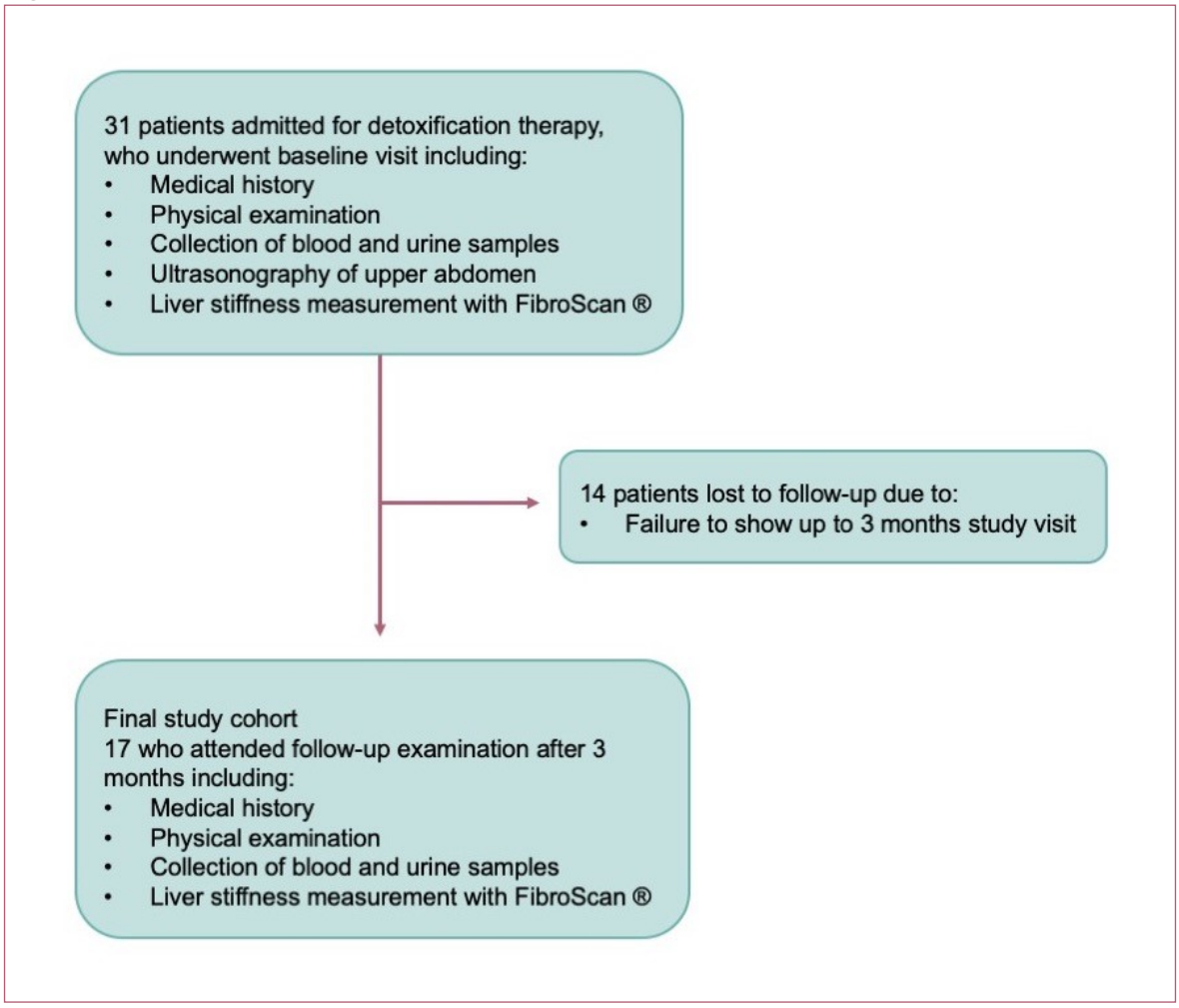
Data are shown as median (Q1; Q3) or as mean (± standard deviation).
BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AP, alkaline phosphatase; GGT, gamma-glutamyl transferase; PT, prothrombin time; PT INR, prothrombine time international normalized ratio; CRP, C reactive protein; CDT, carbohydrate-deficient transferrin; EtG path., pathological values of urinary ethyl glucuronid; FIB-4, fibrosis 4 index; ELF, enhanced liver fibrosis test; LSM, liver stiffness measurement.
*) One patient did not provide a urine sample at baseline visit. Five out of 30 samples showed pathological values of urinary EtG.

During the recruitment period, 31 consecutive patients above the age of 18 years were enrolled. Eleven of 31 individuals were female. The mean age of the initial study population was 48 years, ranging from 29 to 76 years at baseline visit. *Table 3* displays an overview over the patient characteristics at baseline.

3.2 Final study population

At baseline visit, appointments for their first follow-up visit were arranged (3 months after enrolment). Out of 31 enrolled patients, 17 attended the second study visit. As displayed in *Figure 4*, 14 patients left the study by not showing up at their appointment for the first follow up visit. Final statistical analyses were performed with data of the 17 remaining patients.

Figure 4. *Patient flowchart*



3.3 Alcohol consumption

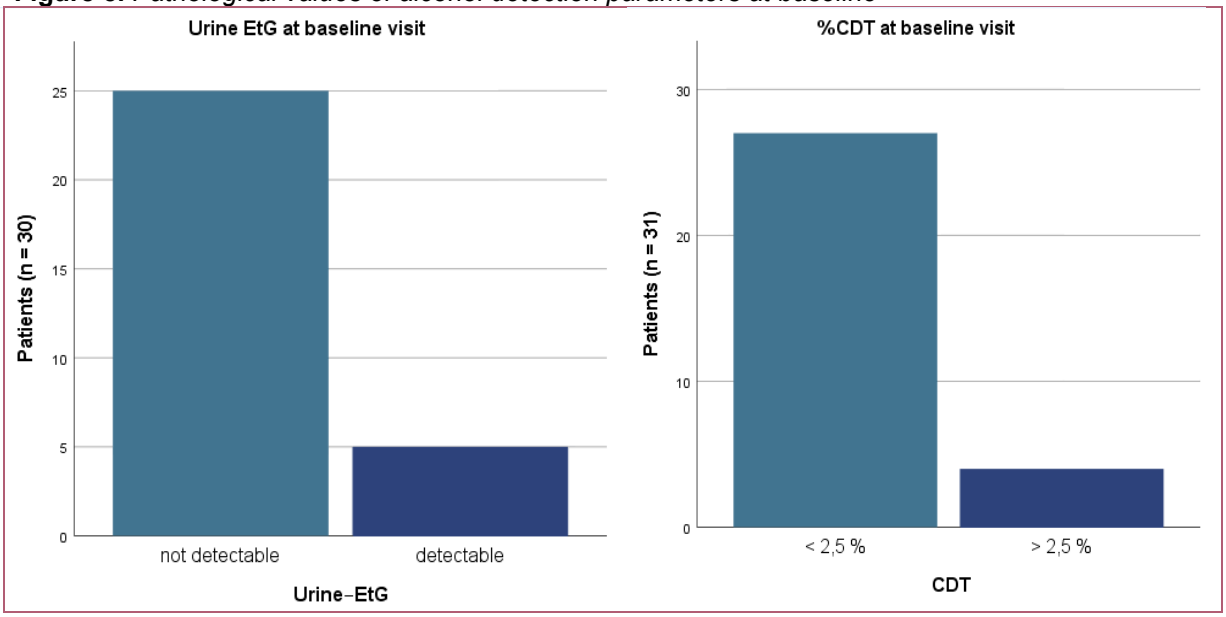
3.3.1 Abstinence at baseline visit

When asked about their medical history and alcohol intake, all 31 patients claimed to be abstinent at baseline visit. According to the information gathered during the interviews, the median of the duration of abstinence was 21 days, ranging from a minimum of seven days to a maximum of 211 days of abstinence prior to baseline visit.

