Non-Alcoholic Fatty Liver Disease
Insights into Alcohol Consumption, Genetics, and Proteomics

Julia Blomdahl
ABSTRACT

NAFLD (Non-Alcoholic Fatty Liver Disease) affects approximately a quarter of the global population and is closely linked to type 2 diabetes mellitus and obesity. The disease spectrum ranges from steatosis and steatohepatitis to fibrosis, cirrhosis, and hepatocellular cancer. However, accurately predicting which patients will experience a progressive disease course remains a significant challenge. The variant gene of PNPLA3 is known to be associated with NAFLD and a more progressive disease, although its precise function remains unclear.

Patients with NAFLD typically consume small to moderate amounts of alcohol, with recommended thresholds set at a maximum of 210 gram per week for males and 140 grams per week in females. However, the impact of alcohol consumption on liver disease in NAFLD remains disputed, with conflicting research findings.

Liver biopsy is considered the gold standard for diagnosing NAFLD. However, due to its impracticality for such a large population with the condition, various non-invasive methods have been explored for diagnosing and evaluating NAFLD.

This thesis aimed to investigate the potential effects of moderate alcohol consumption on NAFLD histology, explore the potential role of variant PNPLA3 in NAFLD, and assess the use of proteomics in classifying fibrosis.

In Papers I and II, moderate alcohol consumption was assessed through questionnaires, clinical interviews, and measurement of the direct alcohol biomarker phosphatidylethanol (PEth). Paper I, a cross-sectional study including 86 participants, showed an association between moderate consumption and advanced fibrosis. Moderate consumption was defined as consuming more than 66 grams of ethanol per week or a PEth-value over 50 ng/mL. Notably, individuals with both moderate alcohol consumption and a diagnosis of type 2 diabetes exhibited significantly more advanced fibrosis. Paper II was a cohort study where 82 participants were followed over 17.2 years. Similarly, participants with moderate alcohol consumption displayed significant fibrosis progression. The strongest association was observed in participants with PEth-value of 48 ng/mL or higher, or those with binge drinking.

In Paper III, the potential role of variant PNPLA3 was explored, exhibiting impaired autophagic flux and reduced lipophagy in variant PNPLA3 cells. Liver biopsies of NAFLD individuals with variant PNPLA3 displayed an accumulation of lipid droplets positive for both PNPLA3 and
LC3 (a common marker of the autophagosome). This suggests that PNPLA3 is part of the lipophagy process, which is impaired in the variant gene and contributes to steatosis.

**Paper IV** examined two independent NAFLD cohorts. In the discovery cohort, 60 participants with biopsy-proven NAFLD were included, while 59 participants were included in the validation cohort. The study evaluated 266 proteins and found that a biomarker model combining ACE2, HGF, and IGFBP-7 distinguished between different fibrosis stages (F0–1 and F2–4) in both cohorts.

In summary, measuring phosphatidylethanol is advisable in NAFLD patient evaluations. Elevated PEth-levels (≥48 ng/mL) or alcohol consumption exceeding 66 grams per week should warrant advice to abstain from alcohol. *PNPLA3* is implicated in NAFLD pathophysiology, potentially through impaired lipophagy. While its clinical application remains uncertain, genetic profiling for NAFLD risk assessment may become part of future non-invasive approaches. Additionally, proteomics holds promise for non-invasive NAFLD assessment, with the combination of ACE2, HGF, and IGFBP-7 identifying significant fibrosis in two separate cohorts.
SVENSK SAMMANFATTNING

Icke-alkoholsakad fettleverssjukdom (NAFLD, från engelskans Non-Alcoholic Fatty Liver Disease), är ett växande problem i hela världen. Uppskattningsvis har mellan 25–30% av befolkningen i västvärlden NAFLD och den är på god väg att bli den vanligaste orsaken till levertransplantation. Sjukdomen är nära kopplad till övervikt och typ 2 diabetes. NAFLD innefattar flera delar och definieras av mer än 5% fettinlagring i levern men kan också akkompanjeras av inflammation, ärrbildning (fibros), cirros (skrumplever) och utveckling av levercancer. Fibros delas in i stadium 0–4, där det fjärde stadiet är synonymt med cirros. Mängden fibros i levern är den tydligast kopplad komponenten till sjuklighet och död i NAFLD. Vanligtvis tar det mellan 7–14 år för ett fibrosstadium att utvecklas men processen kan gå mycket snabbare än så och det är hittills inte möjligt att förutsäga vilka patienter som kommer att ha ett snabbt sjukdomsförlopp. I dagsläget är leverbiopsi den enda metod som med säkerhet kan säga vilket fibrosstadium patienten har. Dock är biopsi behämt med vissa risker samt resurskrävande med tanke på att minst en fjärdedel av befolkningen lider av NAFLD. Därför läggs stora resurser på att utveckla metoder för att icke-invasivt kunna uppskatta fibrosstadium.

Flertalet komponenter bidrar till sjukdomsutvecklingen såsom genetiska mutationer, typ 2 diabetes och fetma. Alkoholkonsumtion har varit omvistat då vissa studier påvisat en positiv effekt avseende sjukdomsutveckling vid måttlig alkoholkonsumtion men andra visat att sjukdomen är mer allvarlig hos de med måttlig alkoholkonsumtion. Definitionsmässigt ska inte alkoholkonsumtionen överstiga 210 gram per vecka för män eller 140 gram per vecka för kvinnor för att man ska kunna ställa diagnosen NAFLD. I standardglas motsvarar det 17,5 standardglas för män respektive cirka 11,5 standardglas för kvinnor.

Denna avhandling omfattar fyra delar och vars syfte var att undersöka hur måttlig alkoholkonsumtion påverkar förekomst av fibros och fibrosutveckling, studera funktionen hos en av de vanligaste genetiska avvikelserna vid NAFLD (genen PNPLA3) samt att mäta proteomik (mätning av protein som förekommer i låga koncentrationer) för att om möjligt skapa en modell för att icke-invasivt förutsäga fibrosstadium hos patienter med NAFLD.

I studie I och II undersökte måttlig alkoholkonsumtion hos 86 respektive 82 studiedeltagare med biopsiverifierad NAFLD. Alkoholkonsumtionen värderades dels genom ett alkoholformulär (AUDIT-C), intervju och genom mätning av alkoholmarkören PEth (fosfatidyletanol) som speglar alkoholkonsumtion de senaste 2–4 veckorna. PEth mäts i klinisk praxis och
svaras ut om värdet är ≥ 0,05 µmol/L. Värden över 0,3 µmol/L motsvarar ett högt alkoholintag (ofta mer än 140 gram/vecka). I studie I, som var en tvärsnittsstudie, sågs det att studiedeltagare som konsumerade mer än 5,5 standardglas per vecka hade högre förekomst av fibrosstadium 3–4. Motsvarande siffror sågs för PEth > 0,07 µmol/L (motsvarande ≥50 ng/mL). Det konstaterades dessutom att studiedeltagare med mättlig alkoholkonsumtion i kombination med typ 2 diabetes hade signifikant högre förekomst av avancerad fibros jämfört med studiedeltagare med låg konsumtion, oavsett om de hade typ 2 diabetes eller ej.

I studie II sågs ett liknande mönster. Studiedeltagarna följdes i 17,2 års tid och under den tiden noterades att deltagare med mättlig alkoholkonsumtion i högre grad utvecklade fibros. Resultaten var mest tydliga hos studiedeltagare med PEth-värde ≥ 0,07 µmol/L (motsvarande ≥48 ng/mL) och de som hade ett periodvis högt dryckesmönster (så kallat "binge drinking", det vill säga att vid minst ett tillfälle per månad inta minst fem standardglas för män, respektive fyra standardglas för kvinnor). Detta var oberoende av ålder, kön, viktuppgång, förekomst av typ 2 diabetes eller leverinflammation.


Sammanfattningsvis har vi studerat flertalet delar avseende diagnostik och sjukdomsutveckling vid NAFLD. Vi har noterat att muterad PNPLA3 kan ha samröre med nedsatt förmåga till att frigöra energi från fettdroppar och istället ansamla dessa. Att en kombinerad modell innehållande biomarkörerna ACE2, HGF och IGFBP-7 kan förutsäga fibrostartum i två oberoende studiepopulationer med NAFLD. Vi har dessutom sett att mättlig alkoholkonsumtion vid NAFLD bidrar till fibrostartum, redan vid mätbart PEth på ≥ 0,07 µmol/L. Förslagsvis bör de accepterade gränsvärdena på 140 och 210 gram alkohol per vecka sänkas för att motverka avancerad fibrostartum hos denna växande patientpopulation.
Non-Alcoholic Fatty Liver Disease
LIST OF PAPERS

I. Moderate alcohol consumption is associated with advanced fibrosis in non-alcoholic fatty liver disease and shows a synergistic effect with type 2 diabetes mellitus.
Blomdahl J, Nasr P, Ekstedt M, Kechagias S
*Metabolism.* 2021 Feb;115:154439.

