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Assessment of Alcohol Consumption in Outpatients with Non-alcoholic Fatty Liver Disease

Dissertation

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vorgelegt von:
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1. Aim of this Thesis

This thesis aims to determinate the average of alcohol consumption in patients with non-alcoholic fatty liver disease (NAFLD) and to investigate the relationship between moderate alcohol consumption and the degree of liver fibrosis in patients with NAFLD.

Firstly, alcohol consumption will be assessed by a three-page questionnaire. In this questionnaire, patients will report their alcohol consumption during three different periods: last three months, last four weeks and last week.

Secondly, the confirmation of the reported alcohol consumption will take place. For this purpose, measurements of direct alcohol markers in hair, blood and urine will be performed.

Finally, the relationship between moderate alcohol consumption and the prevalence of fibrosis in NAFLD will be investigated.

For this purpose, three research questions were formuled:

1. What proportion of abstinence, moderate alcohol consumption and high alcohol consumption can be observed in patients with NAFLD?
2. What is the best method for determining alcohol consumption in patients with NAFLD? (Physicians' Assessments vs questionnaire vs determination of direct alcohol markers)
3. Is occasional alcohol consumption associated with liver fibrosis in patients with NAFLD?

2. Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the most frequent liver diseases worldwide *(Younossi et al., 2018)*. NAFLD was first described by Ludwig in 1980 at the Mayo Clinic and it is defined as the presence of hepatic steatosis in absence of other secondary causes of hepatic fat accumulation *(Marchesini et al., 2016)*. The most common secondary causes of hepatic steatosis are listed in Table 1.

Table 1. Common Causes of Secondary Hepatic Steatosis *(Chalasani et al. 2012)*

Macrovesicular steatosis	Microvesicular steatosis
• Excessive alcohol consumption • Hepatitis C (genotype 3) • Wilson's disease • Lipodystrophy • Starvation • Parenteral nutrition • Abetalipoproteinemia • Medications (e.g., amiodarone, methotrexate, tamoxifen, corticosteroids)	• Reye's syndrome • Medications (valproate, anti-retroviral medicines) • Acute fatty liver of pregnancy • HELLP syndrome • Inborn errors of metabolism (e.g., lecithin cholesterol acyltransferase deficiency, cholesterol ester storage disease, Wolman disease)

NAFLD includes two pathologically distinct conditions: non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) *(Marchesini et al., 2016)*. NAFL is defined as the presence of hepatic steatosis with no evidence of hepatocellular injury, in form of ballooning of the hepatocytes, or no evidence of fibrosis *(Chalasani et al., 2012)*. NASH is the inflammatory subtype of NAFLD and is defined as presence of hepatic steatosis with evidence of hepatocellular injury revealed by ballooned hepatocytes with or without fibrosis *(Chalasani et al., 2012)*. With time NASH can progress to cirrhosis, end-stage liver disease or need for liver transplantation *(Sheka et al., 2020)*.

2.1. Epidemiology of NAFLD

NAFLD is a growing health problem worldwide and has been described as a "global pandemic" *(Derra et al., 2018)*. A recent meta-analyse estimated the worldwide prevalence of NAFLD at about 25%. NAFLD is highly prevalent in all continents, but the highest rates are found in the Middle East and South America (32 and 31%, respectively), and is less common in Africa (14%). The estimated prevalence in each region is illustrated in figure 1 *(Younossi et al., 2016)*.

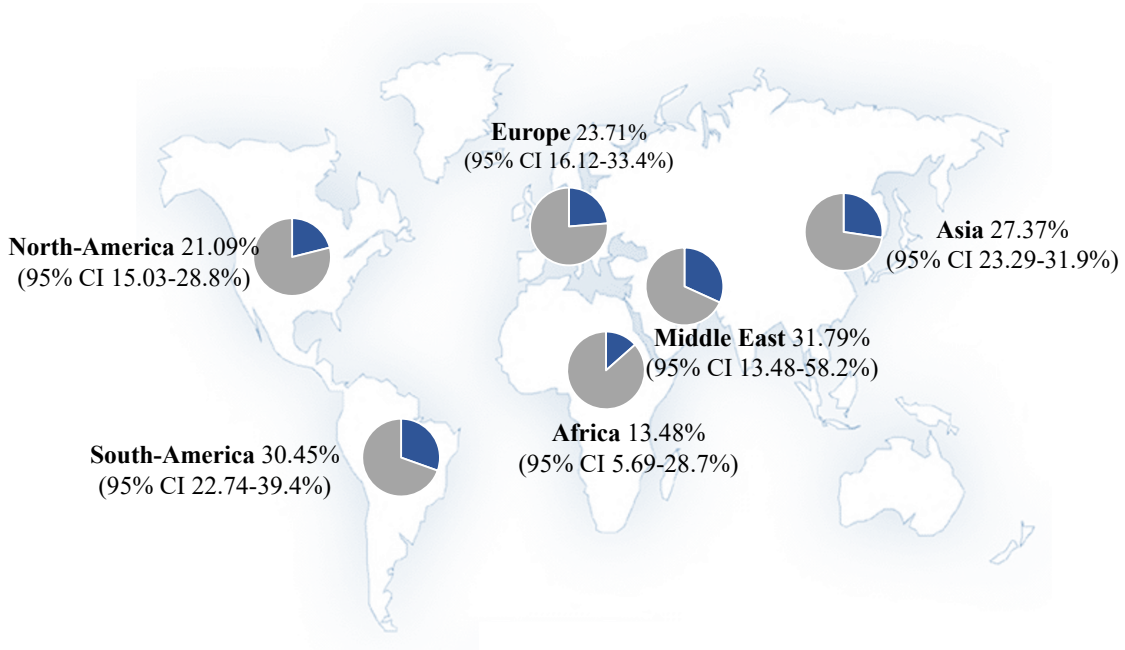

Figure 1. Global NAFLD estimated prevalence (all diagnostic methods) (Younossi et al., 2016).

NALFD patients often present obesity, type 2 diabetes, hypertriglyceridemia, dyslipidaemia, hypertension and metabolic syndrome (Table 2) *(Younossi et al., 2016)*. All of them are well known risk factors for cardiovascular disease.

Table 2. Comorbid conditions associated with NASH (Sheka et al., 2020).

	% Estimated prevalence		
	General USA population	NAFLD	NASH
Hypertriglyceridemia	25.1	40.7	83.3
Obesity	39.8	51.3	81.8
Dyslipidaemia	18.4	69.2	72.1
Metabolic syndrome	34.3	42.5	70.7
Hypertension	29	39.3	68.0
Type 2 diabetes	14	22.5	43.6

NASH and liver-specific disease outcomes are strongly associated with the degree of hepatic fibrosis *(Rinella, 2015)*. Approximately 9% of patients with NASH have fibrosis progression *(Younossi et al., 2016)*.

Hepatocellular carcinoma is a rare complication in NAFL (0.44 per 1000 person-years). In contrast, patients with NASH develop hepatocellular carcinoma at significantly higher rates (5.29 cases per 1000 person-years) *(Younossi et al., 2016)*. Although hepatocellular carcinoma typically develops in the background of cirrhosis, a recent meta-analysis found that in non-cirrhotic patients, those with NASH have a 2.5-fold increased risk of hepatocellular carcinoma compared to other aetiologies of non-cirrhotic liver disease *(Stine et al., 2018)*.

Patients with NAFLD, especially NASH subtype, have higher liver-specific mortality. Nevertheless, cardiovascular disease is the primary cause of death for NAFLD *(Younossi et al., 2016)*.

2.2. Diagnostic of NAFLD

As reported in the last published European Clinical Practice Guideline, NAFLD is characterised by excessive hepatic fat accumulation. This diagnosis requires either histologic demonstration of steatosis >5% of hepatocytes or radiographic demonstration of proton density fat fraction >5.6% *(Marchesini et al., 2016)*.

In order to make a diagnosis of NAFLD it is necessary to exclude other secondary causes of liver damage and the daily alcohol consumption should not exceed 30g for men and 20g for women *(Marchesini et al., 2016)*.

2.2.1. Liver biopsy

Liver biopsy is considered in the currently clinical guidelines as gold standard in the NAFLD diagnostic *(Zhu et al., 2016)*. The definitive classification into NASH or NAFL requires a liver biopsy *(Marchesini et al., 2016)*. Liver biopsy is usually performed percutaneously. In some situations such as severe obesity, thrombocytopenia or coagulation disorders, a percutaneous liver biopsy can be a high risk procedure, so the physician would choose to perform it transjugular, laparoscopic or endoscopic instead *(Andronescu et al., 2018)*. Liver biopsy is typically well tolerated, but it can be painful and complications such as bleeding, infection, bile leak, damage to other organs and rare mortality risk (<0.01%) may occur. On the other hand, the diagnostic integrity can be affected by biopsy adequacy, sampling error or pathologist experience *(Sheka et al., 2020)*.

Figure 2 shows a summary of the possible histological findings in NAFLD. Patients are considered to have NAFL when more than 5% hepatic steatosis is present. Patients are considered to have NASH if steatosis is presented along with hepatocyte ballooning degeneration and lobular inflammation. Over the years, NAFLD may progress to cirrhosis. NASH patients reach this stage in greater proportion (20%) than NAFL patients (4%) *(Sheka et al., 2020)*.

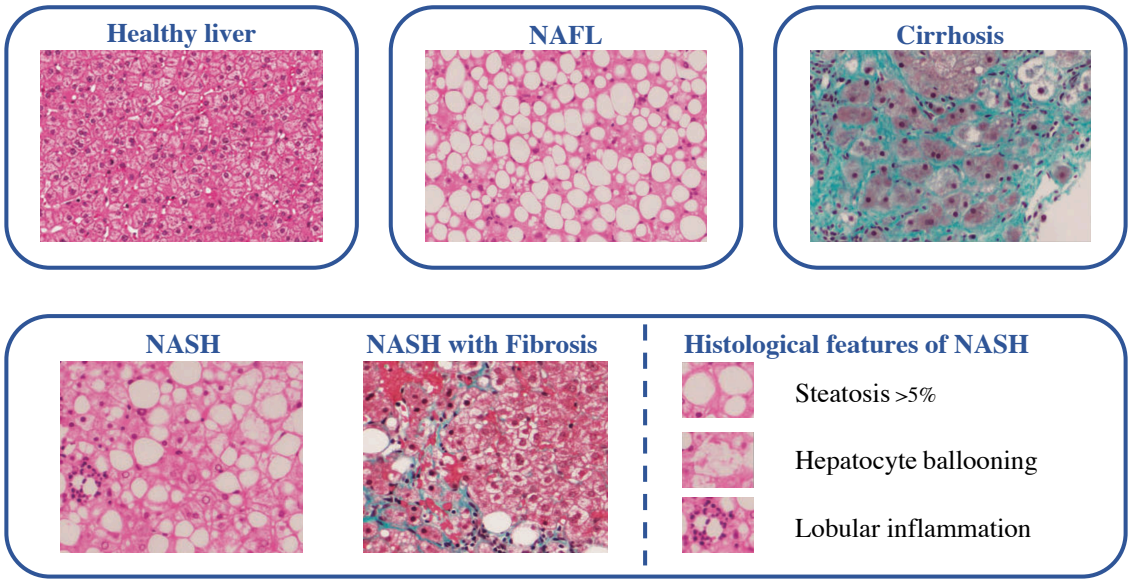

Figure 2. Histologic Features of NAFLD (Sheka et al., 2020).

Table 3. Available multiparametric panels for diagnosing Steatosis or for staging Fibrosis in Patients with NAFLD *(Castera et al., 2019)*.

Index	Age	Sex	BMI	DM	Platelet count	AST level	ALT level	AST/ALT ratio	GGT level	TG level	Other components
Steatosis											
Fatty Liver Index			X						X	X	Waist circumference
Hepatic Steatosis Index			X	X				X			---
SteatoTest	X	X	X				X		X	X	A2M, ApoA1, haptoglobin, total bilirubin, cholesterol, and glucose
Lipid Accumulation Product		X								X	Waist circumference
Index of NASH		X					X			X	Waist-to-hip ratio (only male), and HOMA-IR
NAFLD liver fat score				X				X			Metabolic syndrome and insulin
Fibrosis											
APRI					X	X					---
FIB-4	X				X	X	X				---
FibroTest	X	X	X						X		A2M, ApoA1, haptoglobin and total bilirubin
Fibrometer NAFLD	X				X	X	X				Glucose, ferritin, and body weight
Enhanced Liver Fibrosis score											Hyaluronic acid, PIINP and TIMP-1
Hepacore	X	X							X		A2M, hyaluronic acid and total bilirubin
BARD score			X	X				X			---
NAFLD fibrosis score	X	X		X	X			X			Albumin

BMI (body max index), DM (Diabetes mellitus), AST (Aspartate Aminotransferase), ALT (Alanine Aminotransferase), GGT (γ-glutamyl transferase), TG (triglyceride), A2M (α-2-macroglobulin), ApoA1 (apolipoprotein A1), HOMA-IR (homeostasis model assessment of insulin resistance)

2.2.2. Non-invasive methods

The non-invasive methods available to date are based on two different approaches: one is based on the quantification of serum biomarkers and the other is based on the liver stiffness measurement using either ultrasound- or magnetic-resonance-based elastography techniques *(Castera et al., 2019)*. Several of these techniques might be useful as an alternative to biopsies and assist patients´ follow-up *(Drescher et al., 2019)*.

Serum Biomarkers

During the last decades, different serum biomarkers and clinical parameters have been associated with steatosis or liver fibrosis *(Castera et al., 2019)*. On this basis, a great variety of multiparametric panels and parameter combinations considering serum markers, patient characteristics or comorbidities have been established *(Drescher et al., 2019)*.

Table 3 summarise the current available multiparametric panels. The advantages of analysing serum biomarkers and clinical parameters include their high applicability, their good interlaboratory reproducibility, and their potential widespread availability *(Castera et al., 2019)*. However, none of these multiparametric panels are liver-specific and all of them have limitations and alone are not suitable to replace liver biopsy *(Drescher et al., 2019)*. There are currently no highly sensitive and specific blood tests available to differentiate NASH from NAFL *(Castera et al., 2019)*. Also, there is no specific serum marker to assess hepatic steatosis available *(Drescher et al., 2019)*.

Imaging Techniques

Hepatic fibrosis and hepatic steatosis are two parameters of great importance in NAFLD. Both can be evaluated using image techniques. We can evaluate the hepatic fibrosis using elastography. The hepatic steatosis can be evaluated using ultrasonography or Controlled Attenuation Parameter. The measurement of these parameters with imaging techniques is explained in more detail below.

Elastography is more accurate than other image techniques to evaluate hepatic fibrosis. It is more useful to exclude fibrosis than to confirm it *(Andronescu et al., 2018)*. There are two different elastography techniques: ultrasound- or magnetic resonance-based. The first one uses ultrasound to detect the velocity of the microdisplacements (shear waves) induced in the liver tissue, whereas the other uses the magnetic resonance scanner. The result of them is a liver stiffness measurement, expressed in kilopascals (kPa) or in meters per second. Neither of these modalities has been able to reliably discriminate NASH from NAFL *(Castera et al., 2019)*.

Transient elastography (TE) was the first commercially available ultrasound based elastography technique developed for the measurement of liver stiffness using a dedicated device (FibroScan®, Echosens, Paris, France) *(Castera et al., 2019; de Lédinghen and Vergniol, 2010)*. One of the major advantages of the FibroScan® is that it is a non-invasive method that can instantly and directly measure the elasticity of the liver. It is a simple and low-cost device, that can be used by non-physicians after a short training period *(Sandrin et al., 2003)*.

The FibroScan® (Figure 3) is composed of a probe, a dedicated electronic system, and a control unit. The probe contains a low-frequency vibrator (50 Hz). A single ultrasonic transducer is mounted on the axis of the vibrator and operates at 5 MHz. The single ultrasonic transducer has two functions. It is used as an ultrasonic emitter and receiver and also as a low-frequency-piston-like vibrator to generate a transient vibration. A total of 256 radiofrequency lines are acquired at a repetition frequency of 4000 Hz while a low-frequency elastic wave (shear wave, one period of 1-mm amplitude sinusoid) is sent through the liver by the vibrator (Figure 4). Elasticity is derived from the velocity of the low-frequency elastic wave *(Sandrin et al., 2003)*.

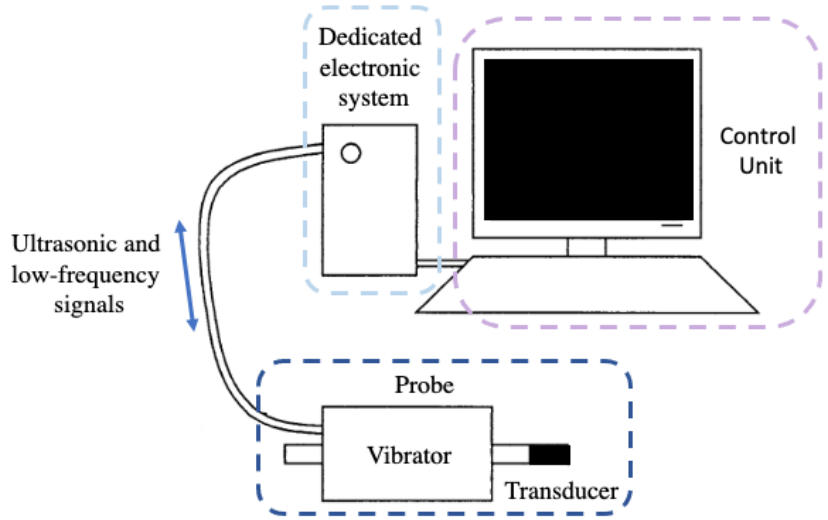

Figure 3. Composition of Fibroscan® (Sandrin et al., 2003)

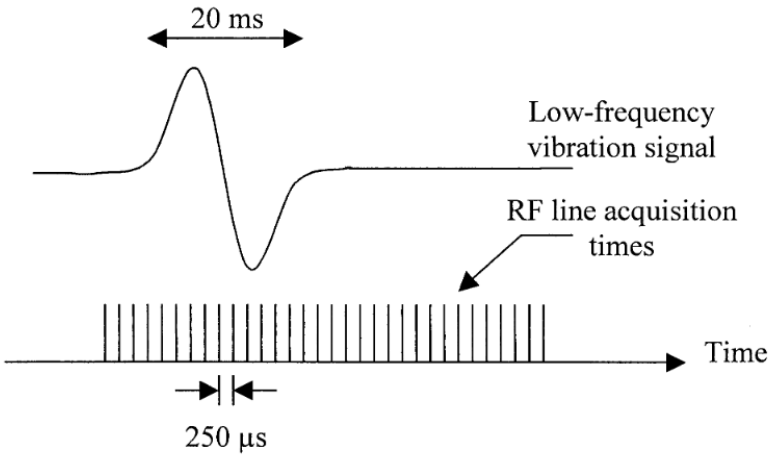

Figure 4. Acquisition sequence (Sandrin et al., 2003). RF (radiofrequency).

The velocity of the measured shear wave is directly related to tissue stiffness, called the elastic modulus, expressed as: *Elastic modulus= 3x(density of tissue)x(shear velocity)x2*. The density of tissue, in our case the liver, is assumed to be constant. *(Castera et al., 2019)*. The stiffer the liver, the faster the shear wave propagates *(Castera et al., 2019; de Lédinghen and Vergniol, 2010)*.

As shown in Figure 5, measurements are performed in the right lobe of the liver through intercostal spaces on fasting patients lying in dorsal decubitus with the right arm in

maximal abduction. TE measures liver stiffness in a volume that approximates a cylinder 1 cm wide and 4 cm long. This volume is at least 100-times bigger than a liver biopsy sample, and this is why it can be considered as more representative of the hepatic parenchyma *(de Lédinghen and Vergniol, 2010)*. The depth of the measurement is between 25 and 65 mm below the skin surface using the M-probe (standard probe) *(Castera et al., 2019; de Lédinghen et al., 2017; de Lédinghen and Vergniol, 2010)* and between 35 and 75 mm using the XL-probe *(Castera et al., 2019; de Lédinghen et al., 2017)*.

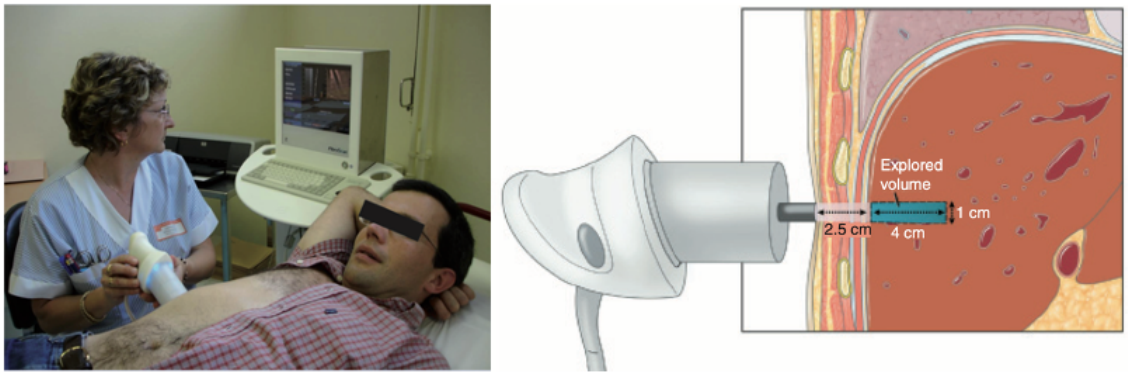

Figure 5. Liver stiffness measurement using FibroScan (de Lédinghen and Vergniol, 2010).

As suggested by the manufacturer, 10 successful measurements should be performed on each patient. The median of these measurements is displayed and used for interpretation. Results are expressed in kPa, and range from 2 to 75 kPa *(Castera et al., 2019)*. Values lower than 5.3 kPa should be considered as normal *(de Lédinghen and Vergniol, 2010)*. A cut-off value of 7.9 kPa has been established for severe fibrosis (sensitivity 91%, specificity 75%, positive predictive value 52% and negative predictive value 95%). Liver stiffness is not affected by hepatic steatosis, necroinflammation or BMI *(de Lédinghen and Vergniol, 2010)*.

Ultrasonography is the most used imaging technique for the diagnosis of hepatic steatosis because it is widely available, well established, well tolerated, and cheap. European guidelines for the management of NAFLD recommend using ultrasonography as first-choice imaging in adults at risk for NAFLD, as it provides additional diagnostic information *(Castera et al., 2019; Marchesini et al., 2016)*. Typical ultrasonography features are hyperechogenicity compared to the right kidney parenchyma, distal attenuation, and the presence of areas of focal sparing *(Castera et al., 2019; Khov et al., 2014)*. The degree of steatosis can be subjectively scored as light, moderate, and severe. Ultrasonography has the limitation that it can only detect steatosis with >2.5%-20% liver fat content and, therefore, a relevant number of patients with steatosis can be missed *(Castera et al., 2019)*.

Controlled Attenuation Parameter (CAP) has been proposed for the non-invasive grading of hepatic steatosis using TE (FibroScan® 502 Touch, Echosens, Paris, France). CAP establish the degree of ultrasound attenuation by hepatic fat *(Castera et al., 2019)*.

The CAP measure takes place on the same liver volume and with the same signal while liver stiffness is measured by FibroScan® *(Castera et al., 2019; Sasso et al., 2010)*. The obtained measurements are processed using a proprietary and sophisticated algorithm to determine whether the acquisition is “valid”, when the shear wave propagates into the

liver, or “invalid”, when the shear wave propagates not completely into the liver or into other organs. FibroScan® only gives the results that have been classified as valid, ensuring that only CAP values of the liver are obtained. Since we are using this guided process, the measurement can be performed by an operator without ultrasound training (de Lédinghen et al., 2017; Sasso et al., 2010).

CAP results are expressed in decibels per meter (dB/m) and range from 100 to 400 dB/m *(Castera et al., 2019; de Lédinghen et al., 2017)*. CAP has been initially only available with the M-probe but has recently also become available with the XL-Probe. CAP can diagnose steatosis, but it cannot accurately differentiate adjacent degrees of steatosis *(Castera et al., 2019; Marchesini et al., 2016)*. CAP is not influenced by liver fibrosis and cirrhosis and it has the ability to quantify and detect hepatic steatosis from 10% of liver fatty infiltration *(Drescher et al., 2019; Sasso et al., 2010)*. A cut-off value of about 300 dB/m has been established as an optimal cut-off for detection of ≥ 5% fat in the liver *(Castera et al., 2019; de Lédinghen et al., 2017)*. The cut-off associated with significant steatosis (>33% of hepatocytes) is >250 dB/m *(Castera et al., 2019)*.

2.3. Alcohol consumption

Alcohol is consumed worldwide but the majority of people in the world abstained from alcohol in the past 12 months *(World Health Organization, 2018)*. This research focuses mainly on the consequences of alcohol consumption in a specific subgroup of German population: NAFLD patients. As there are no available specific statistics on alcohol consumption in this subgroup, the following paragraphs summarises the levels of alcohol consumption, including abstention rates, in Europe and Germany.

The World Health Organization (WHO) estimates that 43% of the world´s population (approximately 2348 billion people) are current drinkers. The term **current drinker** refers to a person who consumes alcohol, regardless of the quantity. Not consuming alcohol is named **abstinence**. Two terms are used in this study to define these subjects depending on when they have stopped drinking. **Lifetime abstainers** are people who have never consumed alcohol and **recent abstainers** are people who previously consumed alcohol but now are abstainers. Table 4 resumes the actual percentage of abstainers in Germany and in the European WHO-Region.

Table 4. Proportion of abstainers in Germany (≥ 15 years old), 2016 *(World Health Organization, 2018)*.

	Germany			European
	Males	Females	Both sexes	WHO Region
Lifetime abstainers	3.7%	12%	7.9%	23.5%
Recent abstainers	7.8%	17.4%	12.7%	16.6%
Total abstainers	11.5%	29.4%	20.6%	40.1%

Since 2000, the proportion of drinkers in the world has decreased by almost 5%. In the same period, this proportion in the European WHO-Region has decreased by about 10%. Despite this, the European WHO-Region still belongs to one of the three WHO-Regions where more than half of the population consumes alcohol (59.9%). The highest alcohol consumption per capita is observed in the WHO-European Region with an average of nearly 21.3 g/day, while the average alcohol consumption in Germany is about 26.5 g/day. Alcohol consumption in the WHO-European Region has shown a negative trend, falling by 5.4 g/day between 2005 and 2016. This downward trend can also be observed in Germany as shown in Figure 6. The most consumed alcoholic beverage in Germany is Beer (53%) followed by wine (28%) and spirits (19%) *(World Health Organization, 2018)*.

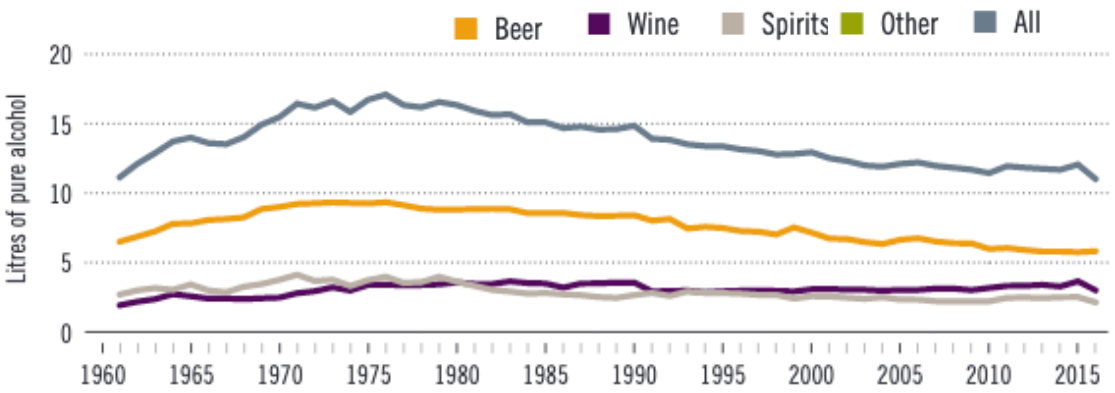

Figure 6. Recorded alcohol per capita (over 15 years old) consumption in Germany, 1961-2016 (World Health Organization, 2018).

2.4. Determination of alcohol consumption (I): Questionnaire

Information on the actual amounts of alcohol consumption can be collected with specifically designed questionnaires such as the Alcohol Use Disorders Identification Test (AUDIT). The AUDIT was developed by the WHO as a simple method of screening for excessive drinking *(Saunders et al., 1993)*.

The AUDIT questionnaire consists of 10 questions about alcohol consumption, drinking behaviour, adverse alcohol-reactions, and alcohol-related problems. It has been proved as a helpfully tool to identify hazardous alcohol use or possible alcohol dependence *(Niemelä, 2016; Saunders et al., 1993)*. Table 5 summarises the purpose of each question in the questionnaire *(World Health Organization, 2001)*.

Table 5. Domains and Item Content of the AUDIT *(World Health Organization, 2001)*.

Domains	Question Number	Item Content
Hazardous alcohol use	1	Frequency of drinking
	2	Typical quantity
	3	Frequency of heavy drinking
Dependence Symptoms	4	Impaired control over drinking
	5	Increased salience of drinking
	6	Morning drinking
Harmful alcohol use	7	Guilt after drinking
	8	Blackouts
	9	Alcohol-related injuries
	10	Others concerned about drinking

The AUDIT is easy to score. Each of the questions has a set of responses to choose from, and each response has a score ranking from 0 to 4 (Table 6). All the response scores should be added to obtain a result (World Health Organization, 2001). The maximum score is 40 points.

Scores of 8 or more are recommended as indicators of hazardous and harmful alcohol use, as well as possible alcohol dependence *(World Health Organization, 2001)*. When a cut-off of 8 or more is used the AUDIT hat a sensitivity of 97% and a specificity of 78% for detecting hazardous alcohol use, and a sensitivity of 95% and specificity of 85% for harmful use of alcohol *(Fiellin et al., 2000)*.

Based on experience gained from the use of the AUDIT, the WHO recommends the following interpretation to AUDIT scores: 8-15 medium level of alcohol problems, 16-19 high level of alcohol problems, 20 or above possible alcohol dependence *(World Health Organization, 2001)*.

When only the first 3 questions of AUDIT are used, it is denominated AUDIT-Consumption (AUDIT-C) questionnaire. It is scored from 0 to 12 points. With ≥ 4 points considered as the better threshold to identify hazardous drinking *(Reinert and Allen, 2007)*.

Table 6. The Alcohol Use Disorders Identification Test *(World Health Organization, 2001)*

		0	1	2	3	4
1	How often do you have a drink containing alcohol?	Never	Monthly or less	2 to 4 times a month	2 to 3 times a week	4 or more times a week
2	How many drinks containing alcohol do you have on a typical day when you are drinking?	1 or 2	3 or 4	5 or 6	7, 8 or 9	10 or more
3	How often during the last year did you have six or more drinks on one occasion?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
4	How often during the last year have you found that you were not able to stop drinking once you had started?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
5	How often during the last year have you failed to do what was normally expected from you because of drinking?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
6	How often during the last year have you needed a first drink in the morning to get yourself going after a heavy drinking session?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
7	How often during the last year have you had a feeling of guilt or remorse after drinking?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
8	How often during the last year have you been unable to remember what happened the night before because you had been drinking?	Never	Less than monthly	Monthly	Weekly	Daily or almost daily
9	Have you or someone else been injured as a result of your drinking?	No		Yes, but not in the last year		Yes, during the last year
10	Has a relative or friend or a doctor or another health worker been concerned about your drinking or suggested you cut down?	No		Yes, but not in the last year		Yes, during the last year

2.5. Determination of alcohol consumption (II): Biomarkers

Several studies have focused on finding objective ways to probe the information provided by patients regarding the amount and frequency of alcohol consumption. The found laboratory parameters have been called alcohol consumption biomarkers. This section presents an overview on the available alcohol biomarkers and their relevance in identifying alcohol consumption. At the end of this section, table 7 summarises the most important information of these biomarkers.