Five out of 30 patients presented with detectable amounts of urinary EtG. In one patient, urinary EtG could not be tested because no urine sample was provided. Ten out of 31 patients presented with CDT values above 1.64%. Three of them showed detectable urinary EtG as well. Seven of them did not present with detectable urinary EtG.

In total, 18 out of 30 patients were confirmed to be abstinent at baseline visit by laboratory testing. In 12 out of 30 patients, alcohol consumption was detected via laboratory testing either by detection of urinary EtG or elevated percentage of CDT.

Figure 5. *Pathological values of alcohol detection parameters at baseline*

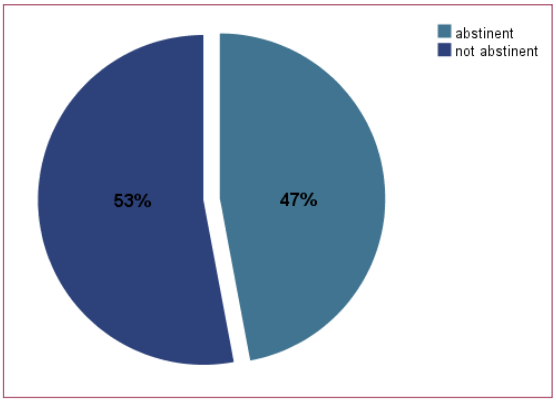


Urinary EtG enables the detection of alcohol consumption up to 80 hours after the last intake (185), whereas %CDT has a half-life of about 2 weeks (186).

3.3.2 Abstinence at 3 months follow up visit

Seventeen patients showed up for their first follow up visit three months after baseline visit. An interview focused especially on their alcohol consumption was conducted with each of the patients. Out of 17 remaining individuals, nine claimed that they had maintained ethanol abstinence during the period between baseline and follow up visits. In one case, no information about the patient's self-report on abstinence was available. However, in that case, urinary EtG was detected. Thus, a lack of abstinence is assumed in said patient. Furthermore, one of the self-reported abstainers could not be confirmed abstinent due to elevated %CDT and EtG values.

Figure 6. *Abstinence at 3 months follow up visit according to laboratory testing*



Hence, according to the results of laboratory testing, eight out of 17 study participants were able to maintain abstinence in the period between their study visits.

3.3.3 Sex-related differences in alcohol intake

Differences in the amount of daily consumed ethanol are displayed in *Table 4*. Welch test was performed to evaluate the difference between the two groups (females and males). As seen in *Table 5*, findings were statistically significant.

Table 4. *Mean ethanol intake per day prior to detoxification*

	Females	Males
Mean	200 (± 73)	383 (± 218)
Min	100	80
Max	300	800

Data are shown in grams per day. Mean values are displayed ± standard deviation.

Table 5. *Welch's t-test: alcohol intake in females vs. males*

	Statistic	df1	df2	Sig.
Welch	11.726	1	25.461	0.002

Sig., significance.

3.4 Accuracy of non-invasive fibrosis blood tests

Using LSM results as a reference, the diagnostic accuracy of ELF test and FIB-4 index was evaluated in the initial study collective of 31 individuals. For the VCTE-based diagnosis of advanced fibrosis (F3, F4), a value of 15 kPa was used as cut-off.

3.4.1 ELF test

To determine the accuracy of ELF test for the diagnosis of advanced fibrosis, ELF results were compared with LSM results above 15 kPa using contingency tables. As seen in *Table 6*, the accuracy of two proposed cut-off values was tested.

Table 6. *Diagnostic accuracy of ELF cut-off values*

ELF cut-off	Sensitivity	Specificity	PPV	NPV
9.8	80.0%	96.2%	80.0%	96.2%
10.5	80.0%	100.0%	100.0%	96.2%

ELF, enhanced liver fibrosis test; PPV, positive predictive value; NPV, negative predictive value.

The results show that in the tested study population, a cut-off value of 10.5, as proposed by *Thiele et al*. (130), is superior to the manufacturer-proposed cut-off value of 9.8.

3.4.2 FIB-4 index

The diagnostic accuracy of the FIB-4 cut-off value of 1.30 was found to be adequate for ruling out advanced fibrosis due to its high specificity and NPV.

Table 7. *Diagnostic accuracy of FIB-4 cut-off value*

FIB-4 cut-off	Sensitivity	Specificity	PPV	NPV
1.30	60.0%	80.8%	37.5%	91.3%

FIB-4, fibrosis-4 index; PPV, positive predictive value; NPV, negative predictive value.

3.4.3 Correlation of non-invasive diagnostic methods

Due to the distribution of parameters, correlation between the non-invasive diagnostic tools was analysed with Spearman correlation test. The results are presented in *Table 8*. ELF score and FIB-4 index correlated better with one another than each test did with liver stiffness, respectively. Liver stiffness correlated slightly better with FIB-4 index than with ELF score.

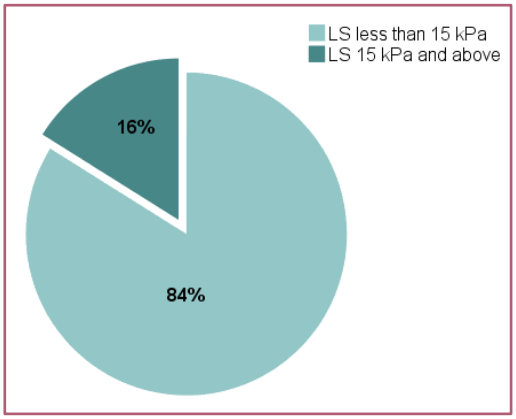
Table 8. *Spearman correlation of ELF score, FIB-4 index and liver stiffness*

		ELF score	FIB-4 index	LS (kPa)
ELF score	Correlation coefficient	1.000	0.522	0.462
	Sig. (2-tailed)	-	0.003	0.009
FIB-4 index	Correlation coefficient	0.522	1.000	0.480
	Sig. (2-tailed)	0.003	-	0.006
LS (kPa)	Correlation coefficient	0.462	0.480	1.000
	Sig. (2-tailed)	0.009	0.006	-

ELF score, enhanced liver fibrosis score; FIB-4, fibrosis 4 index; LS (kPa), liver stiffness (Kilopascals); sig., significance.

3.5 Prevalence of advanced fibrosis detected with LSM

Figure 7. *Prevalence of advanced fibrosis according to LSM*



LS, liver stiffness; kPa, kilopascals.

Five out of 31 patients presented with LSM results above the F3-threshold of 15 kPa. Thus, about 84 % of the initial study population showed no advanced fibrosis.