II. Moderate alcohol consumption is associated with significant fibrosis progression in NAFLD.
Blomdahl J, Nasr P, Ekstedt M, Kechagias S

III. PNPLA3 variant M148 causes resistance to starvation-mediated lipid droplet autophagy in human hepatocytes.

IV. Proteomics to discriminate significant fibrosis in two independent cohorts of biopsy-proven NAFLD.
Manuscript
Non-Alcoholic Fatty Liver Disease
ABBREVIATIONS

\begin{itemize}
\item \textsuperscript{1}H-MRS: Proton magnetic resonance spectroscopy
\item 2D-SWE: Bidimensional shear wave elastography
\item ACE2: Angiotensin converting enzyme-2
\item ADH1B: Alcohol dehydrogenase 1B
\item AFLD: Alcohol-related fatty liver disease
\item AFM: Afamin
\item ALDOB: Fructose-biphosphat ealdolase B
\item ALT: Alanine aminotransferase
\item APOM: Apolipoprotein M
\item APRI: AST to platelet ratio index
\item ARFI: Acoustic radiation force impulse imaging
\item AST: Aspartate aminotransferase
\item AUDIT: Alcohol use disorder identification test
\item AUDIT-C: AUDIT-consumption
\item BAC: Blood alcohol concentration
\item BARD: BMI, AST/ALT-ratio, diabetes
\item BF: Bright field
\item BMI: Body mass index
\item BSA: Bovine serum albumin
\item CAGE: Cut down, annoyed, guilty, eye-opener
\item CAP: Controlled attenuation parameter
\item CDT: Carbohydrate deficient transferrin
\item ChREBP: Carbohydrate-responsive element-binding protein
\item CK18: Cytokeratin 18
\item CYP2E1: Cytochrome P450 2E1
\item DAMP: Danger-associated molecular pattern
\item DMEM: Dulbecco’s modified eagle medium
\item DNL: De novo lipogenesis
\item ELF: Enhanced liver fibrosis
\item ER: Endoplasmic reticulum
\item EtG: Ethyl glucuronide
\item EtS: Ethyl sulphate
\item FAEE: Fatty acid ethyl ester
\item FBS: Fetal bovine serum
\end{itemize}
### Non-Alcoholic Fatty Liver Disease

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>FIB-4</td>
<td>Fibrosis-4</td>
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<td>FLI</td>
<td>Fatty liver index</td>
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<td>FLIP</td>
<td>Fatty liver inhibition progression</td>
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<tr>
<td>FXR</td>
<td>Farnesoid X receptor</td>
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<tr>
<td>GADPH</td>
<td>Glyceraldehyde-3-phosphatase dehydrogenase</td>
</tr>
<tr>
<td>GCKR</td>
<td>Glucokinase regulator</td>
</tr>
<tr>
<td>GDF-15</td>
<td>Growth differentiation factor-15</td>
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<tr>
<td>G-GT</td>
<td>Gamma-glutamyl transferase</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association study</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
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<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
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<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HSC</td>
<td>Hepatic stellate cell</td>
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<tr>
<td>HSD17B13</td>
<td>Hydroxysteroid 17-β dehydrogenase 13</td>
</tr>
<tr>
<td>HTGC</td>
<td>Hepatic triglyceride content</td>
</tr>
<tr>
<td>I148</td>
<td>Wildtype gene of <em>PNPLA3</em></td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IFF</td>
<td>Immunofluorescence fluid</td>
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<tr>
<td>IFG</td>
<td>Impaired fasting glucose</td>
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<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
</tr>
<tr>
<td>IGFBP-7</td>
<td>Insulin-like growth factor-binding protein-7</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun terminal kinase</td>
</tr>
<tr>
<td>LACU</td>
<td>Lifetime alcohol consuming unit</td>
</tr>
<tr>
<td>LAL</td>
<td>Lysosomal acid lipase</td>
</tr>
<tr>
<td>LAMP</td>
<td>Lysosomal-associated membrane protein 1</td>
</tr>
<tr>
<td>LC3</td>
<td>Microtubule-associated protein 1 light chain 3</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography with tandem mass spectrometry</td>
</tr>
<tr>
<td>LD</td>
<td>Lipid droplet</td>
</tr>
<tr>
<td>LDH</td>
<td>Lifetime drinking history</td>
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<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
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<tr>
<td>LGALS3BP</td>
<td>Galectin-3 binding protein</td>
</tr>
<tr>
<td>LOQ</td>
<td>Level of quantification</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>LSM</td>
<td>Liver stiffness measurement</td>
</tr>
<tr>
<td>M148</td>
<td>Variant gene of PNPLA3</td>
</tr>
<tr>
<td>MAC</td>
<td>Moderate alcohol consumption</td>
</tr>
<tr>
<td>MASLD</td>
<td>Metabolic dysfunction-associated steatotic liver disease</td>
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<tr>
<td>MBOAT7</td>
<td>Membrane-bound O-acyltransferase domain-containing 7</td>
</tr>
<tr>
<td>MCV</td>
<td>Mean corpuscular volume</td>
</tr>
<tr>
<td>MetALD</td>
<td>MASLD and increased alcohol intake</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MRE</td>
<td>Magnetic resonance elastography</td>
</tr>
<tr>
<td>mRFP-GFP-LC3</td>
<td>Monomeric red fluorescent protein-green fluorescent protein-LC3</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NAS</td>
<td>NAFLD activity score</td>
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<tr>
<td>NASH</td>
<td>Non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>NFS</td>
<td>NAFLD fibrosis score</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NPX</td>
<td>Normalized protein expression</td>
</tr>
<tr>
<td>OCA</td>
<td>Obeticholic acid</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDFF</td>
<td>Proton density fat fraction</td>
</tr>
<tr>
<td>PEA</td>
<td>Proximity extension assay</td>
</tr>
<tr>
<td>PEth</td>
<td>Phosphatidylethanol</td>
</tr>
<tr>
<td>PIGR</td>
<td>Polymeric immunoglobin receptor</td>
</tr>
<tr>
<td>PIIINP</td>
<td>Procollagen III N-terminal peptide</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PLIN</td>
<td>Perilipin</td>
</tr>
<tr>
<td>PNPLA3</td>
<td>Patatin-like phospholipase domain-containing 3</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptors</td>
</tr>
<tr>
<td>pSWE</td>
<td>Point-shear wave elastography</td>
</tr>
<tr>
<td>RMR</td>
<td>Resting metabolic rate</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SAF</td>
<td>Steatosis, activity, fibrosis</td>
</tr>
<tr>
<td>SCAPIS</td>
<td>Swedish cardiopulmonary bioimage study</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel</td>
</tr>
</tbody>
</table>
### Non-Alcoholic Fatty Liver Disease

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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</thead>
<tbody>
<tr>
<td>SELDI-TOF</td>
<td>Surface-enhanced laser desorption/ionization-time of flight</td>
</tr>
<tr>
<td>SELE</td>
<td>Selectin E</td>
</tr>
<tr>
<td>SIBO</td>
<td>Small intestinal bacterial overgrowth</td>
</tr>
<tr>
<td>SLD</td>
<td>Steatotic liver disease</td>
</tr>
<tr>
<td>SPC</td>
<td>Stereological point counting</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element-binding protein</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>THR-β</td>
<td>Thyroid hormone receptor-β</td>
</tr>
<tr>
<td>TIMP1</td>
<td>Tissue inhibitor of metalloproteinase-1</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TM6SF2</td>
<td>Transmembrane 6 superfamily member 2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>VCTE</td>
<td>Vibration controlled transient elastography</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low-density lipoprotein</td>
</tr>
<tr>
<td>VTN</td>
<td>Vitronectin</td>
</tr>
</tbody>
</table>
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INTRODUCTION

Background
Non-alcoholic fatty liver disease (NAFLD) is a relatively recently defined condition closely associated with obesity and the metabolic syndrome. The term non-alcoholic steatohepatitis (NASH) was first coined by Ludwig et al. in 1980 when they described 20 middle-aged patients without a history of alcohol consumption, whose liver biopsies showed inflammation and fatty infiltration similar to alcohol-related liver disease.

With the growing obesity epidemic, NAFLD has become the most prevalent liver disease in the world, affecting approximately 25% of the population. While the disease can manifest at any age, including in children, it is predominantly seen in middle-aged individuals. NAFLD is a progressive condition that ranges from steatosis (fatty infiltration) to steatohepatitis (with lobular inflammation and ballooning), fibrosis, cirrhosis, and hepatocellular carcinoma. It is estimated that up to 10% of patients experience a progressive disease course leading to end-stage liver disease.

Fibrosis progression is a gradual process, with an average advancement of one fibrosis stage occurring over 7–14 years.

Risk factors for more severe liver disease in NAFLD include a diagnosis of type 2 diabetes mellitus (T2DM), obesity, hypertension, or dyslipidemia.

Diagnosis of NAFLD
NAFLD is histologically characterized by the presence of lipids comprising more than 5% of the liver’s weight. The diagnosis of NAFLD entails the exclusion of other potential liver diseases, including but not limited to viral hepatitis, alcohol-related fatty liver disease (AFLD), Wilson’s disease, and drug-induced steatosis (see Table 1). To distinguish AFLD from NAFLD, limits of daily alcohol consumption have been arbitrarily established; 30 g/day for males and a maximum of 20 g/day for females.

Table 1. Other causes of hepatic steatosis.\textsuperscript{8-11}

<table>
<thead>
<tr>
<th>Drugs and medications</th>
<th>Inborn errors of metabolism</th>
<th>Nutritional</th>
<th>Other</th>
</tr>
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<tbody>
<tr>
<td>Acetylsalicylic acid</td>
<td>Abetalipoproteinemia</td>
<td>Gastric bypass surgery</td>
<td>Acute fatty liver of pregnancy</td>
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<td>Amiodarone</td>
<td>Alpha-1-antitrypsin deficiency</td>
<td>Malnutrition</td>
<td>Alcohol</td>
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<tr>
<td>Antiretroviral drugs</td>
<td>Citrin deficiency</td>
<td>Rapid weight loss</td>
<td>Bacillus cereus</td>
</tr>
<tr>
<td>Cocaine</td>
<td>Congenital disorders of glycosylation</td>
<td>Starvation</td>
<td>Environmental hepatotoxins</td>
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</table>
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Pathophysiology of NAFLD

The pathophysiology of NAFLD is multifactorial, though not yet completely understood. The foundation of the disease involves the development of fat vacuoles in the hepatocytes, whereafter inflammation and fibrosis can develop. A previous theory described the pathophysiology in NAFLD as a “two-hit hypothesis”, where the first hit was the development of steatosis. Then considered a prerequisite for the second hit of inflammation and fibrogenesis. However, this theory is now viewed as overly simplistic, and the prevailing theory is the “multiple-hit hypothesis”, suggesting that multiple factors can occur concurrently and synergistically, see Figure 1. It is recognized that steatosis and steatohepatitis can occur simultaneously, and inflammation may even precede the steatogenic process.