The liver enzymes gamma glutamyl transferase (GGT), aspartate aminotransferase (AST) and alanine aminotransferase (ALT); the mean corpuscular volume (MCV) and carbohydrate-deficient transferrin (CDT) are known as traditional biomarkers of alcohol consumption.

2.5.1. Gamma glutamyl transferase (GGT)

GGT is one of the most established biochemical biomarkers for excessive alcohol consumption *(Conigrave et al., 2003; Niemelä, 2016)*. It is a membrane bound glycoprotein enzyme situated on the cell membrane in several tissues such as liver, kidney, brain, spleen, pancreas and heart *(Conigrave et al., 2003)*. It supports the digestion and is involved in bile production *(Shayani et al., 2019)*. Normal GGT range is up to 45 U/l for females or up to 53 U/l for males *(Spiegel et al., 2008)*.

Even though many factors can lead to an elevated GGT, alcohol is the most common cause. This elevation varies between individuals and also according to the phase in their drinking history. It should be noted that GGT levels can fall in advanced cirrhosis *(Conigrave et al., 2003)*. A positive correlation between ethanol intake and serum GGT activity have been established in many studies. The minimal alcohol consumption required for having elevated GGT is about 60 g/week for women and 74 g/week for men. GGT levels typically rises after heavy alcohol intake that has continued for several weeks, rather than episodic, heavy drinking *(Shayani et al., 2019)*. GGT levels become elevated after 24 hours of heavy alcohol consumption *(Spiegel et al., 2008)* and generally return to normal reference range in 2-6 weeks after abstinence, as half-life of GGT is 14-26 days *(Shayani et al., 2019; Spiegel et al., 2008)*. Consequently, GGT is used regularly both clinically and in research to monitor response to treatment or as an indicator of chronic consumption of alcohol *(Shayani et al., 2019; Conigrave et al., 2003)*.

It is worth to mention that this test has a high rate of false positive results. GGT can be elevated without alcohol consumption in non-alcoholic liver diseases, obesity, pancreatitis, prostate diseases, diabetes, hypertension, hypertriglyceridemia or smoking *(Shayani et al., 2019; Peterson, 2004; Conigrave et al., 2003)*. A wide range of medications affect GGT, particularly those that induce the microsomal enzymes (e.g., anticonvulsants, non-steroidal anti-inflammatories and hormones) *(Conigrave et al., 2003)*.

2.5.2. Serum Aminotransferases (AST and ALT)

AST and ALT were previously known as glutamic oxaloacetic transaminase and glutamic pyruvic transaminase respectively. They are hepatocellular enzymes involved in amino acid metabolism *(Conigrave et al., 2003)*. ALT is found predominantly in the cytosol,

whereas AST activity is highest in the mitochondria *(Shayani et al., 2019; Conigrave et al., 2003)*. AST is present in greatest concentration in the liver, but it also can be found in heart, muscle, kidney, brain, pancreas, lung, leucocytes, and erythrocytes. ALT is found predominantly in hepatocytes and it is less affected by non-hepatic damage *(Niemelä, 2016; Conigrave et al., 2003)*. Consequently, ALT is more specific to alcohol induced liver cell injury compared to AST. Serum amino transferases are good indicators of liver disease when interpreted together *(Shayani et al., 2019)*.

Alcohol is the most common cause of ALT elevation in otherwise healthy people and thus the aminotransferases are usually used in clinical and research context as indicators of hepatic damage from chronic excessive drinking *(Andresen-Streichert* et al., 2018)*. A positive correlation between ethanol intake and ALT/AST ratio have been established in many studies. When aminotransferases are elevated, if the AST/ALT ratio is greater than 2, 90% of the cases are related to alcohol consumption *(Spiegel et al., 2008; Conigrave et al., 2003)*. This increases above 96% if the ratio is > 3 *(Spiegel et al., 2008)*. The minimal alcohol consumption required to elevate aminotransferases is about 40 g/day *(Spiegel et al., 2008)*. Aminotransferases are not increased by a single episode of excessive drinking *(Shayani et al., 2019)*. The levels become elevated after 3 to 7 days of heavy alcohol consumption *(Spiegel et al., 2008)*. ALT/AST generally returns to normal reference range in 2-4 weeks after abstinence *(Jastrzębska et al., 2016)*, as the half-life of AST and ALT are 14-24 hours and 27-57 hours, respectively *(Spiegel et al., 2008)*.

In alcohol dependent patients the elevated aminotransferases reflect liver damage. Because of this, the levels of these enzymes can remain elevated in recent abstinent patients with remaining chronic liver disease *(Shayani et al., 2019)*. When the disease progresses to the state of liver failure the aminotransferases in serum are likely to fail *(Conigrave et al., 2003)*. ALT levels can also increase in extrahepatic conditions such as type 2 diabetes, obesity, weight gain, metabolic syndrome, and insulin resistance *(Shayani et al., 2019; Conigrave et al., 2003)*. Almost any medication can raise aminotransferase levels *(Conigrave et al., 2003)*. Elevated AST/ALT ratios have also been reported from NASH patients with a high fibrosis risk *(Niemelä, 2016)*.

2.5.3. Mean Corpuscular Volume (MCV) of red blood cells

MCV is an index of the average volume of the erythrocytes. When the volume exceeds 94 fl is defined as macrocytosis *(Spiegel et al., 2008)*. Regular alcohol drinking leads to increase in the size of red blood cells *(Shayani et al., 2019)*. Because of this, elevated MCV is the most typical morphologic abnormality associated with excessive alcohol consumption and is often found in persons with alcoholism *(Spiegel et al., 2008)*. Sustained and regular excessive alcohol drinking above 40 g/day appears to be needed to result in elevated MCV levels in absence of other causes of MCV elevation such as folate deficiency, liver disease or bleeding *(Spiegel et al., 2008; Conigrave et al., 2003)*. MCV increases with regular excessive alcohol intake after four to eight weeks. As the life-span of a red blood cell is 120 days, the return of MCV to its normal size can last within two to four months after a person has stopped drinking *(Shayani et al., 2019; Niemelä, 2016; Spiegel et al., 2008; Conigrave et al., 2003)*.

Because patients with disorders unrelated to alcohol use can have elevated MCV, it is not a useful alone as a screening marker for alcohol abuse *(Spiegel et al., 2008)*. Because of its slow response to changes in drinking, MCV is generally unsuitable as a marker of

short-term progress in alcohol consumption *(Conigrave et al., 2003)*. MCV lacks sensitivity when used individually and has limited specificity, as false positive results can be seen in cigarette smokers, liver diseases, vitamin B12 or folic acid deficiency, thyroid disease, various haematological diseases, or in anaemia *(Shayani et al., 2019)*. Additionally, because macrocytosis can persist under strictly controlled alcohol abstinence, MCV is not a reliable clinical indicator of relapse *(Spiegel et al., 2008)*.

2.5.4. Carbohydrate-deficient Transferrin (CDT)

Transferrin is a glycoprotein produced and secreted by the liver *(Staufer and Yegles, 2016)*. Transferrin´s main task is the transport of iron in the body. Normal individual´s transferrin contains four to six sialic acid molecules. Alcohol consumption interferes with the ability of sialic acids to attach to transferrin and causes deficiency of sialic acid content in transferrin, because of this the biomarker is named carbohydrate-deficient transferrin *(Shayani et al., 2019)*. The most accurate way to express CDT level is a percentage of total transferrin concentration *(Spiegel et al., 2008)*. CDT levels above 2.5% of total transferrin concentration are considered in an abnormal range *(Spiegel et al., 2008)*.

The formation of CDT requires an alcohol intake of more than 50 g/day over at least 1 to 2 weeks *(Andresen-Streichert* et al., 2018; Niemelä, 2016; Spiegel et al., 2008)*. After complete abstinence, CDT normalises in two to four weeks *(Shayani et al., 2019; Staufer and Yegles, 2016)*. Consequently, elevated CDT levels are used as a screening marker for continuous heavy drinking pattern *(Staufer and Yegles, 2016; Spiegel et al., 2008)*.

CDT is as a sensitive marker to detect relapse in alcohol dependent people *(Shayani et al., 2019)*. It should be noted that individuals with moderate consumption or episodic drinking pattern could show CDT levels within normal range *(Andresen-Streichert* et al., 2018)*. It is also worth mentioning that studies have found some patients with heavy drinking history who did not show elevated levels of CTD, so the false negative rate of this biomarker should not be underestimated. Nevertheless, CDT shows better performance than other traditional biomarkers *(Shayani et al., 2019)*.

False positive results could be found in patients with liver cirrhosis, primary biliary cirrhosis, hepatitis C infection, smoking, sepsis, anorexia nervosa, or airway diseases *(Staufer and Yegles, 2016)*.

Table 7. Alcohol consumption biomarkers *(Shayani et al., 2019; Andresen-Streichert* et al., 2018; Jastrzębska et al., 2016; Staufer and Yegles, 2016; Cabarcos et al., 2015; Nanau and Neuman, 2015; Spiegel et al., 2008; Peterson, 2004; Conigrave et al., 2003)*.

Biomarker	Source	Se (%)	Sp (%)	Cut-off	MAP	Time to elevation	Window of detection	Drinking Behaviour
Traditional biomarkers								
GGT	Serum/Plasma	37-95	18-93	♂: 53 U/l ♀: 45 U/l	♂: 74 g/week ♀: 60 g/week	24h to 2 weeks	2-6 weeks	Chronic heavy drinking
ALT/AST	Serum/Plasma	15-69	50-95	2	≥ 40 g/day	3-7 days	2-4 weeks	Chronic heavy drinking
MCV	Blood	40-50	80-90	94 fl	≥ 40 g/day	4-8 weeks	8-16 weeks	Chronic heavy drinking
CDT	Serum/Plasma	45-90	70-100	2.5 %	> 50 g/day	1-2 weeks	2-4 weeks	Chronic heavy drinking
Novel biomarkers								
EtOH	Exhalation air	ND	ND	ND	ND	20-35 minutes	10-12 h	Acute alcohol intoxication
	Serum	ND	ND	0.1 g/kg	ND	45 minutes	10-12 h	Acute alcohol intoxication
	Urine	ND	ND	ND	ND	120 minutes	10-12 h	Acute alcohol intoxication
MeOH	Serum	70	98	5 mg/l	ND	45 minutes	Up to 48 h	Recent alcohol consumption
EtG	Blood	ND	ND	ND	1-2 SD	45 minutes	Up to 36 h	Recent alcohol consumption
	Urine	73-75	55-60	0.3 mg/l	~0.1g/kgBW	60 minutes	Up to 5 days	Recent alcohol consumption
	Hair	76	91	7 pg/mg	>20 g/day	ND	3-6 months	Regular alcohol consumption
	Hair	70-90	80-95	30 pg/mg	>60 g/day	ND	3-6 months	Heavy alcohol consumption
PEth	Blood	88-100	48-89	20 ng/ml	28 g/day SDW	90-120 minutes	2-6 weeks	Regular alcohol consumption
	Blood			200 ng/ml	56 g/day SDW			Heavy alcohol consumption

Se: sensitivity, Sp: specificity, MAP: minimal alcohol consumption to positivity, ND: no data, SD: standard drink. g/kgBW: g/kg body weight, SDW: several days per week

Novel alcohol consumption biomarkers are labour parameters that could be determined in biological samples to detect the presence or absence of alcohol as well as alcohol metabolites, which remain longer in the body within a specific window time. Alcohol and its metabolites become present in the body primarily by being absorbed into the bloodstream and then distributed to other matrices via mechanisms such as passive diffusion and ultrafiltration *(Baxter et al., 2017)*.

Blood, breath, urine, and hair are the biological samples commonly used in alcohol testing. The use of one type of biological sample or another will depend on the window of detection of the alcohol consumption of interest in each case. The window of detection of each biological sample can be found in table 8. The longest windows of detection occur in hair, followed by urine, and finally breath and blood *(Baxter et al., 2017)*.

Table 8. General windows of detection across biological samples *(Baxter et al., 2017)*.

	Minutes	Hours	Days	Weeks	Months
Blood	X	X			
Breath	X	X			
Urine		X	X		
Hair				X	X

Maximum detection time should not be de only criteria for choosing a biological sample. Table 9 resumes advantages and disadvantages of the different biological samples.

Table 9. Comparing biological samples *(Baxter et al., 2017)*.

	Blood	Breath	Urine	Hair
Window of detection	1-48 hours	~1h per SD	1-3 days	7-100 days
Primarily detection	Blood alcohol concentration	Blood alcohol concentration	Alcohol metabolite	Alcohol metabolite
Best use	Acute impairment or intoxication	Acute impairment or intoxication	Intermediate term detection	Long-term monitoring (3 months)
Collection	Requires staff trained in phlebotomy	Easily collected	Requires specialized collection facility	Easily collected
Resistance to tampering	High	High	Low	High*
Retesting same sample	Difficult	Generally not possible	Possible	Easy

SD: standard drink. *when chemical untreated

2.5.5. Ethanol (EtOH)

After ingestion of alcohol, EtOH is absorbed via oral, gastric, and small intestinal mucosa. About 2-10% of EtOH is excreted via urine, sweat, and exhalation without being modified. A major part of the consumed EtOH is metabolized to acetaldehyde within the liver especially in case of > 50 grams of alcohol intake or chronic alcohol intake. Acetaldehyde is further metabolized to acetic, which via the citrate cycle and the mitochondrial respiratory chain and is exhaled as CO_2 *(Staufer and Yegles, 2016)*. A

problem is that the ingested ethanol is cleared fairly rapidly from the body at a rate of approximately 0.1 g/kg/h, primarily due to metabolism in the liver, with even more rapid elimination noted in heavy drinkers *(Helander and Eriksson, 2002)*.

EtOH can be detected in **exhaled air**. In the context of EtOH testing, a breath test represents the amount of EtOH present in exhaled breath, which is diffused into the air held in the lungs from pulmonary capillary blood. Bread EtOH concentration can then be used to estimate blood EtOH concentration *(Baxter et al., 2017)*. In volunteer social drinkers, a peak was identified between 20-35 minutes after alcohol ingestion *(Nanau and Neuman, 2015)*. Breath EtOH concentration correlates well with blood EtOH concentration *(Baxter et al., 2017; Staufer and Yegles, 2016)*. Because a person weighting 70 kg can eliminate about 7g of pure EtOH per hour, even after heavy intake of substantial amounts alcohol (corresponding to at least 60-80 g) EtOH will not be detectable after 9-10h *(Helander et al., 1999; Helander and Eriksson, 2002)*. Due to this rapid elimination of EtOH from the body, breath EtOH testing might not be an ideal tool for monitoring abstinence or early detection of relapse. This test is mainly used in the context of drivers accused of driving under the influence of alcohol and needs to be confirmed by a blood EtOH test if positive *(Staufer and Yegles, 2016)*.

EtOH can also be detected in **blood**. EtOH blood concentration is a parameter for recent alcohol consumption and remains positive for only a few hours after alcohol intake. Because of this, blood alcohol level is a useful marker in cases of suspected alcohol intoxication *(Staufer and Yegles, 2016)*.

It is technically possible to perform a direct measurement of EtOH in **urine**. Because of the short detection window in urine of approximately 10-12 hours it is recommended to measure instead other alcohol metabolites in urine in order to achieve a wide detection window *(Baxter et al., 2017)*.

2.5.6. Methanol (MeOH)

MeOH is an alcohol that is present in all alcoholic beverages to a greater or lesser proportion and some MeOH is also formed endogenously *(Roine et al., 1989)*. EtOH and MeOH are oxidized by the same alcohol dehydrogenase enzyme *(Helander and Eriksson, 2002)*, but the affinity for oxidation of EtOH is 10-fold higher than for the oxidation of MeOH. Consequently, EtOH inhibits the oxidation of MeOH and thus the presence of EtOH leads to the successively accumulation of MeOH in blood *(Helander and Eriksson, 2002; Roine et al., 1989)*. Because of the higher affinity of EtOH for the alcohol dehydrogenase and its much higher concentration in blood, MeOH oxidation does not begin until the EtOH level sinks bellow 0.2g/kg *(Haffner et al., 1997)*. Consequently, alcoholics and heavy continuously drinkers are likely to have higher MeOH concentration in blood than non-alcoholics *(Haffner et al., 1997; Roine et al., 1989)*.

MeOH is detectable in blood for up to 2 days but, as explained before, may accumulate in body fluids *(Staufer and Yegles, 2016)*. Several studies used a concentration of 5 mg/l as a threshold to differentiate between patients with and without alcohol dependence with specificity of 98% *(Andresen-Streichert* et al., 2018)*. It is also worth mentioning that MeOH can also be endogenously produced and might show false positive results *(Staufer and Yegles, 2016)*.

2.5.7. Ethyl glucuronide (EtG) in blood, urine (uEtG) and hair (hEtG)

EtG is a direct metabolite of EtOH *(Baxter et al., 2017; Shayani et al., 2019)*. A very small amount (<0.1%) of the consumed EtOH undergoes conjugation reactions with glucuronic acid to produce EtG *(Nanau and Neuman, 2015; Staufer and Yegles, 2016)*. Glucuronic acid is a substance which works to detoxify drugs by turning them into water-soluble compounds that can be easily removed from the body *(Peterson, 2004)*.

It is considered a direct biomarker of alcohol consumption because EtG is only detected when alcohol is consumed. Although rare, it is possible that exposure to ethanol-containing products, such as hand sanitizer, to result in a positive EtG test *(Baxter et al., 2017)*. EtG can be detected in body fluids shortly after alcohol intake up to 80 hours after the complete elimination of alcohol from the body *(Shayani et al., 2019)*. When people test positive for EtG, it is likely that they have consumed alcohol recently, even if there is no EtOH left in their bodies *(Jastrzębska et al., 2016; Cabarcos et al., 2015; Nanau and Neuman, 2015)*. This makes EtG especially useful for detecting drinking relapses *(Jastrzębska et al., 2016)*.

EtG can be detected in **blood** not long after alcohol consumption (<45 minutes) *(Andresen-Streichert* et al., 2018)*. In case of chronic alcohol consumption, EtG peaks 2 to 3.5 hours later in blood and remains in blood up to 36 hours. Measurements in blood are capable of detecting early stages of relapse *(Shayani et al., 2019)*.

uEtG

As the kidneys filter the bloodstream, waste and other by-products, including metabolites, are extracted and eliminated along with water from the body as urine *(Baxter et al., 2017)*. EtG is excreted in urine not long after alcohol consumption (<60 minutes) *(Andresen-Streichert* et al., 2018)*. The uEtG is a test sensitive to recent alcohol consumption of any amount *(Ulwelling and Smith, 2018)*. uEtG concentration peaked approximately 4 hours after EtOH intake *(Shayani et al., 2019)*. Elimination of EtG through the urine represents approximately 0.02-0.06% of the ingested EtOH dose *(Cabarcos et al., 2015)*. uEtG can be detected for up to about 24 hours even after consumption of small quantities of alcohol (~0.1 g/kg body weight), extending it up to 130 hours in case of heavy consumption *(Shayani et al., 2019; Ulwelling and Smith, 2018; Andresen-Streichert* et al., 2018; Baxter et al., 2017; Staufer and Yegles, 2016)*. The longer detection time compared with blood makes uEtG a more sensitive biomarker of recent drinking. Cut-offs of between 0.01 mg/dl and 0.05 ng/dl are recommended to detect heavy drinking *(Shayani et al., 2019)*. uEtG do not allow to distinguish between binge drinking event several days ago and a minor alcohol intake a few hours before the sample was taken *(Andresen-Streichert* et al., 2018)*.

False positive und false negative results may be considered. False negative results might occur in the presence of bacterial degradation in case of urinary tract infections but also might occur because of post collection synthesis of bacteria in the urine. False positive results can be caused by cannabinol, ingestion of high amounts of baker´s yeast, sauerkraut, non-alcoholic beer or alcohol containing mouth washes *(Staufer and Yegles, 2016)*. The intake of larger volume of water results in a steep decrease in uEtG levels and may lead to false negative results. It is important to interpret uEtG levels based on the

urine creatinine levels or to state at least a minimum requirement (usually > 20 mg/dl) *(Andresen-Streichert* et al., 2018)*.

hEtG

EtG can also be detected in hair and thus can help in evaluation of chronic ethanol consumption *(Shayani et al., 2019)*. Hair can be thought as a continuous collection device which absorbs compounds as blood passes through the hair follicle and as sweat gathers and is absorbed around the base of a growing hair shaft. Scalp hair is the most commonly test sample. Scalp hair provides a window of detection of approximately 3 to 6 months *(Baxter et al., 2017)*. Due to the wide detection window short-term reduction in alcohol consumption has no effect on test results *(Andresen-Streichert* et al., 2018)*.

According to the recommendations of the Society of Hair Testing, subjects with hEtG concentrations of <7 pg/mg are regarded as abstinent or very rare drinkers, ≥ 7 pg/mg to 29 pg/mg strongly suggest regular alcohol consumption, and concentrations ≥ 30 pg/mg strongly suggest chronic excessive alcohol intake (≥ 60g/day). These cut-offs are valid for 0-3 cm up to 0-6 cm of proximal scalp hair segments *(Andresen-Streichert* et al., 2018; Kintz, 2015)*.

Chemical treatments such as dyeing, bleaching, perming, and straightening can alter the structure of hair and degrade the hEtG that may be present and give false negative or decreased results *(Baxter et al., 2017; Staufer and Yegles, 2016)*. If hair is collected, patients should be asked about their use of chemical hair treatments at the time of sample collection *(Baxter et al., 2017)*.

2.5.8. Phosphatidylethanol (PEth)

PEth is an abnormal phospholipid formed only in the presence of EtOH via a transphosphatidylation in the cell membrane of peripheral blood cells *(Staufer and Yegles, 2016)*. PEth is not a single molecule, there are several PEth molecule species or homologues. Each homologue contains the same glycerophospholipid central chain, plus two side chains of varying long-chain carboxylic acids *(Andresen-Streichert* et al., 2018; Ulwelling and Smith, 2018; Cabarcos et al., 2015)*. At the present time, over 40 PEth species have been identified in human blood samples *(Hakim et al., 2019; Andresen-Streichert* et al., 2018)*. The most abundant species of PEth in abusive drinkers are 16:0/18:1 and 16:0/18:2, constituting 40% and 20% of total PEth in total blood, respectively *(Hakim et al., 2019; Cabarcos et al., 2015)*. For the detection of alcoholic beverages, the PEth homologue 16:0/18:1 blood level is generally reported as the most sensitive biomarker (sensitivity 86% and specificity 100%) *(Hakim et al., 2019)*.

PEth production begins as soon as EtOH is consumed and peaks within 90 to 120 minutes after alcohol ingestion *(Hakim et al., 2019; Ulwelling and Smith, 2018; Andresen-Streichert* et al., 2018)*. Its elimination half-life in blood is long (3 to 7 days) because of its slow degradation rate and therefore theoretically detectable in blood up to 21-28 days after alcohol consumption. A large detection window up to 3 weeks has also be reported *(Cabarcos et al., 2015; Hakim et al., 2019)*. PEth is found suitable as a biomarker to determine both, currently alcohol consumption and abstinence *(Andresen-Streichert* et al., 2018)*. PEth is also proposed as a long-term alcohol parameter. PEth becomes positive after repeated consumption of ≥ 50 g/day EtOH over a period of 2 to 3 weeks and can be

detected up to 2 weeks after cessation of alcohol consumption, in some cases up to 6 weeks *(Shayani et al., 2019; Staufer and Yegles, 2016)*. Despite of this, PEth can be used to monitor alcohol consumption and can help to identify early signs of harmful alcohol consumption *(Shayani et al., 2019)*.

Currently, no cut-offs have been officially set, but several recommendations exist *(Cabarcos et al., 2015)*. In general, several studies agree on blood concentration of PEth 16:0/18:1 around 200 ng/ml to detect excessive consumers.

Another threshold of 20 ng/ml is usually found in the literature. To exceed this concentration of PEth in blood 21-28 g/day ethanol for women and 35 g/day ethanol for men are required *(Hakim et al., 2019)*. Studies have found no gender differences in the formation of PEth. The key factor seems to be differences in percentages of total body water rather than gender-based differences in the chemistry involved in PEth formation. Women generally have a higher percentage of body fat and correspondingly lower percentage of body water. As alcohol is insoluble in water, the number of drinks needed to obtain a blood alcohol concentration high enough to register a positive PEth will usually be lower for women than for men of the same weight *(Ulwelling and Smith, 2018)*.

A recent meta-analysis *(Ulwelling and Smith, 2018)* propose the following interpretation to 3 different thresholds:

- <20 ng/ml: Abstinence or light drinking (< 28g/day, several days a week)
- 20-200 ng/ml: moderate alcohol consumption (28-56g/day, several days a week). This range also encompasses the WHO "low risk" (males up to 40 g/day and females up to 20g/day) and "medium risk" (males up to 60g/day and females up to 40 g/day).
- >200 ng/ml: excessive alcohol consumption (> 56g/day, several days a week). This range englobes the WHO "high risk" (up to 60 g/day for males and up to 41 g/day for females) and "very high risk" (above 101g/day for males and more than 61 g/day for females).

PEth levels are not affected by gender or age, and in contrast to CDT, PEth is not influenced by the presence of severity of liver disease. This makes PEth a useful tool in monitoring heavy drinkers with hepatic pathology *(Shayani et al., 2019; Staufer and Yegles, 2016)*. PEth has been found to be relatively insensitive to incidental EtOH exposures, such as mouthwash and antibacterial hand sanitizers *(Ulwelling and Smith, 2018)*.

PEth has higher sensitivity and specificity compared to other traditional markers and helps to detect lower alcohol consumption *(Shayani et al., 2019)*. PEth is better than CDT to detect relapse, especially when it comes to quantities of alcohol that are not hight enough to elevate CDT *(Andresen-Streichert* et al., 2018; Shayani et al., 2019)*. PEth is not perfectly correlated with the two other direct tests for alcohol: breath alcohol concentration and uEtG. The lack of a higher correlation among these three direct markers is due to the different detection windows and the different dissipation rates of the measured substances. PEth can be detected for 3 to 4 weeks, long after the EtOH and uEtG metabolites have been metabolized *(Ulwelling and Smith, 2018)*.

2.6. Current research on NAFLD and moderate Alcohol consumption

In this section, the clinical results to date on the impact of alcohol consumption on NAFLD will be summarised.

There are no prospective, randomized trials on moderate alcohol consumption in NAFLD. The highest quality data comes from observational studies. Unfortunately, their conclusions are conflictive. Some studies showed that moderate alcohol consumption has beneficial effects in NAFLD development and progression, while others suggested deterioration of liver steatosis and fibrosis. In addition, there are also studies that found no effect of moderate alcohol consumption in NAFLD. Consequently, the effect of moderate alcohol consumption on NAFLD remains unclear, and further studies are still needed to draw conclusions that could be applied in the clinical practice.

We found 30 papers that were focused on studying the effects of alcohol consumption on NAFLD *(Blomdahl et al., 2021; Åberg et al., 2020; Kashiwagi et al., 2020; Chang et al., 2019; Hajifathalian et al., 2019; Kimura et al., 2018; Ajmera et al., 2018; Mitchell et al., 2018; Yamada et al., 2018; Patel et al., 2017; Hagström et al., 2017; Sogabe et al., 2016; Sookoian et al., 2016; Moriya et al., 2015; Kächele et al., 2015; Takahashi et al., 2015; Hashimoto et al., 2015; Kwon et al., 2014; Dunn et al., 2012; Hamaguchi et al., 2012; Hiramine et al., 2011; Moriya et al., 2011; Ascha et al., 2010; Yamada et al., 2010; Ekstedt et al., 2009; Cotrim et al., 2009; Gunji et al., 2009; Dunn et al., 2008; Suzuki et al., 2007; Dixon et al., 2001)*. The type of study, number of subjects involved and, when applicable, time of follow up in each study is summarised in Table 40 (Summary 9.2).

There is no standardised threshold for moderate alcohol consumption in NAFLD research. The most widely accepted threshold comes from the NAFLD definition itself. According to the current European guideline on NAFLD the cut-off to differentiate NAFLD and alcohol liver disease is ≥ 20 g/day for women and ≥ 30 g/day for men *(Marchesini et al., 2016)*. Because the lack of standardisation, various definitions of moderate alcohol consumption could be found in current publications. Table 41 (Summary 9.3) shows the different alcohol consumption groups used in the current literature. In general, moderate alcohol consumption groups do not exceed an intake of 30 g/day. Eight studies increase the threshold *(Patel et al., 2017; Hashimoto et al., 2015; Takahashi et al., 2015; Hamaguchi et al., 2012; Hiramine et al., 2011; Cotrim et al., 2009; Gunji et al., 2009; Suzuki et al., 2007)*, but some of them have an additional subgroup of light alcohol consumption oft defined as 0-20 g/day *(Hashimoto et al., 2015; Hamaguchi et al., 2012; Hiramine et al., 2011; Gunji et al., 2009; Cotrim et al., 2009; Suzuki et al., 2007)*. Lastly, 11 studies have also looked at the effect of heavy alcohol consumption in NAFLD *(Åberg et al., 2020; Hashimoto et al., 2015; Moriya et al., 2015; Kächele et al., 2015; Takahashi et al., 2015; Hamaguchi et al., 2012; Hiramine et al., 2011; Moriya et al., 2011; Yamada et al., 2010; Gunji et al., 2009; Suzuki et al., 2007)*.

The definition of NAFLD makes mandatory an exhaustively anamnesis regarding alcohol consumption to avoid misclassifications. A standardised tool to assess alcohol consumption in NAFLD does not exist. One problem in moderate alcohol consumption research is that the questionnaires at our disposal have been developed for detecting alcohol abuse and have not been validated to grade modest or moderate alcohol intake. Table 40 (Summary 9.2) summarises the methods used for assessing alcohol consumption in the current literature. Nine studies used a previous validated questionnaire to assess

alcohol consumption *(Åberg et al., 2020; Ajmera et al., 2017; Blomdahl et al., 2021; Chang et al., 2019; Dunn et al., 2012; Ekstedt et al., 2009; Hagström et al., 2017; Kwon et al., 2014; Patel et al., 2017)*, the others do not give detailed information or the information came from patients reports or from a simple questionnaire. Two studies used an alcohol consumption marker, PEth, to probe the information provided by the subjects *(Blomdahl et al., 2021; Hagström et al., 2017)*.

2.6.1. Evidence for protection

The actual evidence for protective effect of moderate alcohol consumption on NAFLD is summarised in table 42 (Supplementary 9.4).