3.6 Alterations of liver- and alcohol-related parameters

Table 9. *Progression of parameters*

	Baseline visit	3 months follow up visit	*p*-value
AST (U/L)	26 (20; 44)	20 (17; 29)	0.093
ALT (U/L)	26 (17; 39)	18 (15; 23)	0.020
GGT (U/L)	60 (30; 152)	28 (20; 75)	0.070
Bilirubin (mg/dL)	0.41 (0.26; 0.63)	0.40 (0.34; 0.74)	0.124
MCV (fL)	90.3 (± 7)	87.4 (± 6.5)	0.016
%CDT (%)	1.56 (1.26; 2,12)	1.96 (1.11; 2.12)	0.381
FIB-4	1.20 (± 0.58)	1.27 (± 0.59)	0.558
ELF*	9.09 (8.32; 9.84)	8.54 (8.02; 9.69)	0.179
LS (kPa)	6.4 (4.8; 14.8)	4.8 (4.1; 6.8)	0.057

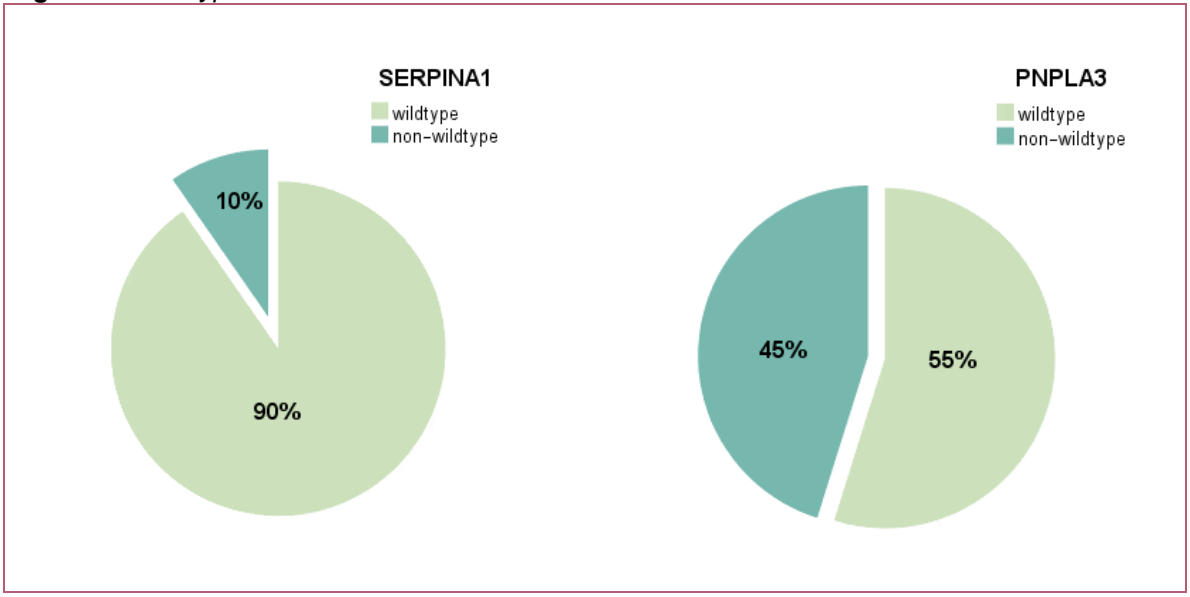
Data are shown as median (Q1; Q3) or as mean (± standard deviation).
AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; MCV, mean corpuscular volume; CDT, carbohydrate-deficient transferrin; FIB-4, fibrosis 4 index; ELF, enhanced liver fibrosis score; LSM, liver stiffness measurement.
For better comparability, bilirubin and ELF are displayed as median in both visits (Q1; Q2) despite the normal distribution of bilirubin at baseline visit and ELF at 3 months follow up visit.
*) ELF test values of 16 out of 17 patients were used for the analysis due to one case of missing data at 3 months follow up visit.

As shown in *Table 9*, a decrease of liver transaminases was found at the follow up visit. ALT decrease was significant with a significance level set at 5%. GGT median decreased by more than 50 percent. However, this finding was not statistically significant. MCV dropped significantly with a p-value of 0.016, whereas bilirubin values remained almost constant. Interestingly, an increase of %CDT and also FIB-4 index was found, whilst ELF score and LS dropped after a period of 3 months.

3.7 Genetics

In the initial study population (n = 31), genetic analyses regarding mutations of SERPINA1 and PNPLA3 were performed. In absolute numbers, 28 individuals had wildtype alleles of SERPINA1 and 17 patients had wildtype alleles of PNPLA3. The approximate percentages of wildtype vs. non-wildtype alleles are displayed in *Figure 8*.

Figure 8. *Genotypes of SERPINA1 and PNPLA3*



Differences in ELF score, FIB-4 index and LS between groups with wildtype and non-wildtype alleles in PNPLA3 and SERPINA1 were analysed.

As shown in *Table 10* and *Figure 9*, the patient subgroup carrying either a homo- or heterozygous mutation in PNPLA3 (I148M), had lower values of ELF score, FIB-4 index and LS. However, these findings are not statistically significant (see *Table 11*).

Table 10. *ELF score, FIB-4 index and liver stiffness, by PNPLA3 genotype*

	ELF in WT	ELF in n-WT	FIB-4 in WT	FIB-4 in n-WT	LS (kPa) in WT	LS (kPa) in n-WT
Patients	n = 17	n = 14	n = 17	n = 14	n = 17	n = 14
Median	9.02	8.49	1.13	0.85	6.4	5.6
Min	7.54	7.57	0.55	0.37	3.2	2.8
Max	14.31	13.04	2.46	1.87	75	75
Q1	8.31	8.03	0.83	0.7	4.2	4
Q3	9.81	9.03	1.37	1.35	15.9	7.2

ELF, Enhanced liver fibrosis score; FIB-4 index, Fibrosis-4 index; LS, liver stiffness; Max, maximum; Min, minimum; WT, wildtype genotype of PNPLA3; n-WT, non-wildtype genotype of PNPLA3 (homo- or heterozygous for I148M allele)

Table 11. *Mann-Whitney U test for ELF score, FIB-4 index and Liver stiffness, by PNPLA3 genotype*

	Genotype	n	Mean rank	Mann-Whitney U	asympt. sig. (2-tailed)
ELF score	WT	17	17.71	90.00	0.250
	n-WT	14	13.93		
FIB-4 index	WT	17	17.65	91.00	0.266
	n-WT	14	14.00		
Liver stiffness	WT	17	17.44	94.50	0.336
	n-WT	14	14.25		

n, number of patients; WT, wildtype PNPLA3; n-WT, non-wildtype PNPLA3 (homozygous and heterozygous for PNPLA3 I148M allele); asympt. sig., asymptotic significance, 2-tailed.

To further differentiate between genotypic variants, the cohort (n = 31) was divided into three groups by PNPLA3 genotype: wildtype (n = 17), heterozygous for I148M (n = 9) and homozygous for I148M (n = 5). *Tables 12-14* display the results of the descriptive analyses.