The development of insulin resistance, a crucial factor in NAFLD pathophysiology, is influenced by both genetic factors and dietary habits. Insulin resistance leads to adipose tissue lipolysis, increased influx of fatty acids into the liver, and enhanced de novo lipogenesis.
Introduction

Figure 1. Pathophysiology of NAFLD.
Abbreviations: IR, insulin resistance; FFA, free fatty acids; ChREBP, carbohydrate-responsive element-binding protein; SREBP-1, sterol regulatory element-binding protein; DNL, de novo lipogenesis; TAG, triacylglycerol; ROS, reactive oxygen species; ER, endoplasmic reticulum; HSC, hepatic stellate cell; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor kappa B; TGF-β, transforming growth factor-β; TLR, toll-like receptor; LPS, lipopolysaccharides.

Steatosis and lipotoxicity
Normally, there is a balance in the liver between lipid uptake and disposal. Lipid uptake consists of the influx of free fatty acids (FFAs), which can be derived from degradation of food in the gastrointestinal tract or from breakdown of adipose tissue. FFAs can be esterified with glycerol to produce triacylglycerol (TAG) or with cholesterol to produce cholesterol esters. TAG and cholesterol esters can be stored in the liver as lipid droplets or released into the bloodstream as VLDLs. Hepatocytes can also synthesize new FFAs through de novo lipogenesis, which utilizes acetyl-CoA, usually derived from carbohydrates. For energy production, FFAs can be degraded in the liver through β-oxidation.\textsuperscript{12,14}

Adipose tissue lipolysis, triggered by insulin resistance, leads to the breakdown and inflammation of adipose tissue, releasing FFAs from
Non-Alcoholic Fatty Liver Disease

adipocytes and leading to a surplus in the liver. Approximately 60% of hepatic triglyceride content in NAFLD is derived from FFAs through lipolysis, about 25% from de novo lipogenesis, and 15% from dietary sources. Triglyceride accumulation itself is not toxic and is considered a potential defense mechanism against an increased influx of FFAs.12,13

Several transcription factors, such as SREBP-1 and ChREBP, can stimulate hepatic de novo lipogenesis. ChREBP is activated by glucose, while SREBP-1 is activated by insulin.12,14 Additionally, impaired β-oxidation in insulin resistance inhibits the breakdown of fatty acids. Excessive carbohydrate intake, particularly products high in fructose, contributes to steatosis. Glucose is converted into lipogenic molecules like acetyl-CoA, which then forms malonyl-CoA and palmitic acid, a precursor to triglyceride fatty acids.14,15 Fructose consumption appears to have a greater impact on lipid metabolism compared to glucose.16

An excess of FFAs also promotes the synthesis of other lipids besides TAG, including phospholipids, free cholesterol, and ceramides. These additional lipids, which are more toxic, contribute to lipotoxicity, a mechanism in which metabolites of free fatty acids are believed to induce hepatotoxicity. They can affect the function of intracellular organelles such as the mitochondria and the endoplasmic reticulum (ER) or interact with proinflammatory pathways.17 Saturated FFAs, such as stearate and palmitate, are considered to have a greater impact on lipotoxicity compared to unsaturated FFAs.17,18

Inflammation and Steatohepatitis
Multiple factors contribute to the inflammatory state observed in NASH, including mitochondrial dysfunction, adipose tissue dysfunction, activation of inflammasomes, cytokines, and endotoxins derived from an altered gut microbiota.12 Although these pathways have been described in various studies, their relative importance and interplay remain undetermined. Histologically, NASH is characterized by lobular inflammation (infiltration of leukocytes) and ballooning (enlarged, degenerating hepatocytes).

Several types of leukocytes are associated with NASH pathophysiology. Kupffer cells, resident macrophages in the sinusoidal lumen, can express proinflammatory cytokines and chemokines (such as TNF-α and IL-6) upon activation.18 Both Kupffer cells and recruited macrophages can express TGF-β, a central cytokine that activates stellate cells.18 Neutrophils also influence disease progression through hepatic infiltration and secretion of various cytokines.19 Other components of the innate and adaptive immune system play a role in NASH pathogenesis but can have either proinflammatory or anti-inflammatory functions.

Peroxisome proliferator-activated receptors (PPARs) are involved in regulating lipid and glucose metabolism as well as inflammation and fibrosis.20 Three isotypes have been identified: PPAR-α, PPAR-β/δ, and PPAR-γ.20 PPAR-γ can inhibit the activation of HSC and regulate metabolism in
Introduction

Adipose tissue. PPAR-β/δ can suppress pro-inflammatory macrophages and instead promote an anti-inflammatory phenotype. PPAR-α can improve lipid metabolism by regulating FFA transport and β-oxidation. The involvement of PPAR-receptors in NAFLD pathophysiology is not yet fully understood.

Mitochondria have a threshold for lipid flux that they can handle. When the influx exceeds this threshold, lipid-derived toxic metabolites like ceramides are generated, and reactive oxygen species (ROS) are produced. This triggers inflammatory pathways and contributes to the necroinflammation seen in NASH. Ceramides are normally a part of sphingomyelin, a structural lipid in nerve tissue membranes, but they also possess signaling properties and can promote apoptosis. Mitochondria also contain a significant amount of CYP2E1, an enzyme that can oxidize FFAs and ethanol into by-products. These by-products can potentially impair mitochondrial function and have been implicated in the pathophysiology of NAFLD.

ER stress activates the “unfolded protein response” (UPR), an adaptive response to alleviate ER stress by reducing protein synthesis, increasing protein folding, and enhancing protein degradation. In NAFLD, various factors can trigger UPR, including oxidative stress, mitochondrial injury, and hyperglycemia. UPR subsequently activates c-jun terminal kinase (JNK), itself an activator of apoptosis and inflammation. UPR also enhances the activation of SREBP-1C, further maintaining lipid accumulation in NAFLD. In general, ROS production and ER stress contribute to inflammation by increasing the secretion of TNFα and NF-κB and initiating cell death.

The gut microbiota can be considered an endocrine organ due to its close relationship with the host and its influence on the development of various chronic diseases. Altered microbiota and dysbiosis have been linked to NAFLD development in animal models and are believed to contribute to NAFLD in humans. Changes in the gut microbiota can compromise the intestinal barrier, resulting in the release of lipopolysaccharides (LPS) into the portal vein and the liver. LPS (as well as FFAs) activate toll-like receptors (TLRs) that initiate inflammation.

Inflammasomes are complexes located in the cytoplasm that, upon activation, trigger the expression of proinflammatory and profibrotic characteristics. They are considered to be involved in the pathogenesis of NAFLD and can be activated by LPS and various ligands (such as danger-associated molecular patterns, DAMPs).

Bile acids are synthesized in the liver and secreted as part of bile, essential for the breakdown of dietary lipids. Additionally, they function as signaling molecules, interacting with receptors such as FXR. FXR activation can stimulate β-oxidation and reduce de novo lipogenesis. FXR activation is also implicated in reducing inflammation and fibrosis. It is hypothesized that patients with NAFLD have impaired bile acid metabolism, and changes in bile acid composition are associated with disease progression.
Adipose tissue acts as an endocrine organ and secretes adipokines, a type of cytokine, to regulate metabolism. The major two adipokines are adiponectin and leptin. Adiponectin is a specific adipokine that plays a role in regulating insulin sensitivity, fatty acid oxidation, and inhibiting the accumulation of FFAs.\textsuperscript{14} Leptin is also secreted by adipose tissue and can activate hepatic stellate cells.\textsuperscript{14} Low levels of adiponectin and higher serum levels of leptin have been described in NAFLD patients.\textsuperscript{26}

**Fibrosis**

The activation of hepatic stellate cells constitutes a key mechanism underlying fibrosis development in NAFLD. When activated, the stellate cell transforms from a storage cell, primarily storing vitamin A, to acquiring fibrogenic properties. Activated stellate cells, also known as myofibroblasts, have the capacity to produce and deposit extracellular matrix components, including collagen, a major constituent of fibrotic tissue, in response to injury. Various factors, such as ROS and oxidized LDL particles, can trigger the activation of hepatic stellate cells.\textsuperscript{12} Additionally, hepatic stellate cells can express chemokines and adhesion molecules, facilitating the recruitment of Kupffer cells and other macrophages, thereby maintaining disease progression.\textsuperscript{27}

While there are components within the immune system capable of resolving fibrosis, the precise mechanisms involved remain to be fully elucidated. Matrix metalloproteinases (such as MMP-9 and MMP-13), secreted by macrophages, have been identified as possessing antifibrotic properties and the ability to promote fibrolysis.\textsuperscript{27}

**Autophagy and lipophagy**

Lipophagy represents a specific form of autophagy responsible for regulating lipid content. Autophagy is a cellular process where intracellular components are degraded within lysosomes. Impaired lipophagy has been associated with steatosis and lipotoxicity.\textsuperscript{28,29}

Lipids are typically stored in intracellular lipid droplets (LDs), which consist of lipid esters enclosed by a monolayer of phospholipids and are coated with perilipins, structural proteins. Cells can activate the degradation of LDs in response to a catabolic state and also when faced with an increased influx of lipids, preventing excessive LD storage that could otherwise compromise cell structure.\textsuperscript{28} The breakdown of LDs is believed to occur through both lipolysis (mediated by lipases) and lipophagy. During autophagy (and lipophagy), part of an organelle or lipid droplet is separated by a double-membrane structure, forming an autophagosome. Subsequently, the autophagosome fuses with a lysosome, creating an autolysosome that degrades the enclosed material. Lipophagy is particularly initiated during starvation or when the cell encounters an increased amount of lipids.\textsuperscript{28}

Common perilipins that coat LDs include PLIN1 and PLIN2.\textsuperscript{30} Other proteins coating LDs are PNPLA2 and PNPLA3.\textsuperscript{31} LC3 (microtubule-
associated protein 1 light chain 3) is a part of the autophagy process and becomes lipidated to form LC3-II when autophagy is initiated, serving as a marker for the autophagosome. LAMP1 (lysosomal-associated membrane protein 1) is a common marker for lysosomes.\textsuperscript{32}