The first paper on the effects of moderate alcohol consumption in NAFLD was a cross-sectional study published in 2001. It analysed the impact of alcohol consumption in 108 patients whose liver disease was diagnosed by laparoscopic liver biopsy taken during a planned surgery for severe obesity.
It concluded that moderate alcohol consumption was associated with decreased risk of NASH (odds ratio (OR) 0.35, 95% confidence interval (CI) 0.12-1, p=0.04). This effect was no longer significant after controlling for possible confounders such as diabetes or insulin resistance.
The authors suggested that moderate alcohol consumption seemed to reduce the risk of NAFLD possibly by reducing insulin resistance *(Dixon et al., 2001)*.

The second publication was in 2007. It was a cross-sectional study that analysed the association between alcohol consumption and elevated aminotransferase levels in 1,177 males without any form of chronic liver disease, undergoing annual check-ups with a follow-up of 5 years.
As expected, excessive alcohol consumption (≥ 280g/week) was associated with increased odds of hypertransaminasemia compared with none or minimal (0-70 g/week) alcohol consumption (adjusted odds ratio (aOR) 1.4, 95% CI 1.1-1.93, p=0.023). Surprisingly, in the younger group moderate alcohol consumption (140-280 g/week) was associated with decreased odds (aOR 0.5, 95% CI 0.3-0.9, p=0.032) of hypertransaminasemia, while the same amount of alcohol consumption was associated in the older group with increased odds (aOR 1.6, 95% CI 1.1-2.3, p=0.014). In the older group, only light alcohol consumption (70-140 g/week) was associated with decreased odds (aOR 0.6, 95%CI 0.4-1.0, p=0.036) of hypertransaminasemia.
During a 5-year follow-up of 326 subjects without NAFLD or altered liver enzymes at baseline, moderate alcohol consumption (140-280 g/week) was associated with decreased incidence of hypertransaminasemia (aOR0.4, 95% CI 0.1-0.9, p=0.02) versus non-drinkers or minimal alcohol consumption.
The authors concluded that light to moderate alcohol consumption may protect against development of hypertransaminasemia among males without other liver conditions *(Suzuki et al., 2007)*.

One study carried out in 5,599 asymptomatic Japanese men used ultrasound to assess hepatic steatosis. It found that light (40-140 g/week) and moderate (140-280 g/week) alcohol consumption significantly and independency reduced the likelihood of hepatic steatosis compared with abstinence (light: OR 0.82, 95% CI 0.68-0.99, p=0.044 and moderate: 0.75 95% CI 0.61-0.93, p=0.008) *(Gunji et al., 2009)*.

A cross-sectional study of 63,447 subjects that also had a longitudinal retrospective part which followed 10,424 subjects over 5 years used ultrasound to assess hepatic steatosis. They found that the prevalence of steatosis has an inverse association with the alcohol consumption ($p < 0.05$).
In the 5 years follow-up they found that the risk of newly developed hepatic steatosis was significantly lower in males with a daily moderate (aOR 0.72, 95% CI 0.58-0.89) or daily heavy alcohol consumption (aOR 0.65, 95% CI 0.50-0.85) than in non-drinkers.
They conclude that alcohol drinking may not be a major risk for fatty liver in Japanese undergoing health check-up *(Yamada et al., 2010)*.

Hamaguchi et al. conducted in 2012 a cross sectional-study in 18,571 Japanese asymptomatic subjects. They divided them into four groups according to the amount of alcohol consumption: none-minimal drinkers, light drinkers (40-140 g/week), moderate drinkers (140-280 g/week) and excess drinkers (above 280 g/week).
They found that the prevalence of hepatic steatosis decreased with light (OR 0.54, 95% CI 0.34-0.88, p=0.012) or moderate (OR 0.43 95% CI 0.21-0.88, p=0.021) alcohol consumption in women. Surprisingly, they found that the prevalence of hepatic steatosis also decreased in men regardless the amount of alcohol consumption (light: OR 0.69 95% CI 0.6-0.79, moderate: OR 0.72, 95% CI 0.63-0.83, excess: 0.74 95% CI 0.64-0.85, p<0.001) *(Hamaguchi et al., 2012)*.

Another study conducted by Moriya et al. showed results in the line with the previous study. They conducted a longitudinal study with a retrospective cohort of 5,297 Japanese asymptomatic subjects with follow-up from 2004 to 2006.
They found that the alcohol consumption of 0.1-69.9 g/week and 70-139.9 g/week in women was inversely associated with fatty liver after adjusting for obesity, exercise, and smoking (OR 0.71, 95% CI 0.52-0.96 and OR 0.67, 95% CI 0.45-0.98, respectively).
They also found that alcohol consumption was inversely associated with fatty liver regardless the amount of alcohol consumption in men, even after adjusting for obesity, exercise, and smoking (0.1-69.9 g/week: OR 0.79, 95%CI 0.68-0.90, 70-139.9 g/week: OR 0.73, 95% CI 0.63-0.84, 140-279.9 g/week: OR 0.69, 95% CI 0.60-0.79 and ≥ 280g/week: OR 9.68, 95% CI 0.58-0.79) *(Moriya et al., 2015)*.

One study was conducted in 8,029 patients and put the focus on the differences in alcohol consumption between obese (BMI ≥ 25 kg/m^2 in Japan) and non-obese patients.
Moderate alcohol consumption, defined as 20-50 g/day, was a significant negative risk factor for hepatic steatosis in obese subjects (OR 0.39 vs 0.74 in non-obese).
Not surprisingly, alcohol consumption above 50 g/day was a significant risk factor for hepatic steatosis in women (OR 3.35) and in non-obese man (OR 1.29), but it was a significant negative risk factor in obese males (OR 0.62).
They concluded that the influence of alcohol intake on fatty liver differed depending on the level of alcohol consumption, gender, and the presence of obesity, showing biphasic effects *(Takahashi et al., 2015)*.

A longitudinal retrospective study with a follow-up of 10 years carried out in 5,437 asymptomatic Japanese population used ultrasound to assess hepatic steatosis.
It found that the adjusted hazard risk of light (40-140 g/week) and moderate (140-280 g/week) alcohol consumption for development fatty liver in men were 0.72 (95% CI 0.60-0.86, p<0.001) and 0.69 (95% CI 0.57-0.84, p<0.001), respectively. However, they were no significant in women.

They concluded that the newly onset of fatty liver was significantly lower in apparently healthy men who consume light to moderate alcohol *(Hashimoto et al., 2015)*.

Another study was conducted in 432 subjects using ultrasound to assess hepatic steatosis. They found that the presence of NAFLD was markedly reduced in subjects drinking 0-20 g/day (19%), compared to non-drinkers (35%) and heavy drinkers (20-40 g/day: 34%, 40-60 g/day: 38,6%, >60g/day: 44.9%) *(Kächele et al., 2015)*.

Cohorts with **biopsy assessed** NAFLD were also used to define the role of modest alcohol consumption.
Dunn et al. conducted liver biopsies in 582 subjects and found that modest drinkers, defined as up to 20 g/day, had lower odds of having diagnosis of NASH (OR 0.56, 95% CI 0.39-0.84) compared to non-drinkers. Regarding the histological parameters, they found that modest drinkers had lower odds for fibrosis (OR 0.56, 95% CI 0.41-0.77) and ballooning hepatocellular injury (OR 0.66, 95% CI 0.48-0.92) than lifetime non-drinkers *(Dunn et al., 2012)*.
A cross sectional-study based on 77 subjects founded that lifetime alcohol consumption above 24 grams-years was associated with less severe disease (OR 0.25, 95% CI 0.07-0.97, p=0.046) and that patients who consumed above 24 grams-years had significantly lower fibrosis scores on liver histology (1.2 ± 1.0 vs 1.8 ± 1.2, p=0.03) *(Kwon et al., 2014)*.
In line with these results, a study with 139 subjects found that a lifetime alcohol consumption between 3.1-13.3 units of alcohol per week had the lower risk of fibrosis (aOR 0.23, 95% CI 0.08-0.66, p=0.006) in NAFLD. They also found that an increase in median weekly alcohol consumption to a maximum of 13 drinks per week was associated with lower fibrosis stage (aOR for each incremental unit 0.86, 95% CI 0.76-0.97, p=0.017). A recent stablished alcohol consumption marker, PEth, was used to study the effect of alcohol consumption in patients with PEth ≥ 0.3 µmol/l. Subjects with PEth above this cut-off indicates an alcohol consumption above moderate intake. As expected, they found that these patients had higher ORs for a higher fibrosis stage (aOR 2.77, 95% CI 1.01-7.59, p=0.047) *(Hagström et al., 2017)*.
Finally, another cross-sectional study with 178 subjects also compared the histological differences. They found that the ballooning (aOR 5.6, 95% CI 0.36-0.91, p=0.017) and fibrosis scores (aOR 0.71, 95% CI 0.51-0.98, p=0.035) were significantly lower in the patients with alcohol consumption up to 20 g/day than in the abstainers. They found no differences regarding steatosis (p=0.433) or inflammation (p=0.871). They also used gene expression analysis in a subgroup of 20 liver biopsies (10 light consumption and 10 abstainers) that revealed a marked inhibition of the pathways involved in the immune response in the light alcohol consumption group than in the abstainers *(Yamada et al., 2018)*.

The influence of **drinking pattern** on the association of modest alcohol consumption with NAFLD was also investigated in two studies.
Moriya et al. conducted a study in 2011 which involved 7,112 subjects. The diagnosis of hepatic steatosis was assessed by ultrasonography. They found a significant inverse correlation between drinking frequency and the prevalence of fatty liver (1-3d/week: 38%, 4-6d/week: 29%, daily: 16%, p<0.001). They also found that drinking less than 20 grams on 1-3 days per week was associated with low prevalence of fatty liver (aOR 0.47, 95% CI 0.23-0.96) *(Moriya et al., 2011)*.

In line to the results of the study described previously, a cross-sectional study of 9,886 asymptomatic Japanese men found that the prevalence of hepatic steatosis was inversely associated with the frequency of alcohol consumption (>21 days pro month, OR 0.62, 95% CI 0.53-0.71) but not with the volume of alcohol consumed *(Hiramine et al., 2011)*.

The effect of the alcohol consumption regarding the type of **alcoholic beverage** was also studied in two papers.
Firstly, Dunn et al. suggested in 2008 that wine consumption up to one serving per day, compared to non-alcohol consumption, is associated with lower prevalence of suspected NALFD (aOR 0.51, 95% CI 0.30-0.85) assessed by elevation of ALT in serum. They also studied the impact the same amount of alcohol consumption (up to one serving per day) of modest beer, liquor or mixed dinking, founding no significant association *(Dunn et al., 2008)*.
The other study was conducted by Mitchell et al. It englobed 187 subjects with NAFL assessed by liver biopsy. They found that exclusive wine drinkers had lower mean fibrosis stage (0.8±1.1 vs lifetime abstinence 1.6±1.6, $p<0.05$) and lower odds of advanced fibrosis (OR 0.20, 95% CI 0.06-0.69, $p=0.01$), compared to lifetime abstinence subjects. They also found that modest alcohol consumption (1-70 g/week) was associated with lower mean fibrosis stage (0.9±1.1 vs lifetime abstainers: 1.6±1.6, $p<0.05$) and a decreased risk of advanced fibrosis (OR 0.33, 95% CI 0.14-0.78, $p=0.001$) compared to lifetime abstainers *(Mitchell et al., 2018)*.

To date exist to the best of our knowledge, a single **meta-analysis** that tried to summarise the available evidence on the association between alcohol intake and NAFLD or NASH. They perform two analyses, one in 43,175 NAFLD subjects which showed that modest alcohol consumption was associated with a significant protection from the odds of having NAFLD (OR 0.69, 95% CI 0.65-0.73, $p<0.001$) and another with 822 NASH subjects which showed that modest alcohol consumption was found to have a significant protective effect on the development of NASH (OR 0.50, 95% CI 0.34-0.74, $p<0.001$) *(Sookoian et al., 2014)*.

A single study tested the association of modest alcohol consumption on **survival** in NAFLD. It was conducted by Hajifathalian et al. in 2019. It englobed 4,568 subjects with 70-month follow-up. It found, as expected, that drinking more than 21 g/day showed harmful effect on overall mortality (adjusted hazard risk (aHR) 1.45, 95%CI 1.01-2.10, $p=0.047$) compared to non-drinking. On the other hand, modest alcohol consumption between 7 and 21 g/day showed decreased risk of overall mortality compared to non-drinking (aHR 0.64, 95%CI 0.42-0.97, $p=0.035$) *(Hajifathalian et al., 2019)*.

2.6.2. Evidence for detrimental effects.

The actual evidence for detrimental effect of moderate alcohol consumption on NAFLD is summarised in table 43 (Supplementary 9.5).

The first paper that showed detrimental effect of moderate alcohol consumption in NAFLD was published in 2009. It analysed the impact of alcohol consumption in 71 patients with repeated liver biopsies. They found that the proportion of patients reporting heavy episodic drinking at least once monthly was higher among those with significant fibrosis progression (47% vs 11%, $p=0.003$). They conclude that moderate alcohol

consumption was associated with fibrosis progression in NAFLD and recommended to advise this patients to avoid heavy episodic drinking *(Ekstedt et al., 2009)*.

Another two studies based on analysis of **liver biopsies** also showed detrimental effect of moderate alcohol consumption in NAFLD. One was conducted in 285 NAFLD patients. They found that modest alcohol consumption (<10 g/day in women and < 20 g/day in men) compared to no use of alcohol was associated with less improvement in steatosis (0.49 vs 0.30, p=0.04) and level of AST (+2 U/L vs -7 U/l, p=0.04) as well as lower odds of NASH resolution (aOR 0.32, 95% CI 0.11-0.92, p=0.04) *(Ajmera et al., 2018)*. The other study was conducted in 86 NAFLD patients conclude that moderate alcohol consumption was associated with advanced fibrosis (aOR 5.5-9.7 95% CI 1.05-69.6) *(Blomdahl et al., 2021)*.

The impact of moderate alcohol consumption and the risk of **hepatocellular carcinoma** was also studied in NAFLD patients. One study was conducted in 510 cirrhosis patients (NAFLD and hepatitis C). They found alcohol consumption to be the most significant modifiable risk factor associated with risk of hepatocellular carcinoma development (p=0.002) and compared to non-drinkers, patients who reported any regular alcohol consumption were at greater risk for hepatocellular carcinoma development (HR 3.6, 95% CI 1.5-8.3, p=0.003) *(Ascha et al., 2010)*. Another study conducted in 301 NAFDL patients found that a mild drink habit (<20 g/day) appears to be a risk for hepatocarcinogenesis in NAFLD patients with advanced fibrosis (F3-4) (RR 4.83, 95% CI 1.01-23, p=0.04) *(Kimura et al., 2018)*.

Since **non-invasive methods** became available to avoid the use of liver biopsies three studies used this new multiparametric panels, showing detrimental effect of moderate alcohol consumption in NAFLD. A large cohort study conducted in middle-aged NAFLD subjects found that moderate alcohol consumption (up to 20 g/day on women and 30 g/day on men) was significantly and independently associated with worsening non-invasive markers of fibrosis. FIB-4 was found to be worse in light drinkers vs. non-drinkers (1.06, 95% CI 0.98-1.16), and moderate drinkers vs. non-drinkers (1.29, 95% CI 1.18-1.40). NFS was also worse in light drinkers vs. non-drinkers (1.09, 95% CI 0.1.02-1.16), and moderate drinkers vs. non-drinkers (1.31, 95% CI 1.23-1.40) *(Chang et al., 2019)*. Another large cohort study used the Fatty Liver Index and showed that consuming 10-19 g/day alcohol in general or 0-9 g/day as non-wine beverages doubled the risk for advanced liver disease compared to lifetime abstainers *(Åberg et al., 2018)*. Lastly, another study conducted in 286 NAFLD subjects found that moderate alcohol consumption (7-20.0 drinks/week in men and 7-13.9 drinks week in women) had a significant association with intermediate-high grade of FIB-4 (OR 1.87, 95% CI 1.21-2.89, p=0.005) or NFS (OR 2.91, 95% CI 1.72-4.94, p<0.001) compared to non-drinkers *(Kashiwagi et al., 2020)*.

2.6.3. No evidence for protection or detrimental effect

Not all studies published to date report benefits or detrimental effect of moderate alcohol consumption on NAFLD. These studies are summarised in table 43 (Supplementary 9.5).

One study used **transient elastography** to assess liver fibrosis in 151 diabetic patients with NAFLD. They found that light or moderate alcohol consumption was not

significantly associated with liver fibrosis, defined as kPa above 8.2 kPa *(Patel et al., 2017)*.

A novel approach, using **mendelian randomisation of a genetic variant** (rs1229984 A;G) of the alcohol dehydrogenase was conducted in liver biopsies of 466 subjects. Since the carriers of A-allele consumed significantly lower amounts of alcohol compared with no carriers (p=0.03), this parameter can be used as a marker of genetic predisposition to lower alcohol consumption. They showed that carriers of A-allele had lower degree of histological steatosis (1.76±0.83 vs 2.19±0.78, p=0.03) and lower scores of lobular inflammation (0.54±0.65 vs. 0.95±0.92, p=0.02) and NAFLD-Activity Score (2.9±1.4 vs. 3.7±1.4, p=0.015) compared with non-carriers, suggesting no benefit on moderate alcohol consumption in NAFLD severity *(Sookoian et al., 2016)*.

One study found no significant difference in the prevalence of fatty liver (defined as **ALT** elevation) in women with metabolic syndrome between light drinkers and non-drinkers and they suggested that other factors such as BMI, waist circumference, visceral fat type, and lifestyle-related disease may be more important than low alcohol consumption for the prevalence of fatty liver *(Sogabe et al., 2016)*.

Lastly, one study conducted in 132 morbidly obese patients suggested that light to moderate alcohol consumption may have a protection effect against insulin resistance but it had no impact on the severity of activity and stage of liver disease assessed with liver biopsies *(Cotrim et al., 2009)*.

3. Materials and Methods

3.1. Patients

We offered participation in a prospective observational study to adult patients – who fulfilled at least one of the inclusion criteria described below – presenting between February and August 2018 to the NAFLD Outpatient Clinic at the University Medical Hospital Hamburg-Eppendorf (UKE) (Hamburg, Germany). The local ethics committee approved the study (PV5068), and all patients gave written informed consent for participation.

The main inclusion criteria were as follow:

1. Histological confirmation of NAFLD.
2. Assessment hepatic steatosis by liver ultrasound or Controlled Attenuation Parameter (CAP) with the exclusion of other chronic liver diseases or steatosis-inducing drugs.
3. First consultation in our NAFLD outpatient Clinic for further clarification of persistent high liver parameters.

3.2. Physicians Assessments

All patients were evaluated by their hepatologist at the UKE, who conducted a non-structured interview concerning the recent alcohol consumption without having access to the results of neither the patient's questionnaire nor laboratory tests.

3.3. Patient's Questionnaire

The alcohol intake was assessed using a 3-page questionnaire (supplementary 9.1) with an adapted Alcohol Use Disorders Identification Test (AUDIT) *(World Health Organization, 2001)*. In this tool, patients reported alcohol consumption during three different periods (last three months, last four weeks and last week).

The questionnaire starts with three questions about alcohol consumption in three different time periods. These three questions are a variation of the first question of the AUDIT, which aims to better determine alcohol consumption in last 3 months, last 4 weeks and last week.

In case of an affirmative answer to the last week alcohol consumption question, subjects were asked to give the precise number of alcoholic beverages consumed last week subdivided into four different categories: 300 ml beer, 200 ml wine/sparkling wine, 50 ml liqueur and 50 ml spirits. This is an extra question that does not belong to the AUDIT. The aim of this question is to better estimate the amount of alcohol consumed last week. The next questions correspond to the AUDIT questionnaire which was explained in detail before (Introduction 2.4).

The following seven questions seek information that could explain false negative results in alcohol markers: consumption of non-alcoholic beer, malt beer, hair treatments, hidden alcohol (food/drink/medication), mouthwash, or hand disinfection.

Finally, subjects were briefly asked about their family status, professional life, and smoking habits.

3.4. Determination of the amount of alcohol consumption with the questionnaire information.

3.4.1. Last three months and last four weeks of alcohol intake

The question about the last three months and four weeks of alcohol intake had five possible answers:

1. No alcohol consumption.
2. Once per month.
3. Twice to four times per month.
4. Twice to three times per week.
5. Four times or more per week.

Additionally, another question asked about the number of drinks containing alcohol that the patient drink in a typical drinking day. It had five possible answers:

1. One to two alcoholic beverages on a drinking day.
2. Three to four alcoholic beverages on a drinking day.
3. Five to six alcoholic beverages on a drinking day.
4. Seven to nine alcoholic beverages on a drinking day.
5. Ten or more alcoholic beverages on a drinking day.

Using the information obtained from these two questions, we extrapolated an approximate consumption of alcohol in grams of ethanol per day in the following described way:

- For the calculations, we assume that a month has exactly four weeks.
- The number of drinking days per month (DDM) was calculated using the question about alcohol intake. Since the answers comprised a time interval, we assumed an averaged DDM as described in Table 10.
- Using the question about the number of drinks consumed on a typical drinking day, we calculated de standard drinks (SD) on a typical drinking day. Since the answers covered an interval, we assumed an average of the number of SD as described in Table 11.
- Each SD was assumed to contain on average 14 grams of ethanol.
- Finally, the reported alcohol consumption in grams of ethanol was calculated using the two following equations:

$$g\ alcohol/week = \frac{DDM\ x\ SD\ X\ 14}{4}$$

$$g\ alcohol/day = \frac{\frac{DDM\ x\ SD\ x\ 14}{4}}{7}$$

Table 10. Number of drinking days for the calculation of the reported alcohol consumption

Reported DDM	Assumed DDM
No alcohol	0
1x Month	1
2-4x Month	3
2-3x week	10
≥ 4x week	16

Table 11. Standard drinks for the calculation of the reported alcohol consumption

Reported number of drinks	Assumed SD
0	0
1-2	1,5
3-4	3,5
5-6	5,5
7-9	8
≥10	10

3.4.2. Alcohol intake in the preceding week

In the questionnaire, patients were first asked about any alcohol consumption in the preceding week. In case of an affirmative answer, patients were asked to give a precise number of alcoholic beverages consumed last week subdivided into five different categories: 300 ml beer, 200 ml wine, 200 ml sparkling wine, 50 ml liqueur and 50 ml spirits. For further calculations sparkling wine was considered within the category wine and liqueur was considered withing the category spirits. As a result, we have three different categories: 200 ml wine, 300 ml beer and 50 ml spirituous. The ethanol content of each type of beverage used in this study is described in table 12.

Table 12. Assumed grams of ethanol for each reported alcoholic beverage

	Volume of alcohol	Grams of ethanol
Wine (200 ml)	11%	17.6
Beer (300 ml)	4.8%	11.52
Spirituous (50 ml)	33%	13.2

The reported alcohol consumption in grams of ethanol was calculated using the two following equations:

$$g\ ethanol/week = (n°wine\ x\ 17.6) + (n°\ beer\ x\ 11.52) + (n°\ spirituous\ x\ 13.2)$$

$$g\ ethanol/day = \frac{(n°\ wine\ x17.6) + (n°\ beer\ x\ 11.52) + (n°\ spirituous\ x\ 13.2)}{7}$$

3.4.3. Alcohol abstinence

All subjects who reported alcohol abstinence during the last three-months were telephonically interviewed to accurately classify them into either lifetime abstinence or recent abstinence. Recent abstinence is defined as subjects who have consumed alcohol in varying degrees and at some point of their life have stopped drinking. It should be

noted that subjects with heavy alcohol consumption who are currently abstinent are not included in this study because they had alcoholic liver disease and not NAFLD.

3.4. Determination of alcohol consumption markers

A classification based only on self-reported alcohol consumption has the risk of misclassifying subjects who underreport or even deny their alcohol intake. To avoid this bias, the following alcohol-related biomarkers were determined.

3.4.1. Phosphatidylethanol (PEth)

As explained in the introduction, PEth production begins as soon as alcohol is consumed and peaks within 90-120 minutes after its ingestion *(Andresen-Streichert* et al., 2018; Hakim et al., 2019; Ulwelling and Smith, 2018)*. Because of its slow degradation rate PEth is detectable in blood up to 2 weeks after cessation of alcohol consumption, in some cases PEth could be detected up to 6 weeks *(Shayani et al., 2019; Staufer and Yegles, 2016)*. A recent meta-analysis proposes a threshold of 20 ng/ml. Using this cut-off PEth seems to be able to detect regularly moderate alcohol consumption (28 grams per day several days a week) *(Ulwelling and Smith, 2018*).

PEth can be determined from whole blood samples or dried blood spots (DBS). Considering the confirmation that DBS can be stored at room temperature for 30 days without a significant decrease in PEth, we have opted for this method to avoid the high PEth-instability in normal blood samples *(Faller et al., 2013, 2011)*.

A modification from a previously validated method using online solid-phase extraction followed by liquid chromatography-tandem mass spectrometry (online-SE-LC-MS/MS) in whole blood samples was used for the DBS analysis *(Schröck et al., 2017)*. In summary, blood samples were drawn and five spots of 20 µl of whole blood each were prepared on the day of sampling. After drying for at least 4 hours, the DBS were stored at room temperature with desiccant until analysis.

For this study, we analysed six different homologues of PEth (16.0/18:1, 16:0/18:2, 16:0/20:4, 18:0/18:1, 18:0/18:2 and 18:1/19:1). For further analysis, only the results of the two most used homologues in currently PEth research (16:0/18:1 and 16:0/18:2) were used.

The calibration range was between 10-1000 ng/ml. The limit of detection (LOD) and quantification (LOQ) were 4 ng/ml and 9 ng/ml, respectively *(Aboutara et al., 2021)*. Using the proposed threshold in a recent meta-analyse *(Ulwelling and Smith, 2018)*, concentrations above 20 ng/ml were regarded as positive.

3.4.2. Hair ethyl glucuronide (hEtG)

For hEtG determination, at least a 3 cm long and 0.5 cm thick hair strand was collected from the occiput by cutting the hair close to the skin from three different areas, to avoid inaccurate results due to low scalp perfusion. The proximal 3 cm long hair segment was analysed.

A previously validated method by liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used for the hEtG analysis *(Mueller et al., 2017)*. The LOD and LOQ were 1.7 and 4.7 pg/mg, respectively.

Concentrations above 7 pg/mg were considered positive. According to international standards *(Kintz, 2015)*, the following cut-off values for hEtG were applied:

- Abstinence or rare drinking: <7 pg/mg
- Repeated alcohol consumption: 7-30 pg/mg
- Chronic excessive alcohol consumption: ≥ 30 pg/mg.

3.4.3. Urinary ethyl glucuronide (uEtG)

uEtG was determined by a previously validated method *(Staufer et al., 2011)*. The urinary samples were screened using an enzyme immunoassay. In the case of positive test results (cut-off >0.3 mg/dl to increase specificity and prevent false-positive test results), the same urine samples were retested by LC-MS/MS for confirmation. The LOQ was 0.1 mg/L. Results above 0.3 mg/dl were treated as positive.

3.4.4. Classic alcohol consumption markers (CDT, MeOH, EtOH)

The percentage quantification of **CDT** was analysed using high-performance liquid chromatography. A value higher than 2.3% was regarded as positive.

MeOH was determined using headspace gas chromatography with flame ionisation detection. Results above 5 mg/l were considered positive.

The analysis of **EtOH** was performed with flame ionisation detection. Results above 0.1 g/kg were considered positive.

3.5. Serum biochemistry, anthropometric measurements, and medication

Venous blood samples were extracted the day of informed consent for participation in this study, and mean corpuscular volume (MCV), haemoglobin A1c (HbA1c), albumin, gamma-glutamyltransferase (GGT), AST, ALT, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), International Normalized Ratio (INR), and Quick were measured using the standard techniques in the UKE laboratory.

Body mass index (BMI) was calculated as weight (in kilograms) divided by the square of the height (in meters). The patient´s medication (in case of diabetes mellitus, hypertension, or hypercholesterolemia) was obtained from the clinical documentation of the hepatologist at the day of the blood extraction.

3.6. Definition of NAFLD

The diagnosis of NAFLD was established by the results of CAP, abdominal ultrasonography, or liver biopsy. Liver stiffness was measured using transient elastography.

The transient elastography and CAP were measured using FibroScan®. The transient elastography is a non-invasive method for measuring liver stiffness. CAP is also a non-invasive method for measuring ultrasonic attenuation at 3.5 MHz on the signals acquired by the FibroScan®. A trained nurse performed the FibroScan® in patients in a morning fasting state. The measurement place was the median axillary line on the first intercostal space under the liver upper limit, with the patient lying in dorsal decubitus. In the case that the measurement with the M-sonde was considered unsuccessful (for example, in obese patients) a second measurement with the XL-sonde was tried. Liver stiffness results with less than four valid measurements or an interquartile range (IQR) above 30% were classified as non-valid. CAP was obtained only when the associated liver stiffness results were valid and used the same signals as the one used to measure the liver stiffness. The final liver stiffness and CAP results were expressed in kPa and dB/m, respectively. Liver stiffness above 7.9 kPa was defined as severe fibrosis. CAP above 300 dB/m was considered as a diagnosis of steatosis.

All liver ultrasounds were performed in the Department of Ultrasound of the Medical Clinic of the UKE by skilled physicians. NAFLD was assessed concerning the presence of typical signs of steatosis (bright hepatic echoes, increased hepatic echogenicity and vascular blurring of portal or hepatic vein) and other pathologies. The differences between ultrasonography equipment and examiners can potentially cause inaccurate quantification of steatosis severity; therefore, we did not use this data in our statistical analysis.

Liver biopsy was performed only to the subgroup of patients with a clinical indication for this procedure. All liver biopsies were sent to expert liver pathologists at the UKE for diagnosis and scoring according to the NAFLD Activity Score (NAS).

For this study, the patients were classified as follows:

- Confirmed NAFLD (histologically confirmed).
- Probable NAFLD (steatosis in CAP and/or sonography).
- Unclear hepatopathy (normal CAP, normal sonography and not performed liver biopsy).
- Other diagnoses (strong suspicion of alcohol-related fatty liver disease or histological confirmation of other liver disease).

3.7. Subgroups

According to the reported alcohol consumption and the additional telephonic information regarding abstinence, the patients were classified into three subgroups: lifetime abstinence, recent abstinence and occasional drinkers.

3.8. Statistical analysis

Statistical calculations were conducted with SPSS® Statistics version 24.0.0.0 (IBM Corp., Armonk, New York, USA). P-values lesser than 0.05 were considered significant.

Differences between the three subgroups regarding metric variables were calculated using either the parametrical one-way ANOVA or the non-parametrical Kruskal-Wallis H test.

When a post-hoc test has been necessary, it has been performed by the Bonferroni method.

To compare nominal (categorical) variables, the Chi-square or the Fischer´s test, if necessary, were used.

We used a receiver operating characteristic curve (ROC curve) to compare the sensitivity and specificity of the different screening methods for alcohol consumption and the Hanley & McNail method to compare the ROC-Curves.

The correlation between reported alcohol consumption and PEth was calculating using the Spearman correlation method.

4. Results

4.1. Patient selection: cohort after exclusion criteria

Between February and August 2018, a total of 129 subjects were enrolled in this study after giving informed consent. We excluded 41 participants (21 women and 20 men) in whom the diagnosis of NAFLD was not certain after investigations were performed in our outpatient clinic (Figure 7).