Table 12. *ELF score, by wildtype PNPLA3, heterzygous and homozygous alleles of PNPLA3 I148M*

	ELF in WT	ELF in HTZ	ELF in HMZ	
Patients	n = 17	n = 9	n = 5	
Median	9.02	8.68	8.30	
Min	7.54	7.83	7.57	
Max	14.31	9.71	13.04	ELF, Enhanced liver fibrosis score; Max, maximum; Min, minimum; Q1, quartile 1; Q3, quartile 3; WT, wildtype genotype of PNPLA3; HTZ, heterozygous for I148M; HMZ, homozygous for I148M.
Q1	8.31	8.14	7.79	
Q3	9.81	9.09	11.00	

Table 13. *FIB-4 index, by wildtype PNPLA3, heterozygous and homozygous alleles of PNPLA3 I148M*

	FIB-4 in WT	FIB-4 in HTZ	FIB-4 in HMZ	
Patients	n = 17	n = 9	n = 5	
Median	1.13	0.76	1.07	
Min	0.55	0.37	0.70	
Max	2.46	1.63	1.87	FIB-4, Fibrosis-4 index; Max, maximum; Min, minimum; Q1, quartile 1; Q3, quartile 3; WT, wildtype genotype of PNPLA3; HTZ, heterozygous for I148M; HMZ, homozygous for I148M.
Q1	0.83	0.65	0.76	
Q3	1.37	1.43	1.49	

Table 14. *Liver stiffness, by wildtype PNPLA3, heterozygous and homozygous alleles of PNPLA3 I148M*

	LS in WT	LS in HTZ	LS in HMZ	
Patients	n = 17	n = 9	n = 5	
Median	6.4	4.8	5.9	
Min	3.2	3.5	2.8	
Max	75.0	10.4	75.0	LS, liver stiffness (kPa); Max, maximum; Min, minimum; Q1, quartile 1; Q3, quartile 3; WT, wildtype genotype of PNPLA3; HTZ, heterozygous for I148M; HMZ, homozygous for I148M.
Q1	4.2	4.0	4.2	
Q3	15.9	6.0	42.3	

In the subgroup heterozygous for I148M, median values of FIB-4 index and LS were lower than in the other subgroups (*Tables 12-14*). ELF, however, was found lowest in the homozygous subgroup. All parameters had lower median values in the homozygous subgroup compared to the subgroup with the wildtype PNPLA3 genotype.

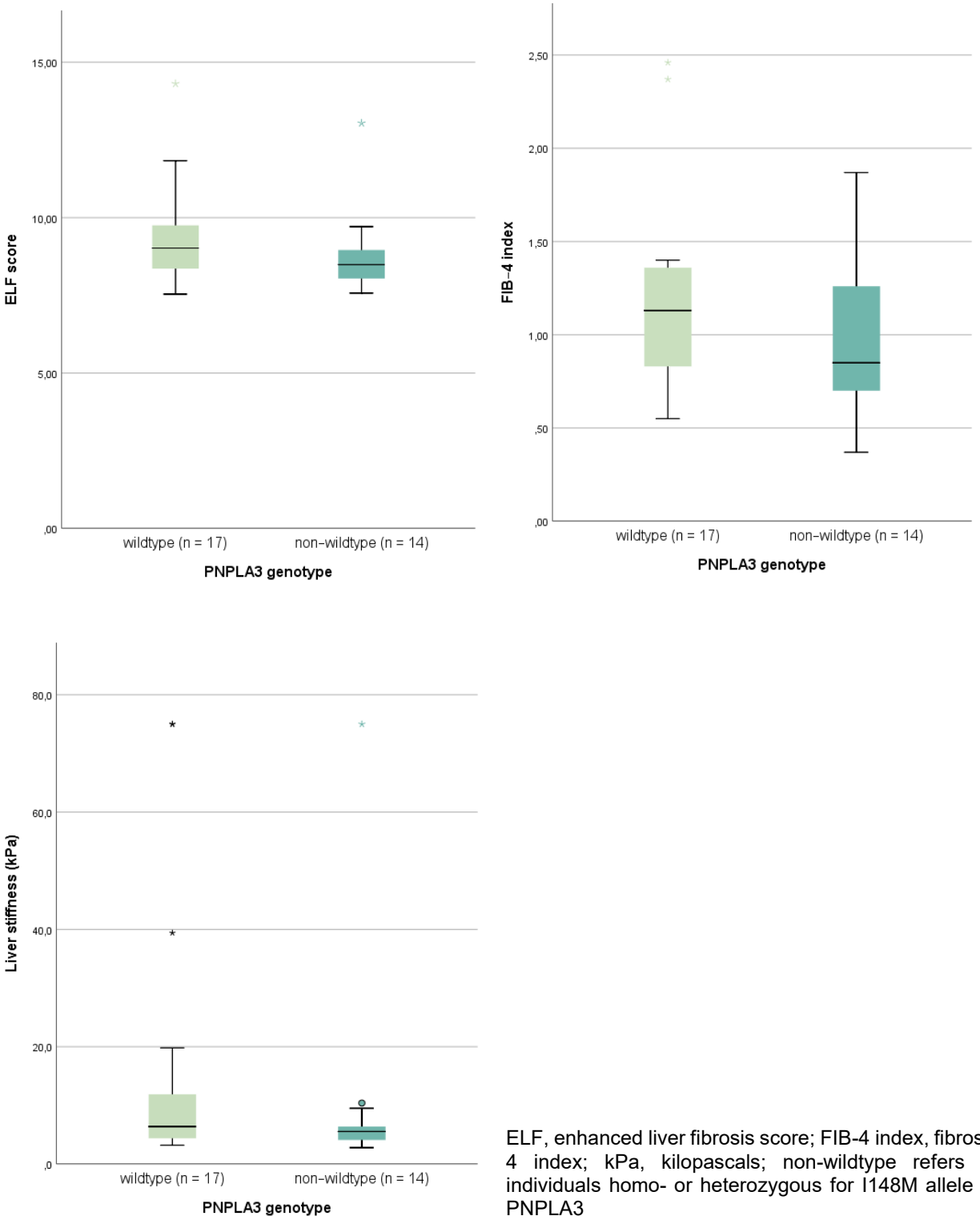
Kruskal-Wallis test was performed in order to determine differences between the three subgroups. The results are displayed in *Table 15*. None of the results are statistically significant.

Table 15. *Kruskal-Wallis test for ELF score, FIB-4 index and Liver stiffness, by PNPLA3 genotype*

	Genotype	n	Mean rank	Kruskal-Wallis H	asympt. sig.
ELF score	WT	17	17.71	1.335	0.513
	HTZ	9	14.11		
	HMZ	5	13.60		
FIB-4 index	WT	17	17.65	1.612	0.447
	HTZ	9	12.89		
	HMZ	5	16.00		
Liver stiffness	WT	17	17.44	1.661	0.436
	HTZ	9	12.72		
	HMZ	5	17.00		