**Liver biopsy and histopathology**

A liver biopsy is the gold standard method to diagnose NAFLD. The presence of macrovesicular steatosis is a key finding. It can be accompanied by lobular inflammation, hepatocellular ballooning, and Mallory-Denk (intracytoplasmic inclusion) bodies. Non-alcoholic steatohepatitis (NASH) is defined by the presence of steatosis, lobular inflammation, and ballooning, see Table 2.\textsuperscript{33}

Another histological feature of NAFLD is the presence of fibrosis. Using the Kleiner classification,\textsuperscript{34} fibrosis is staged 0–4, where stage 4 is equal to cirrhosis, see Table 2. Several other histological features are described in NAFLD, such as portal inflammation, acidophil bodies (i.e., apoptotic bodies), glycogenated nuclei, and megamitochondria.\textsuperscript{34} However, these are seldom described in clinical research.

| Table 2. List of histological definitions in NAFLD.\textsuperscript{34} |
|-------------------------|---|---|
| **Histological feature** | **Scoring** | **Definition** |
| Steatosis | Grade | |
| 0 | <5% |
| 1 | 5–33% |
| 2 | >33–66% |
| 3 | >66% |
| Lobular inflammation | 0 | No foci |
| 1 | <2 foci per 200x field |
| 2 | 2–4 foci per 200x field |
| 3 | >4 foci per 200x field |
| Ballooning | 0 | None |
| 1 | Few balloon cells |
| 2 | Prominent ballooning |
| Fibrosis | Stage | |
| 0 | None |
| 1 | Perisinusoidal |
| 2 | Perisinusoidal and portal |
| 3 | Bridging fibrosis |
| 4 | Cirrhosis |

Liver biopsy is not without complications. Besides procedural discomfort, clinically significant bleeding occurs in approximately 1 in 500 biopsies, resulting in reduced blood pressure, tachycardia, or pain, without requiring intervention or transfusion. The risk of major bleeding is about 1 in 2,500 to 1 in 10,000. Mortality rates are fortunately low, at approximately ≤1 in 10,000.\textsuperscript{35} Most liver biopsy needles have a diameter of 1.6 mm and a length
Thus a biopsy samples only a small part of the liver, approximately \(1/50,000\) of its volume. Sampling variability is a concern, as demonstrated by Ratziu et al., who performed a study where two biopsy samples were collected at the same time from patients with NAFLD. The study revealed a sampling variability of 41% for one-stage fibrosis, but only a 12% risk of a difference of two or more stages between the two samples.

Histopathological assessment also has its limitations. The interobserver agreement for steatosis is approximately \(\kappa=0.64–0.79\), \(\kappa=0.60–0.84\) for fibrosis, and \(\kappa=0.33–0.45\) for inflammation.\(^{34,38}\) Even intraobserver agreement, where the same pathologist reviewed the slide a second time but was blinded, has limitations with a \(\kappa=0.63–0.83\) for steatosis, \(\kappa=0.43–0.81\) for inflammation, and \(\kappa=0.67–0.85\) for fibrosis stage.\(^{34,38}\)

To address the variability in histopathological assessment, Kleiner et al.\(^{34}\) developed the NAFLD activity score (NAS), a system for evaluating biopsies before and after therapeutic interventions. The NAS ranges from 0–8 and is the sum of scores for steatosis (0–3), lobular inflammation (0–3), and ballooning (0–2). Additionally, Bedossa et al.\(^{39}\) further developed a scoring system for NASH diagnosis, creating the SAF score (Steatosis, Activity, Fibrosis) to standardize assessment among pathologists. The SAF score combines fibrosis, steatosis, ballooning, and lobular inflammation. To diagnose NASH, each component of steatosis, ballooning, and lobular inflammation must attain at least grade 1. Lobular inflammation is divided into three stages, with stage 2 representing both stages 2–3 according to the Kleiner classification. Based on the SAF score, they developed the FLIP algorithm, a histological algorithm for diagnosing NASH more readily, see Figure 2. Interobserver agreement for activity using the SAF score is \(\kappa=0.75.\)\(^{40}\)

**Figure 2.** The FLIP algorithm for diagnosing NASH.
Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; FLIP, fatty liver inhibition progression.
Non-invasive diagnosis and staging

Given the unfeasibility of performing a liver biopsy on approximately a quarter of the population, new non-invasive methods have emerged to diagnose and arbitrarily stage NAFLD. The following section outlines non-invasive scoring used to estimate significant or advanced fibrosis, steatosis, and NASH.

Non-invasive scores (blood-based/serum tests)

Non-invasive scoring tests, comprising blood-based or serum tests, are commonly used in primary care settings to determine which patients should be referred for assessment by a hepatologist. These tests combine blood tests, patient information, and algorithms to assess the likelihood of a patient having a high risk of significant fibrosis (F2–4) or advanced fibrosis (F3–4). While several scoring tests have been developed, the NAFLD fibrosis score (NFS) and FIB-4 are commonly used in routine practice. Other tests exist, but they either perform inferiorly to FIB-4 and/or NFS, or involve more advanced testing and blood work that is not typically conducted in NAFLD patients (see Table 3 for examples). The non-invasive scoring systems have an average PPV of approximately 50%, but often exhibit a NPV of 80–90%, rendering them useful in excluding advanced fibrosis.

### Table 3. Example of non-invasive scores for estimation of significant or advanced fibrosis.

<table>
<thead>
<tr>
<th>Test</th>
<th>Included items</th>
<th>AUR-OC</th>
<th>PPV</th>
<th>NPV</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIB-4[41]</td>
<td>Age, ALT, AST, platelet count</td>
<td>0.80</td>
<td>43%</td>
<td>90%</td>
<td>74%</td>
<td>71%</td>
</tr>
<tr>
<td>NAFLD Fibrosis Score[40]</td>
<td>Age, BMI, diabetes, ALT, AST, platelet count, albumin</td>
<td>0.82</td>
<td>56%</td>
<td>93%</td>
<td>82%</td>
<td>77%</td>
</tr>
<tr>
<td>AST/ALT-ratio[43]</td>
<td>AST, ALT</td>
<td>0.83</td>
<td>44%</td>
<td>93%</td>
<td>74%</td>
<td>78%</td>
</tr>
<tr>
<td>APRI[44]</td>
<td>AST, platelet count</td>
<td>0.79</td>
<td>47%</td>
<td>91%</td>
<td>72%</td>
<td>77%</td>
</tr>
<tr>
<td>BARD[44]</td>
<td>BMI, ALT, AST, diabetes</td>
<td>0.70</td>
<td>37%</td>
<td>87%</td>
<td>60%</td>
<td>72%</td>
</tr>
<tr>
<td>FibroTest®[45]</td>
<td>α2-macroglobulin, haptoglobin, G-GT, bilirubin, apolipoprotein A1, age, gender</td>
<td>0.81</td>
<td>54%</td>
<td>90%</td>
<td>77%</td>
<td>77%</td>
</tr>
<tr>
<td>Hepascore[44]</td>
<td>α2-macroglobulin, hyaluronic acid, bilirubin, G-GT, age, gender</td>
<td>0.81</td>
<td>57%</td>
<td>92%</td>
<td>76%</td>
<td>84%</td>
</tr>
<tr>
<td>FibroMeter NAFLD[40]</td>
<td>Glucose, AST, ALT, ferritin, platelet count, body weight, age</td>
<td>0.94</td>
<td>88%</td>
<td>92%</td>
<td>79%</td>
<td>96%</td>
</tr>
<tr>
<td>ELF™ test[47,48]</td>
<td>Hyaluronic acid, TIMP1, PHINP</td>
<td>0.87</td>
<td>80%</td>
<td>98%</td>
<td>78%</td>
<td>76%</td>
</tr>
</tbody>
</table>
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FIB-4 index = age*AST (IU/L) / platelet count (*10^9/L) * √ALT (IU/L). FIB-4 values in the table are for cut-off <1.3 (for the estimation of F0–2 vs. F3–4).\textsuperscript{22} NAFLD fibrosis score = -1.675 + 0.037*age (years) + 0.094*BMI (kg/m²) + 1.13*IFG/diabetes (yes=1, no=0) + 0.99*AST/ALT-ratio - 0.013*platelet count (*10^9/L) - 0.66*albumin (g/dL). NAFLD Fibrosis score values in the table are for cut-off -1.455.\textsuperscript{22} AST/ALT-ratio cut-off 0.8.\textsuperscript{22} APRI = AST (IU/L) / platelet count (*10^9/L) *100. Values in the table are for cut-off 0.54.\textsuperscript{22} BARD score (scale 0–4) = BMI ≥28 = 1 point; AST/ALT-ratio ≥0.8 = 2 points; diabetes mellitus = 1 point. Values in the table are for cut-off 2.\textsuperscript{22} Values in the table for FibroTest® correspond to a cut-off 0.30 for the likelihood of F2–4. \textsuperscript{22} Values in the table for Hepascore are for cut-off 0.37.\textsuperscript{22} PPV and NPV for the ELF™ test are sourced from \textsuperscript{47}, while AUROC, sensitivity, and specificity are derived from a pooled analysis in \textsuperscript{48}. Abbreviations: FIB-4, fibrosis-4; ALT, alanine aminotransferase; AST, Aspartate aminotransferase; BMI, Body mass index; APRI, AST to platelet ratio index; BARD, BMI, AST/ALT-ratio, diabetes; G-GT, Gamma-glutamyl transferase; ELF, Enhanced liver fibrosis; TIMP1, tissue inhibitor of metalloproteinase-1; PIINP, procollagen III N-terminal peptide; IFG, impaired fasting glucose.

Steatosis

Ultrasonography
Ultrasonography has the advantage of high accessibility, but its diagnostic performance varies. In the case of mild steatosis (up to 29% steatosis), only two-thirds of the patients can be detected using ultrasound. For patients with steatosis up to 10 %, ultrasound fails to detect any cases.\textsuperscript{49} Additionally, the method suffers from low inter- and intra-observer agreement on the presence of steatosis (κ=0.43 and κ=0.54, respectively).\textsuperscript{50} Moreover, ultrasound is not suitable for diagnosing inflammation or staging fibrosis.