Figure 7. Patient selection criteria to evaluate the impact of alcohol consumption on NAFLD

Firstly, we excluded twenty-one patients with diagnosis of unclear hepatopathy without significant steatosis (CAP < 300 dB/m or no signs of hepatic steatosis in ultrasonography and/or histology). Finally, we excluded other twenty patients who were diagnosed with other underlying liver diseases (13 alcohol liver disease, 1 autoimmune hepatitis, 1 haemochromatosis, 1 toxic cirrhosis, 1 primary biliary cirrhosis, 1 Wilson´s disease, 1 cryptogenic cirrhosis, and 1 proximal myotonic myopathy).

After the exclusion criteria, eighty-eight patients remain in this study. Fifty-five patients (62.5%) were classified as occasional drinkers and thirty-three as abstainers. Eighteen of the abstinence patients (20.5%) were subclassified as lifetime abstainers and fifteen (17%) as subjects with recent abstinence (Figure 8). Table 13 summarises the subgroup´s characteristics.

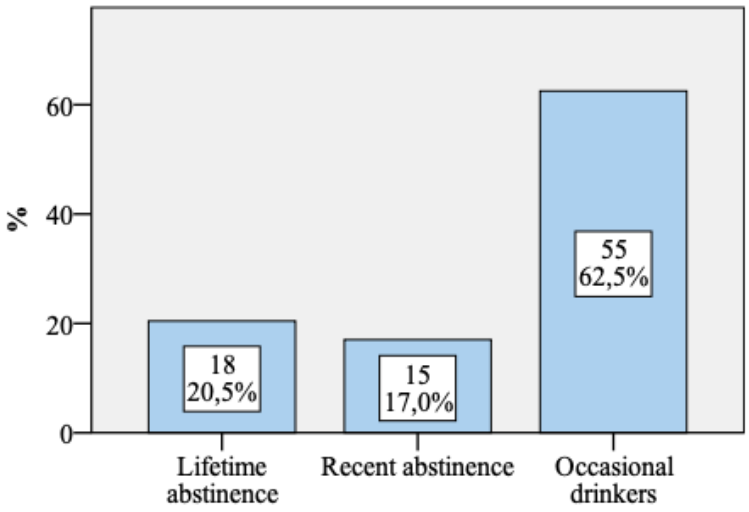

Figure 8. Subgroups according to reported alcohol consumption

Table 13. Characteristics of the study population (I)

	Total (n=88)	Lifetime abstinence (n=18)	Recent abstinence (n=15)	Occasional drinkers (n=55)	p-value
Age (years)	52 (±14)	53.2 (±16.4)	56.9 (±13.3)	50.3 (±13.7)	0.008
Gender, male (% males)	44 (50%)	8 (44.4%)	7 (46.7%)	29 (52.7%)	0.798
BMI (kg/m^2)	31.3 (±5.8)	30.9 (±4.0)	31.9 (±5)	31.3 (±6.5)	0.877
HbA1c (%)	5.9 (±1.1)	6 (±1.4)	6.4 (±1.5)	5.7 (±0.8)	0.162
Creatinine (mg/dl)	0.9 (±0.2)	0.9 (±0.2)	1 (±0.3)	0.9 (±0.2)	0.659
Cholesterol (mg/dl)	201 (±42.4)	183.5 (±47.8)*	184.3 (±41.7)	211.4 (±37.9)*	0.012
TG (mg/dl)	205.1 (±102)	245.2 (±153.6)	174.2 (±68.5)	200.2 (±84.7)	0.503
HDL (mg/dl)	47.8 (±13.2)	43.2 (±9.3)	49.9 (±13.7)	48.7 (±14)	0.241
LDL (mg/dl)	114.3 (±40.2)	100.2 (±39.4)	100.9 (±47.1)	122.3 (±36.7)	0.055
Smokers					0.252
Non-smoker	54 (69.2%)	8 (57.1%)	11 (84.6%)	35 (68.6%)	
Ex-smoker	12 (15.4%)	4 (28.6%)	0	10 (19.6%)	
Active smoker	12 (15.4%)	4 (13.3%)	2 (15.4%)	6 (11.8%)	
Diabetes therapy					0.097
No	73 (83%)	12 (66.7%)	10 (66.7%)	51 (92.7%)	
OAD	10 (11.4%)	4 (22.2%)	3 (20%)	3 (5.5%)	
Insulin	2 (2.3%)	1 (5.6%)	1 (6.7%)	0	
OAD + Insulin	3 (3.4%)	1 (5.6%)	1 (6.7%)	1 (1.8%)	
Insulin therapy					0.125
No	83 (94.3%)	16 (88.9%)	13 (86.7%)	54 (98.2%)	
Yes	5 (5.7%)	2 (11.1%)	2 (13.3%)	1 (1.8%)	
Antihypertensive drugs					0.725
No	50 (56.8%)	9 (50%)	8 (53.3%)	33 (60%)	
Yes	38 (43.2%)	9 (50%)	7 (46.7%)	22 (40%)	
Lipid lowering drugs					0.052
No	72 (81.8%)	15 (83.3%)	9 (60%)	48 (87.3%)	
Yes	16 (18.2%)	3 (16.7%)	6 (40%)	7 (12.7%)	

Data are represented as mean (±SD) or n (%). OAD: oral anti-diabetic. *p<0.05 lifetime abstinence vs occasional drinkers

The first step of the research was to compare each subgroup with each other to know if there were significant differences between the three groups. Firstly, a Kolmogorov-Smirnov test was used to know if the distribution of each variable was parametric or non-parametric. Depending on the outcome, One-way ANOVA was used in parametric variables and Kruskal-Wallis H in non-parametric variables to reveal significant differences concerning the patients` characteristic between groups.

The International Obesity Task Force definition of obesity sets **BMI** cut-off points of 25 kg/m^2 for adult overweight and 30 kg/m^2 for obesity *(Mathus-Vliegen et al., 2012)*. According to this definition, the mean of our study population was found to be obese with an average BMI of 31.3 ± 5.8 kg/m^2. No significant differences were found between the subgroups concerning the mean BMI (p=0.877).

The patients in our study have an average **HbA1c** of 5.9 ± 1.1%. This value indicates that the mean of our study population is classified as prediabetes, defined as HbA1c values between 5.7% and 6.4% by many international diabetes guidelines *(Nauck et al., 2020)*. No significant differences were found between the subgroups concerning the median HbA1c (p=0.162).

The patients in our study have an average **creatinine** of 0.9 ± 0.2 mg/dl. This value is within the reference range from our laboratory, so from this, we can conclude that the patients in our study presented a normal renal function. No significant differences were found between the subgroups concerning the mean creatinine (p=0.66)

The average **HDL** is 47.8 ± 13.2 mg/dl, which corresponds to a normal range from our laboratory. No significant differences were found between the subgroups concerning the median HDL (p=0.241).

According to the last classification of hypertriglyceridemia of the European Atherosclerosis Society and the European Society of Cardiology values of **TG** under 150 mg/dl are regarded as normal, between 150 and 880 mg/dl are defined as hypertriglyceridemia and above 880 mg/dl are defined as severe hypertriglyceridemia. According to this definition the mean of our study population has hypertriglyceridemia with an average TG of 205.1 ± 102 mg/dl. There was no statistical difference between the subgroups concerning the median TG (p=0.503). Hypertriglyceridemia has been found in more than 80% of people who are overweight or obese *(Simha, 2020)*. As explained above, the subjects in this study are defined in median as obese, so it is not surprising to found high TG levels.

The average **LDL** was 114.3 ± 40.2 mg/dl, which corresponds to the normal range from our laboratory. It is slightly below 115 mg/dl, which is the recommended threshold of the currently European guideline for the management of dyslipidaemia *(Mach et al., 2020)*. There was no statistical difference between the subgroups concerning the median LDL (p=0.055).

The average **cholesterol** was 201 ± 42.4 mg/dl for the whole study population. The initial conducted ANOVA test showed a potential difference in the median of cholesterol (p=0.012) between the subgroups. All the groups were compared with each other. The fact that some of the comparisons may be found significant only by randomness must be considered. Because of that, a post-hoc test was carried out to discard this randomness.

We decided to use the Bonferroni post-hoc method since it is one of the most rigorous withing this type of analysis. When a result is found to be still significant despite applying this procedure, nobody will doubt the statistical credibility of its conclusions. This analysis showed that there was a statistically significant difference (p=0.047) in cholesterol mean between lifetime abstinence (183.5 ± 47.8 mg/dl) and modest drinkers (211.4 ± 37.9 mg/dl) (Figure 9).

According to the currently European guideline for the management of dyslipidaemia, total plasma cholesterol should be below 190 mg/dl *(Mach et al., 2020)*. Lifetime abstinence and recent abstainers have a total plasma cholesterol under this threshold (183.5 ± 47.8 mg/dl and 184.3 ± 41.7 mg/dl, respectively). In contrast, modest drinkers have mean total plasma cholesterol of 211.4 ± 37.9 mg/dl. This is above the recommended threshold and this subgroup would meet in mean of cholesterol the criteria of hypercholesterolaemia.

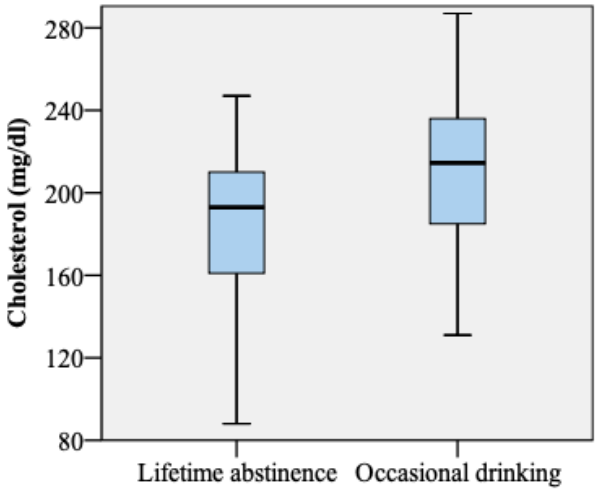

Figure 9. Cholesterol Lifetime abstinence vs occasional drinking.

The subgroups have not differed significantly in the proportion of **smoking** (p=0.252), intake of **diabetes drugs** (p=0.097), **insulin** (p=0.125), **antihypertensive drugs** (p=0.725) or **lipid lowering drugs** (p=0.052). However, two non-significant trends should be noted. Firstly, a lower proportion of occasional drinkers have antidiabetic treatment. Lastly, recent abstinence subjects appear to have higher proportion of lipid lowering drugs. As described above, this subgroup meets in median of cholesterol the criteria of hypercholesterolemia, so the higher proportion of treatment could be explained by pharmacological intervention of this issue.

To exclude that other factors may cause differences in the severity of liver disease between the subgroups, the sociodemographic data such as age and social coexistence were analysed in detail.

The **age** range of the cohort was between 24 and 78 years, and the average age was 52 years. There was no significant difference (p=0.234) between the subgroups concerning the mean age.

Also, the **gender** distribution was similar in the subgroups (Table 14). Forty-four females (50%) were included in this study. There was no significant difference (p=0.798) between the gender proportion of all subgroups.

Table 14. Gender distribution

	Male	Female	Total
Lifetime abstinence	8 (44.4%)	10 (55.6%)	18 (20.5%)
Recent abstinence	7 (46.7%)	8 (53.3%)	15 (17%)
Occasional drinkers	29 (52.7%)	26 (47.3%)	55 (62.5%)
	44	44	88

A total of 79 (89.8%) subjects gave us information about their current **family status** (Table 19). There was no significant difference (p=0.795) between all subgroups.

Table 15. Social coexistence

	Alone	Partner and children	With partner	Other	
Lifetime abstinence	2 (14.3%)	6 (42.9%)	5 (35.7%)	1 (7.1%)	14
Recent abstinence	2 (14.3%)	3 (21.4%)	8 (57.1%)	1 (7.1%)	14
Occasional drinkers	9 (17.6%)	11 (21.6%)	28 (54.9%)	3 (5.9%)	51
	13	20	41	5	79

Table 16 summarises data on the **professional life** of 75 subjects (85.2%) who reported that information in the questionnaire. The proportion of unemployed patients is significative higher among the recent abstinence patients (p<0.05).

Table 16. Professional life

	Fully employed	Part-time	Retired	Unemployed	
Lifetime abstinence	7 (53.8%)	0	5 (38.5%)	1 (7.7%)	13
Recent abstinence	4 (30.8%)	0	6 (46.2%)	3 (23.1%)*	13
Occasional drinkers	32 (65.3%)	5 (10.2%)	11 (22.4%)	1 (2%)	49
	43	5	22	5	75

* p<0.05

Mean **reported alcohol consumption** in the occasional drinkers in our study was 3.8 ± 4.9 g/day the last three months, 4±4.6 g/day the last four weeks and 4.2±5.1 g/day the last week, which is a very low intake and is within the WHO parameters of non-harmful alcohol consumption.

Subjects were asked to indicate the sort of **alcoholic beverages** consumed last week. The four available answers were: no consumption, beer, wine, or spirits. 83 subjects (94.3%) provided us this information in the questionnaire (Table 17). 34 subjects (40.9%) have reported consumption of at least one sort of alcoholic beverage last week. 13 subjects (38.2%) reported consumption of different sorts of alcohol beverages last week.

The most consumed alcoholic beverage was beer (45.2%), followed by wine (40.5%) and spirits (14.3%). From the 21 subjects who consumed only one type of alcoholic beverage, beer (61.9%) was the most common, followed by wine (23.8%) and spirits (14.3%).

Table 17. Alcohol beverage consumed last week

	Beer	Wine	Spirits	
Reported consumption	19 (45.2%)	17 (40.5%)	6 (14.3%)	
Exclusive drinkers	13 (61.9%)	5 (23.8%)	3 (14.3%)	21 (61.8%)
Mix drinkers				13 (38.2%)

Highlights:

- Moderate alcohol consumption was common in NAFLD (62.5%).
- Obesity was common in NAFLD (median BMI of 31.3±5.8 kg/m^2).
- Hypertriglyceridemia was common in NAFLD (205.1±102 mg/dl).
- Upper normal limit of LDL was common in NAFLD (114.3±40.2 mg/dl).
- Significative higher cholesterol levels have been found in occasional drinkers compared with lifetime abstinence (211.4 ± 37.9 vs 183.5 ±47.8 mg/dl, p=0.012, respectively).

4.2. Evaluation of alcohol consumption in NAFLD

To evaluate the effect of alcohol consumption in subjects with NAFLD, it is necessary to know the amount of alcohol consumed. As explained in the introduction, there are several ways at our disposal to accurately quantify the alcohol consumption. This section presents the results of the different methods used in this study: Physicians´ assessments, questionnaire, AUDIT, AUDIT-C and alcohol consumption markers.

4.2.1. Physicians´ assessments

All patients were evaluated by their hepatologist. Recent and past alcohol consumption is a standard question in the routine anamnesis in all consultations in our hepatology department to exclude alcohol liver disease. The limitation of this information is that it is not collected in a standardised way, so it only gives a general knowledge of alcohol consumption. We consider it as an overview of alcohol consumption in the last 3 months to be able to make comparisons with the other methods used in this study.

An assessment of the alcohol consumption by the treating physician (Figure 10) was retrospectively found in eighty-eight (100%) patient charts. According to these, the alcohol consumption by the physician was classified into five different categories: abstinence, rarely consumption, moderate consumption, and regular consumption. To compare the questionnaire and the physician´s assessments, we assume the following consumption for each of the categories:

- Rarely consumption: once per month or less
- Occasional consumption: twice to four times monthly
- Moderate consumption: twice to three times weekly
- Regular consumption: four times or often per week

According to physicians´ assessments most subjects (51.1%) were classified abstinent. Seventeen (19.3%) had rarely alcohol consumption, fourteen (15.9%) occasional consumption, ten (11.4%) moderate consumption and two (2.3%) regular consumption.

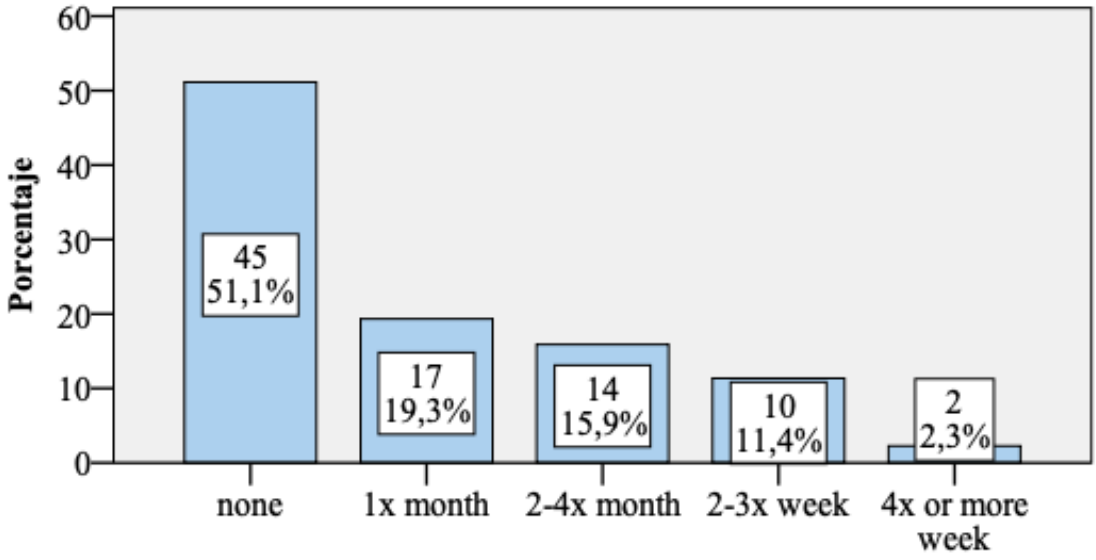

Figure 10. Physician´s assessment

4.2.2. Reported alcohol consumption (Questionnaire)

As explained in the introduction, the questionnaire answered by our subjects contains a variant of the first question of the AUDIT to get a better overview of alcohol consumption in different time periods: last 3 months, last four weeks and last week. This section describes the results of these questions.

Last three months

Firstly, we asked the patients to indicate alcohol consumption during last three months (Figure 11). Eighty-one subjects (92%) answered this question. In response to this question, most subjects (41.5%) reported no alcohol consumption last three months. Seventeen (20.7%) reported alcohol consumption monthly or less, nineteen (23.2%) two to four times a week, ten (12.2%) two to three times a week and two (2.4%) four times or often weekly.

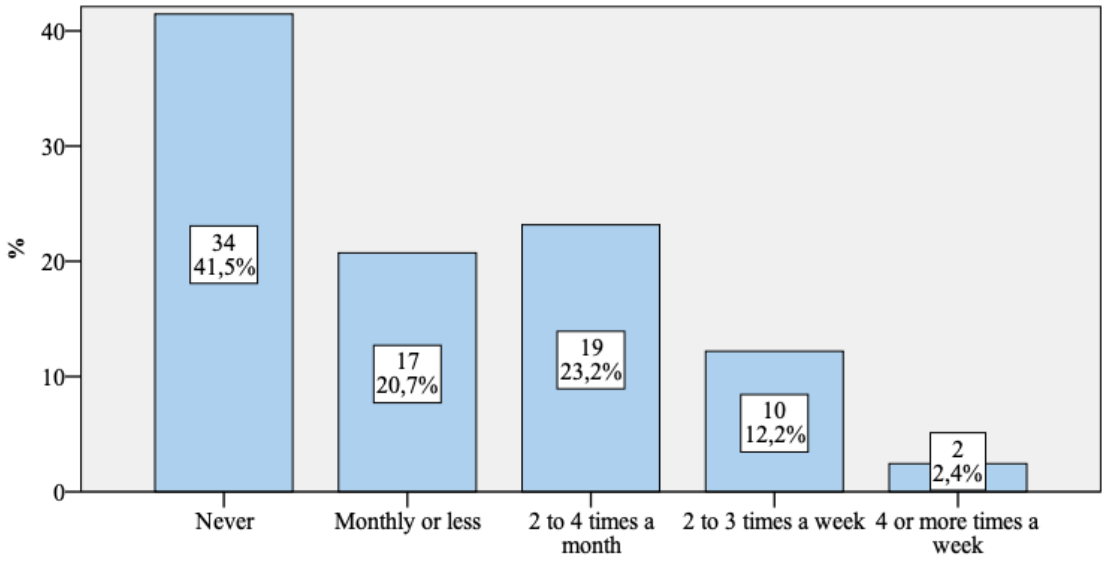

Figure 11. Reported alcohol consumption last 3 months

In the previous section we assumed that the physicians´ assessments would reflect last three months alcohol consumption for comparisons. A graphical representation of the comparison between physicians´ assessments and questionnaire is shown in figure 12 below. A statistical comparison between these two methods of determining alcohol consumption can be found in chapter 4.3.1. and 4.3.2.

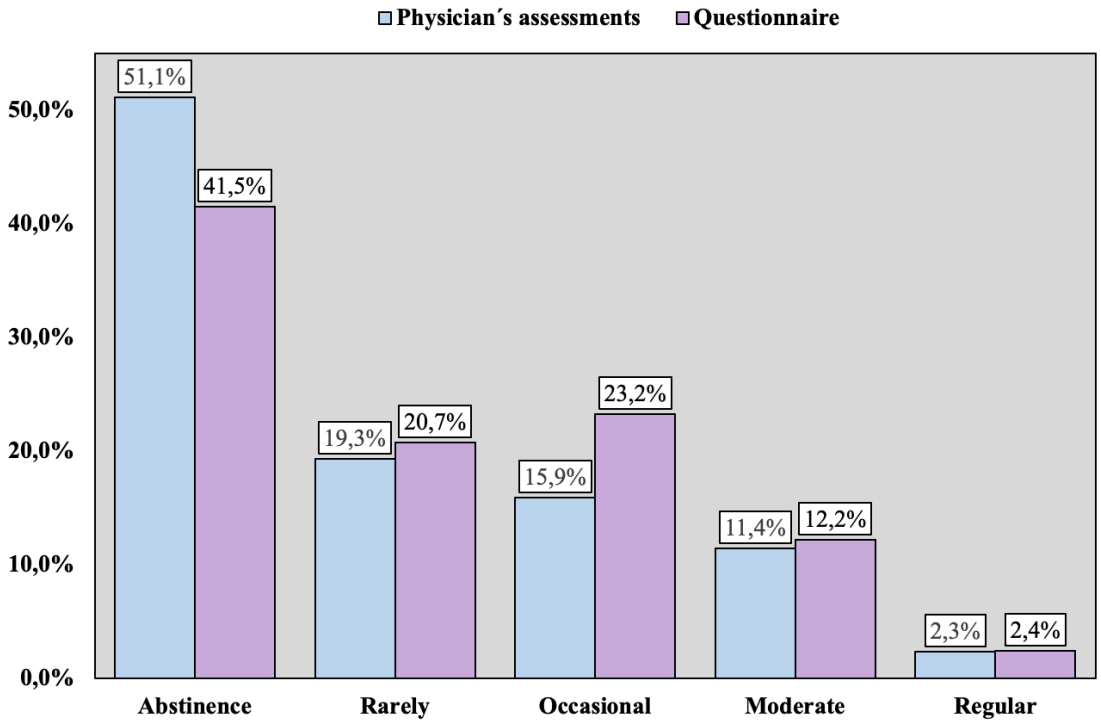

Figure 12. Physician´s assessments vs 3-month-questionnaire

Last four weeks

Secondly, we asked the patients to indicate alcohol consumption during last four weeks (Figure 13). Eighty-one subjects (93.2%) answered this question. In response to this question, most subjects (47.6%) reported no alcohol consumption last four weeks. Eighteen (22%) reported alcohol consumption once a month or less, thirteen (15.9%) two to four times a month, nine (11%) two to three times a week, and three (3.7%) four or more times per week.

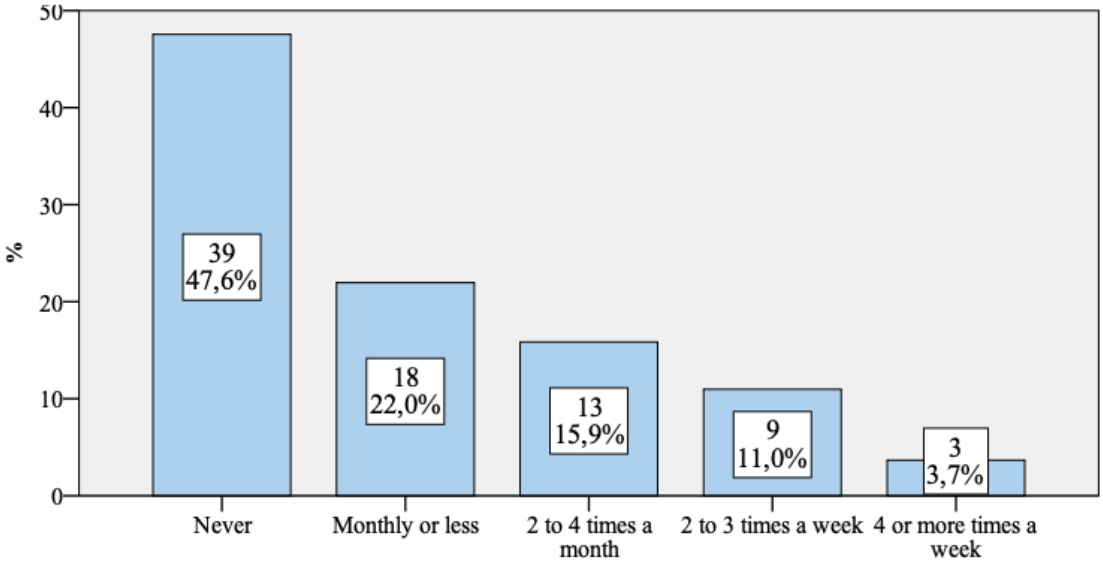

Figure 13. Reported alcohol consumption last 4 weeks

Last week

Finally, the subjects were asked to inform us whether they had consumed alcohol the last week or not (Figure 14). In response to this question, most subjects (59%) denied alcohol consumption last week.

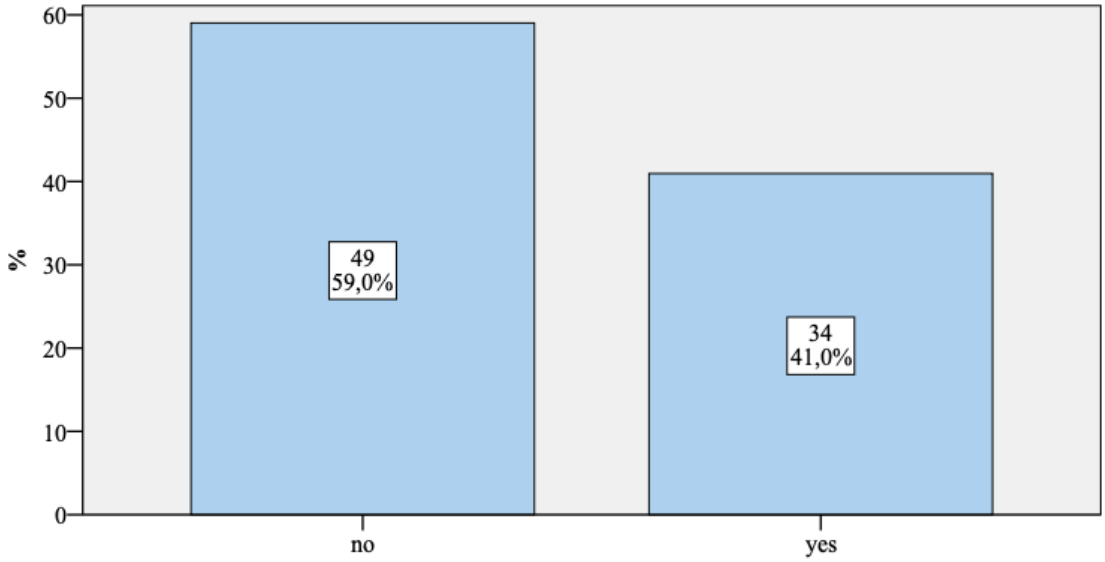

Figure 14. Reported alcohol consumption last week

According to the results of the questionnaire we can see that the percentage of subjects who report no alcohol consumption has an upward trend (last 3 months, last 4 weeks and last week: 41.5% vs. 47% vs. 59%, respectively). It is possible that subjects have reduced the alcohol consumption prior to the consultation with their hepatologist.

4.2.3. Reported alcohol consumption: AUDIT-C and AUDIT

Information about actual amounts of alcohol consumption can also be collected using specifically designed questionnaires. In our study we used two validated questionnaires: AUDIT-C and AUDIT. They were explained in detail in the introduction.

AUDIT-C

As explained in the introduction, AUDIT-C uses only the first 3 questions of AUDIT. It is scored from 0 to 12 points. The better cut-off to identify hazardous drinking is set in ≥ 4 points *(Reinert and Allen, 2007)*.

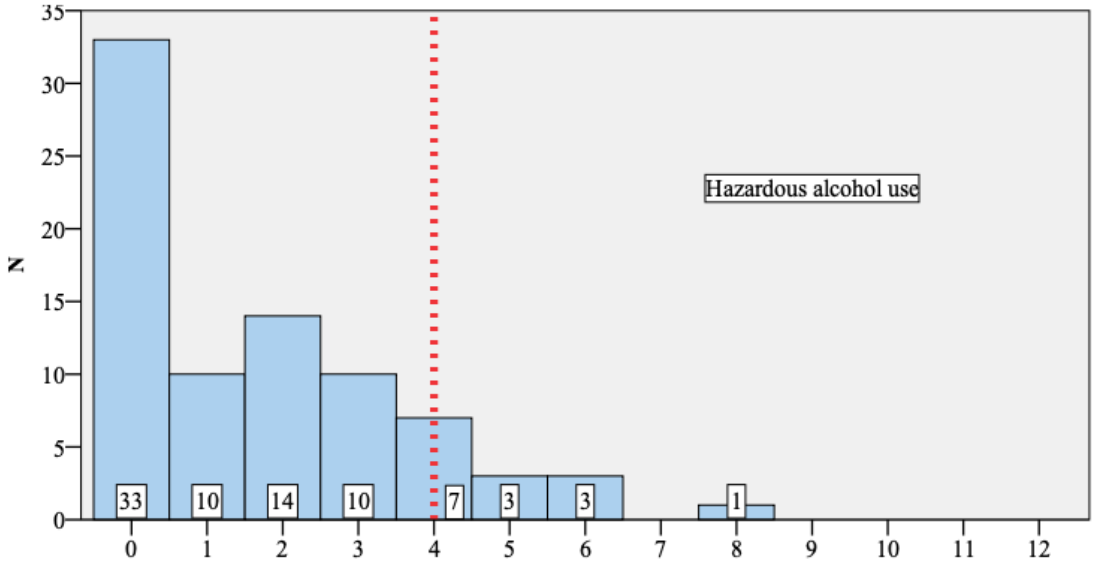

Figure 15. AUDIT-C. Dotted red line: cut-off 4 for hazardous alcohol use.

Eighty-one subjects (92.3%) answered the AUDIT-C questions. The AUDIT-C results are reflected in figure 15. Most subjects were within the normal scores. Fourteen subjects (17.3%) exceeded the 4-points cut-off, which may indicate that these subjects have hazardous alcohol use. These subjects are summarised in Table 18. AUDIT-C was the only positive alcohol consumption marker in three subjects (Pat. No. 53, Pat. Nr. 69 and 79).