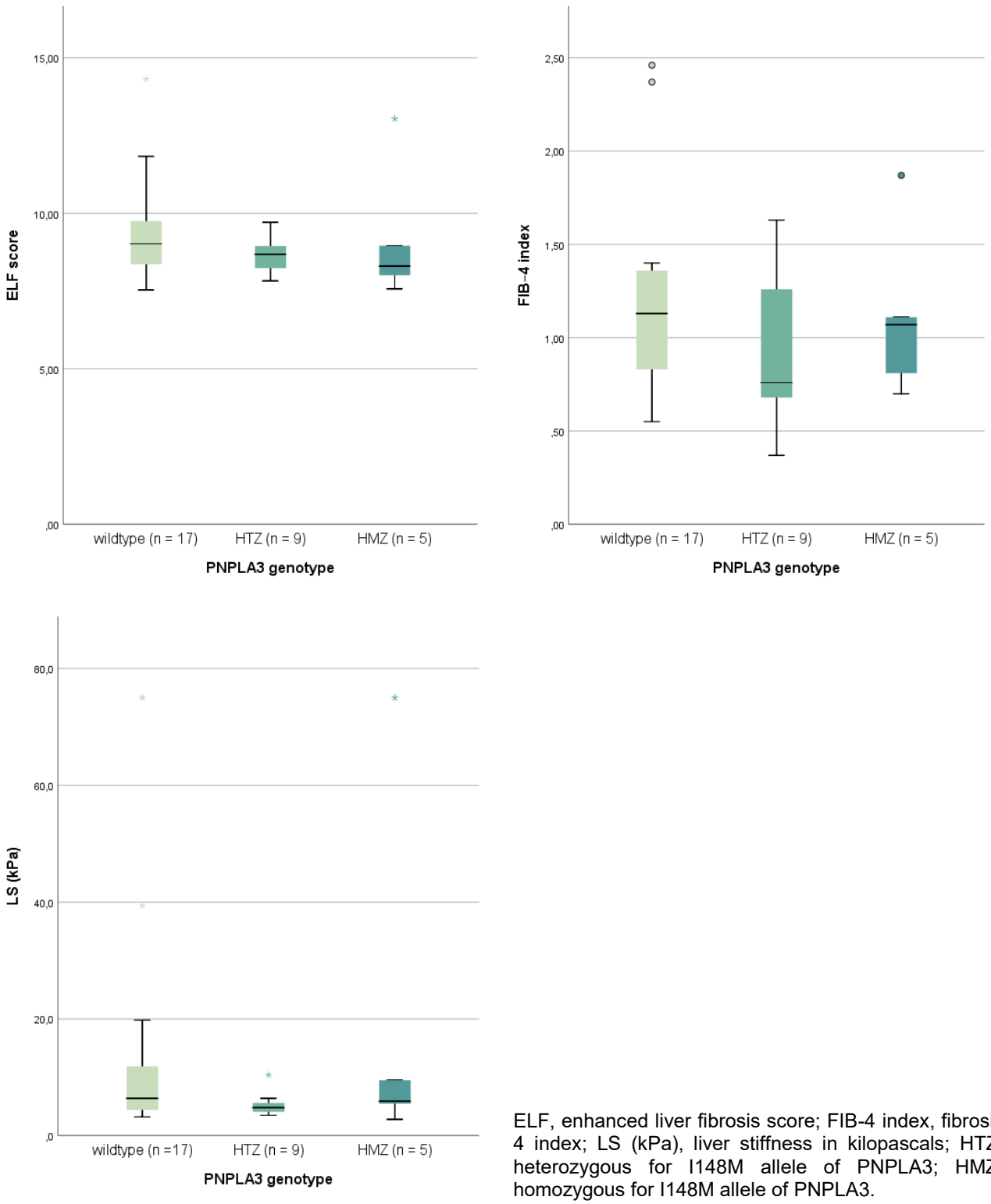
n, number of patients; WT, wildtype PNPLA3; HTZ, heterozygous for I148M allele; HMZ, homozygous for I148M allele; asympt. sig., asymptotic significance.

Figure 9. *Differences in ELF, FIB-4 index and LS, by PNPLA3 wildtype vs. non-wildtype*



ELF, enhanced liver fibrosis score; FIB-4 index, fibrosis 4 index; kPa, kilopascals; non-wildtype refers to individuals homo- or heterozygous for I148M allele of PNPLA3

Figure 10. *Differences in ELF score, FIB-4 index and LS, by PNPLA3 wildtype vs. heterozygous for I148M vs. homozygous for I148M*



ELF, enhanced liver fibrosis score; FIB-4 index, fibrosis 4 index; LS (kPa), liver stiffness in kilopascals; HTZ, heterozygous for I148M allele of PNPLA3; HMZ, homozygous for I148M allele of PNPLA3.

Table 16. *ELF score, FIB-4 index and liver stiffness, by SERPINA1 genotype*

	ELF in WT	ELF in n-WT	FIB-4 in WT	FIB-4 in n-WT	LS (kPa) in WT	LS (kPa) in n-WT
Patients	n = 28	n = 3	n = 28	n = 3	n = 28	n = 3
Mean	9.16	8.89	1.15	0.66	13.8	16.1
SD	1.64	0.77	0.49	0.27	21.9	20.2
Median	8.68	8.68	1.09	0.73	5.8	5.6
Min	7.54	8.25	0.55	0.37	2.8	3.2
Max	14.31	9.75	2.46	0.89	75.0	39.4
Q1	8.09	-	0.77	-	4.2	-
Q3	9.67	-	1.38	-	9.7	-

ELF, Enhanced liver fibrosis score; FIB-4 index, Fibrosis-4 index; LS, liver stiffness; Max, maximum; Min, minimum; SD, standard deviation; WT, wildtype genotype of SERPINA1; n-WT, non-wildtype genotype of SERPINA1.

3.8 Histological results in patients with LS above 15 kPa

Patients with an LSM result of 15 kPa and above at baseline visit were offered a liver biopsy. Two patients consented to the performance of liver biopsy and histological examination. *Table 17* displays the histological results of said patients.

Table 17. *Results of histological examination*

Liver stiffness	Description	Fibrosis stage
75 kPa	Cirrhotic conversion of the liver with pericellular fibrosis in the presence of minimal steatosis of the liver. In HE stain, no alterations as sign of steatohepatitis detectable. Berlin blue stain negative.	**F4**
75 kPa	Signs of steatohepatitis in the presence of incomplete cirrhotic conversion of the liver tissue, accompanied by focal pericellular fibrosis. Berlin blue stain negative.	**F4**

4 Discussion

4.1 Study limitations

For the most part, our findings were compatible with those of previous research groups mentioned in the introduction of this thesis. However, many of our findings were not of statistical significance. This can be explained by the small size of our study population of initially 31 patients at baseline and the final study cohort of 17 individuals, respectively. Thus, our results regarding diagnostic accuracy of non-invasive methods and the role of different genotypes in fibrosis development can be interpreted as indications, but due to an insufficient sample size, they do not allow for general conclusions.

Regarding the drinking behavior and abstinence, it needs to be mentioned that our data on the daily consumption of ethanol prior to detoxification rely on the reports of our patients in their interviews at the baseline visit. It is important to note that drinking patterns vary a lot concerning the types of drinks and the amount of every type of drink consumed. In most cases, drinking patterns are not identical every day and they also vary strongly over the course of time. Thus, our calculations of daily intake based on the patients' reports must be seen as estimated, average values.

Another limitation of our study is the heterogeneous duration of abstinence prior to baseline visit, with a median duration of 21 days, ranging from 7 to 211 days. Patients were sent to the Liver clinic by our collaborating institution at State Hospital Graz II regardless of their period of abstinence. We did not determine a certain period of abstinence in our study protocol. The abstinence period was not taken into account in our statistical calculations. Additionally, in some cases, patients were not able to recall the exact number of days they had been abstinent at the time of their enrolment. Thus, our calculations based on patients' reports are potentially fraught with information bias.

Regarding the evaluation of noninvasive tests, it should be noted that liver histology was not available as reference test since our protocol did not include liver biopsy for all patients.