Controlled Attenuation Parameter (CAP)
CAP is a parameter obtained through FibroScan® that measures ultrasound attenuation and provides an estimation of steatosis. CAP values range between 100–400 dB/m.\textsuperscript{51} Currently used cut-offs for CAP are based on a meta-analysis that included approximately 2,800 patients with liver diseases, mostly NAFLD and viral hepatitis,\textsuperscript{52} see Table 4.

<table>
<thead>
<tr>
<th>Steatosis grade</th>
<th>Cut-off (dB/m)</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥1</td>
<td>248</td>
<td>69%</td>
<td>82%</td>
</tr>
<tr>
<td>≥2</td>
<td>268</td>
<td>77%</td>
<td>81%</td>
</tr>
<tr>
<td>≥3</td>
<td>280</td>
<td>88%</td>
<td>78%</td>
</tr>
</tbody>
</table>

Proton density fat fraction
Proton density fat fraction (PDFF) is an MRI-based method for determining hepatic triglyceride content (HTGC) and correlates rather well with
steatosis based on liver biopsy. However, PDFF does not have the capability to detect the presence of NASH. The commonly used cut-offs for the presence of steatosis are usually 5 or 5.56%, but HTGC as low as 3% has been found to correspond well with biopsy-verified steatosis.

**SteatoTest**
The SteatoTest is a panel of 12 clinical variables and biomarkers used to diagnose steatosis non-invasively. Included variables are ALT, α₂-macroglobulin, apolipoprotein AI, haptoglobin, bilirubin, G-GT, cholesterol, triglycerides, glucose, age, gender, and BMI. AUROC for steatosis ranges from 0.72 to 0.86.

**Fatty Liver Index**
The Fatty Liver Index (FLI) is an algorithm that utilizes BMI, waist circumference, triglycerides, and G-GT compared with ultrasonography for the diagnosis of steatosis in NAFLD. It demonstrates good accuracy in diagnosing NAFLD but has limited value since the algorithm is compared with a diagnosis based on ultrasonography.

**NASH**
NASH is generally challenging to diagnose non-invasively, as most techniques are unable to differentiate between steatosis and signs of inflammation. The most validated tool for NASH is the measurement of cytokeratin-18 fragments (CK18). CK18 is a marker of hepatocyte apoptosis and shows an AUROC of 0.82, sensitivity of 78%, and specificity of 86% for diagnosing NASH. However, measurement of CK18 is rarely performed in the clinical setting.

**Fibrosis**
There are multiple methods to estimate fibrosis in patients with NAFLD. However, many are used exclusively in tertiary settings and are not commonly available.

**Transient elastography**
Twenty years ago, Sandrin et al. described transient elastography, a non-invasive method to estimate fibrosis stage in HCV patients. It assesses liver stiffness by measuring the speed of an induced shear wave, providing an estimation of fibrosis stage. Vibration-controlled transient elastography (VCTE) has since become a widely used examination in hepatology departments. For reliable measurements, 10 valid shots are required, with an interquartile range (IQR) of 30% or less and a success rate of at least 60%. Stiffness measurement ranges from 2.5–75 kPa. Approximately 3–5% of patients will have a failed exam, where no valid shots are obtained. This
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failure is often influenced by high BMI (>30 kg/m²), although operator experience can also impact the success rate (with a cut-off of 500 exams or more).62,63 Around 15–20% of patients may have an unreliable exam (e.g., less than 10 valid shots, high IQR, or low success rate), even though the development of the XL-probe have resulted in more valid measurements.63 However, when comparing the M-probe and XL-probe in the same patient, XL-probe values are on average 1.7 kPa lower.63 Recent food intake is a potential pitfall as it can increase the liver stiffness measurement (LSM). Accurate measurements require a fasting period of at least 3 hours.64 Congestive heart failure and severe hepatic inflammation can also lead to falsely elevated LSM-values.65

Different cut-offs for LSM have been proposed, resulting in different detection rates for advanced fibrosis. According to the new Baveno VII criteria, an LSM of <10 kPa is suggested to rule out compensated advanced chronic liver disease, although this cut-off is not specific to NAFLD.66 Using a cut-off of <8 kPa VCTE show an NPV of 95.5% for advanced fibrosis (F3-4) in NAFLD.67 In patients with morbid obesity (BMI ≥ 40 kg/m²) a cut-off of 12.8 kPa show an NPV of 95.5% for significant fibrosis (F2-4).68 However, the PPV remains between 40–60% for detecting advanced fibrosis or cirrhosis.69

In addition to VCTE, liver stiffness can also be measured using ultrasonography with specific techniques, such as bidimensional shear wave elastography (2D-SWE) and point-shear wave elastography (pSWE), also known as ARFI (acoustic radiation force impulse imaging).64,69 These techniques require prior knowledge of general ultrasonography, which is a limitation.

Magnetic resonance elastography

Magnetic resonance elastography (MRE) utilizes magnetic resonance imaging to estimate liver stiffness. This technique requires both specific hardware and software, making it less accessible.53 A circular disc that generates shear waves is positioned on the patient’s upper right quadrant of the abdomen.53 Provided that no general contraindication for MRI apply, failure rates of MRE are substantially lower than VCTE (approximately 8%).63 A cut-off of ≥3.0 kPa can be utilized to determine the presence of advanced fibrosis (F3-F4), yielding an AUROC of 0.87, sensitivity of 78%, and specificity of 80%.65

Clinical characteristics and risk factors

To date, the natural history of NAFLD is not entirely known, and identifying patients with a more progressive disease has proven challenging.4,5
Many studies investigating the natural history of NAFLD have been performed at tertiary centers, resulting in a potential bias toward patients with more advanced liver disease. However, it is established that individuals with NAFLD have higher mortality rates compared to the general population, mostly attributed to cardiovascular disease, cancer, and liver-related causes.\textsuperscript{70,71}

NAFLD is sometimes described as a component of the metabolic syndrome, although it is not included as an official diagnostic criterion. The metabolic syndrome is defined as having at least three of the following criteria: elevated fasting plasma glucose (\(>110\) mg/dL); elevated blood pressure (\(>130\) mmHg systolic; \(>85\) mmHg diastolic); waist circumference >88 cm in women, and >102 cm in men; elevated triglycerides or low HDL levels.\textsuperscript{72} Globally, approximately 40\% of adults are overweight (BMI 25–29 kg/m\(^2\)) and 12\% have obesity (BMI \(\geq 30\) kg/m\(^2\)).\textsuperscript{73,74} In the United States, obesity rates are even higher, reaching 38\%.\textsuperscript{75} Obesity is more prevalent among women (15\% vs. 11\% in men).\textsuperscript{76} Nearly 10\% of the global population is diagnosed with type 2 diabetes.\textsuperscript{76} Around 70\% of patients with T2DM and up to 90\% of patients with morbid obesity who undergo bariatric surgery have NAFLD.\textsuperscript{77} Most individuals diagnosed with NAFLD are between 40–60 years of age, and while it appears to be slightly more common in men, the data vary across studies.\textsuperscript{78} Many are diagnosed due to elevated liver enzymes, although up to 50\% of patients with NASH have normal ALT levels.\textsuperscript{58} Another common finding in NAFLD is the presence of steatosis on routine imaging, such as ultrasonography or computed tomography. NAFLD often remains asymptomatic, making it easily overlooked and diagnosed at late stages. However, certain patient groups are more susceptible to NAFLD. Obesity is a significant risk factor, and NAFLD is associated with an increased risk of T2DM.\textsuperscript{79} Approximately 50\% of NAFLD patients are obese, and 20\% have T2DM. Among patients with NASH, 80\% are obese, and over 40\% have T2DM.\textsuperscript{2} Hyperlipidemia is prevalent in 70\% of patients, and 40\% of NAFLD patients have hypertension and/or metabolic syndrome, while these conditions are present in 70\% of individuals with NASH.\textsuperscript{2} Other diseases associated with NAFLD include polycystic ovarian syndrome, obstructive sleep apnea, and hypothyroidism.\textsuperscript{80–82}

Despite the common occurrence of overweight and obesity in NAFLD, a subgroup known as “lean NAFLD” has been identified. This refers to patients with a “normal BMI” (i.e., <25 kg/m\(^2\)) but with all liver-related components of NAFLD. Typically, they have a sedentary lifestyle and exhibit other features of the metabolic syndrome. It is estimated that up to 10–20\% of the population can belong to this patient group.\textsuperscript{83}
Histologic progression
The histologic foundation of NAFLD is macrovesicular steatosis, but it also encompasses steatohepatitis and fibrosis. Approximately 25% of adults suffer from NAFLD, with 5–6% having NASH and 1–2% developing cirrhosis, with a risk of HCC development. Among individuals with NAFLD, at least 25% progress to NASH, and of those with NASH, 25% progress to cirrhosis, see Figure 3. Fibrosis progression can occur regardless of baseline histology, with both simple steatosis and baseline NASH experiencing fibrosis progression. A few studies have performed paired liver biopsies with varying follow-up intervals. Singh et al. summarized the findings in a meta-analysis of 11 cohort studies of patients with biopsy-proven NAFLD, including a total of 411 participants. At baseline, 35.8%, 32.5%, 16.7%, 9.3%, and 5.7% had fibrosis stage 0, 1, 2, 3, and 4, respectively. One-third of the patients progressed in fibrosis stage. The annual fibrosis progression rate was found to be 0.07 stages in patients with simple steatosis at baseline, and 0.14 stages in patients with NASH (and fibrosis stage 0) at baseline. This corresponds to a one-stage progression rate of 14.3 years and 7.1 years, respectively. However, they also identified a subgroup of patients known as rapid progressors, defined as patients with fibrosis stage 0 at inclusion but who had developed F3–4 in the follow-up biopsy. Approximately, a fifth of the patients (17.2–18.2%) who developed fibrosis were identified as rapid progressors, developing stage 3–4 fibrosis over the course of 5.9 years. Even patients with isolated steatosis (i.e., without histological signs of inflammation) can progress to advanced fibrosis, although at a lower rate (approximately 5%). For the majority of patients with F0–1, it is estimated to take around 30–35 years to develop decompensated liver disease. Presence of NASH does not seem to influence the risk of severe liver disease.