One of these subjects only detected by AUDIT-C (Pat. No. 53) reported 2.3 and 5.4 grams of alcohol consumption last 4 weeks and last week respectively, which might have been too low intake for detection by PEth and uEtG. uEtG detects alcohol consumption up to the last 5 days. If the alcohol consumption was 6- or 7-days prior test, it could be another reason why uEtG was negative. Since the subject did not give a hair sample, the reported alcohol consumption of 2.3 g/day last three months could not be verified with one alcohol consumption marker.

Another of these subjects only detected by AUDIT-C (Pat. No. 79) reported no alcohol consumption last 4 weeks and last week, which is consistent with the negative results of PEth and uEtG. The subject reported alcohol consumption of 2.3 g/day last three months, which might have been to low intake for detection by hEtG. hEtG values below 7 pg/mg can indicate abstinence or very rare alcohol consumption *(Andresen-Streichert* et al., 2018; Kintz, 2015)*. It can thus be suggested that this subject rarely consumes alcohol and

has also ceased consumption in the last four weeks, and therefore all the alcohol consumption markers are negative.

The last subjects only detected by AUDIT-C (Pat. Nr. 69) reported no alcohol consumption last four weeks and last week, which is consistent with the negative results of uEtG and PEth. The subject reported alcohol consumption of 2.3 g/day last three months, which might have been to low intake for detection by hEtG. As already explained with Pat. Nr. 79 it could also suggest a rarely alcohol consumption. PEth was detected in blood but the cut-off was not reached in both homologues. It have been reported that PEth can be detected up to 6 weeks after alcohol intake *(Shayani et al., 2019; Staufer and Yegles, 2016)*, this explains the PEth detection in blood as the subject has reported alcohol consumption at some point in the last 3 months.

Table 18. Subjects with AUDIT-C ≥ 4 points

Pat. No.	Self-report (g/day)			Physician	A-C	A	Alcohol consumption markers				
	3M	4W	1W				A/A Ratio	uEtG (mg/l)	hEtG (pg/mg)	PEth I (ng/ml)	PEth II (ng/ml)
6	17.5	17.5	14.8	++	8	8	0.41	0.53	NA	7.4	19.1
33	17.5	17.5	11.5	++	5	9	0.50	2	NA	169.6	229.9
40	17.5	17.5	10.1	++	4	15	0.51	2	NA	118.7	238.6
43	7.5	2.3	0	+	6	13	0.73	0	NA	93.4	146.3
53	2.3	2.3	5.4	+	4	4	0.95	0	NA	0	0
69	2.3	0	0	++	4	6	0.58	0	NA	11.8	18.8
71	2.3	12	15.1	++	4	4	0.77	0	NA	54.8	69.5
79	2.3	0	0	+	4	5	0.71	0	0	0	0
83	12	12	14.9	+++	4	4	0.70	0	0.8	15.7	28.1
89	12	12	17.6	+++	4	4	1.13	0.32	0	19.8	26.8
94	7.5	7.5	12.5	++	5	6	1.15	0	NA	22.2	19
111	8.3	8.3	0	±	6	9	0.27	0	NA	25.8	32.1
112	8.3	8.3	3.3	+	5	10	0.47	0	NA	50.9	53.6
117	5.3	1.8	7.5	-	6	12	0.53	0	NA	172.9	193.5

Grey: positive alcohol consumption marker. Pink: reported abstinence. Orange: only positive
NA: no sample or no data available.
A-C: AUDIT-C-Questionnaire. A: AUDIT-Questionnaire. A/A Ratio: AST/ALT Ratio.
Physician: - (abstinence), ± (rarely), + (occasional), ++ (moderate) and +++ (regularly).
PEth I (homologue 16:0/18:2) and PEth II (homologue 16:0/18:1)

Whenever AUDIT was positive so was AUDIT-C, making the use of the two questionnaires redundant since AUDIT did not provide us with any additional relevant information. AUDIT-C was positive in six subjects in which AUDIT was negative. These six subjects had reported alcohol consumption at some point in the last 3 months. In three cases, AUDIT-C was the only positive parameter, as explained before (Pat. N. 53, 69 and 79). In three cases PEth and AUDIT-C were the only positive markers (Pat. N. 71, 83 and 94). In one case AUDIT-C was positive with PEth and slightly positive uEtG (Pat. N. 89). These findings suggest that AUDIT-C might be better than AUDIT in the assessment of moderate alcohol consumption in subjects with NAFLD.

AUDIT

AUDIT consists of 10 questions about alcohol consumption, drinking behaviour, adverse alcohol-reactions, and alcohol-related problems. Each of the questions has a set of responses to choose from, and each response has a score ranking from 0 to 4. All the response scores should be added to obtain a result. The maximum score is 40 points. The

WHO recommends the following AUDIT interpretation *(World Health Organization, 2001)*:

- 8-15 points: medium level of alcohol problems
- 16-19 points: high level of alcohol problems
- 20 or above: possible alcohol dependence

Eighty-one subjects (92.3%) answered the AUDIT questions. The AUDIT results are reflected in figure 16. Most subjects were within the normal scores. Seven patients (8.6%) exceeded the 8-points cut-off, which may indicate that these subjects have a hazardous alcohol use. These subjects are resumed in table 19. No subject exceeded the 16-points cut-off. AUDIT was never the only alcohol consumption parameter in our study.

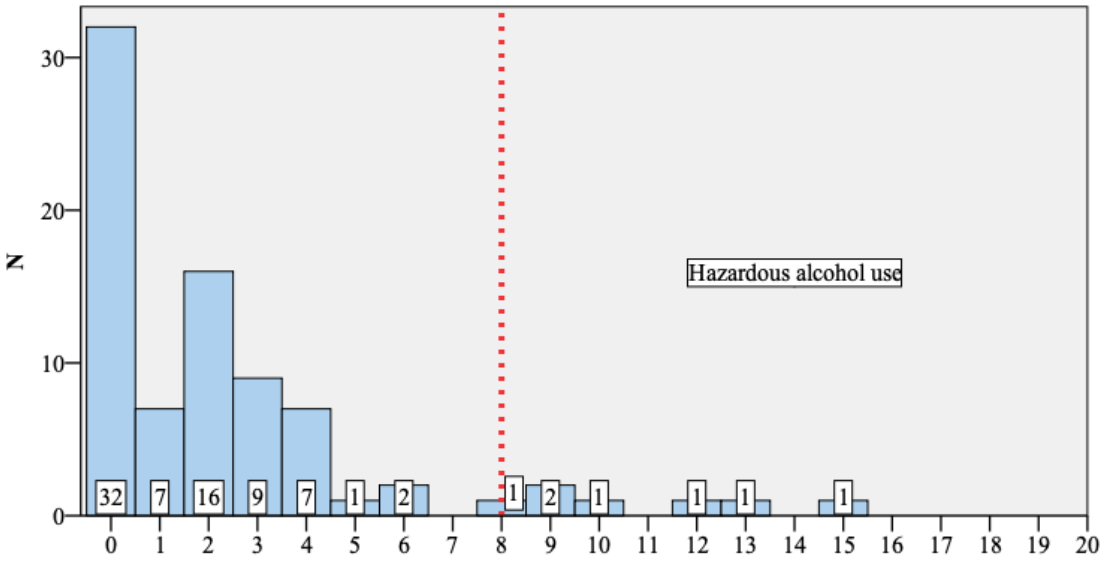

Figure 16. AUDIT. Dotted red line: cut-off 8 for hazardous alcohol use

Table 19. Subjects with AUDIT ≥ 8

Pat. No.	Self-report (g/day)			Physician	A-C	A	Alcohol consumption markers				
	3M	4W	1W				A/A Ratio	uEtG (mg/l)	hEtG (pg/mg)	PEth I (ng/ml)	PEth II (ng/ml)
6	17.5	17.5	14.8	++	8	8	0.41	0.53	NA	7.4	19.1
33	17.5	17.5	11.5	++	5	9	0.50	2	NA	169.6	229.9
40	17.5	17.5	10.1	++	4	15	0.51	2	NA	118.7	238.6
43	7.5	2.3	0	+	6	13	0.73	0	NA	93.4	146.3
111	8.3	8.3	0	±	6	9	0.27	0	NA	25.8	32.1
112	8.3	8.3	3.3	+	5	10	0.47	0	NA	50.9	53.6
117	5.3	1.8	7.5	-	6	12	0.53	0	NA	172.9	193.5

Grey: positive alcohol consumption marker. Pink: reported abstinence.
NA: no sample or no data available.
A-C: AUDIT-C-Questionnaire. A: AUDIT-Questionnaire. A/A Ratio: AST/ALT Ratio.
Physician: P, - (abstinence), ± (rarely), + (occasional), ++ (moderate) and +++ (regularly).
PEth I (homologue 16:0/18:2) and PEth II (homologue 16:0/18:1)

4.2.4. Results of alcohol consumption markers

Blood samples from 88 patients were analysed for EtOH and MeOH (100%), 87 for PEth (98.9%) and 86 for CDT (97.7%). Urine samples were available in 87 cases (98.9%). A hair sample of minimum 3 cm length could only be obtained from 18 subjects (20.5%) due to either lack of feasibility because of short hair (n=12) or either objection of the patient or non-available samples (n=58). Table 20 shows the cut-off for each alcohol consumption marker used in this study.

Table 20. Cut-off of alcohol consumption markers

	Cut-off
AST/ALT ratio	2
EtOH (g/kg)	0.1
MeOH (mg/l)	5
CDT (%)	2.3
uEtG (mg/l)	0.3
hEtG (pg/mg)	7
PEth 16:0/18:2 (ng/ml)	20
PEth 16:0/18:1 (ng/ml)	20

Table 21 shows the median and standard deviation of the alcohol consumption markers determined in this study. PEth levels are as expected significant higher in occasional drinkers ($p<0.001$). We could expect to also find significant higher levels of uEtG in occasional drinkers. The differences are almost statistically significant with $p=0.07$. This can be explained by the fact that having only 8 positive uEtG samples is not powerful enough to obtain significant results. The same explanation applies to hEtG for which we only have eighteen samples of whom only one is positive.

Table 21. Alcohol consumption markers

	N	Total (n=88)	Lifetime abstinence (n=18)	Recent abstinence (n=15)	Occasional drinkers (n=55)	p-value
AST/ALT ratio	88	0.8±0.5	0.8±0.4	0.9±0.5	0.7±0.4	0.132
EtOH (g/kg)	88	0±0	0±0	0±0	0±0	0.999
MeOH (ng/l)	88	0.1±0.4	0.1±0.4	0.2±0.6	0.1±0.3	0.754
CDT (%)	86	0.8±0.4	0.9±0.4	0.9±0.4	0.8±0.5	0.823
uEtG (mg/l)	87	0.1±0.5	0±0	0±0	0.2±0.6	0.07
hEtG (pg/mg)	18	4.5±18.8	0±0	0±0	6.7±23.1	0.589
PEth 16:0/18:2 (ng/ml)	87	24.6±79.3	0±0*	0±0*	39.7±98*	<0.001
PEth 16:0/18:1 (ng/ml)	87	47.6±129.4	0±0*	0±0*	76.8±249.4*	<0.001

Data are represented as mean (±SD). *p<0.05 lifetime abstinence vs occasional drinkers. **p<0.05 recent abstinence vs occasional drinkers

Eighteen subjects (20.5%) tested positive for at least one alcohol consumption marker. A graphic representation of the number of positive markers and the amount of exclusively positive markers can be found in figure 17. PEth was the most frequent positive alcohol consumption marker, found to be positive in 16 subjects and was the only positive marker in 10 subjects. In two subjects, uEtG was the only positive marker indicating very recently alcohol consumption. In two cases the AST/ALT ratio was the only positive marker. This may be caused in 90% of all cases by alcohol consumption, but we must not forget that elevated AST/ALT ratios have also been reported from NASH patients with a high

fibrosis risk *(Niemelä, 2016)*. More detailed explanations of each of these cases can be found in the corresponding explanation for each alcohol consumption marker.

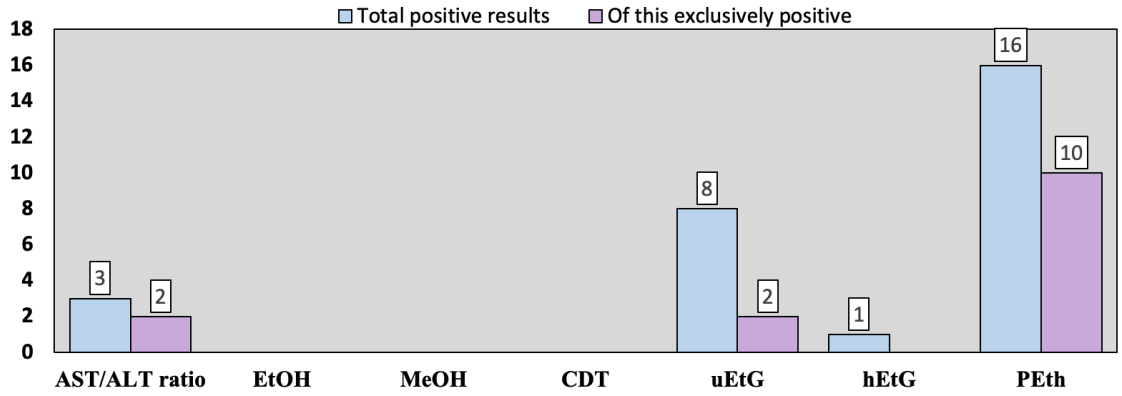

Figure 17. Distribution of alcohol consumption markers in patients with evidence of alcohol intake

The above data do not consider the use of two WHO-validated questionaries on alcohol consumption: AUDIT and AUDIT-C. Graphical representation of the alcohol consumption markers together with the AUDIT and AUDIT-C questionnaires can be found in figure 18. Taking this into account three more subjects were detected, making a total of twenty-one subjects (28.4%) who tested positive for at least one alcohol consumption marker.

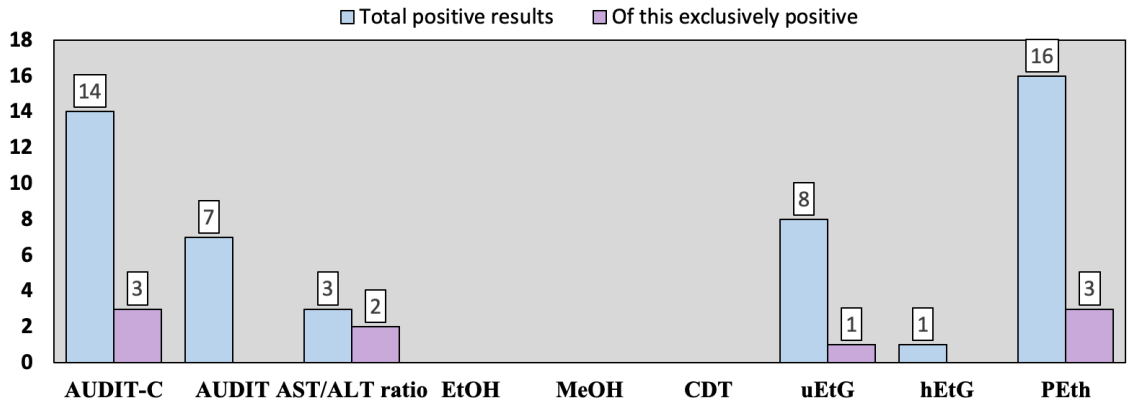

Figure 18. Distribution of alcohol consumption markers, AUDIT-C and AUDIT questionnaires in patients with evidence of alcohol intake

PEth remains as the most frequent positive alcohol consumption marker followed by AUDIT-C. The use of AUDIT-C reduces the number of exclusive positive PEth from 10 to 3 subjects and the number of exclusive positive uEtG from 2 to 1 subject. AUDIT-C was found to be positive in 14 subjects and was the only positive alcohol marker in 3 subjects (detailed explanation in section 4.2.3 above). The two cases where AST/ALT ratio was the only positive marker remain same as without using AUDIT-C. As explained above, this might be due to alcohol consumption, but high fibrosis risk may also play a role here.

CDT

None of the subjects tested positive for **CDT** using 2.3% as threshold for positive results. As explained in the introduction, the formation of CDT requires an alcohol intake of more than 50 g/day over at least 1 to 2 weeks *(Andresen-Streichert* et al., 2018; Niemelä, 2016; Spiegel et al., 2008)*. This marker was not considered to be a useful indicator of alcohol consumption in our study. It was to be expected that this marker was always negative in our subjects, since the NAFLD definition excludes excessive alcohol consumption *(Marchesini et al., 2016)* and continuous heavy drinking is what this marker would have detected *(Spiegel et al., 2008; Staufer and Yegles, 2016)*. The use of this parameter was thought to be an extra control in order not to include subjects with excessive alcohol consumption who had reported abstinence or underreported their alcohol consumption.

EtOH

None of the subjects tested positive for **EtOH** using 0.1 g/kg as threshold for positive test result. As explained in the introduction, EtOH in blood is a parameter for recent alcohol consumption and remains positive only 10 to 12 hours after alcohol intake. This marker was not considered to be a useful indicator of alcohol consumption in our study. It was expected that this marker was always negative in our subjects since this marker is often used in cases of suspected alcohol intoxication *(Staufer and Yegles, 2016)*. If a subject had would have presented with a positive EtOH would be considered a subject with an acute alcohol problem and would be classified instead as alcohol liver disease. The use of this parameter was also thought to be an extra control in order not to include subjects with excessive alcohol consumption who had reported abstinence or underreported their alcohol consumption.

MeOH

None of the subjects tested positive for **MeOH**, using 5 mg/l as the threshold for a positive test result. As explained in the introduction, MeOH is detectable in blood for up to 2 days after alcohol consumption *(Staufer and Yegles, 2016)*. This marker was not considered to be a useful indicator of alcohol consumption in our study. In case of lowering the cut-off value for a positive MeOH test result to 3 mg/l, as used in some other institutions, MeOH results would remain negative. So, in this study the determination of this alcohol consumption marker was, as expected since it detects recent heavy alcohol consumption *(Haffner et al., 1997; Roine et al., 1989)*, not useful to prove or disprove information given in the questionnaires.

AST/ALT Ratio

The aminotransferases are traditionally used as indicators of hepatic damage from chronic excessive drinking *(Andresen-Streichert* et al., 2018)*. When aminotransferases are elevated, if the **AST/ALT ratio** is higher than 2.0, 90% of the cases are due to alcohol consumption *(Conigrave et al., 2003; Spiegel et al., 2008)*. Alcohol consumption above 40 g of alcohol per day is known to make this marker positive *(Spiegel et al., 2008)*. Regarding the subjects of this study, it should be emphasized that elevated AST/ALT ratios have also been reported from NASH subjects with a high fibrosis risk *(Niemelä,*

2016). In our study, only three subjects had AST/ALT ratios above 2. These subjects are summarised in Table 22.

In two cases, AST/ALT ratio was the only positive alcohol consumption marker. One subject (Pat. No. 28) acknowledged regularly modest alcohol consumption, which is not in concordance with the alcohol consumption that this marker usually detects. However, the transient elastography was 9.5 kPa, which is defined as a severe fibrosis. This elevated AST/ALT ratio may reflect the patient´s fibrosis rather than an elevated alcohol consumption, although both may be the cause of this elevation due the regularly reported alcohol consumption, although in moderate quantity, by this subject.

Another subject (Pat. No. 26) reported abstinence, which was also reflected in the AUDIT and AUDIT-C questionnaires. The physician had not suspected recent alcohol consumption. No other alcohol consumption marker was found positive in this subject. The transient elastography was 15.4 kPa, which is defined as a severe fibrosis. Making us assume this elevated AST/ALT ratio reflects the patient´s fibrosis, assuming taking the patient´s abstinence as trustworthy answer.

In summary, the AST/ALT ratio has not allowed us to detect alcohol consumption in NAFLD subjects where this ratio has been the only positive marker. In the third case (Pat. No. 85) in which this marker was positive, other markers also detected the alcohol consumption that this marker could have detected. It should be noted that this patient has also been diagnosticated with fibrosis by liver biopsy, so this index could be also reflecting the patient´s fibrosis and not the alcohol consumption.

These findings suggest that AST/ALT ratio would be more associated with high level of fibrosis. One possible implication of this is that AST/ALT ratio should not be used as an alcohol consumption marker in NAFLD due to this confounding factor.

Table 22. Subjects with positive AST/ALT Ratio

Pat. No.	Self-report (g/day)			P	A-C	A	LB	kPa	Alcohol consumption markers				
	3M	4W	1W						A/A Ratio	uEtG (mg/l)	hEtG (pg/mg)	PEth I (ng/ml)	PEth II (ng/ml)
26	0	0	0	-	0	0	2/4	15.4	2.37	0	NA	0	0
28	2.3	2.3	3.3	±	2	2	NA	9.5	3.04	0	NA	0	0
85	7.5	7.5	11.9	++	3	4	2/4	NA	2.07	2	79.92	299.9	764.4

Grey: positive alcohol consumption marker. Pink: reported abstinence. Orange: only positive
NA: no sample or no data available. LB: fibrosis score in liver biopsy. kPa: transient elastography.
A-C: AUDIT-C-Questionnaire. A: AUDIT-Questionnaire. A/A Ratio: AST/ALT Ratio.
Physician: P, - (abstinence), ± (rarely), + (occasional), ++ (moderate) and +++ (regularly).
PEth I (homologue 16:0/18:2) and PEth II (homologue 16:0/18:1)

uEtG

As explained in the introduction, **uEtG** is useful to detect recent alcohol consumption of any amount *(Ulwelling and Smith, 2018)*. uEtG concentration peaked approximately 4 hours after EtOH intake and can be detected up to 5 days *(Andresen-Streichert* et al., 2018; Baxter et al., 2017; Shayani et al., 2019; Staufer and Yegles, 2016; Ulwelling and Smith, 2018)*. One limitation is that it does not allow to distinguish between a binge drinking event several days ago and a minor alcohol intake few hours before the sample was taken *(Andresen-Streichert* et al., 2018)*. The Cut-off used in this study was 0.3

mg/l. Urine creatinine levels were also determined to rule out possible false negatives. No false negatives were identified.

Eighty-seven subjects (98.9%) provided a urine sample. Most subjects were tested negative. Eight subjects (9.1%) had uEtG values above the threshold of 0.3 mg/l. These subjects are summarised in Table 23. Six positive uEtG were confirmed by at least another alcohol consumption marker. One positive uEtG (Pat. No. 6) was confirmed by a validated questionnaire. uEtG was the only positive alcohol consumption marker in one subject (Pat. No. 32).

Pat. No. 32 reported 4.2 g/day alcohol consumption last week. This is consistent with a positive uEtG result it alcohol consumption up to 5 days of any amount. The alcohol intake last week (4.2 g/day) and last 4 weeks (0.8 g/day) might have been too low for detection by PEth, but it was expected to detect PEth below the threshold. Since the subject did not give a hair sample, the reported last three months alcohol consumption of 0.8 g/day could not be verified with another alcohol consumption marker.

Table 23. Subjects with positive uEtG

Pat. No.	Self-report (g/day)			Physician	A-C	A	Alcohol consumption markers				
	3M	4W	1W				A/A Ratio	uEtG (mg/l)	hEtG (pg/mg)	PEth I (ng/ml)	PEth II (ng/ml)
6	17.5	17.5	14.8	++	8	8	0.41	0.53	NA	7.4	19.1
32	0.8	0.8	4.2	+	1	1	0.67	0.31	NA	0	0
33	17.5	17.5	11.5	++	5	9	0.50	2	NA	169.6	229.9
40	17.5	17.5	10.1	++	4	15	0.51	2	NA	118.7	238.6
80	0	0	0	-	0	0	0.48	2	NA	400.8	543.1
82	NA	NA	NA	-	NA	NA	0.91	2	NA	497.6	1597.2
85	7.5	7.5	11.9	++	3	4	2.07	2	79.92	299.9	764.4
89	12	12	17.6	+++	4	4	1.13	0.32	0	19.8	26.8

Grey: positive alcohol consumption marker. Pink: reported abstinence. Orange: only positive
NA: no sample or no data available.
A-C: AUDIT-C-Questionnaire. A: AUDIT-Questionnaire. A/A Ratio: AST/ALT Ratio.
Physician: - (abstinence), ± (rarely), + (occasional), ++ (moderate) and +++ (regularly).
PEth I (homologue 16:0/18:2) and PEth II (homologue 16:0/18:1)

hEtG

The determination of **hEtG** give information about chronic alcohol consumption up to 3 to 6 months *(Baxter et al., 2017)*. In this study a cut-off of 7 pg/mg was used, which is the currently recommendation of the Society of Hair testing. Values above this threshold strongly suggest regular alcohol consumption.

In our study only eight hair samples were collected. Of these, only one was positive. In Pat. No. 58 the hEtG concentration was >30 pg/mg, which indicates excessive alcohol intake of >60 g ethanol per day up to 3 months (Table 24). Both homologues of PEth above 200 ng/ml also suggest excessive alcohol consumption of >56 g/day. uEtG and AST/ALT ratio were also found positive. Subject and physician reported moderate alcohol consumption. This data suggests that the amount of alcohol consumption might be underestimated by the physician and the subject.

Table 24. Subject with positive hEtG

Pat. No.	Self-report (g/day)			Physician	A-C	A	Alcohol consumption markers				
	3M	4W	1W				A/A Ratio	uEtG (mg/l)	hEtG (pg/mg)	PEth I (ng/ml)	PEth II (ng/ml)
58	7.5	7.5	11.9	++	3	4	2.07	2	79.92	299.9	764.4

Grey: positive alcohol consumption marker. Pink: reported abstinence. NA: no sample or no data available.
A-C: AUDIT-C-Questionnaire. A: AUDIT-Questionnaire. A/A Ratio: AST/ALT Ratio.
Physician: - (abstinence), ± (rarely), + (occasional), ++ (moderate) and +++ (regularly).
PEth I (homologue 16:0/18:2) and PEth II (homologue 16:0/18:1)

PEth

As explained in the introduction, **PEth** is useful to detect current alcohol consumption *(Andresen-Streichert* et al., 2018)*. PEth concentration peaked approximately 90 to 120 minutes after EtOH intake *(Andresen-Streichert* et al., 2018; Hakim et al., 2019; Ulwelling and Smith, 2018)* and can be detected up to 3 to 6 weeks *(Hakim et al., 2019; Shayani et al., 2019; Staufer and Yegles, 2016; Cabarcos et al., 2015)*. The cut-off used in this study was 20 ng/ml. Values above this threshold strongly suggest moderate alcohol consumption above 28-56 g/day, several days per week *(Ulwelling and Smith, 2018)*. As explained in materials and methods, we considered the **PEth** alcohol marker to be positive when at least one of the determined PEth homologues was positive.

PEth could be determined in eighty-seven subjects (98.9%). Most subjects were tested negative. Sixteen subjects (18.4%) had at least one PEth homologue above the threshold of 20 ng/ml. These subjects are summarised in table 25. Six positive PEth were confirmed by at least another alcohol consumption marker. Seven positive PEth were confirmed by a validated questionnaire. PEth was the only positive alcohol consumption marker in three subjects (Pat. No. 45, 78 and 109).

Pat. No. 45 reported 16.6 g/day alcohol consumption last week and 5.3 g/day last 4 weeks. This is consistent with a positive PEth result since PEth detects current alcohol consumption up to 3 to 6 weeks. Since uEtG detects alcohol consumption up to 5 days, the negative uEtG may indicate that the alcohol consumption las week has taken place 6 or 7 days before sampling. Since the subject did not give a hair sample, the reported last 3 months alcohol consumption of 5.3 g/day could not be verified with another alcohol consumption marker.

Pat. No. 78 did not answer the questionnaire, but the physician suspected rare alcohol consumption. A probable explanation for PEth levels above the threshold might be that the subject consumes alcohol regularly and did not want to report it in the questionnaire.

Pat. No. 109 reported 3.8 g/day alcohol consumption last week and 2.3 g/day last four weeks. This is consistent with a positive PEth result since PEth detects current alcohol consumption up to 3 to 6 weeks. Since uEtG detects alcohol consumption up to 5 days, the negative uEtG may indicate that the alcohol consumption last week has taken place 6 or 7 days before sampling. Since the subject did not give a hair sample, the reported last 3 months alcohol consumption of 2.3 g/day could not be verified with another alcohol consumption marker.

Table 25. Subjects with positive PEth

Pat. No.	Self-report (g/day)			Physician	A-C	A	Alcohol consumption markers				
	3M	4W	1W				A/A Ratio	uEtG (mg/l)	hEtG (pg/mg)	PEth I (ng/ml)	PEth II (ng/ml)
33	17.5	17.5	11.5	++	5	9	0.50	2	NA	169.6	229.9
40	17.5	17.5	10.1	++	4	15	0.51	2	NA	118.7	238.6
43	7.5	2.3	0	+	6	13	0.73	0	NA	93.4	146.3
45	5.3	5.3	16.6	+	3	4	0.50	0	NA	42.2	33.1
71	2.3	12	15.1	++	4	4	0.77	0	NA	54.8	69.5
78	NA	NA	NA	±	NA	NA	0.63	0	NA	62.9	61.2
80	0	0	0	-	0	0	0.48	2	NA	400.8	543.1
82	NA	NA	NA	-	NA	NA	0.91	2	NA	497.6	1597.2
83	12	12	14.9	+++	4	4	0.70	0	0.8	15.7	28.1
85	7.5	7.5	11.9	++	3	4	2.07	2	79.92	299.9	764.4
89	12	12	17.6	+++	4	4	1.13	0.32	0	19.8	26.8
94	7.5	7.5	12.5	++	5	6	1.15	0	NA	22.2	19
109	2.3	2.3	3.8	+	3	3	0.51	0	NA	20	16.5
111	8.3	8.3	0	±	6	9	0.27	0	NA	25.8	32.1
112	8.3	8.3	3.3	+	5	10	0.47	0	NA	50.9	53.6
117	5.3	1.8	7.5	-	6	12	0.53	0	NA	172.9	193.5

Grey: positive alcohol consumption marker. Pink: reported abstinence. Orange: only positive
NA: no sample or no data available.
A-C: AUDIT-C-Questionnaire. A: AUDIT-Questionnaire. A/A Ratio: AST/ALT Ratio.
Physician: - (abstinence), ± (rarely), + (occasional), ++ (moderate) and +++ (regularly).
PEth I (homologue 16:0/18:2) and PEth II (homologue 16:0/18:1)

Table 26 lists five PEth negative subjects who were positive for other alcohol consumption markers.

Pat. No. 6 reported 14.8 g/day alcohol consumption last week. This is consistent with a positive uEtG result since positive uEtG result since it detects alcohol consumption up to 5 days of any amount. The alcohol intake last week (14.8 g/day) and last 4 weeks (17.5 g/day) might have been too low for detection by PEth but, as expected, PEth blood levels below the threshold were found. Since the subject did not give a hair sample, the reported last 3 months alcohol consumption of 17.5 g/day could not be verified with another alcohol consumption marker.

In one case the reported alcohol consumption was only detected by a slightly positive uEtG (Pat. No. 32). This subject is explained in the uEtG section above.

In three cases, alcohol consumption was not detected by PEth, but only by a positive AUDIT-C (Pat. No. 53, 69 and 79). These three subjects are explained in the AUDIT-C section above.

In case of lowering the cut-off value for a positive PEth to 15 mg/l, Pat. No. 6 and 69 would be positive and three subjects would remain negative.