4.2 Results

4.2.1 Drinking behavior and abstinence

As proposed in previous epidemiological studies about AUD and ALD, male drinkers consume a higher amount of ethanol per day than females. Our results are compatible with those of previous research, since in our study population, the male subgroup had a mean daily ethanol intake of 383 (± 218) grams per day, whereas the female study participants drank 200 (± 73) grams of pure ethanol per day. These mean values were calculated from patients' reports about their most recent daily alcohol intake prior to detoxification.
At the baseline visit some patients claimed to be abstinent when they were not, as we detected with our laboratory tests. This behavior probably lies in the social stigma associated with addiction resulting in the feeling of shame and the need to lie about their incapability to stay abstinent.

4.2.2 Non-invasive fibrosis tests

The results on the diagnostic accuracy of ELF test show that in our study cohort, a cut-off value of 10.5, as proposed by *Thiele et al*. (130), is superior to the manufacturer-proposed cut-off value of 9.8. We used a liver stiffness of ≥15 kPa as a non-invasive reference for the detection of advanced fibrosis (≥ F3).

Thiele et al. calculated the diagnostic accuracy for the 10.5 cut-off with a 79 % sensitivity, 91% specificity, PPV of 71 % and NPV of 94 %. For the same threshold, we found the ELF score to be an accurate diagnostic tool with a sensitivity of 80 %, a specificity of 100 %, PPV of 100 % and NPV of 96.2 %. The manufacturer proposed threshold of 9.8 performed with the same sensitivity and NPV, but had poorer results regarding specificity and PPV (see *Table 6*).

These results indicate that the cut-off value for advanced fibrosis proposed by *Thiele et al*. should be preferred over the threshold proposed by the manufacturer. However, our cohort was rather small. Hence, studies with larger sample size and liver histology as reference test need to be conducted to confirm these findings with certainty in order to implement this cut-off for the clinical routine in the diagnosis and treatment of patients with ALD.

In our correlation analysis, we found that the tools based on serum parameters (ELF and FIB-4) correlated better with one another than each of them does with liver stiffness, respectively.

4.2.3 Prevalence of advanced fibrosis and results at follow up visit

At the initial study visit, in 16 % of our patients advanced fibrosis was diagnosed using LSM with a threshold of 15 kPa. As *Seitz et al*. mentioned in their *Nature Reviews Disease Primer* on ALD, a subset of 10 to 20 % of heavy alcohol abusers will develop advanced ALD (42). Our results on the prevalence of advanced fibrosis in long term alcoholics are compatible with their estimation, although in the mentioned publication the entities included in the term 'advanced ALD' are not precisely defined. As for the progression of liver parameters and liver stiffness, some findings of previous studies (142,143,171) were confirmed by our results. Median values of AST and ALT as well as GGT declined after 3 months of abstinence. GGT median even dropped by more than half from 60 to 28 U/L. This most likely results from a less active metabolic activity of the liver tissue due to the alcohol abstinence.

Liver stiffness declined from a median of 6.4 kPa to a median of 4.8 ($p = 0.057$). Since this finding was accompanied by a decrease of AST (from a median of 26 U/L to a median of 20 U/L, $p = 0.093$), we assume that the contribution of inflammation to increased liver stiffness suggested by *Mueller et al* (142), can be confirmed.

4.2.4 The role of PNPLA3 and SERPINA1

In our study population (n = 31), 45 % of the participants presented with a non-wildtype variant of PNPLA3 and 10 % tested with a non-wildtype variant of SERPINA1, respectively. Regarding PNPLA3, our analyses on potential differences of ELF, FIB-4 index and LS in the subgroups by genotype showed that all three parameters have lower median values in the subgroup with the non-wildtype variant I148M. The results were not statistically significant. However, these findings indicate that a mutation of PNPLA3 might be associated with a steatosis-dominant phenotype of ALD. We further divided the non-wildtype group of our cohort in subgroups by heterogeneous versus homogenous occurrence of the I148M mutation. This led to three subgroups by PNPLA3 genotype: wildtype (n = 17), heterozygous for I148M (n = 9) and homozygous for I148M (n = 5). The subgroup heterozygous for I148M showed the lowest median values for FIB-4 (0.76) and LS (4.8 kPa), whereas the median of ELF (8.30) was lowest in the homozygous subgroup. The wildtype subgroup showed the

highest median values for ELF, LS and FIB-4, respectively. We expected the group with the homozygous genotype for the variant I148M to be associated with a lower median value of liver stiffness. Thus, our findings were contradictory to our expectation after the first comparison (wildtype vs. non-wildtype). In the homozygous group (n = 5), one patient presented with a LS of 75 kPa. In the subgroup carrying wildtype PNPLA3 (n = 17), two patients had LS results of 75 kPa. Outliers have a bigger impact the smaller the sample size is. However, in contrast to the mean value, the median is not skewed significantly by outliers. Thus, our findings cannot simply be explained by the distribution of the subgroups.

Regarding SERPINA1 genotypes, we found that the mean values of ELF and FIB-4 were lower in non-wildtype carriers than in patients with the wildtype genotype. Liver stiffness was higher in patients carrying non-wildtype SERPINA1 alleles. However, due to the sample size of non-wildtype carriers (n = 3), our results do not allow for a sensible conclusion regarding an association between SERPINA1 genotypic variants and the severity of liver fibrosis in patients with ALD.

4.2.5 Histology

Consistent with the suggestions of previous studies, our results of the histological examination performed by the hepatopathologist were compatible with the FibroScan® LSM results of 75 kPa (highest measurable value). *Table 18* displays the previously proposed cut-offs for the diagnosis of F4 in the currently available literature.

Table 18. *Proposed cut-off values for the diagnosis of F4*

	Mueller et al. (142)	**Trabut et al. (143)**	**Thiele et al. (130)**	**Thiele et al. (144)**
Cut-off F4	> 12,5 kPa	≥ 19,5 kPa	≥ 19,7 kPa	-

kPa, kilopascals

4.3 Conclusion

About 16% of asymptomatic alcoholics already showed signs of advanced fibrosis. Assuming similar percentages in larger populations, referring patients with alcohol abuse or addiction to a liver clinic might be an important motivational measure in their treatment. ELF and FIB-4 are adequate non-invasive tools for the detection of advanced fibrosis. Median values of AST, ALT and GGT decreased after 3 months of abstinence, as well as ELF and liver stiffness. The role of PNPLA3 and SERPINA1 genotypes could not be determined adequately in this study.

4.4 Outlook

This thesis was conducted with a study collective of 31 patients. A larger cohort needs to be investigated in order to generate reliable results on the topic. I conducted my thesis within the ALD-DETOX study run by Dr. Rudolf Stauber at the Department of Gastroenterology and Hepatology at the Medical University of Graz. After the recruitment of the 31 participants I included in my analyses, the recruitment period for ALD-DETOX is being continued. Further research on the topic will be conducted.

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6 Appendix

6.1 Ethics Committee

This project |Non-invasive diagnosis of hepatic fibrosis in patients during alcohol detoxification therapy) was approved by the Ethics Committee of the Medical University of Graz prior to initiating the study.
Number of ethics approval: 31-177 ex 18/19.

6.2 Informed Consent Form

The informed consent form used during this study is attached in the following pages.

PatientInneninformation[1] und Einwilligungserklärung zur Teilnahme an der klinischen Studie

Nichtinvasive Diagnostik der Leberfibrose[2] bei Patienten unter stationärer Alkoholentzugstherapie

Sehr geehrte Teilnehmerin, sehr geehrter Teilnehmer!

Wir laden Sie ein an der oben genannten klinischen Studie teilzunehmen. Die Aufklärung darüber erfolgt in einem ausführlichen ärztlichen Gespräch.