Figure 3. Histological progression of NAFLD and NASH. Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma.
Prognosis and risk factors
Unsurprisingly, older age (>60 years) has been associated with a higher prevalence of advanced fibrosis, along with obesity and an AST/ALT-ratio >1.87,88 In the aforementioned study by Singh et al.,5 a meta-analysis of baseline variables of possible risk factors for fibrosis progression was performed, but no statistically significant associations were found. However, T2DM and obesity have previously been identified as risk factors for developing more severe liver disease (HR 2.25 and HR 1.20, respectively).6 The yearly incidence of HCC in patients with cirrhosis is approximately 1–2%.89 There is also an increased risk of non-cirrhotic HCC in patients with NAFLD, with an aOR of approximately 4 compared to HCV.89

Mortality and morbidity
NAFLD is one of the leading causes of liver-related mortality and is among the primary causes of hepatocellular cancer-associated liver transplantations.78,90 Patients with NAFLD have increased all-cause mortality compared to the general population, with cardiovascular disease being the most prevalent cause of death.4,77 They also face an elevated risk of morbidity from cardiovascular conditions such as myocardial infarction, ischemic stroke, and atrial fibrillation.81 In a study by Adams et al.,79 patients with NAFLD were followed for 7.6 years, revealing a mortality ratio of 1.34 compared to the general population. The most common causes of death were cancer (28%), ischemic heart disease (25%), and liver disease (13%). In the general population, liver disease was the 13th leading cause of death. Dam-Larsen et al.91 reported similar mortality rates among their NAFLD population compared to the general population but contributed the deaths to cardiovascular disease and malignancy. Sanyal et al.92 tracked 1,773 patients with NAFLD over 4 years, observing increased all-cause mortality and liver-related complications with increasing fibrosis stage. Fibrosis stage has emerged as the sole histological feature that predicts mortality, with a significant exponential increase in mortality risk by more than 40-fold between F0 and F4.86,93,94 Approximately 1–10% of NAFLD patients progress to end-stage liver disease, and it is the fastest-growing indication for liver transplantation.4,95

Genetics
In 2008, Romeo and colleagues96 performed a genome-wide association study (GWAS) on participants from the Dallas Heart Study to examine the relationship between single nucleotide polymorphisms (SNPs) and hepatic fat content measured by 1H-MRS. Their findings revealed a significant association between a variant of the patatin-like phospholipase domain-containing 3 (PNPLA3) gene and liver fat content, as well as liver
Non-Alcoholic Fatty Liver Disease

inflammation. Since then, PNPLA3 has been recognized as the most influential genetic variant in NAFLD, contributing to an increased risk of a more severe disease course and linking it to NASH, fibrosis, and HCC.97-99 Several genetic studies in NAFLD have been conducted, uncovering additional polymorphisms associated with NAFLD and its severity, as detailed below.

There is also a strong genetic predisposition for developing NAFLD, with a higher prevalence observed among individuals who have a first-degree relative with the disease. Genetic components are estimated to contribute to up to 50% of disease occurrence.100

PNPLA3

PNPLA3, located on chromosome 22, codes for the protein adiponutrin, which consists of 481 amino acids. Also known as PNPLA3, adiponutrin plays a role in lipid metabolism, although its exact mechanism is not fully understood.101,102 The variant form of PNPLA3 gene (rs738409) results in a cytosine-to-guanine shift, leading to the substitution of isoleucine with methionine at position 148. It is suggested that PNPLA3 functions as a hydrolase enzyme for triglycerides and retinyl esters, with the variant PNPLA3 being a “loss-of-function” mutation. Another hypothesis proposes that it acts as a transacylase and that the variant gene has a “gain-of-function” effect and is involved in lipogenesis.103 PNPLA3 is abundant in human hepatocytes and localizes to LDs, and accumulates in variant M148.99

Interestingly, PNPLA3 knockout mice do not exhibit increased levels of TAG in the liver. However, overexpression of M148 and knock-in mice with variant PNPLA3 result in steatosis, whereas overexpression of wildtype PNPLA3 does not.103

The prevalence of the PNPLA3 variant gene varies among ethnic groups, with the highest prevalence found in Hispanics (57%). Among Europeans, 23% carry the variant gene, while only 14% of Africans inherit it.98 Notably, hepatic lipid content associated with variant PNPLA3 appears to be independent of body composition and the presence of T2DM. Furthermore, the increased risk of NASH conferred by the PNPLA3 variant is observed regardless of the severity of steatosis.101,104

TM6SF2

A variant of Transmembrane 6 Superfamily Member 2 (TM6SF2) is linked to an increased risk of steatosis and HCC in NAFLD.98,105 This genetic variant involves an adenine-to-guanine substitution at position 167 (rs58542926) on chromosome 19, resulting in the replacement of glutamate with lysine. The frequency of this polymorphism ranges from 3–8%, with the highest prevalence observed in Europeans and Asians.98
normal function of TM6SF2 is to regulate the enrichment of triglycerides in VLDL and secretion of lipoproteins. The genetic variant results in decreased lipid secretion and concomitant lipid accumulation and steatosis. However, it also contributes to lower levels of circulating lipids and individuals carrying the variant gene have a reduced risk of cardiovascular disease.

**MBOAT7**
Membrane-bound O-acyltransferase domain-containing 7, involved in the phospholipid metabolism known as the Lands cycle, exhibits a variant gene associated with a down-regulation of the protein and an elevated risk of NAFLD, disease severity, and an increased likelihood of developing HCC. The variant gene results in defective phospholipid metabolism, ultimately leading to an increase in TAG production and steatosis.

**GCKR**
Glucokinase regulator (GCKR) regulates the influx of glucose in the hepatocytes and plays a role in adjusting de novo lipogenesis. The genetic variant, a loss-of-function mutation (rs1260326), leads to increased glucose intake and increased production of malonyl-CoA, resulting in hepatic lipid accumulation, and has been linked to NAFLD. However, it also results in reduced plasma glucose levels and improved insulin sensitivity.

**HSD17B13**
This gene is located on chromosome 4 and encodes an enzyme called 17-beta hydroxysteroid dehydrogenase 13, which is localized on lipid droplets. It normally stabilizes lipid droplets and is implicated in the activation of hepatic stellate cells. The variant gene is a “loss-of-function” mutation and has been linked to protection against inflammation and fibrosis. However, the exact mechanism of this protective effect remains unclear, as other studies have suggested that a favorable effect is only observed in combination with other clinical factors and in conjunction with the wild-type PNPLA3 allele.

**Proteomics in NAFLD**
In the context of non-invasive diagnosis and enhanced comprehension of NAFLD and its stages, various “omics” have been investigated, including genomics, proteomics, transcriptomics, metabolomics, and lipidomics. The basis for “omics” is to measure small molecules (such as proteins and lipids) in various diseases to explore the pathophysiology and possible pathways of disease mechanisms.
Proteomics entails the measurement of various small protein molecules that reflect both the current and past cellular environments. Analyzing proteomics in NAFLD emerged in 2005, when Younossi et al.\textsuperscript{109} identified 12 distinct protein peaks that distinguished NAFLD from obese controls. With the use of SELDI-TOF technology they were unable to identify the corresponding proteins. However, technological advancements have since progressed, enabling the identification of thousands of proteins from a patient. Niu et al.\textsuperscript{110} performed a noteworthy study comparing proteome profiles of NAFLD patients with and without cirrhosis, comparing them to matched controls. In total they included 48 participants divided into three cohorts, the first cohort included patients with NAFLD without diabetes (n=10) and their matched controls (n=10), the second consisted of patients with NAFLD and T2DM (n=10) compared to matched controls with T2DM (n=8). Lastly, in their third cohort were ten NAFLD patients with cirrhosis. They found numerous proteins to be downregulated in cirrhotic patients, many involved in coagulation. Several proteins were also upregulated, many involved in immune system regulation. These proteins were compared between the groups and resulted in six proteins significantly different in all stages of NAFLD compared to controls (ALDOB, APOM, LGALS3BP, PIGR, VTN, and AFM).

Developments in technology since the early 21\textsuperscript{st} century have enabled the exploration of different “omics”. However, none of them have been integrated into clinical practice or utilized for clinical decision-making. Several studies have detected alterations in the proteomic profiles of patients with NAFLD, but few have been replicated. This underscores the complexity of the field and the dynamic nature of cellular environments.\textsuperscript{111,112}

Alcohol consumption in NAFLD

Assessment of alcohol consumption
The amount of consumed alcohol is what distinguishes NAFLD from AFLD, and accurate assessment of a patient’s alcohol habits is of paramount importance. However, relying on interviews or questionnaires for assessment carries the risk of recall bias and underreporting. Indirect laboratory markers (such as MCV, G-GT, AST, ALT, and CDT) lack reliable sensitivity and specificity, even for detecting chronic alcohol overconsumption (see Table 5). Significant elevation of these indirect markers occurs only with high alcohol intake, and they are insufficient to differentiate between abstinence, low-to-moderate and heavy alcohol consumption. Interestingly, Staufer et al.\textsuperscript{113} discovered that 10% of patients classified as NAFLD (all reported alcohol consumption of less than 210 grams/week in males and less
than 140 grams/week in females) had elevated EtG-levels in their hair, indicating excessive alcohol consumption. After further discussion, all patients confirmed excessive alcohol intake. This finding emphasizes the need of using direct biomarkers when evaluating alcohol consumption in NAFLD patients, particularly in clinical trials.

The threshold between NAFLD and AFLD is an alcohol consumption of less than 210 grams/week in males and 140 grams/week in females. However, few patients estimate their alcohol consumption in exact grams. A simpler way to describe their previous intake is by standard drinks. Nevertheless, the estimation of alcohol content in a standard drink varies across countries, ranging from 9.5 grams in the UK to 23.5 grams in Japan. In many European countries, a standard drink is considered to contain 12 grams of ethanol, see Figure 4.114,115

Figure 4. Overview of standard drinks.
Abbreviations: alc/vol, alcohol by volume.