Table 26. Patients with at least one positive alcohol consumption marker but negative PEth

Pat. No.	Self-report (g/day)			Physician	A-C	A	Alcohol consumption markers				
	3M	4W	1W				A/A Ratio	uEtG (mg/l)	hEtG (pg/mg)	PEth I (ng/ml)	PEth II (ng/ml)
6	17.5	17.5	14.8	+	8	8	0.41	0.53	NA	7.4	19.1
32	0.8	0.8	4.2	+	1	1	0.67	0.31	NA	0	0
53	2.3	2.3	5.4	+	4	4	0.95	0	NA	0	0
69	2.3	0	0	+	4	6	0.58	0	NA	11.8	18.8
79	2.3	0	0	+	4	5	0.71	0	0	0	0

Grey: positive alcohol consumption marker. Pink: reported abstinence. Orange: only positive
NA: no sample or no data available.
A-C: AUDIT-C-Questionnaire. A: AUDIT-Questionnaire. A/A Ratio: AST/ALT Ratio.
Physician: - (abstinence), ± (rarely), + (occasional), ++ (moderate) and +++ (regularly).
PEth I (homologue 16:0/18:2) and PEth II (homologue 16:0/18:1)

Highlights:

- **ALT/AST ratio** may be more associated with advanced fibrosis than with alcohol consumption in NAFLD and thus it could be a non-reliable alcohol consumption marker in NAFLD
- **EtOH**, **MeOH** and **CDT** were not useful detecting alcohol consumption in NAFLD subjects, as expected since they detect heavy alcohol consumption.
- **uEtG** was once the only positive marker in one subject who reported moderate alcohol consumption.
- We do not have enough hair samples to draw definitive conclusions about **hEtG**. We must say that in some cases it was not possible to obtain a hair sample as the subject's hair was shorter than 3 cm, which was common in men. On the other hand, the subjects refused to give hair samples in greater proportion than urine or blood as they considered it more intrusive due to the cosmetic consequences.
- **AUDIT-C** seems to be a good instrument in the assessment of alcohol consumption in NAFLD. The use of **AUDIT** in NAFLD seems not to provide any additional relevant information.
- **PEth** was the most frequent alcohol consumption marker and seems to be a good instrument in the assessment of alcohol consumption in NAFLD. In case of lowering the cut-off from 20 to 15 ng/ml, moderate alcohol consumption would be detected in two additional subjects.

4.3. Diagnostic value of alcohol consumption markers, questionnaires, and physicians´ assessments in NAFLD subjects.

In order to calculate the sensibility and specificity in this study it was necessary to define a gold standard for "true consumption" and "true abstinence" to compare the different methods used to determinate alcohol consumption. We also needed a gold standard to avoid biases that could happen by using only the alcohol consumption markers, only the patient's questionnaire or only the physicians´ assessments as a reference.

The information provided by patients´ questionnaires and the alcohol consumption markers englobes different time periods. On the other hand, the alcohol consumption markers have also different detection windows. To be able to make a better comparison of our tests, we have created three separate gold standards: last week, last four weeks and last three months. The classification criteria for the three gold standards are summarised in Figure 19.

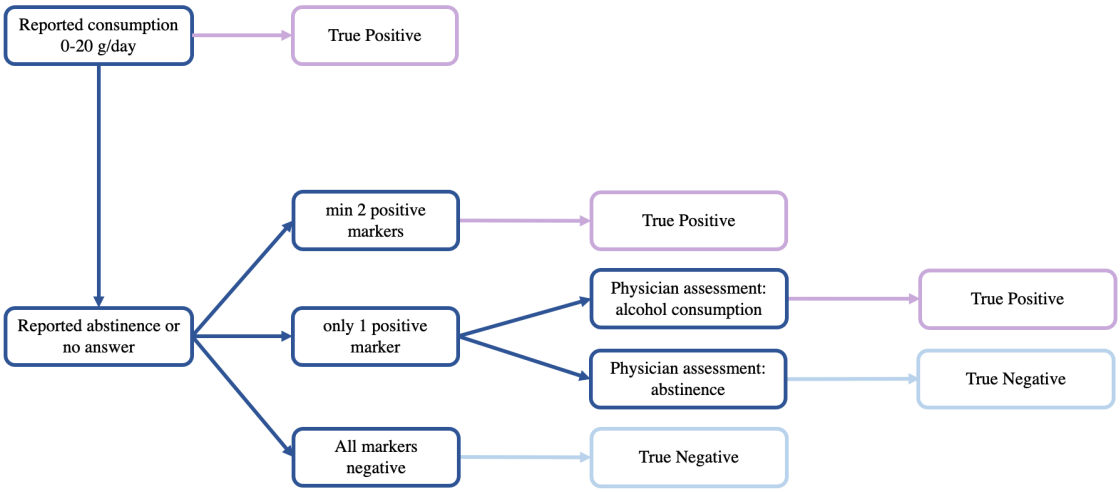

Figure 19. Gold standard classification criteria into true positive or true negative

In this study, following the current NAFLD definition in the European guidelines *(Marchesini et al., 2016)*, subjects who reported more than 20g/day in women and 30 g/day in men were excluded from the study as they do not fit in NAFLD definition. The maximal reported alcohol consumption was 17.6 g/day for the last week and 17.5 g/day for the last four weeks and three months.

Reported alcohol consumption under 20 g of ethanol per day

A subject that reported any alcohol consumption under 20 g/day was considered as a **true positive**. The subjects have actively and consciously participated in the study and gave us freely this information. There is no reason to assume that the provided information may be false, so there is no need for second criteria to reaffirm the veracity of the report.

Table 27 includes all subjects who have reported alcohol consumption and consequently classified as true positives, where no positive alcohol consumption marker was found. Not all patients on the table are classified as true positive for all the three gold standards. Patients who have reported abstinence for one of the categories have been classified according the criteria explained below.

Table 27. Reported alcohol consumption with all alcohol consumption markers negative.

Pat. No.	Self-report (g/day)			Physician	A-C	A	Alcohol consumption markers				
	3M	4W	1W				A/A Ratio	uEtG (mg/l)	hEtG (pg/mg)	PEth I (ng/ml)	PEth II (ng/ml)
12	0.8	0.8	0	+	2	2	0.47	0	NA	0	0
16	2.3	2.25	1.7	-	3	3	0.34	0	NA	9.6	12.5
20	0.8	0.8	1.7	±	2	2	0.38	0	NA	0	0
23	2.3	0.8	2.5	±	2	2	0.89	0	NA	16.6	13.7
25	2.3	0	1.7	+	3	3	0.48	0	NA	0	0
27	0.8	0.8	8.8	+	2	2	1.18	0	NA	16	13.7
28	0.8	0.8	0.8	-	1	1	0.55	0	NA	0	0
35	0.8	0.8	1.9	-	1	2	0.55	0	NA	0	0
36	2.3	2.3	2.5	+	2	2	0.54	0	NA	0	0
37	7.5	7.5	0	++	3	3	0.98	0	NA	0	0
38	2.3	2.3	3.3	±	2	2	3.04	0	NA	0	0
46	2.3	2.3	0	+	2	2	0.52	0	NA	0	0
51	7.5	7.5	4.9	++	3	3	0.63	0	NA	0	0
62	0.8	0	0	-	1	3	0.49	0	NA	0	0
64	2.3	0	0	+	2	2	0.42	0	NA	0	0
68	0.8	0.8	3.3	±	1	1	0.63	0	NA	0	0
73	0.75	0.8	5.0	±	2	2	0.65	0	NA	0	0
75	0.8	0.8	0	+	0	0	0.70	0	NA	0	0
81	0.8	0.8	0	+	0	0	1.12	0	0	0	0
87	2.3	2.3	3.3	+	2	2	0.72	0	NA	0	0
90	0.8	0.8	3.6	±	2	2	0.42	0	0	0	0
93	7.5	7.5	7.5	++	3	3	0.80	0	NA	0	0
95	0.8	0.8	2..5	±	1	1	0.37	0	NA	0	0
101	0.8	0.8	0	±	2	2	0.84	NA	NA	0	0
104	2.3	2.3	3.3	+	3	3	0.32	0	NA	13	14.4
105	0.8	2.3	2.5	±	2	2	0.71	0	0	0	0
107	0.8	0.8	1.7	±	1	1	0.52	0	0	0	0
108	7.5	7.5	5.0	±	3	3	0.45	0	0	0	0
113	0	2.3	0	+	2	2	0.51	0	NA	0	0
115	0.8	0.8	0	±	1	1	0.71	0	NA	0	0
126	0	0.8	0	-	1	1	0.81	0	0	0	0

Grey: positive alcohol consumption marker. Pink: reported abstinence.
NA: no sample or no data available.
A-C: AUDIT-C-Questionnaire. A: AUDIT-Questionnaire. A/A Ratio: AST/ALT Ratio.
Physician: - (abstinence), ± (rarely), + (occasional), ++ (moderate) and +++ (regularly).
PEth I (homologue 16:0/18:2) and PEth II (homologue 16:0/18:1)

Reported abstinence or not answered

If the subject reported abstinence or did not answer the questionnaire we needed additional criteria to consider the subject as a true positive or true negative. Alcohol consumption markers have been used as the second condition. If all alcohol consumptions markers are negative, the subject was considered as a **true negative**. A patient who has these results would be a candidate on a liver transplant list or could have their driving license returned after withdrawal due to excess of alcohol. We found these two conditions strict enough to classify these patients as true negatives.

If two or more of the alcohol consumption markers turns out to be positive, the subject was considered as a **true positive**. Finding two positive alcohol consumption markers is strict enough to classify these subjects as true positives and suggests that the subject falsely reported abstinence or refused to report alcohol consumption.

If only one alcohol consumption marker was positive and the subject reported abstinence or did not answer the questionnaire, additional criteria was needed to consider the subject as a true positive or true negative. Physician´s assessment has been used as the third condition. If the physician reported rarely, occasional, moderate or regular alcohol consumption, the patient was considered **true positive**.

Table 28 shows the three subjects classified as true positive using the above explained criteria.

Pat. No. 69 and 79 were classified with these criteria as a true positive for the last 4 weeks and last week gold standard. Since they have reported alcohol consumption in the last 3 months, they had already been classified as true positives due patient report.

Pat. No. 78 did not answer the questionnaire and presented a positive alcohol consumption marker (PEth), so the hepatologist register of alcohol consumption in patient´s chart classified this subject as true positive.

Table 28. True positives by reported abstinence or no answer, one positive alcohol consumption marker and physician-reported rarely, occasional, moderate, or regularly alcohol consumption.

Pat. No.	Self-report (g/day)			Physician	A-C	A	Alcohol consumption markers				
	3M	4W	1W				A/A Ratio	uEtG (mg/l)	hEtG (pg/mg)	PEth I (ng/ml)	PEth II (ng/ml)
69	2.3	0	0	++	4	6	0.58	0	NA	11.8	18.8
78	NA	NA	NA	±	NA	NA	0.63	0	NA	62.9	61.2
79	8.3	0	0	+	4	5	0.71	0	0	0	0

Grey: positive alcohol consumption marker. Pink: reported abstinence. Orange: only positive
NA: no sample or no data available.
A-C: AUDIT-C-Questionnaire. A: AUDIT-Questionnaire. A/A Ratio: AST/ALT Ratio.
Physician: - (abstinence), ± (rarely), + (occasional), ++ (moderate) and +++ (regularly).
PEth I (homologue 16:0/18:2) and PEth II (homologue 16:0/18:1)

After following the criteria mentioned before, if the physician reported abstinence the patient was considered **true negative**. No subject was classified as a true negative using this criterion.

4.3.1. Sensibility and specificity

In order to compare the different tests regarding their diagnostic value, we calculated their sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV). The results are summarized in Table 29.

In our study only 18 patients agreed to provide a hair sample for hEtG determination. Due to the low number of samples, we have not compared hEtG with the rest of the test used in the study. As we saw in the previous section, AUDIT does not provide extra information with respect to AUDIT-C, so we did not compare it with the rest of the tests used in this study.

For the physician´s assessments we established two cut-offs. One cut-off was established in abstinence vs. any alcohol consumption. The other cut-off was established in

abstinence and rarely alcohol consumption (e.g., once a year, marginally on holidays or special celebrations) vs. occasional, moderate, or regularly alcohol consumption.

Table 29. Sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) sorted by detecting window.

	N	Se	Sp	PPV	NPV
Last three months					
hEtG	*18*	*0.11*	*1*	*1*	*0.53*
Physician$^{\text{Abstinence vs consume}}$	88	0.78	0.92	0.93	0.76
Physician$^{\text{Minimal vs. substancial consume}}$	88	0.49	0.97	0.96	0.58
Questionnaire$^{\text{3 months}}$	82	0.98	1	1	0.97
AUDIT-C	81	0.29	1	1	0.48
AUDIT	81	0.14	1	1	0.43
Last four weeks					
PEth$^{\text{4 weeks}}$ > 20 ng/ml	87	0.33	1	1	0.55
Questionnaire$^{\text{4 weeks}}$	82	0.93	1	1	0.92
Last week					
PEth$^{\text{1 week}}$ > 20 ng/ml	87	0.39	1	1	0.65
uEtG	87	0.20	1	1	0.59
Questionnaire$^{\text{1 week}}$	83	0.87	1	1	0.90

Concerning the alcohol consumption in the **last three months**, the highest sensitivity was found for the questionnaire (98%) while the highest specificity (100%) was found for the questionnaire and AUDIT-C.

The physician´s assessment was often imprecise in terms of exact period of time and amount of alcohol consumption but presenting an overall sensitivity of 78% and specificity of 92% regarding abstinence vs. alcohol consumption in the entire study population.

Concerning the alcohol consumption in the **last four weeks**, the highest sensitivity (93%) was found in the questionnaire. Concerning the specificity, both the alcohol consumption marker PEth and the questionnaire showed the highest values (100%).

Concerning the alcohol consumption in the **last week**, the highest sensitivity (87%) was found for the questionnaire, followed by the alcohol marker PEth (39%), and lastly for the alcohol marker uEtG (20%). Concerning the specificity, both the alcohol consumption markers and the questionnaire showed the highest values (100%).

4.3.2. ROC curves of the alcohol consumption markers

Table 30 summarises the results of the calculations of the area under the ROC curve (AUROC) for each alcohol consumption marker.

Table 30. Area under the ROC curve for each alcohol consumption marker.

	AUROC (95% IC)	AUROC (p-value)
Last three months		
hEtG	*0.61 (0.34-0.89)*	*0.46*
Physicians´ assessment	0.92 (0.76-1)	0.005
AUDIT-C	0.93 (0.80-1)	0.004
Last four weeks		
PEth 16:0/18:2	0.72 (0.62-0.83)	<0.001
PEth 16:0/18:1	0.72 (0.62-0.83)	<0.001
Last week		
PEth 16:0/18:2	0.77 (0.66-0.87)	<0.001
PEth 16:0/18:1	0.77 (0.66-0.87)	<0.001
uEtG	0.60 (0.48-0.72)	0.12

We have three tests covering the **last three months** which can be used to calculate the AUROC: hEtG, physicians' assessment and AUDIT-C.

Since only eighteen hEtG determinations were made in the study, the calculation of AUROC is, as expected, inconclusive. Future studies with greater number of hair samples should be carried out to calculate the best cut-off for detection of moderate alcohol consumption in NAFLD.

The ROC curves from AUDIT-C and physicians´ assessments are represented in Figure 20. The calculated AUROC was in both methods above 0.9, indicating that they are excellent tools for the discrimination of moderate alcohol consumption in last 3 months.

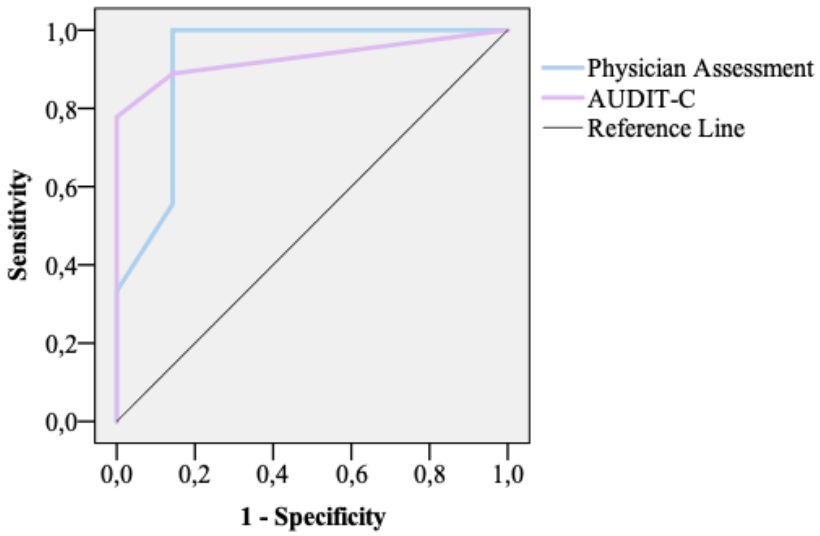

Figure 20. ROC curve of physicians´ assessment and AUDIT-C for the last 3 months. For a better overview the zero point is shifted in the graphic.

AUDIT-C is a validated questionnaire for detection of hazardous alcohol consumption with a 4 points cut-off. In this study we want to detect light to moderate alcohol consumption. The analysis of the different cut-offs is shown in Table 31. Using AUDIT-C with a cut-off of 2 points rises the sensibility to 79% maintaining a 100% specificity. The use of cut-off of 1 point would be optimal, with a sensibility and specificity of 94%. This suggest that AUDIT-C might be useful in NAFLD to determinate light to moderate alcohol consumption with a threshold of 1 point. Further studies are required to confirm

whether AUDIT-C could be useful in this population to assess non-hazardous alcohol consumption.

Table 31. Sensibility (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) of AUDIT-C with different cut-offs.

	Se	Sp	PPV	NPV
Last three months				
AUDIT-C (≥ 4: hazardous alcohol consumption)	0.29	1	1	0.48
AUDIT-C (≥ 3)	0.49	1	1	0.56
AUDIT-C (≥ 2)	0.78	1	1	0.74
AUDIT-C (≥ 1)	0.94	0.94	0.96	0.91

In order to evaluate alcohol consumption in the **last four weeks**, PEth was the only marker with a corresponding detecting window. As described in the material and methods section, in this study we determined six different PEth homologues. Only the most extended used homologues in the actual literature (16:0/18:2 and 16:00/18:1) were used for the calculation of the ROC curves. The ROC curves from both homologues are represented in Figure 21.

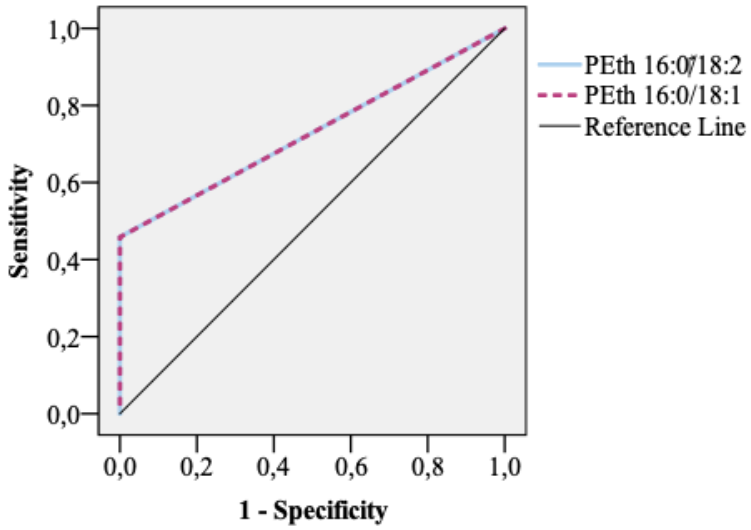

Figure 21. ROC curve of both PEth homologues for the last 4 weeks. For a better overview the zero point is shifted in the graphic.

The alcohol consumption marker PEth with a AUROC light above 0.7 is a good tool and can be used on those subjects where we suspect the reliability of the questionnaire to reaffirm the veracity of the report. There were no differences between the two homologues used. Future studies should focus on determining which of the various available homologues should be used to simplify this diagnostic method.

The scientific community has not yet agreed on the standard cut-off for PEth. 20 ng/ml is the most widely used in the published studies to date. In our study we want to be able to distinguish abstinence from light to moderate alcohol consumption. Table 32 shows the results of sensitivity and specificity as we lower the cut-off. Regardless the cut-off, a sensitivity of 50% could not be reached, while maintaining the specificity at 100%.

Table 32. Sensibility (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) of PEth with different cut-offs for last 4 weeks.

	Se	Sp	PPV	NPV
Last four weeks				
$PEth^{4\ weeks} > 10$ ng/ml	0.47	1	1	0.62
$PEth^{4\ weeks} > 15$ ng/ml	0.42	1	1	0.58
$PEth^{4\ weeks} > 20$ ng/ml	0.33	1	1	0.55

To evaluate the alcohol consumption in the **last week**, we have three different alcohol consumption markers available that have a corresponding detecting window: both PEth homologues and uEtG. Figure 22 represented the ROC curves of these alcohol consumption markers.

PEth has a AUROC above 0.7 and is also a good tool in the determination of the last week alcohol consumption in NAFLD. PEth can be used in those cases where the veracity of the questionnaire is not certain to reaffirm the veracity of the report. There were no differences between the two homologues. Future studies should focus on determining which of the various available homologues should be used to simplify this diagnostic method.

Since the AUROC confidence interval of uEtG contains 0.5 this marker was found not significant for the determination of alcohol consumption in last week in subjects with NAFLD (p=0.12). Future studies with more subjects should be conducted to conclude whether the use of this marker is reliable in detecting alcohol consumption in NAFLD.

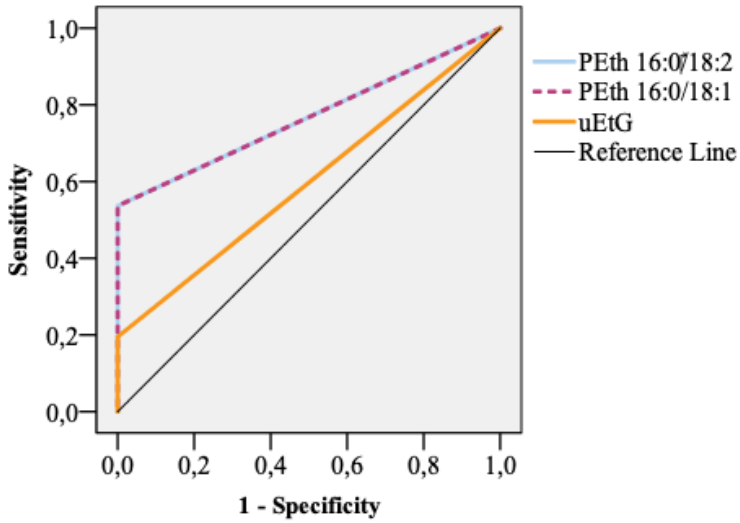

Figure 22. ROC curves both PEth homologues and uEtG for the last week. For a better overview the zero point is shifted in the graphic.

The scientific community has not yet agreed on the standard cut-off for PEth. 20 ng/ml is the most widely used in the published studies to date. In our study we want to be able to distinguish abstinence from light to moderate alcohol consumption. Table 33 shows the results of sensitivity and specificity as we lower the cut-off point. Only reducing the cut-off to 10 ng/ml a sensitivity of 55% could be reached maintaining the specificity at 100%.

Table 33. Sensibility (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) of PEth with different cut-offs for last week.

	Se	Sp	PPV	NPV
Last one week				
PEth $^{\text{last week}}$ > 10 ng/ml	0.55	1	1	0.72
PEth $^{\text{last week}}$ > 15 ng/ml	0.49	1	1	0.69
PEth $^{\text{last week}}$ > 20 ng/ml	0.39	1	1	0.65

4.3.2. Correlation between alcohol intake and PEth.

Finally, we calculated the correlation between reported alcohol consumption (last week and last four weeks) and both PEth homologues (Table 34). We can conclude that both PEth homologues have a good correlation with the reported alcohol consumption last week and last four weeks (r=0.67, p<0.001). Subjects who did not report their alcohol consumption and those who reported no consumption but in whom two positive alcohol consumption markers or one positive alcohol consumption marker plus positive physicians' assessment was found were excluded from this calculation.

Table 34. Spearman correlation (r) between reported alcohol consumption and PEth homologues

	PEth 16:0/18:2	PEth 16:0/18:1
Reported alcohol consumption $^{\text{last week}}$	0.665*	0.668*
Reported alcohol consumption $^{\text{last week}}$	0.665*	0.668*

*p<0.001

Highlights:

- A **simple questionnaire** is a useful and excellent tool in the determination of alcohol consumption in NAFLD:
  - Last 3 months: sensitivity 98%, specificity 100%.
  - Last 4 weeks: sensitivity 99%, specificity 100%.
  - Last week: sensitivity 87%, specificity 100%.
- **AUDIT** and **AUDIT-C** with the stablished thresholds were not useful as screening methods for light to moderate alcohol consumption in NAFLD because of the low sensitivity (14% and 29% respectively). Lowering AUDIT-C cut off to 1 or 2 helps to improve the sensitivity (94% and 78%, respectively) but future studies should confirm the applicability of these cut-offs in NALFD.
- If verification of the questionnaire is needed **PEth** is also a good method to determinate moderate alcohol consumption in the last four weeks in subjects with NAFLD.
  - Last 4 weeks: sensitivity 33%, specificity 100%, AUROC 0.72.
  - Last week: sensitivity 39%, specificity 100%, AUROC 0.76.
- Reducing the **PEth** cut-off at 10 ng/ml raises the sensitivity (47% last 4 weeks and 55% last week) maintaining 100% specificity.
- **PEth** showed a good correlation with the reported alcohol consumption last week and last four weeks (0.67, p<0.01)
- **uEtG** was found not useful (p=0.12) in the determination of light to moderate alcohol consumption in NAFLD.

Table 35. Liver parameters in the population and subgroups.

	Total (n=88)	Lifetime abstinence (n=18)	Recent abstinence (n=15)	Occasional drinkers (n=55)	p-value
MCV (fl)	87.6 (±5.9)	84.9 (±6)	89.8 (±7.0)	87.9 (±5.2)	0.091
Albumin (g/l)	39.8 (±3.3)	39.8 (±1.9)	38.2 (±4.9)	40.3 (±2.9)	0.076
Bilirubin (mg/dl)	0.7 (±0.4)	0.6 (±0.5)	1 (±0.7)	0.6 (±0.3)	0.137
AST (U/l)	40.7 (±26.8)	48.6 (±38.5)	45.7 (±26.4)	36.8 (±21.6)	0.109
ALT (U/l)	62.4 (±41.8)	59 (±22.4)	65.6 (±49.7)	62.7 (±44.8)	0.862
GGT (U/l)	141.9 (±150.5)	99 (±85.4)	130 (±110.4)	159.1 (±173.4)	0.375
INR	1 (±0.1)	1 (±0.1)	1.1 (±0.1)*	1 (±0.1)*	0.030
Quick (%)	95.9 (±12.2)	92.8 (±12.2)	87 (±15.2)*	99.4 (±9.8)*	0.001
Liver stiffness (kPa)	10 (±10.6)	13.4 (±11.5)**	17 (±19.9)	7.1 (±3.7)**	0.037
Liver stiffness (kPa)	6.5 (4.9-9.1)	7 (6.4-20.5)**	7.3 (4.7-28.1)	7.1 (4.8-8.1)**	0.037
CAP (dB/m)	326 (±57.2)	326.5 (±60.7)	327.5 (±82.5)	325.5 (±49.8)	0.780

Data are represented as mean (±SD), n (%) or median (interquartile range), *p<0.05 recent abstinence vs occasional drinkers **p<0.005 lifetime abstinence vs occasional drinkers

4.4. Impact of alcohol consumption in NAFLD-Patients

One of the main highlights of this study was to determine if the moderate alcohol consumption influenced the liver function and, more particularly, the fibrosis in patients with NAFLD. Table 35 summarised the detailed liver parameters for the entire study population and for each subgroup.

The average of **MCV** (87.6 ± 5.9 fl), **albumin** (39.8 ± 3.3 g/l) and **bilirubin** (0.7 ± 0.4 mg/dl) were within the normal range. There was no statistical difference between the subgroups concerning the median of MCV (p=0.091), albumin (p=0.076) and bilirubin (p=0.137).

The average of all the **liver enzymes** (AST 40.7 ± 26.8 U/l, ALT 62.4 ± 41.8 U/l and GGT 141.9 ± 150.5 U/l) was above the normal range. There was no statistical difference between the subgroups concerning the median of liver enzymes (p=0.109, 0.862 and 0.375, respectively). Since all our subjects have NAFLD it is not surprising to find high liver enzymes in all subgroups *(Marchesini et al., 2016)*.

A central role of the liver function is the coagulation. That is the reason for our analysis in detail with INR and Quick, since they are the worldwide standard parameters to assess coagulation.

The average **INR** was 1 ± 0.1 and the average **Quick** was 95.9 ±12.2%, which are within the normal range of our laboratory. The initial conducted test was Kruskal-Wallis for INR and one-way ANOVA for Quick. The reason of choosing these tests was that the INR distribution was non-parametric, and the INR distribution was parametric.

The initial tests suggested statistical differences in INR (p=0.017) and Quick (p=0.001) between subgroups. The post-hoc analyse showed that there were statistically significant differences (INR: p=0.030; Quick: p=0.01) between recent abstinence (Quick: 87 ±15.2%, INR: 1.1 ±0.1) and occasional drinkers (Quick: 99.4 ± 9.8%, INR: 1±0.1). Box plot of Quick and INR can be found in Figure 23 and Figure 24, respectively.

Examining the INR box plot in figure 24, we can see that 0.9, 1.1 and 1.2 values are taken as being outside the interquartile range for occasional drinkers, because of a higher number of subjects with INR of 1.0. This explains why the Quick and INR appear to give contradictory results, as Quick seems to be rising while INR is falling in occasional drinkers. Despite these “statistical relevant” differences, the values are within the normal range and therefore they have no clinical relevance and can be assumed as a mathematical curiosity.

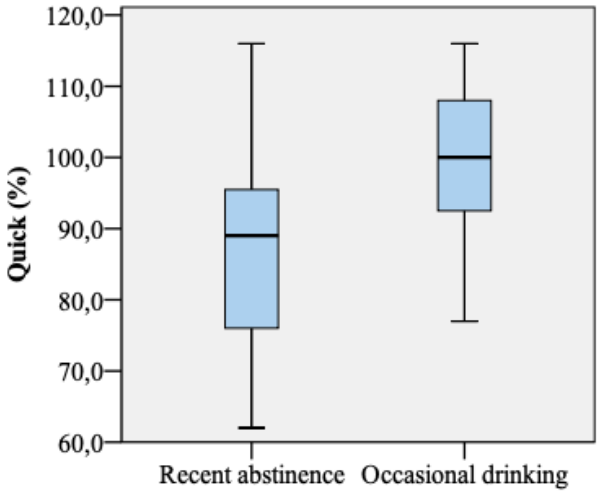

Figure 23. Quick recent abstinence vs occasional drinkers

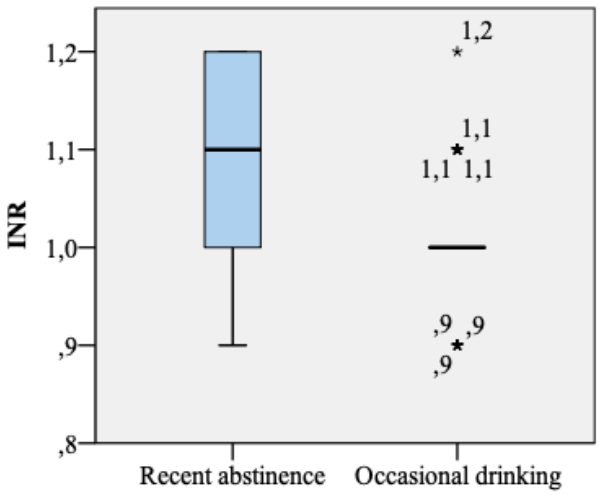

Figure 24. INR: recent abstinence vs occasional drinkers

As explained in the chapter Materials and Methods, the diagnosis of NAFLD in this study was carried out by non-invasive methods using ultrasonography and FibroScan®. For the statistical calculations, we decided to use the data provided by FibroScan ®, given that the results are quantitative measures of liver stiffness and CAP that allow more accurate comparisons between subgroups.