Ihre Teilnahme an dieser klinischen Studie erfolgt freiwillig. Sie können jederzeit ohne Angabe von Gründen aus der Studie ausscheiden. Die Ablehnung der Teilnahme oder ein vorzeitiges Ausscheiden aus dieser Studie hat keine nachteiligen Folgen für Ihre medizinische Betreuung.

Klinische Studien sind notwendig, um verlässliche neue medizinische Forschungsergebnisse zu gewinnen. Unverzichtbare Voraussetzung für die Durchführung einer klinischen Studie ist jedoch, dass Sie Ihr Einverständnis zur Teilnahme an dieser klinischen Studie schriftlich erklären. Bitte lesen Sie den folgenden Text als Ergänzung zum Informationsgespräch mit Ihrem Arzt sorgfältig durch und zögern Sie nicht Fragen zu stellen.

Bitte unterschreiben Sie die Einwilligungserklärung nur

- wenn Sie Art und Ablauf der klinischen Studie vollständig verstanden haben,
- wenn Sie bereit sind, der Teilnahme zuzustimmen und
- wenn Sie sich über Ihre Rechte als Teilnehmer an dieser klinischen Studie im Klaren sind.

Zu dieser klinischen Studie, sowie zur Patienteninformation und Einwilligungserklärung wurde von der zuständigen Ethikkommission eine befürwortende Stellungnahme abgegeben.

1. Was ist der Zweck der klinischen Studie?

Der Zweck dieser klinischen Studie ist die frühzeitige Erkennung von ernsten Leberschäden als Folge von übermäßigem Alkoholkonsum. Eine alkoholische Lebererkrankung wird üblicherweise erst dann erkannt, wenn sie schon weit fortgeschritten ist; die Behandlungsmöglichkeiten sind in diesen Fällen sehr begrenzt.

[1] Wegen der besseren Lesbarkeit wird im weiteren Text zum Teil auf die gleichzeitige Verwendung weiblicher und männlicher Personenbegriffe verzichtet. Gemeint und angesprochen sind – sofern zutreffend – immer beide Geschlechter.

[2] Leberfibrose: Vernarbung der Leber durch Bindegewebe; Vorstufe von Leberzirrhose.

Frühe Stadien eines alkoholischen Leberschadens können sich hingegen völlig zurückbilden, wenn auf Alkohol verzichtet wird.

Wir möchten in dieser klinischen Studie herausfinden, wie häufig bei Alkoholabhängigkeit bereits ein Leberschaden vorliegt, wie schwer dieser ist, und wie sich der Verzicht auf Alkohol auf diesen Schaden auswirkt.

2. Wie läuft die klinische Studie ab?

Diese Studie wird an der Leberambulanz der Universitätsklinik für Innere Medizin Graz durchgeführt und es werden insgesamt ungefähr *100* Personen daran teilnehmen. Ihre Teilnahme wird bis zu 12 Monate dauern.

Folgende Maßnahmen werden ausschließlich aus Studiengründen durchgeführt:

Sie werden gebeten, uns einige Fragen zu beantworten, eine Harnprobe abzugeben und wir werden folgende Untersuchungen durchführen:

- Messung der Körpergröße und des Gewichts
- Messung des Blutdrucks
- Blutabnahme
- Ultraschalluntersuchung des Oberbauchs
- Spezielle Ultraschalluntersuchung mit FibroScan® (Messung der Lebersteifigkeit)

Sie werden gebeten, hierzu in die Leberambulanz zu kommen. Für die Erstuntersuchung ist ein Patiententransport vom Zentrum für Suchtmedizin zur Leberambulanz und zurück verfügbar. Weitere Kontrolltermine nach 3, 6 und 12 Monaten werden in der Leberambulanz vereinbart. Die Einhaltung der Besuchstermine, einschließlich der Anweisungen des Studienarztes, ist von entscheidender Bedeutung für den Erfolg dieser klinischen Studie.

3. Worin liegt der Nutzen einer Teilnahme an der Klinischen Studie?

Die Studienteilnahme kann für Sie nützlich sein, da wir Ihnen darüber Auskunft geben, ob ein Leberschaden vorliegt und, wenn ja, wie fortgeschritten die Schädigung ist. Diese Information kann für Sie eine zusätzliche Motivation zur Alkoholabstinenz bedeuten. Darüber hinaus können die angebotenen Kontrolltermine bei der Nachsorge Ihrer Erkrankung von Nutzen sein.

4. Gibt es Risiken, Beschwerden und Begleiterscheinungen?

Die im Rahmen dieser klinischen Prüfung durchgeführten Blutabnahmen können selten zu Beschwerden führen (z.B. Schmerzen an der Einstichstelle, Bluterguss). Wir werden pro Besuch ungefähr 20 ml Blut (entspricht ca. 2 Esslöffeln) abnehmen.

5. Was ist zu tun beim Auftreten von Symptomen, Begleiterscheinungen und/oder Verletzungen?

Sollten im Verlauf der klinischen Studie irgendwelche Symptome, Begleiterscheinungen oder Verletzungen auftreten, müssen Sie diese Ihrem Arzt mitteilen, bei schwerwiegenden Begleiterscheinungen umgehend, ggf. telefonisch (Telefonnummern, etc. siehe unten).

6. Wann wird die klinische Studie vorzeitig beendet?

Sie können jederzeit auch ohne Angabe von Gründen, Ihre Teilnahmebereitschaft widerrufen und aus der klinischen Studie ausscheiden ohne dass Ihnen dadurch irgendwelche Nachteile für Ihre weitere medizinische Betreuung entstehen.

Ihr Studienarzt wird Sie über alle neuen Erkenntnisse, die in Bezug auf diese klinische Studie bekannt werden, und für Sie wesentlich werden könnten, umgehend informieren. Auf dieser Basis können Sie dann Ihre Entscheidung zur **weiteren** Teilnahme an dieser klinischen Studie neu überdenken.

Es ist aber auch möglich, dass Ihr Studienarzt entscheidet, Ihre Teilnahme an der klinischen Studie vorzeitig zu beenden, ohne vorher Ihr Einverständnis einzuholen. Die Gründe hierfür können sein:

a) Sie können den Erfordernissen der Klinischen Studie nicht entsprechen;

b) Ihr Studienarzt hat den Eindruck, dass eine weitere Teilnahme an der klinischen Studie nicht in Ihrem Interesse ist;

Sofern Sie sich dazu entschließen, vorzeitig aus der klinischen Prüfung auszuscheiden, oder Ihre Teilnahme aus einem der oben genannten Gründe vorzeitig beendet wird, ist es für Ihre eigene Sicherheit wichtig, dass Sie sich einer normalen Kontrolluntersuchung unterziehen. Diese besteht meistens aus einer körperlichen Untersuchung sowie aus Laboruntersuchungen.

7. Datenschutz

Bei den Daten, die über Sie im Rahmen dieser klinischen Studie erhoben und verarbeitet werden, ist grundsätzlich zu unterscheiden zwischen

1) jenen personenbezogenen Daten, anhand derer Sie direkt identifizierbar sind (z.B. Name, Geburtsdatum, Adresse, Bildaufnahmen...),

2) pseudonymisierten (verschlüsselten) personenbezogenen Daten, bei denen alle Informationen, die direkte Rückschlüsse auf Ihre Identität zulassen, durch einen Code (z. B. eine Zahl) ersetzt bzw. (z.B. im Fall von Bildaufnahmen) unkenntlich gemacht werden. Dies bewirkt, dass die Daten ohne Hinzuziehung zusätzlicher Informationen und ohne unverhältnismäßig großen Aufwand nicht mehr Ihrer Person zugeordnet werden können und

3) anonymisierten Daten, bei denen eine Rückführung auf Ihre Person nicht mehr möglich ist.