Questionnaires
Several questionnaires have been developed to assess an individual’s alcohol consumption. Among these, the most used are the Alcohol Use Disorders Identification Test (AUDIT), CAGE, and Lifetime drinking history (LDH).

The AUDIT questionnaire consists of 10 questions, with each question scoring between 0–4 points. A score of ≥8 points indicates heavy alcohol consumption.116 The sensitivity for detecting heavy alcohol consumption ranges from 51–96%, with a specificity of 75–96%.117 A shorter version of AUDIT, known as AUDIT-C (C referring to consumption) has been constructed. It consists of the first three questions from AUDIT, focusing on the quantity of alcohol consumed rather than signs of dependence.118

CAGE comprises 4 questions, and a positive result indicating heavy alcohol consumption is obtained if there are two or more positive answers. All four questions focus on signs of alcohol dependence, with a sensitivity ranging from 58–100% and specificity of 61–80%.117,119

Lifetime Drinking History (LDH) is a comprehensive questionnaire that covers the individual’s alcohol consumption from the time the person first started drinking alcohol up to the present day. It includes questions
about amounts consumed, types of beverages, and frequency, and has a high test-retest reliability of 0.80.\textsuperscript{120}

**Indirect biomarkers of alcohol use**

**Table 5.** Indirect biomarkers of alcohol use.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Sensitivity*</th>
<th>Specificity*</th>
<th>Normalization after abstinence</th>
<th>Confounders</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDT</td>
<td>39%</td>
<td>70–100%</td>
<td>2–3 weeks</td>
<td>Gene variants</td>
</tr>
<tr>
<td>G-GT</td>
<td>52%</td>
<td>18–93%</td>
<td>2–6 weeks</td>
<td>Obesity, biliary obstruction, non-specific liver injury</td>
</tr>
<tr>
<td>AST</td>
<td>39%</td>
<td>47–68%</td>
<td>2–3 weeks</td>
<td>Non-specific hepatocyte injury, liver fibrosis, muscle injury</td>
</tr>
<tr>
<td>MCV</td>
<td>40%</td>
<td>80–90%</td>
<td>2–4 months</td>
<td>Vitamin deficiency, hematological disease, hypothyroidism</td>
</tr>
</tbody>
</table>

Abbreviations: CDT, carbohydrate-deficient transferrin; G-GT, gamma-glutamyl transferase; AST, aspartate aminotransferase; MCV, mean corpuscular volume.

* For chronic alcohol overconsumption.\textsuperscript{121,122}

G-GT is a membrane-bound glycoprotein enzyme used in glutathione metabolism. It can be elevated in chronic alcohol overconsumption, although it has limited sensitivity and several confounders.\textsuperscript{121} CDT, on the other hand, is a specific type of transferrin that undergoes a structural change (loss of a carbohydrate side chain [sialic acid residue]) in response to high alcohol consumption. CDT levels typically rise when alcohol consumption reaches at least 50–80 grams/day for 2–3 weeks.\textsuperscript{121}

Other laboratory markers that can be affected by chronic overconsumption include platelets (typically low but exhibit thrombocytosis after abstinence), increased HDL (which decreases after about a week of abstinence), ferritin, urate, IgA, and albumin (which can be increased in cases of overconsumption without liver disease, while it is typically low in cases with liver disease). Mentioned markers are generally unaffected in cases of low-to-moderate consumption.\textsuperscript{121}

**Direct biomarkers of alcohol use**

Except for ethanol itself, all direct biomarkers measurable in humans are products of phase II-reactions during the degradation of ethanol, see Figure 5. The most common biomarkers are phosphatidylethanol (PEth), ethyl glucuronide (EtG), ethyl sulphate (EtS), and fatty acid ethyl esters (FAEEs).
**Introduction**

Ethanol can be detected in blood, breath analysis, and urine. Generally, ethanol is eliminated at a constant rate, typically around 7 grams of ethanol per hour. However, individuals with heavy alcohol use eliminate ethanol up to 1.5 times faster than non-drinkers. After approximately 12 hours from the last ingestion, ethanol is usually not detectable in urine. Depending on the proportion of body-water and -fat, the same dose of ethanol can produce different blood alcohol concentrations (BAC). Generally, women, who tend to have a higher percentage of body fat, may reach a higher BAC with the same ethanol dose. Ethanol can also diffuse into alveolar air, leading to detectable alcohol in breath. The relationship between breath and blood concentration is approximately 1:2100. BAC is also influenced by the quantity and rate of alcohol consumed, as well as the presence of food in the stomach.

**Ethanol**

**Figure 5.** Overview of ethanol metabolism. Adapted from 124.

Abbreviations: PEth, phosphatidylethanol; EtG, ethyl glucuronide; EtS, ethyl sulphate; FAEE, fatty acid ethyl ester; CYP2E1, cytochrome P450 2E1.

**Phosphatidylethanol**

Phosphatidylethanol (PEth) was first described in detail in 1983 by Alling et al. They found an abnormal phospholipid in rats after being fed or injected with ethanol. PEth is a cell membrane phospholipid which only forms in the presence of ethanol. Phosphatidylcholine is normally transformed into phosphatidic acid (PA) by phospholipase D (PLD). Normally, PLD uses water as a substrate for the reaction but prefers ethanol by a
factor of several hundred and subsequently forms PEth, which is more stable than PA and accumulates in cells. PEth consists of a group of glycerophospholipids with a phosphoethanol head and two carboxylic acid chains. Humans have at least 48 isoforms of PEth, each with a specific composition of side chains. The most abundant forms in humans are PEth 16:0/18:1 and 16:0/18:2 (16 and 18 indicating the number of carbons in each side chain, and 0 and 1 representing the number of double bonds in each carboxylic acid side chain). PEth 16:0/18:1 constitutes approximately 40-45% of total PEth in whole blood and is the form commonly measured. PEth accumulates in cell membranes and is most easily detected in whole blood. It has a half-life of four days, although with large inter-individual variability, and it remains detectable for 2-4 weeks after alcohol consumption ceases. Measured PEth-value corresponds best to the drinking habits in the last two weeks. Studies have shown PEth to have a high sensitivity, ranging from 95–100% and a specificity of 100%. Cut-off values are not internationally established but a level above 20–80 ng/mL indicates social drinking, while levels surpassing 210 ng/mL is suggestive of alcohol overconsumption. A value over 700 ng/mL has also been proposed for detection of heavy alcohol use. In Sweden, the most abundant form of PEth (16:0/18:1) is measured in routine clinical practice using LC-MS/MS. The detection level is set at 35 ng/mL (0.05 µmol/L), indicating low-to-no consumption, while values exceeding 210 ng/mL (0.3 µmol/L) suggest previous high alcohol consumption. No false positive results for PEth have been reported, and its detection is not influenced by gender or concurrent liver disease. It is important to note that if a blood sample contains ethanol (suggesting very recent ingestion) and is insufficiently stored, in vitro formation of PEth can occur, affecting analytical results. As such, blood samples for analysis of PEth should be drawn in sober patients and stored at -80°C.

Minimal exposure to alcohol, such as alcohol-based mouthwash and hand sanitizer, does not elevate PEth. However, a single ingestion of alcohol can elevate PEth to measurable levels. In a previous experiment of a single dose-intake of alcohol after 2 weeks of abstinence, PEth 16:0/18:1 ranged from 37.2–122 ng/mL. Participants ingested alcohol to achieve a BAC of 1 g/kg (which corresponded to 3–6 standard drinks), and PEth remained detectable for 3–12 days following the event. A similar experiment was conducted by Gnann et al., where participants ingested alcohol for five consecutive days to reach a BAC of 1 g/kg during each session. PEth 16:0/18:1 became detectable within the first eight hours of drinking and reached levels of 74–237 ng/mL after the initial days of drinking. Unfortunately, there is no linear relationship between the amount of alcohol ingested and PEth levels, but a dose-response relationship exists. Despite large inter-individual variations, an increase in alcohol consumption by
Introducing approximately 1.5 standard drinks per day raises PEth by about 70 ng/mL (equal to 0.10 μmol/L). Furthermore, decreasing consumption by 1.5 drinks per day leads to a corresponding decrease in PEth levels.

**Ethyl glucuronide**

EtG is a phase II-metabolite of ethanol, only formed in the presence of ethanol. It constitutes less than 0.1% of the ethanol metabolism. It can be measured in blood, urine, and hair. EtG can be measured in urine for up to 5 days after cessation of drinking. Measuring EtG in a 3-6 cm strand of hair can detect alcohol consumption from the last three months. EtG in blood can be detected up to 2-4 days after a drinking episode, depending on the amount ingested. A urinary tract infection with E. coli can falsely lower EtG levels by hydrolyzing EtG. Impaired renal function can also prolong the detection time of EtG in urine. Dyeing, bleaching, and perming the hair can falsely lower EtG detection in hair strands. A few cases have reported measurable EtG in hair after using lotions or hairspray containing alcohol. However, hair color, melanin content, or gender does not influence the results. Use of hand sanitizers or alcohol-based mouthwash can lead to positive EtG levels.

**Ethyl sulphate**

EtS is another phase II-metabolite of ethanol, only formed in the presence of ethanol. It can be measured in blood and urine, with detection times similar to EtG. Renal impairment can result in a longer detection time in urine. Unlike EtG, EtS it is not falsely decreased due to urinary tract infections.

**Fatty acid ethyl esters**

FAEEs consist of a group of free fatty acids esterified in the presence of ethanol. The most common ones are ethyl myristate, ethyl palmitate, ethyl stearate and ethyl oleate. FAEE is best measured in hair, but using different hair products containing alcohol can influence its formation and produce false positive FAEE results.