As a reminder of what is explained in the introduction, CAP is a non-invasive method to assess hepatic steatosis which establish the degree of ultrasound attenuation by hepatic fat *(Castera et al., 2019)*. Results are expressed as dB/m and range from 100 to 400 dB/m *(Castera et al., 2019; de Lédinghen et al., 2017)*. A cut-off value of 300 dB/m has been established as an optimal cut-off for detection of ≥ 5% fat in the liver.

The average **CAP** was 326 ± 57.2 dB/m. This result is expected since this data corroborates the diagnosis of steatosis in our cohort. There were no significative differences between the subgroups concerning the median of CAP (p=0.780). CAP is only able to diagnose steatosis but cannot differentiate between adjacent degrees of steatosis *(Castera et al., 2019; Marchesini et al., 2016)*. Consequently, information regarding degrees of steatosis could not be assessed in our study.

Briefly remembering the introduction, the liver fibrosis can be evaluated using non-invasive techniques. In our study we used a ultrasound-based elastography, named transient elastography, which uses shear waves to measure liver stiffness. Results are expressed in kPa, and range from 2 to 75 kPa *(Castera et al., 2019)*. A cut-off of 7.9 kPa has been established for severe fibrosis *(de Lédinghen and Vergniol, 2010)*.

The average **liver stiffness** was 10 ± 10.6 kPa, which corresponds to a severe fibrosis stage. The initial conducted Kruskal-Wallis test suggested statistical differences in the liver stiffness (p=0.037) between the subgroups. The post-hoc analysis showed that there were statistical differences (p=0.044) between lifetime abstinence (7 (6.4-20.5) kPa) and occasional drinking (7.1 (4.8-8.1) kPa) (Figure 25). However, a non-significant trend (p=0.08) between recent abstinence and occasional drinkers (7.7 (4.7-28.1) vs. 7.1 (4.8-8.1) kPa, respectively) should also be noted.

These findings suggest that occasional alcohol consumption throughout a lifetime was not harmful and even appeared to be associated with lower liver stiffness than lifetime alcohol abstinence.

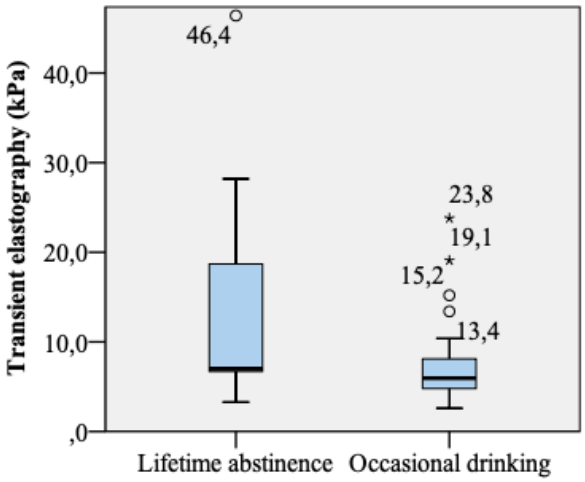

Figure 25. Liver stiffness (kPa) lifetime abstinence vs. occasional drinkers

Highlights:

- Occasional alcohol consumption throughout lifetime was not found to be harmful and even appeared to be associated with lower liver stiffness compared to lifetime alcohol abstinence.
- Further longitudinal studies are needed to evaluate how moderate alcohol consumption affects NAFLD progression.

5. Discussion

Alcohol consumption is a well-known dose-dependent risk factor of alcoholic liver disease for both, women and men *(Becker et al., 1996)*. The threshold for developing cirrhosis and non-cirrhotic liver damage was found to be up to 30 g/day in the general population *(Bellentani et al., 1997)*. NAFLD patients often present obesity *(Younossi et al., 2016)* and BMI above normal range has also been related with liver disease and some studies also shown evidence of a supra-additive interaction between drinking > 30 g/day and high BMI in liver damage *(Hart et al., 2010)*. The diagnosis of NAFLD requires not to exceed 30g/day for men and 20 g/day for women *(Marchesini et al., 2016)*, thus NAFLD subjects do not exceed per definition the established thresholds for hepatic damage due alcohol intake. The effect of alcohol intake under this threshold is often defined as moderate alcohol consumption and the effect of it on NAFLD remains controversial. NAFLD is a growing health issue worldwide and it is the leading cause of chronic liver disease in USA and Europe *(Derra et al., 2018; Younossi et al., 2018)*.

Consequently, determining the effect of moderate alcohol consumption in the progression of liver fibrosis in NAFLD is determinant since liver fibrosis is strongly associated with the liver-specific disease outcomes *(Rinella, 2015)*. And that is exactly the purpose of the current study. Our most significant finding is that moderate alcohol consumption up to 20 g/day throughout lifetime appeared to be associated with lower liver stiffness compared to lifetime alcohol abstinence.

The effects of moderate alcohol consumption in NAFLD have been studied during the last ten years and the answer to this question remains still unclear. Most of the published studies suggest a protective role of moderate alcohol consumption in NAFLD. On the other hand, the most recent studies showed detrimental effect of moderate alcohol consumption.

A correct diagnosis of NAFLD requires an accurate assessment of the amount of alcohol intake. Specific designed questionnaires can be used to collect information on the actual amounts of alcohol consumed. The main problem is that these questionnaires, such as AUDIT, have been developed as screening methods for excessive drinking *(Saunders et al., 1993)* and their applicability to assess moderate alcohol consumption has never been investigated.

People consuming alcohol may tend to underreport their consumption and recall bias in observational studies should not be forgotten. Consequently, more objective methods to assess the real alcohol consumption or to validate patients´ reports are needed. Alcohol consumption markers to provide more accurate information regarding alcohol intake are commonly used in liver transplantation. PEth is one of this markers and it was recently used in two studies of moderate alcohol consumption and NAFLD as a method to validate subjects´ reports *(Blomdahl et al., 2021; Hagström et al., 2017)*. The knowledge to date only allows to make broad generalizations between PEth values and the quantity, frequency, and recency of the alcohol consumption *(Ulwelling and Smith, 2018)*. In our study, subjects reported low average alcohol consumption under 28 g/week, and the level of PEth in all participants were rather low (mean value 39.7 ng/ml), indicating that the assessment of alcohol consumption with a simple questionnaire and AUDIT-C was reliable.

To the best of our knowledge, only two other studies have evaluated PEth in NAFLD *(Blomdahl et al., 2021; Hagström et al., 2017)*. They reported that PEth ≥ 50 and ≥ 211 ng/ml, respectively, were significantly associated with higher fibrosis stage. These both studies used liver biopsies to determine grade of hepatic fibrosis. In contrast, our study used a non-invasive determination method (transient elastography) that do not distinguish between different degrees of fibrosis, not allowing us to draw conclusions about it. A recent meta-analyse *(Ulwelling and Smith, 2018)* proposed values above 200 ng/ml to be interpreted as excessive alcohol consumption, so that the higher fibrosis stage found by Hagström et al., 2017, could be explained due to alcohol liver damage. The amount of alcohol consumption needed to elevate PEth above 50 ng/ml remains unclear, therefore the higher fibrosis stage reported by Blomdahl et al., 2001 could be due to a higher alcohol intake, thus corresponding to alcohol liver disease and not NAFLD.

Liver biopsy remains as gold standard in NAFLD diagnosis. Our finding agrees with plenty of liver biopsy-based studies that showed that moderate alcohol consumption seems to be associated with less degree of fibrosis *(Mitchell et al., 2018; Yamada et al., 2018; Hagström et al., 2017; Kwon et al., 2014; Dunn et al., 2012)*. There are also two biopsy-based studied that shows detrimental effect of moderate alcohol consumption in liver fibrosis in NAFLD subjects. One of them showed that moderate alcohol consumption was associated with advanced fibrosis and they found the highest risk for advanced fibrosis in type 2 diabetes patients *(Blomdahl et al., 2021)*. If we compare both study populations, our study have much lower rates of type 2 diabetes subjects (17% vs. 48.8%). Since alcohol consumption seems to have a synergic effect with insulin resistance *(Blomdahl et al., 2021)*, this could explain the differences between the conclusions of our studies. The other biopsy-based study showed that heavy episodic drinking (at least one per month) was associated with significant fibrosis progression *(Ekstedt et al., 2009)*. Our questionnaire did not ask about the drinking pattern, thus conclusions regarding it could not be made with our data.

Patel et al. 2017 conducted a cross-sectional study in 151 diabetic patients with NAFLD and concluded that light or moderate alcohol consumption were not significantly associated with liver fibrosis *(Patel et al., 2017)*. This is the only published study to date that also used transient elastography as method to assess liver stiffness and our conclusions differ. There is a known synergic negative effect between insulin resistance and alcohol consumption *(Blomdahl et al., 2021)*, and the main difference between our studies resides in the study populations, while their study was conducted exclusively on diabetic patients, they represent only 17% of our cohort. The relatively low rate of diabetic patients in our study and their even distribution between subgroups may have helped to control the synergic effect with insulin resistance, allowing us to observe the protective effect of moderate alcohol consumption on liver stiffness.

Recently, new non-invasive fibrosis indices have been developed. To date two studies assessed the impact of alcohol consumption on two non-invasive liver fibrosis indices: Fibrosis-4-Index and NAFLD fibrosis score *(Chang et al., 2019; Kashiwagi et al., 2020)*. Both found that moderate alcohol consumption was associated with worse non-invasive markers of fibrosis. These new fibrosis indices are excellent in ruling out significant fibrosis *(Sheka et al., 2020)* but compared with liver biopsy they could not differentiate fibrosis grading *(Drescher et al., 2019)*. To the best of our knowledge, there is not a proved positive correlation between fibrosis-index and histopathological fibrosis degree.

The main strength of this study is the use of objective markers for alcohol consumption to confirm our patients´ statements (PEth, hEtG, uEtG, CDT, MeOH and EtOH). For the first time, all this alcohol consumption markers were used together to assess alcohol consumption in NAFLD patients. PEth was the most frequent positive alcohol consumption marker, found to be positive in 16 subjects and it was the only positive marker in 10 subjects. These rates are in line with previous published data regarding transplant setting *(Barrio et al., 2020; Andresen-Streichert et al., 2017)*.

Our study found PEth to be a good tool to detect moderate alcohol consumption in NAFLD subjects (AUROC 0.7). A controlled drinking study in healthy volunteers reported a higher PEth AUROC (0.92) to discriminate between abstinence and moderate daily consumption of red wine during last 3 months *(Kechagias et al., 2015)*. This difference could be explained due different time periods (3 months vs. 1 to 3 weeks in our study) and the fact that our study population is not based on healthy subjects and also have an heterogenous alcohol consumption pattern (type of alcoholic beverages, amount and frequency). Our AUROC is also lower as the described for the detection of alcohol misuse with PEth in organ donors (AUROC 0.89) *(Lowery et al., 2018)* and in critically ill patients (AUROC 0.93) *(Afshar et al., 2017)*. These differences were already expected since our study were trying to reliably detect moderate alcohol consumption instead of misuse in our population.

Using a PEth cut-off of 20 ng/ml our study found a sensibility of 33-39% and specificity of 100% to detect moderate alcohol consumption. These results agree with the reported in a drinking study in healthy volunteers for a PEth cut-off of 28 ng/ml (sensitivity 28% and specificity 100%) *(Kechagias et al., 2015)*. Our study also found a strong correlation between PEth and the reported alcohol consumption in NAFLD subjects (r=0.67). These findings are in line with the correlation found in a drinking study in healthy volunteers (r=0.62) *(Kechagias et al., 2015)*, and in a study in alcohol-dependent patients under pharmacologically therapy to reduce their alcohol consumption (r=0.52-0.56) *(Walther et al., 2015)*. In summary, our study can be used to improve the interpretation of PEth in NAFLD subjects, but further studies are still needed in order to establish optimal thresholds to detect moderate alcohol consumption and further determine how accurate is the relation between PEth values and the amount of alcohol consumed in NALFD.

Although the findings of our study should be interpreted with caution, another strength is that we included a relatively high proportion of lifetime abstainers (20%), which were reliably classified due to subject´s report, all negative consumption markers, and telephonic interview. Lastly, we have consistently excluded participants who have other common causes of chronic liver disease, reducing possible confounders.

Finally, several important weaknesses need to be considered. First, it was a small single centre study with a small study sample of 88 participants and, consequently, our findings may not be representative of the entire NAFLD population. Second, the use of a cross-sectional design does not allow us to make temporal or causal relationships between moderate alcohol consumption and NAFLD. Third, our study did not use the gold standard (liver biopsy) to assess hepatic steatosis and fibrosis, but it used a wide accepted non-invasive method (Fibroscan®) instead. We were unable to investigate the impact of moderate alcohol consumption in steatosis and fibrosis grading since this method does not accurately differentiate between its different grades. Finally, we could not evaluate

the effect of binge drinking, total caloric intake, dietary pattern, physical activity, or caffeine intake as the data were not available.

Conclusions

Although limited by a small sample size, this study suggests that occasional alcohol consumption throughout lifetime is not harmful and even appears to be associated with lower liver stiffness comparing to lifetime alcohol abstinence. Further prospective studies assessing alcohol consumption using sensitive direct biomarkers (e.g. PEth) and lifetime drinking habits questionnaires (e.g. Skinner lifetime drinking history) are needed to evaluate how moderate alcohol consumption affects NAFLD progression.

6. Abstract

Title:
Is occasional alcohol consumption associated with the presence of liver fibrosis in patients with non-alcoholic fatty liver disease?

Background & Aims:
The impact of alcohol consumption on the non-alcoholic fatty liver disease (NAFLD) is controversial. Recent studies have suggested that light to moderate alcohol consumption might be associated with a lower risk of fibrosis progression in NAFLD. This study investigates the association of alcohol consumption with the prevalence of fibrosis in patients with NAFLD confirming the patient´s statement on alcohol intake by determining a set of direct alcohol markers.

Methods:
NAFLD patients were prospectively recruited at the outpatient general Hepatology Clinic at the University Medical Hospital Hamburg-Eppendorf between February and August 2018. The alcohol intake was assessed using a questionnaire. To confirm patients´ statements, direct alcohol markers including Phosphatidylethanol (PEth), ethyl glucuronide in the hair (hEtG) and urine (uEtG), carbohydrate-deficient transferrin (CDT), methanol (MeOH) and ethanol (EtOH) were determined. Liver stiffness and Controlled Attenuation Parameter (CAP) were measured using FibroScan.

Results:
After informed consent, a total of 88 patients were included in this study. According to patient´s statements and results of alcohol markers patients were classified as lifetime abstinence (LTA, n=18, 20.5%), recent abstinence (RA, n=15, 17%) or occasional drinkers (OD, n=55, 62.5%) with ethanol intake ≤ 20 and ≤ 30 g EtOH daily in females and males, respectively. The average reported alcohol consumption of OD was low (28g EtOH weekly). In all patients with reported LTA or RA, all direct alcohol markers tested negative, confirming the truth of patients' statements. In 32.7% (18/55) of the OD; at least one positive direct alcohol marker indicating recent alcohol consumption was found (88.9% (16/18) PEth, 44.4% (8/18) uEtG and 5.6% (1/18) hEtG).
There was no statistical difference between patients with LTA, RA or OD concerning the number of patients with IDDM, BMI, or concerning gender, age, CAP, HbA1c, Bilirubin, GOT, GPT, GGT, Creatinine, LDL-cholesterol, and Triglyceride. The median liver stiffness in LTA was significantly higher than in OD (7 (6.4-20.5) vs. 5.95 (4.8-8.1) kPa, p=0.04), while there was no difference between RA and LTA and between RA and OD.

Conclusions:
Consumption of alcohol was common in NAFLD patients. Occasional alcohol consumption throughout a lifetime showed no detrimental effect and even appeared to be associated with lower liver stiffness than lifetime alcohol abstinence. Further longitudinal studies are needed to evaluate how moderate alcohol consumption affects NAFLD progression.

7. Abbreviations

aHR	Adjusted hazard risk
ALT	Alanine aminotransferase
aOR	Adjusted odds ratio
AST	Aspartate aminotransferase
AUDIT	Alcohol use disorders identification test
AUROC	Area under ROC curve
BMI	Body mass index
CAP	Controlled attenuation parameter
CDT	Carbohydrate-deficient transferrin
CI	Confidence interval
EtG	Ethyl glucuronide
EtOH	Ethanol
GGT	Gamma glutamyl transferase
HbA1c	Haemoglobin A1c
HDL	High density lipoprotein cholesterol
hEtG	Ethyl glucuronide in hair
INR	International normalized ratio
kPa	Kilopascals
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDL	Low density protein cholesterol
LOD	Limit of detection
LOQ	Limit of qualification
MCV	Mean corpuscular volume
MeOH	Methanol
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
OR	Odds ratio
PEth	Phosphatidylethanol
ROC curve	Receiver operating characteristic curve
TE	Transient elastography
TG	
uEtG	Ethyl glucuronide in urine
WHO	World Health Organization

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9. Supplementary

- **Questionnaire** (German)
- **Table 40.** Current research on NAFLD and moderate alcohol consumption: type of study, number of subjects, number of women in each study, follow-up, method of assessing alcohol consumption, and use of alcohol consumption markers.
- **Table 41.** Alcohol consumption groups used in each study and proportion of subjects in each group.
- **Table 36.** Summary of studies suggesting a protective effect of moderate alcohol intake on NAFLD prevalence or NAFLD progression
- **Table 43.** Summary of studies suggesting a detrimental effect or no effect of moderate alcohol intake on NAFLD prevalence or NAFLD progression

9.1. Questionnaire (German)

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Fragebogen zum Alkoholkonsum

Datum:

Name: Geburtsdatum:

Körpergröße:

Körpergewicht:

Wie oft haben Sie in den letzten 3 Monaten Alkohol konsumiert?

O Nie | O Etwa 1 mal pro Monat | O 2-4 mal pro Monat | O 2-3 mal pro Woche | O 4 mal oder öfter pro Woche

Wie oft haben Sie Alkohol in den letzten 4 Wochen konsumiert?

O Nie | O 1 mal | O 2-4 mal pro Monat | O 2-3 mal pro Woche | O 4 mal oder öfter pro Woche

Haben Sie in der letzten Woche Alkohol getrunken?

O Ja O Nein

Falls ja, was haben Sie in der letzten Woche konsumiert?

O Bier Anzahl der Flaschen (0,3 Liter):
O Wein/Sekt Anzahl der Gläser (0,2 Liter):
O Likör Anzahl der Gläser (0,05 Liter):
O Schnaps Anzahl der Gläser (0,05 Liter):

Wenn Sie an einem Tag Alkohol trinken, wie viel alkoholhaltige Getränke trinken Sie dann typischerweise?

O 1 oder 2 **O** 3 oder 4 **O** 5 oder 6 **O** 7 oder 8 **O** 10 oder mehr

	Nie	**Seltener als einmal pro Monat**	**Einmal im Monat**	**Einmal pro Woche**	**Täglich oder fast täglich**
e) Wie oft haben Sie im letzten Jahr mehr als 6 alkoholische Getränke getrunken?	O	O	O	O	O
f) Wie oft haben Sie im letzten Jahr festgestellt, dass Sie mehr getrunken haben, als Sie eigentlich wollten?	O	O	O	O	O
g) Wie oft haben Sie im letzten Jahr im Zusammenhang mit dem Alkoholtrinken eine Aufgabe nicht erledigt, die man eigentlich von Ihnen erwartet hatte?	O	O	O	O	O
h) Wie oft haben Sie im letzten Jahr morgens Alkohol getrunken, um in Schwung zu kommen?	O	O	O	O	O
i) Wie oft fühlten Sie sich im letzten Jahr schuldig oder hatten ein schlechtes Gewissen aufgrund Ihres Alkoholtrinkens ?	O	O	O	O	O
j) Wie oft im letzten Jahr waren Sie aufgrund des Alkoholtrinkens nicht in der Lage, sich an Ereignisse der letzten Nacht zu erinnern?	O	O	O	O	O

Würden Sie oder jemand anderes schon einmal verletzt, weil Sie Alkohol getrunken haben?
O Ja O Nein

Hat sich schon einmal ein verwandter, ein Freund, ein Arzt oder jemand anderes über Ihr Alkoholtrinken Sorgen gemacht oder Ihnen vorgeschlagen, weniger zu trinken?
O Ja O Nein

Haben Sie in den letzten 6 Monaten regelmäßig alkoholfreies Bier getrunken?
O Ja O Nein
Wenn ja wie viel und wann zuletzt ?

Haben Sie in den letzten 6 Monaten regelmäßig Malzbier getrunken?
O Ja O Nein
Wenn ja wie viel und wann zuletzt ?

Haben Sie sich in den letzten 3 Monaten die Haare gefärbt/getönt oder sich eine Dauerwelle anfertigen lassen?
O Ja O Nein

Benutzen Sie regelmäßig Haarspray, Haarschaum oder Haarwasser?
O Ja O Nein

Bitte kreuzen Sie an, was Sie in der letzten **Woche** zu sich genommen haben:

O Alkoholhaltige Pralinen/Eis/Süßspeisen
O Alkoholhaltige Saucen/Suppen O Alkoholfreies Bier/Wein/Sekt
O Mehr als 1 Liter Obstsaft pro Tag Oandere alkoholhaltige Lebensmittel

Haben Sie in der letzten Woche außer den von uns verschriebenen Medikamente mit möglichen alkoholischen Bestandteilen, wie z.B. Hustensaft oder pflanzliche Tropfen eingenommen?
O Ja Welche ? O Nein

Haben Sie in der letzten **Woche** Gebrauch gemacht von:

O Mundwasser (Wenn möglich, bitte Produkt und Marke angeben)
O Haarwasser (Wenn möglich, bitte Produkt und Marke angeben)
O Händedesinfektionsmittel (Wenn möglich, bitte Produkt und Marke angeben)

Außerdem würden wir Sie gerne noch um folgende Angaben bitten:
In meinem Haushalt lebe ich

O allein. O mit Partner und Kindern.
O mit Partner. O anderes

Ich bin zurzeit

O voll berufstätig O eingeschränkt berufstätig
O berentet O arbeitslos

Rauchen Sie und wenn ja wie viel?
O nein O mittlerweile nicht mehr
O ja Schachteln pro Tag:
Anzahl der Jahre:

9.2. Current research on NAFLD: type of study, number of subjects, number of women in each study, follow-up, and method of assessing alcohol consumption.

Table 37. Current research on NAFLD and moderate alcohol consumption: type of study, number of subjects, number of women in each study, follow-up, and method of assessing alcohol consumption.

Study	Type of study	Patients (n)	♀ (n)	Follow up	Assessment consumption			TQ	ACM
					SR	I	DP		
Blomdahl et al., 2021	Cross-sectional cohort	86	25	-	✓	✓		AUDIT-C	PEth
Åberg et al. 2020	Cross-sectional and longitudinal (R)	27,774 8,345	? 3,297	- 11,1 y	✓	✓	✓	Standarized procedures of the MONICA	
Kashiwagi et al. 2020	Cross-sectional cohort	268	68	-	✓	✓	✓		
Hajifathalian et al. 2019	Longitudinal (R)	4,568	2,622	70 m	✓				
Chang et al. 2019	Longitudinal (R)	58,927	10,606	4.9 y	✓		✓	CAGE	
Kimura et al. 2018	Longitudinal (P)	301	165	6 y	✓	✓			
Ajmera et al. 2018	Longitudinal (R)	285	200	4.26 v	✓		✓	AUDIT, AUDIT-C, SKINNER	
Mitchell et al. 2018	Cross-sectional cohort	187	116	-	✓	✓	✓	SDRA	
Yamada et al. 2018	Cross-sectional	178	87	-	✓				
Patel et al. 2017	Cross-sectional cohort	151	96	-	✓			AUDIT and intake preceding 5 years	
Hagström et al. 2017	Cross-sectional cohort	139	56	-	✓			AUDIT and SKINNER	PEth
Sogabe et al. 2016	Cross-sectional	1,141	1,141	-	✓			Standardised SR-questionnaire	
Sookoian et al. 2016	Cross-sectional	466	301	-	✓	✓	✓		
Moriya et al. 2015	Longitudinal (R)	5297	1524	ND	✓		✓		
Kächele et al. 2015	Cross-sectional	432	250	-		✓			
Takahashi et al. 2015	Cross-sectional	8,029	2,284	-		✓			
Hashimoto et al. 2015	Longitudinal (R)	5,437	1,990	10 y	✓				
Kwon et al. 2014	Cross-sectional cohort	77	43	-	✓			SKINNER	
Hamaguchi et al. 2012	Cross-sectional	18,571	7,721	-	✓				
Dunn et al. 2012	Cross-sectional cohort	582	384	-	✓			AUDIT and LTDH	
Hiramine et al. 2011	Cross-sectional cohort	9,886	0	-	✓				
Moriya et al. 2011	Cross-sectional	7,112	2,155	-	✓		✓		

Ascha et al. 2010	Longitudinal (P)	510	183	3.2 y	✓		✓	
Yamada et al. 2010	Cross-sectional and Longitudinal (R)	63,447 10,424	31,009 5,529	- 5 y	✓	✓		
Ekstedt et al. 2009	Longitudinal	71	20	13.8 y	✓		✓	AUDIT-C
Cotrim et al. 2009	Cross-sectional	132	91	-		✓	✓	Interview: patients and relatives
Gunji et al. 2009	Cross-sectional	5,599	0	-	✓	✓		
Dunn et al. 2008	Cross-sectional	11,754	6886	-		✓		
Suziki et al. 2007	Cross-sectional and Longitudinal (R)	1,177 326	0 0	- 5 y	✓			
Dixon et al. 2001	Cross-sectional cohort	108	82	-	✓	✓		

SR: self-report, I: interview, DP: drinking patterns, TQ: Type of questionnaire, ACM: alcohol consumption marker, R: retrospective, P: prospective, y: years, months, v: visits. AUDIT: alcohol use disorder identification test, AUDIT-C: alcohol use disorder identification test-consumption, SKINNER: Skinner Lifetime Drinking History, LTDH: lifetime drinking history questionnaire, SDRA: seven-day recall measurement of alcohol consumption, SR: self response

Blue: exclusive male/female population. Rosa: studies suggesting protective effect of moderate alcohol consumption in NAFLD. Orange: studies suggesting detriment/no effect of moderate alcohol consumption in NAFLD.

9.3. Alcohol consumption groups used in current research in NAFLD

Table 38. Alcohol consumption groups used in each study and proportion of subjects in each group.

Study	Alcohol consumption groups	ABS	LT-ABS
Blomdahl et al., 2021	**Low-** vs. **moderate consumption**: >66g/w (AUDIT-C), > 96g/w (interview) and PEth ≥ 50 ng/ml.	✗	✗
Åberg et al. 2020	**Lifetime abstainers**, **0-9**, **10-19**, **20-29**, **30-39**, and **40-49** g/d.	✓	✓
Kashiwagi et al. 2020	Alcohol intake: **non-**, **light-**: 0.1-6.9 drinks/w and **moderate-drinkers**: ♂: 7-20.9 and ♀: 7-13.9 drinks/w Drinking pattern: **non-drinking**, **0.1-4** and **5-7** days/w	✓	✗
Hajifathalian et al. 2019	**Non-drinking**: < 7 g/day, **modest drinking**: 7-21 g/day, **more than modest drinking**: ≥21 g/day	✗	✗
Chang et al. 2019	**Non-drinking**, **light drinking**: 1-10 g/d and **moderate drinking**: 10-20 g/d (♀), 10-30 g/d (♂)	✓	✗
Kimura et al. 2018	**Non-drinking** vs **mild drinking**: < 20g/d	✗	✗
Ajmera et al. 2018	**Lifetime non-drinking** vs **modest drinking**: <10 g/d (♀), < 20g/d (♂)	✓	✓
Mitchell et al. 2018	**Lifetime abstainers**, **modest drinkers**: <70 g/week, **moderate drinkers**: ≥70g/week	✓	✓
Yamada et al., 2018	**Non-drinkers**: 0 g/day, **light drinking**: 0-20 g/day	✓	✗
Patel et al., 2017	**Lifetime non-drinkers**, **light drinkers**: always ≤ 20g/d and **moderate drinkers**: any period >20 g/d	✓	✓
Hagström et al., 2017	**Below-** (13.2 g/week), **above median lifetime alcohol intake** (13.2 g/week)	✗	✗
Sogabe et al. 2016	**Non-drinkers**: under 120g/year and **Light drinkers**: 0-20 g/drinking day	✗	✗
Sookoian et al. 2016	**Lower exposure to alcohol**: rs1229984: AG + AA vs **higher exposure to alcohol**: rs1229984: GG	✗	✗
Moriya et al. 2015	Alcohol intake: **non-drinkers**, **0.1-69.9** g/w, **70-139.9** g/w, **140-279.9** g/w, **≥ 280** g/w Drinking pattern: **non-drinker**, **1-3** drinking days/w, **4-6** drinking days/w, **7** drinking days/w	✓	✗
Kächele et al. 2015	**Non-drinkers**, **>0-20** g/d, **>20-40** g/d, **>40-60** g/d, **>60** g/d.	✓	✗
Takahashi et al. 2015	**Non-drinkers**: <20 g/d, **moderate drinkers**: 20-50 g/d, **heavy drinkers**: >50 g/d	✗	✗
Hashimoto et al. 2015	**None or minimal**: <40g/w, **light-**: 40-140 g/w, **moderate-**: 140-280 g/w, **heavy consumption**: >280 g/w	✗	✗
Kwon et al. 2014	Lifetime alcohol intake: **<24 gram-years**, **≥ 24 gram-years** Lifetime alcohol intake: **Lifetime abstinence**, **alcohol users**	✓	✓
Hamaguchi et al. 2012	**None to minimal-**: <40 g/w, **light-**: 40-140 g/w, **moderate-**: 140-280 g/w, **excess drinkers**: >280 g/week	✗	✗
Dunn et al. 2012	**Lifetime non-drinkers**, **modest drinkers**: 0-20 g/day	✓	✓
Hiramine et al. 2011	**Never-drinker**: 0 g/d, **light drinker**: 0 - <20 g/d, **moderate drinkers**: 20-59 g/d, **heavy drinkers**: ≥ 60 g/d	✓	✗

Moriya et al. 2011	Alcohol intake: **non-drinkers**, **0.1-69.9** g/w, **70-139.9** g/w, **140-279.9** g/w**, ≥ 280** g/w Drinking pattern: **non-drinker**, **1-3 days/w**, **4-6 days/w** and **7 days/w**.	✓	✗
Ascha et al. 2010	**Never drinker**, **Social alcohol intake**: ≤ 2 drinks/d or 3-6 drinks daily on weekends, **significant alcohol intake**: > 2 drinks/d or >6 drinks daily on weekends for the past 5 years, **Formerly significant alcohol intake**: more than social alcohol intake within the past 5 years	✓	✗
Yamada et al. 2010	**Non-drinker**, **occasional drinkers**, **daily moderate drinkers** (~23 g/d), **daily heavy drinkers**: (≥ 46g/d)	✓	✗
Ekstedt et al. 2009	ND	✗	✗
Cotrim et al. 2009	**Non-drinker, Light drinker**: < 20 g/d, **moderate drinker**: 20-40 g/d	✓	✗
Gunji et al. 2009	Alcohol intake: **Non-drinker**: <40g/w, **light-**: 40-140 g/w, **moderate-**: 140-280 g/w, **Heavy drinker**: >280 g/w Drinking pattern: **1 days/month**, **11-20 days/month**, **> 20 days/month**	✗	✗
Dunn et al. 2008	Alcohol intake: **non-drinker, modest drinker**: up to 1 alcoholic beverage per day Beverage preference: **modest wine drinker**, **modest beer drinker**, **modest liquor drinker**, **modest mixed drinker**	✓	✗
Suzuki et al. 2007	**None or minimal**: <70 g/w, **light**: 70-140 g/w, **moderate**: 140-280 g/week, **excess drinkers**: ≥ 250 g/w	✗	✗
Dixon et al. 2001	**Non-drinker**, **< 20**, **20-100**, **100-200** g/w	✓	✗

I: alcohol intake, DP: drinking pattern, B: alcohol beverage, TDD; typical drinking day, LT: lifetime alcohol consumption, SKINNER: Skinner lifetime Drinking History questionnaire. AUDIT: alcohol use disorder identification test, w: week, d: day

Rosa: studies suggesting protective effect of moderate alcohol consumption in NAFLD. Orange: studies suggesting detriment/no effect of moderate alcohol consumption in NAFLD.