Der Code zur Verschlüsselung wird von den verschlüsselten Datensätzen streng getrennt und nur an Ihrem Prüfzentrum aufbewahrt.

Zugang zu Ihren nicht verschlüsselten Daten haben der Prüfarzt und andere Mitarbeiter des Studienzentrums, die an der klinischen Studie oder Ihrer medizinischen Versorgung mitwirken. Die Daten sind gegen unbefugten Zugriff geschützt. Zusätzlich können autorisierte und zur Verschwiegenheit verpflichtete Beauftragte der Medizinischen Universität Graz sowie Beauftragte von in- und/oder ausländischen Gesundheitsbehörden und jeweils zuständige Ethikkommissionen in die nicht verschlüsselten Daten Einsicht nehmen, soweit dies für die Überprüfung der ordnungsgemäßen Durchführung der klinischen Studie notwendig bzw. vorgeschrieben ist.

Eine Weitergabe der Daten erfolgt nur in verschlüsselter oder anonymisierter Form. Auch für etwaige Publikationen werden nur die verschlüsselten oder anonymisierten Daten verwendet.

Sämtliche Personen, die Zugang zu Ihren verschlüsselten und nicht verschlüsselten Daten erhalten, unterliegen im Umgang mit den Daten der Datenschutz-Grundverordnung (DSGVO) sowie den österreichischen Anpassungsvorschriften in der jeweils gültigen Fassung.

Im Rahmen dieser klinischen Studie ist keine Weitergabe von Daten in Länder außerhalb der EU vorgesehen.

Sie können Ihre Einwilligung zur Erhebung und Verarbeitung Ihrer Daten jederzeit widerrufen. Nach Ihrem Widerruf werden keine weiteren Daten mehr über Sie erhoben. Die bis zum Widerruf erhobenen Daten können allerdings weiter im Rahmen dieser klinischen Studie verwendet werden.

Aufgrund der gesetzlichen Vorgaben haben Sie außerdem, sofern dies nicht die Durchführung der klinischen Studie voraussichtlich unmöglich macht oder ernsthaft beeinträchtigt, das Recht auf Einsicht in die Ihre Person betreffenden Daten und die Möglichkeit der Berichtigung, falls Sie Fehler feststellen.

Sie haben auch das Recht, bei der österreichischen Datenschutzbehörde eine Beschwerde über den Umgang mit Ihren Daten einzubringen (www.dsb.gv.at).

Die voraussichtliche Dauer der klinischen Studie ist 24 Monate. Die Dauer der Speicherung Ihrer Daten über das Ende der klinischen Studie hinaus ist durch Rechtsvorschriften geregelt.

Falls Sie Fragen zum Umgang mit Ihren Daten in dieser klinischen Studie haben, wenden Sie sich zunächst an Ihren Prüfarzt. Dieser kann Ihr Anliegen ggf. an die Personen, die am Studienzentrum für den Datenschutz verantwortlich sind, weiterleiten.

Datenschutzbeauftragte/r der Medizinischen Universität Graz: datenschutz@medunigraz.at

8. Entstehen für die Teilnehmer Kosten? Gibt es einen Kostenersatz oder eine Vergütung?

Durch Ihre Teilnahme an dieser klinischen Studie entstehen für Sie keine zusätzlichen Kosten. Eine Vergütung ist nicht vorgesehen.

9. Möglichkeit zur Diskussion weiterer Fragen

Für weitere Fragen im Zusammenhang mit dieser klinischen Studie stehen Ihnen Ihr Studienarzt und seine Mitarbeiter gern zur Verfügung. Auch Fragen, die Ihre Rechte als Patient und Teilnehmer an dieser klinischen Studie betreffen, werden Ihnen gerne beantwortet. Sobald allgemeine Ergebnisse dieser klinischen Studie vorliegen, können Sie ebenfalls darüber informiert werden, falls Sie dieses wünschen.

Name der Kontaktperson: Univ.-Prof. Dr. Rudolf Stauber (Med. Universität Graz)

Ständig erreichbar unter: 0316-385-80268 bzw. -12731

Name der Kontaktperson: OA Dr. Werner Heran (LKH Graz II, Standort Süd)

Ständig erreichbar unter: 0316-2191-2236

10. Aufbewahrung von Proben

Nach dem Ende der Studie möchten wir die übrigen Proben gerne für weitere Forschungsprojekte auf dem Gebiet der Hepatologie (Lebererkrankungen) verwenden. Für jedes dieser Forschungsprojekte wird zuvor die Zustimmung der Ethikkommission eingeholt. Die Proben werden in der Biobank der Leberambulanz für 10 Jahre gelagert. Sie können jederzeit verlangen, dass Ihre Proben vernichtet werden. Verantwortlich für die Lagerung und Vernichtung der Proben ist Prof. Dr. Rudolf Stauber (Leiter der Leberambulanz).

11. Einwilligungserklärung

Name des Patienten:

Geb.Datum:

Ich erkläre mich bereit, an der klinischen Studie *Nichtinvasive Diagnostik der Leberfibrose bei Patienten unter stationärer Alkoholentzugstherapie* teilzunehmen.

Ich bin von Herrn/Frau ..... ausführlich und verständlich über die klinische Studie, mögliche Belastungen und Risiken, sowie über Wesen, Bedeutung und Tragweite der klinischen Studie, sich für mich daraus ergebenden Anforderungen aufgeklärt worden. Ich habe darüber hinaus den Text dieser

Patientenaufklärung und Einwilligungserklärung, die insgesamt *6* Seiten umfasst gelesen. Aufgetretene Fragen wurden mir vom Studienarzt verständlich und genügend beantwortet. Ich hatte ausreichend Zeit, mich zu entscheiden. Ich habe zurzeit keine weiteren Fragen mehr.

Ich werde den ärztlichen Anordnungen, die für die Durchführung der klinischen Studie erforderlich sind, Folge leisten, behalte mir jedoch das Recht vor, meine freiwillige Mitwirkung jederzeit zu beenden, ohne dass mir daraus Nachteile für meine weitere medizinische Betreuung entstehen.

Ich stimme ausdrücklich zu, dass meine im Rahmen dieser klinischen Studie erhobenen Daten wie im Abschnitt „Datenschutz“ dieses Dokuments beschrieben verwendet werden.

Ich stimme zu, dass meine Proben zu Forschungszwecken in der Biobank der Leberambulanz aufbewahrt werden:

ja nein

Für den Fall, dass ich aus der Studie ausscheide, bin ich einverstanden, dass meine Proben weiterhin aufbewahrt und analysiert werden, wie in dieser Information beschrieben:

ja nein

Eine Kopie dieser Patienteninformation und Einwilligungserklärung habe ich erhalten. Das Original verbleibt beim Studienarzt.

.....
(Datum und Unterschrift des Patienten)

.....
(Datum, Name und Unterschrift des verantwortlichen Prüfarztes)

(Der Patient erhält eine unterschriebene Kopie der Patienteninformation und Einwilligungserklärung, das Original verbleibt im Studienordner des Studienarztes.)