9.4. Summary of studies suggesting a protective effect of moderate alcohol intake of NAFLD prevalence or NAFLD progression

Table 39. Summary of studies suggesting a protective effect of moderate alcohol intake on NAFLD prevalence or NAFLD progression

Study	Outcome measure	Results
Dixon et al. 2001	LB	**Subjects**: 108 obese patients (BMI >35 kg/m^2) (Australia). **Groups**: alcohol intake. No consumption, <20, 20-100, 100-200 g/week. **Results**: Moderate alcohol consumption was associated with decreased risk of NASH (OR 0.35, 95%CI 0.12-1.00, p=0.04) and diabetes (OR 0.18, 95% CI 0.12-1). The effect of alcohol on NASH was not significant after controlling for diabetes or insulin resistance. **Conclusion**: insulin resistance and systemic hypertension, features of the metabolic syndrome, are independently associated with advanced forms of NAFLD. Moderate alcohol consumption seems to reduce the risk of NAFLD in the severely obese, possibly by reducing insulin resistance.
Suzuki et al. 2007	Blood: ALT	**Subjects**: cross-sectional 1,177 men, 5-year longitudinal: 326 men (Japan). **Groups**: alcohol intake. None or minimal (0-70 g/week), light (70-140 g/week), moderate (140-280 g/week) and excess drinkers (>280 g/week). **Results**: Excess alcohol consumption was associated with increased odds of hypertransaminasemia versus none or minimal consumption (aOR 1.4, 95%CI 1.1-1.93, p=,0.023) There was significant interaction between age group and alcohol consumption (p<0.01). In the younger group, moderate consumption was associated with decreased odds (aOR 0.5, 95%CI 0.3-0.9, p=0.032), while in the older group, light consumption was associated with decreased odds (aOR 0.6, 95%CI 0.4-1.0, p=0.036) and excess consumption was associated with increased odds (aOR 1.6, 95% CI 1.1-2.3, p=0.014) of hypertransaminasemia. During follow-up, moderate consumption was associated with decreased incidence of hypertransaminasemia versus none or minimal consumption (aOR0.4, 95%CI 0.1-0.9, p=0.02) **Conclusion**: light to moderate alcohol consumption may protect against the development of hypertransaminasemia among male subjects without other liver conditions. Further studies are required before recommending light to moderate alcohol consumption.
Dunn et al. 2008	Blood: ALT	**Subjects**: 11,754 subjects with suspected NAFLD (elevated ALT) (USA). **Groups**: alcohol intake last month and beverage preference. Non-drinkers, modest wine drinkers, modest beer drinkers, modest liquor drinkers and modest mixed drinkers. Modest drinker (up to one drink per day) **Results**: Modest wine consumption up to one serving per day compared to non-alcohol use is associated with a lower prevalence of suspected NAFLD (aOR 0.51, 95% CI 0.33-0.79).

		Conclusion: modest wine consumption is associated with reduced prevalence of suspected NAFLD. The current study supports the safety of one glass of wine per day for cardioprotection in patients at risk for both coronary heart disease and NAFLD.
Gunji et al. 2009	US	**Subjects**: 5,599 asymptomatic men (Japan). **Groups**: hepatic steatosis (sonography). Fatty liver vs. non-fatty liver. **Results**: Light (40-140 g/week) and moderate (140-280 g/week) alcohol consumption significantly and independently reduced the likelihood of hepatic steatosis (light: OR 0.82, 95%CI 0.68-0.99, p=0.044, moderate: OR 0.75, 95%CI 0.61-0.93, p=0.008) **Conclusion**: the prevalence of fatty liver was significantly and independently decreased hy light and moderate alcohol consumption in men of an asymptomatic Japanese population.
Yamada et al. 2010	US	**Subjects**: cross-sectional: 63,447 asymptomatic subjects. Longitudinal: 10,424 asymptomatic subjects (Japan). **Groups**: alcohol intake. Non-drinkers, occasional drinkers, daily moderate drinkers (~ 23 g/day) and daily heavy drinkers (>46 g/day). **Results**: Cross-sectional: The prevalence of hepatic steatosis in non- (♂: 28.5%, ♀: 12.4%), occasional (♂: 27.5%, ♀: 7.7%), daily moderate (♂: 18.7%, ♀:5.4%) and daily heavy drinkers (♂:19.1%, ♀:6.7%) has an inverse association (p≤0.05). Longitudinal: The risk of newly developed hepatic steatosis was significantly lower in daily moderate (aOR 0.72, 95%CI 0.58-0.89) and daily heavy (aOR 0.65, 95%CI 0.50-0.85) drinkers than non-drinkers in men. **Conclusion**: alcohol drinking may not be a major risk for fatty liver in Japanese undergoing a health check-up.
Moriya et al. 2011	US	**Subjects**: 7,112 asymptomatic subjects (Japan). **Groups**: drinking frequency. Non-drinker, 1-3 days/week, 4-6 days/week and 7 days/week. **Results**: Alcohol consumption was inversely associated with fatty liver (aOR 0.54, 95%CI 0.46-0.63). There was a significant inverse correlation between drinking frequency and the prevalence of fatty liver (1-3 days/week: 38%, 4-6 days/week: 29%, daily drinking: 16%, p<0.001). Drinking less than 20g on 1-3 days/week was associated with low prevalence of fatty liver (aOR 0.47, 95%CI 0.23-0.96) **Conclusion**: alcohol consumption appears to protect against NAFLD.
Hiramine et al. 2011	US	**Subjects**: 9,886 asymptomatic subjects (Japan) **Groups**: Alcohol intake: never-drinker: 0 g/day, light drinker: 0 - <20 g/day, moderate drinkers: 20-59 g/day, heavy drinkers: ≥ 60 g/day. **Results**: The prevalence of fatty liver displayed a U-sharped-curve across the categories of daily alcohol consumption (non-drinkers: 44,7%, light: 39.3%, moderate: 35.9%, heavy: 40.1%, p<0.001). The prevalence of fatty liver was associated inversely with light (OR 0.71, 95% CI 0.59-0.86) and moderate (OR 0.55, 95% CI 0.32-0.62) alcohol consumption as determined by multivariate analysis after adjusting for potential confounding variables. Examination of drinking patterns (frequency and volume) revealed that the prevalence of fatty liver was inversely associated with the frequency of alcohol consumption (≥ 21 days/month, OR 0.62, 95%CI 0.53-0.71) but not with the volume of alcohol consumed.

		Conclusion: our observations suggest that alcohol consumption plays a protective role against fatty liver in men, and consistent alcohol consumption may contribute to this favourable effect.
Dunn et al. 2012	LB	**Subjects**: 582 NAFLD subjects (USA). **Groups**: lifetime alcohol intake. Lifetime non-drinkers and modest drinkers. **Results**: Modest drinkers compared to non-drinkers had lower odds of having a diagnosis of NASH (OR 0.56, 95% CI 0.39-0.84, p=0.002). Modest drinkers had a significantly lower odds for fibrosis (OR 0.56, 95% CI 0.41-0.77) and ballooning hepatocellular injury (OR 0.66, 95% CI 0.48-0.92) than lifetime non-drinkers. **Conclusion**: In a large, well-characterized population with biopsy proven NAFLD, modest alcohol consumption was associated with lesser degree of severity as determined by lower odds of the key features that comprise a diagnosis of steaohepatitis, as well as fibrosis. These findings demonstrate that need for prospective studies and coordinated consensus on alcohol consumption recommendations in NAFLD
Hamaguchi et al. 2012	US	**Subjects**: 18,571 asymptomatic subjects (Japan). **Groups**: alcohol intake last month. Non or minimal alcohol consumption (<40 g/week), light alcohol consumption (40-140 g/week), moderate alcohol consumption (140-280 g/week) and excess alcohol consumption (> 280 g/week). **Results**: The prevalence of fatty liver decreased in men and women with light to moderate alcohol consumption. The OR of fatty liver was clearly <1.0 in men with any level of alcohol consumption (light: OR 0.69 95%CI 0.6-0.79, moderate: OR 0.72, 95%CI 0.63-0.83, excess: 0.74 95%CI 0.64-0.85, p<0.001) and in women with light (OR 0.54, 95%CI 0.34-0.88, p=0.012) to moderate (OR 0.43 95%CI 0.21-0.88, p=0.021) alcohol consumption. **Conclusion**: Light to moderate alcohol consumption has a favourable effect for fatty liver, but not for metabolic syndrome in Japanese men and women.
Kwon et al. 2014	LB	**Subjects**: 77 NAFLD subjects (USA). **Groups**: lifetime alcohol intake. A: threshold 24 g/years. B: Lifetime abstinence vs alcohol use **Results**: Alcohol consumption ≥ 24 gram-years was associated with less severe disease (OR 0.26, 95%CI 0.07-0.97, p=0.046). Patients who consumed ≥ 24 gram-years had significantly lower fibrosis scores on liver histology (1.2 ± 1.0 vs 1.8 ± 1.2, p=0.03) **Conclusion**: Some degree of regular alcohol consumption over the course of a lifetime compared to minimal intake appears to have a protective effect on the histological severity of liver disease among patients with strictly defined NAFLD.
Hashimoto et al. 2015	US	**Subjects**: 5,437 asymptomatic subjects (Japan). **Groups**: alcohol intake last month. None or minimal alcohol consumption (<40 g/week), light alcohol consumption (40-140 g/week), moderate alcohol consumption (140-280 g/week) and heavy alcohol consumption (>280 g/week) **Results**: In men, the adjusted hazard risk of light and moderate alcohol consumption for the development of fatty liver were 0.72 (95% CI 0.60-0.86, p<0.001) and 0.69 (95%CI 0.57-0.84, p<0.001), respectively. However, they were not significant in women.

		Conclusion: The newly onset of fatty liver was significantly repressed in apparently healthy men who consume light to moderate alcohol.
Takahashi et al. 2015	US	**Subjects**: 8029 asymptomatic subjects (Japan). **Groups**: alcohol intake last year. Non-drinkers (<20 g/day), moderate drinkers (20-50 g/day) or heavy drinkers (>50 g/day) **Results**: Heavy alcohol intake was a significant risk factor for fatty liver in women (OR 3.35) Moderate alcohol intake was a significant negative risk factor for hepatic steatosis in obese subjects (BMI ≥ 25 kg/m^2) (OR 0.74 non-obese vs 0.39 obese) Heavy alcohol intake (>50g/day) was a significant negative risk factor in obese males (OR 0.62) Heavy alcohol intake was a risk factor in non-obese males (OR 1.29) and in all females (non-obese: OR 2.22, obese 6.6) **Conclusion**: The influence of alcohol intake on fatty liver differed depending on the level of alcohol consumption, gender, and the presence of obesity, and showed biphasic effects.
Kächele et al. 2015	US	**Subjects**: 432 population-based subjects (Germany). **Groups**: alcohol intake last week. Non-drinkers, <20 g/day, 20-40 g/day, 40-60 g/day, >60 g/day. **Results**: Presence of fatty liver disease was markedly reduced in subjects drinking 0-20 g/day (19%), compared to non-drinkers (35%) and heavy drinkers (20-40 g/day: 34%, 40-60 g/day: 38.6%, >60g/day: 44.9%) **Conclusion**: based on data from a population-based sample, there is no evidence for a link between fatty liver disease, alcohol consumption, and inflammatory cardiovascular risk markers. However, larger prospective studies are needed to confirm this.
Moriya et al. 2015	US	**Subjects**: 5,297 asymptomatic subjects (Japan). **Groups**: alcohol intake: non-drinkers, 0.1-69.9 g/week, 70-139.9 g/week, 140-279.9 g/week and ≥ 280 g/week. **Results**: In men, drinking 0.1-69.9 g/week (OR 0.79, 95%CI 0.68-0.90), 70-139.9 g/week (OR 0.73, 95%CI 0.63-0.84), 140-279.9 g/week (OR 0.69, 95%CI 0.60-0.79) and drinking ≥ 280g/week (OR 9.68, 95%CI 0.58-0.79) were inversely associated with hepatic steatosis after adjusting for obesity, exercise, and smoking. In women, drinking 0.1-69.9 g/week (OR 0.71, 95%CI 0.52-0.96) and drinking 70-139.9 g/week (OR 0.67, 95%CI 0.45-0.98) were inversely associated with fatty liver after adjusting for obesity, exercise, and smoking. **Conclusion**: light to moderate alcohol consumption, or even somewhat excessive amounts especially in men, was likely to protect most individuals against fatty liver over time.
Hagström et al. 2017	LB	**Subjects**: 139 NAFLD subjects (Sweden). **Groups**: lifetime alcohol intake. Above and below median lifetime alcohol consumption (1.1. units/week). **Results**: An increase in median weekly alcohol consumption to a maximum of 13 drinks per week was associated with lower fibrosis stage (aOR for each incremental unit 0.86, 95%CI 0.76-0.97, p=0.017). The lowest risk for fibrosis was found with the lowest odd seen in the top quartile of alcohol consumption (3.1-13.3 units of alcohol per week) (aOR 0.23, 95% CI 0.08-0.66, p=0.006)

		Subjects with PEth ≥ 0.3µmol/l had a higher ORs for a higher fibrosis stage (aOR 2.77, 95% CI 1.01-7.59, p=0.047) **Conclusion**: lifetime alcohol consumption with up to 13 units per week is associated with lower fibrosis stage in NAFLD. Elevated PEth is associated with higher stages of fibrosis.
Yamada et al. 2018	LB	**Subjects**: 178 NAFLD subjects (Japan). **Groups**: alcohol intake. Non-alcohol and light alcohol consumption (≤20 g/day) **Results**: No significant differences in steatosis (p=0.433) or inflammation (p=0.871) score were noted among the groups. The ballooning (aOR 0.56, 95%CI 0.36-0.91, p=0.017) and fibrosis (aOR 0.71, 95%CI 0.51-0.98, p=0.035) scores were significantly lower in the light alcohol consumer group than in the non-alcohol group. Gene expression analysis revealed a marked inhibition of the pathways involved in the immune response in the light alcohol group compared to that in the non-alcohol group. **Conclusion**: Light alcohol consumption might supress activity of non-alcoholic steatohepatitis by reducing gene expression levels involved in the immune response. This inhibition in gene expression was associated with a lowering of liver fibrosis and hepatocellular injury.
Mitchell et al. 2018	LB	**Subjects**: 187 NAFLD subjects (Australia). **Groups**: alcohol intake last week. Abstinent, <70 g/week, ≥ 70 g/week. **Results**: Modest alcohol consumption (1-70 g/week) was associated with lower mean fibrosis stage compared to lifetime abstainers (modest: 0.9±1.1 vs lifetime abstainers: 1.6±1.6, p<0.05) and a decreased risk of advanced fibrosis (OR 0.33, 95% CI 0.14-0.78, p=0.001). Exclusive wine drinkers but not exclusive beer drinkers, had lower mean fibrosis stage (exclusive wine: 0.8±1.1 vs lifetime abstinent: 1.6±1.6 p<0.05) and lower odds of advanced fibrosis (OR 0.20, 95% CI 0.06-0.69, p=0.01), compared to lifetime abstinent subjects. **Conclusion**: modest (1-70 g/week) alcohol consumption, particularly wine in a non-binge pattern, is associated with lower fibrosis in patients with NAFLD. Prospective longitudinal studies into fibrosis progression, cardiovascular outcomes, and mortality are required before clinical recommendations can be made.
Hajifathalian et al. 2019	HSI	**Subjects**: 4568 NAFLD subjects (USA). **Groups**: alcohol intake last 12 months. Non-drinkers: <0.5 drinks/day, modest drinkers: 0.5-1.5 drinks/day, ≥ 1.5 drinks/day. **Results**: Drinking 0.5-1.5 drinks per day decreased the risk of overall mortality by 41% (aHR 0.64, 95%CI 0.42-0.97, p=0.035) compared to not drinking. Drinking ≥ 1.5 drinks per day showed a significative effect on mortality (aHR 1.45, 95%CI 1.01-2.10, p=0.047). **Conclusion**: among patients with NAFLD modest alcohol consumption is associated with a significant decrease in all-cause mortality, while drinking ≥ 1.5 drinks per day is associated with an increase in mortality. These results help to inform the discussion of potential risk and benefits of alcohol use in patietns with NAFLD

LB: liver biopsy, US: ultrasonography, NIM: non-invasive markers, aOR: adjusted odds ratio, OR: odds ratio, CI: confidence interval, aHR: adjusted hazard risk

9.5. Summary of studies suggesting a detrimental effect or no effect of moderate alcohol intake on NAFLD or NAFLD progression

Table 40. Summary of studies suggesting a detrimental effect or no effect of moderate alcohol intake on NAFLD prevalence or NAFLD progression

Study	Outcome measure	Results
Ekstedt et al. 2009	Repeated LB	**Subjects**: 71 NAFLD patients (Sweden). **Groups**: significant progression vs. no significant progression in fibrosis stage. **Results**: The proportion of patients reporting heavy episodic drinking at least one a month was higher among those with significant fibrosis progression (47% vs 11%. p=0.003) and a trend towards higher weekly alcohol consumption was also seen (38 vs. 17 g/week, p=0.061). Heavy alcohol drinking (p<0.001) and insulin resistance (p<0.01) were independently associated with significant fibrose. **Conclusion**: moderate alcohol consumption, consistent with the diagnosis of NAFLD to be set, is associated with fibrosis progression in NAFLD. These patients should be advised to refrain from heavy episodic drinking.
Cotrim et al. 2009	LB	**Subjects**: 132 morbidly obese patients (BMI >40 kg/m^2 or BMI >30 kg/m^2 with others associated conditions such as hypertension, diabetes, hyperlipidaemia, or sleep apnoea) (Brazil). **Groups**: alcohol intake. G1 (20-40 g/day), G2 (<20 g/day), and G3 (no intake) **Results**: The presence of insulin resistance was similar in G1 and G3 (81.3 and 78.7%, respectively) but significantly less in G2 (54%, p<0.05) in severely obese patients. Light to moderate alcohol consumption did not correlate with the severity of NAFLD in morbidly obese patients undergoing bariatric surgery. **Conclusion**: the results suggest that light to moderate alcohol consumption may have a protection effect against insulin resistance in severely obese patients. However, it had no impact on the severity of activity and stage of liver disease.
Ascha et al. 2010	CT AFP	**Subjects**: 510 cirrhosis patients (USA). **Groups**: 195 NASH-cirrhosis and 315 HCV-cirrhosis. **Results**: The median follow-up was 3.2 years, during which 12.8% of NASH-cirrhotic and 20.3% of HCV-cirrhotic patients developed hepatocellular carcinoma (p=0.03). Yearly cumulative incidence of hepatocellular carcinoma was found to be 2.6% in patients with NASH-cirrhosis, compared with 4% in patients with HCV-cirrhosis (p=0.09) Multivariate regression analysis revealed that older age (p=0.006) and alcohol consumption (p=0.002) were independent variables associated with development of hepatocellular carcinoma in patients with NASH-cirrhosis. Compared with non-drinkers, patients who reported any regular alcohol consumption were at greater risk for hepatocellular carcinoma development (HR 3.6, 95% CI 1.5-8.3, p=0.003) **Conclusion**: patients with NASH cirrhosis have a greatly increased risk of liver cancer. Alcohol consumption, a modifiable risk factor, appears to be the most significant factor associated with risk of hepatocellular carcinoma development in our study population.

Sogabe et al. 2016	Blood: ALT	**Subjects**: 1141 women with fatty liver (ultrasound) (Japan). **Groups**: alcohol intake. Non-drinkers vs. light drinkers. **Results**: There was no significant difference in the prevalence of NAFLD and ALT between light drinkers and non-drinkers. BMI, waist circumference, diastolic blood pressure, triglyceride, uric acid, impaired glucose tolerance, and visceral fat type were significant predictors of the prevalence of fatty liver with ALT elevation in logistic regression analysis. **Conclusion**: there was no significant difference in the prevalence of fatty liver with ALT elevation in females with metabolic syndrome between light drinkers and non-drinkers, suggesting that other factors such as BMI, waist circumference, visceral fat type, and lifestyle-related disease may be more important than low alcohol consumption for the prevalence of fatty liver with ALT elevation.
Sookoian et al. 2016	LB	**Subjects**: 266 NAFLD patients (Argentina). **Groups**: Genetic variant (rs1229984 A;G) in the alcohol dehydrogenase. Carriers at least one A-allele (AG + AA) vs carriers both G-Allele (GG) **Results**: Carriers of A-allele consumed significantly lower amounts of alcohol compared with non-carriers (2.3±5.3 vs 8.18±21 g/day, p=0.03). Carriers of the A-allele had lower degree of histological steatosis (1.76±0.83 vs 2.19±0.78, p=0.03) and lower scores of lobular inflammation (0.54±0.65 vs. 0.95±0.92, p=0.02) and NAFLD-Activity Score (2.9±1.4 vs. 3.7±1.4, p=0.015) compared with non-carriers. **Conclusion**: mendelian randomisation analysis suggests no beneficial effect of moderate alcohol consumption on NAFLD disease severity.
Patel et al. 2017	TE	**Subjects**: 151 patients with NAFLD and type 2 diabetes mellitus (Australia). **Groups**: alcohol intake last 5 years. Lifetime non-drinkers, light-drinkers (median 4.8 g/week), and moderate drinkers (median 66.7 g/week). **Results**: Compared to lifetime non-drinkers, light drinkers had 1.79 (95%CI 0.67-4.82, p=0.247) and moderate drinkers had 0.91 (95% CI 0.27-3.10, p=0.881) times the odds of having liver stiffness measurements ≥ 8.2 kPa (adjusted for age, gender, and body mass index) **Conclusion**: in diabetic patients with NAFLD, light or moderate alcohol consumption was not significantly associated with liver fibrosis.
Kimura et al. 2018	US or CT AFP	**Subjects**: 301 NAFLD patients (Japan). **Groups**: alcohol intake last 2 years. Mild drinking vs. non-drinking (<20g/day) **Results**: Over 6 years of observation, the HCC appearance rate was significantly higher in the mild drinking group (6.5% vs. 1.4%, p=0.02). Hepatic advanced fibrosis (F3-4) (RR 11.60, 95%CI 2.36-56.9, p<0.01), diabetes mellitus (RR 89.50, 95%CI 6.01-1331.2, p<0.01) and serum triglyceride (RR 0.98, 95%CI 0.95-0.99, p=0.04) were factors significantly related to HCC in all NAFLD patients, while the effect of drinking habit was marginal (RR 4.43, 95%CI 0.88-22.4, p=0.07). In patients with advanced fibrose (F3-F4) a drinking habit (RR 4.83, 95%CI 1.01-23, p=0.04), alpha-fetoprotein (RR 1.23, 95%CI 1.04-1.44, p=0.001) and diabetes mellitus (RR 12, 95%CI 1.20-119.66, p=0.03) were identified as significant contributors to HCC occurrence.

		Conclusion: a mild drink habit appears to be a risk factor for hepatocarcinogenesis in NAFLD patients, especially those with advanced fibrosis.
Ajmera et al. 2018	Paired LB	**Subjects**: 285 NAFLD patients no receiving pharmacologic therapy (USA). **Groups**: lifetime alcohol consumption. Lifetime non-drinkers vs. modest drinkers. **Results**: During a mean follow-up of 47 months between biopsies, non-drinkers had a greater mean reduction in steatosis grade than modest drinkers (reduction of 0.49 and 0.30, respectively, p=0.04) and a greater reduction in meal level of aspartate transaminase (reduction of 7U/l vs increase of 2 U/L, respectively, p=0.04). Modest drinkers had significantly lower odds of NASH resolution compared to non-drinkers (aOR 0.32, 95% CI 0.11-0.92, p=0.04). **Conclusion**: in a longitudinal analysis of liver biopsies from patients with NAFLD not receiving pharmacologic therapy, modest alcohol use was associated with less improvement in steatosis and level of aspartate transaminase, as well as lower odds of NASH resolution, compared to no use of alcohol.
Chang et al. 2019	FIB-4 and NFS	**Subjects**: 58927 subjects with NAFLD (South Korea) **Groups**: alcohol consumption. Non-drinkers, light drinkers (<10gday) vs. moderate drinkers (♀: 10-20 g/day, ♂: 10-30 g/day). **Results**: The aHR for worsening of FIB-4 comparing light-drinkers and moderate drinkers with non-drinkers were 1.06 (95%CI 0.98-1.16) and 1.29 (1.18-1.40), respectively. Using NFS, corresponding aHR comparing light drinkers and moderate drinkers with non-drinkers were 1.09 (1.02-1.16) and 1.31 (1.23-1.40), respectively. The association of moderate drinkers with worsening of either FIB-4 or NFS remain significant after introducing alcohol use and confounder treated as time-varying covariates. **Conclusion**: in this large-scale cohort of young and middle-aged individuals with NAFLD, non-heavy alcohol consumption, especially moderate alcohol consumption, was significantly and independently associated with worsening of non-invasive markers of fibrosis, indicating that even moderate alcohol consumption might be harmful.
Åberg et al. 2020	FLI	**Subjects**: 8345 subjects with fatty liver disease (FLI ≥ 60) (Finland). **Groups**: alcohol intake previous year. Lifetime abstainers, 0-9, 10-19, 20-29, 30-39 and 40-49 g/day. **Results**: Alcohol consumption showed a dose-dependent risk increase for incident advanced liver disease and malignancies. Consuming 10-19 g/day of alcohol in general or 0-9 g/day as non-wine beverages doubled the risk for advanced liver disease compared to lifetime abstainers. Alcohol intake up to 49 g/day was associated with a 22-40% reduction of incident cardiovascular disease, only in never smokers. A J-shaped association between alcohol intake and all-cause death with a maximal risk reduction of 21% (95% CI 5-34%) at alcohol intake of 0-9 g/day compared to lifetime abstainers was observed but only evident in never smokers. Alcohol intake >30 g/day yielded increased risk estimates for mortality compared to lifetime abstainers.

		Conclusion: even low alcohol intake in NAFLD is associated with increased risks for advanced liver disease and cancer. Low to moderate alcohol use is associated with reduced mortality and cardiovascular disease risk but only among never smokers.
Kashiwagi et al. 2020	FIB-4 and NFS	**Subjects**: 286 NAFLD subjects (Japan). **Groups**: alcohol intake (Non-drinkers, light drinking: 0.1-6.9 drinks/week and moderate alcohol consumption: ♂: 7-20.9 drinks/week and ♀: 7-13.9 drinks/week) and drinking pattern (non-drinking, 0.1-4 days/week and 5-7 days/week) **Results**: Moderate alcohol consumption had a significant association with intermediate-high grade of FIB-4 (OR 1.87, 95% CI 1.21-2.89, p=0.005) or NFS (OR 2.91, 95%CI 1.72-4.94, p<0.001) compared to non-drinkers. **Conclusion**: non-heavy drinking might not reduce the risk of cardiovascular disease in NAFLD subjects. On the contrary, even moderate drinking could promote hepatic fibrosis. Thus, NAFLD drinkers should not be recommended for even a moderate amount of alcohol.
Blomdahl et al., 2021	LB	**Subjects**: 86 NAFLD patients (Sweden). **Groups**: fibrosis stage. F0-2 and F3-4. **Results**: Average weekly alcohol consumption was higher in the group with advanced fibrosis. Moderate alcohol consumption, independently of the method of assessment, was associated with increased probability of advanced fibrosis (adjusted OR 5.5-9.7, 95% CI 1.05-69.6). Patients with type 2 diabetes mellitus consuming moderate amounts of alcohol had a significantly higher rate of advanced fibrosis compared with those consuming low amounts (50-60% vs 2.2-21.6%, p < 0.05). **Conclusion**: Moderate alcohol consumption, irrespective of assessment method (clinical interview, AUDIT-C, and PEth), was associated with advanced fibrosis. PEth in blood ≥ 50 ng/ml may be a biological marker indicating increased risk for advanced fibrosis in NAFLD. Patients with T2DM consuming moderate amounts of alcohol had the highest risk of advanced fibrosis, indicating a synerstic effect of insulin resistance and alcohol on the histopathological progression of NAFLD.

LB: liver biopsy, US: ultrasonography, CT: computer tomography, TE: transient elastography, AFP: alpha fetoprotein, FLI: Fatty Liver Index (BMI, waist circumference, GGT, triglycerides), FIB-4: Fibrosis-4 index for liver fibrosis (age, AST, platelet count and ALT), NFS: NAFLD fibrosis score (age, BMI, impaired fasting glucose or diabetes, AST, ALT, platelets, albumin), aOR: adjusted odds ratio, OR: odds ratio, CC: correlation coefficient, CI: confidence interval, aHR: adjusted hazard risk, RR: risk ratio. Grey: no effect in NAFLD. Orange: detriment effect in NAFLD.

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11. Curriculum Vitae

Berufserfahrung

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04/2017 – 12/2019	Assistenzärztin Gastroenterologie und Hepatologie am UKE-Eppendorf, Hamburg
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Publikationen

Amadea Mosebach, Nadine Aboutara, Maria Rodriguez Lago, Alexander Müller, Melanie Lang, Lutz Fischer, Stefanie Iwersen-Bergmann, Martina Sterneck, 2020. Impaired diagnostic accuracy of hair ethyl glucuronide testing in patients with renal dysfunction. Forensic Sci Int 2020 Dec; 317:110518. doi: 10.1016/j.forsciint.2020.110518.

Sprachkenntnisse

Deutsch	Verhandlungssicher
Englisch	Fließend
Spanisch	Muttersprache

12. Eidesstattliche Erklärung

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

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