**Moderate alcohol consumption and prevalence of NAFLD**

Multiple studies utilizing ultrasonography for NAFLD diagnosis have demonstrated a reduced prevalence of steatosis among individuals with moderate alcohol consumption (MAC) compared to abstainers. However, the definition of “moderate” varies considerably across studies, ranging from 70 grams/week to 280 grams/week.
Moderate alcohol consumption and severity of NAFLD

There is a limited number of studies of biopsy-verified NAFLD and moderate alcohol consumption. To date, eleven cross-sectional and four cohort studies have investigated the potential impact of alcohol consumption on NAFLD histology (see Table 6 and Table 7).

Table 6. Cross-sectional studies of biopsy-verified NAFLD and MAC.

<table>
<thead>
<tr>
<th>Authors, year</th>
<th>Sample size</th>
<th>Definition of MAC</th>
<th>Assessment type</th>
<th>Focus</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dixon et al. 2001</td>
<td>105</td>
<td>&lt;200 g/week</td>
<td>Interview, questionnaire</td>
<td>Liver histology</td>
<td>No significant difference in NASH</td>
</tr>
<tr>
<td>Cotrim et al. 2009</td>
<td>132</td>
<td>&lt;280 g/week</td>
<td>Interview</td>
<td>Liver histology</td>
<td>No difference in liver histology</td>
</tr>
<tr>
<td>Dunn et al. 2012</td>
<td>582</td>
<td>&lt;140 g/week</td>
<td>Questionnaire</td>
<td>Liver histology, steatohepatitis</td>
<td>Less steatohepatitis and fibrosis in MAC</td>
</tr>
<tr>
<td>Kwon et al. 2014</td>
<td>77</td>
<td>&lt;40 g/week</td>
<td>Questionnaire</td>
<td>Liver histology, lifetime consumption</td>
<td>Less advanced fibrosis in MAC</td>
</tr>
<tr>
<td>Sookoian et al. 2016</td>
<td>266</td>
<td>210 g/week (male) 140 g/week (female) + gene carriers</td>
<td>Interview</td>
<td>Genetic carriers as a measure of alcohol consumption, no protective association of MAC on histology</td>
<td>Higher rate of steatosis and inflammation in non-carriers (i.e., drinkers). No difference in fibrosis stage</td>
</tr>
<tr>
<td>Hagström et al. 2017</td>
<td>120</td>
<td>168 g/week</td>
<td>Questionnaire, PEth</td>
<td>Liver histology</td>
<td>Reduced risk of fibrosis in MAC. Elevated PEth levels increased the risk of significant fibrosis</td>
</tr>
<tr>
<td>Yamada et al. 2018</td>
<td>178</td>
<td>≤140 g/week</td>
<td>Questionnaire</td>
<td>Liver histology</td>
<td>Lower fibrosis stage in MAC</td>
</tr>
<tr>
<td>Mitchell et al. 2018</td>
<td>187</td>
<td>210 g/week (male) 140 g/week (female)</td>
<td>Interview, questionnaire</td>
<td>Fibrosis, binge-drinking, type of alcohol</td>
<td>Less fibrosis if consuming wine &lt;70 g/w, and in non-binge drinkers</td>
</tr>
</tbody>
</table>
**Introduction**

<table>
<thead>
<tr>
<th>Authors, year</th>
<th>Sample size</th>
<th>Definition of MAC</th>
<th>Assessment type</th>
<th>Focus</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tan et al. 2020</td>
<td>71</td>
<td>&lt;21 units/week (male) &lt;14 units/week (female)</td>
<td>Interview</td>
<td>Liver histology</td>
<td>No difference in prevalence of advanced fibrosis</td>
</tr>
<tr>
<td>Vilar-Gomez et al. 2020</td>
<td>1,153</td>
<td>&lt;196 g/week (male) &lt;98 g/week (female)</td>
<td>Questionnaire</td>
<td>Liver histology and ADH1B alleles</td>
<td>aOR 0.68 for fibrosis stage comparing non-drinkers with MAC</td>
</tr>
<tr>
<td>Staufer et al. 2022</td>
<td>57</td>
<td>210 g/week (male) 140 g/week (female)</td>
<td>Questionnaire, EtG</td>
<td>Alcohol detection in NAFLD</td>
<td>Subgroup analysis showed no significant difference in fibrosis stage</td>
</tr>
</tbody>
</table>

Abbreviations: MAC, moderate alcohol consumption; NASH, non-alcoholic steatohepatitis; PEth, phosphatidylethanol; ADH1B, alcohol dehydrogenase 1B; EtG, ethyl glucuronide.

**Table 7.** Cohort studies of biopsy-verified NAFLD and MAC.

In summary, cross-sectional studies present conflicting evidence regarding MAC. A total of 2,928 participants were investigated in eleven studies.\(^{153,144}\) Among them, five studies found no significant difference in histology (fibrosis stage and/or NASH) when comparing moderate consumption with no-to-low consumption. Six studies indicated more advanced fibrosis in non-drinkers compared with moderate consumers. Only one study reported more severe steatosis and inflammation in drinkers, but they did not observe a statistically significant difference in fibrosis stage.\(^{148}\) Notably, the definition of moderate alcohol consumption varied across studies.
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ranging from 40–280 grams per week. Only two studies included alcohol biomarkers (PETH and EtG) to estimate consumption, all others relied on clinical interviews and/or questionnaires.

In contrast, when analyzing available cohort studies, a different pattern emerges. Although only four studies in total, moderate consumption was associated with HCC and cirrhosis. Binge drinking was also linked to more advanced fibrosis, while non-drinkers displayed less severe histology compared to moderate consumers.154–157

Interestingly, the cross-sectional study by Dunn et al.146 and the cohort study by Ajmera et al.158 are both based on participants from the NASH CRN cohort (included 582 and 285 participants, respectively). The cross-sectional study found less fibrosis in moderate drinkers, whereas the cohort study showed more severe histology among moderate drinkers. This suggests that follow-up studies are necessary for a comprehensive assessment of alcohol’s impact on liver histology.

When examining other evidence on the effect of moderate alcohol consumption on NAFLD without biopsies, the results remain inconsistent. Chang et al.158 performed a large cohort study of 190,048 individuals undergoing health check-ups with a median follow-up of 4.1 years. Steatosis was diagnosed by ultrasound and FIB-4 served as a proxy for fibrosis. They observed a lower incidence of steatosis in light-to-moderate drinkers compared to non-drinkers. However, the risk of developing steatosis, along with increased FIB-4 scores, was elevated in light and moderate drinkers compared to non-drinkers (HR 1.16–1.65). Åberg et al.159 conducted a large population-based study on alcohol consumption in fatty liver disease. Although the diagnosis of fatty liver was based on a Fatty liver index >60, they identified an increased risk of liver disease with any alcohol use (but not statistically significant in the 0–9 grams/day) compared to lifetime abstainers. Another interesting study performed by Ferri and colleagues160 focused on lifetime alcohol consumption in 276 patients at a hepatology unit. They quantified alcohol consumption into “LACU” (Lifetime Alcohol Consuming Unit) and converted a weekly consumption of 70 grams of ethanol over a year to 1 LACU. They found that light consumers (averaging 8.7 LACU over their lifetime) had a lower prevalence of cirrhosis and HCC compared to both abstainers and moderate drinkers. Moderate users had an average lifetime consumption of 80 LACU. Evidently, consumption of up to 70 grams/week has not been proven to associate with worse liver-related outcomes in NAFLD, compared to consumption close to the “allowed” threshold (i.e., less than 140-210 grams/week).

The type of beverage consumed (e.g., beer, wine, or spirits) is rarely reported in studies. In the aforementioned study, beer consumption was associated with less advanced fibrosis, whereas Dunn et al. found exclusive
wine consumption to be significantly less associated with prevalence of NAFLD.\(^{150,161}\)

**Moderate alcohol consumption and morbidity and mortality in NAFLD**

In the general population, alcohol consumption exhibits a dose-response relationship with the risk of liver cirrhosis.\(^{162}\) Cardiovascular disease is the leading cause of death among NAFLD patients. Contrary to the idea in the general population, moderate alcohol consumption is not associated with reduced risk of cardiovascular disease in NAFLD patients.\(^{163}\) In the study by Åberg et al., they also observed an increased risk of cancer in the low alcohol consumption group (0–9 g/day) compared to lifetime abstainers.\(^{159}\) However, in their fully adjusted model, alcohol consumption of 0–9 grams/day was associated with lower all-cause mortality (HR 0.79). Hajifathalian et al.\(^{164}\) reported similar results in their study, where NAFLD participants consuming 0.5–1.5 drinks per day had decreased overall mortality compared to abstainers (HR 0.64). Drinking ≥1.5 drinks per day had a detrimental effect on mortality with an HR of 1.45.

Ultimately, the impact of moderate alcohol consumption in NAFLD pathophysiology remains unclear and has the potential to influence outcomes in various ways, not only histologically but also in non-liver related illnesses and mortality.

**Treatment of NAFLD**

At present, there are no pharmaceutical treatments available for NAFLD or NASH. Typically, advise given upon a NAFLD diagnosis revolves around achieving a weight loss of at least 5–10% through dietary modifications and exercise. Notably, a 10% reduction in weight has proven to have a significant impact on liver histology. After one year, 90% of patients achieved resolution of NASH, and over 80% showed improvement in fibrosis by at least one stage.\(^{165}\)

However, maintaining weight loss over an extended period has proven to be a challenge, as demonstrated in a study conducted by Fotherill and colleagues.\(^{166}\) The resting metabolic rate (RMR) of the participants decreased as their weight decreased. However, it is noteworthy that this reduced RMR persisted after 6 years, even as participants regained weight, thereby making it difficult to sustain weight loss over the long term. Bariatric surgery has been studied in NAFLD, with Roux-en-Y-bypass being the most used method. It has been associated with histological improvement, with NASH resolution observed in up to 85% of cases and one-third of patients experiencing an improvement in fibrosis stage one year after the procedure.\(^{167}\)
Several pharmacological studies have focused on improvement of NASH as their primary endpoint, with some also aiming for a combined improvement or “no worsening” of fibrosis. Consequently, participants in clinical trials undergo liver biopsies both upon enrollment and for evaluation after treatment. However, it is important to note that none of these treatments have received approval for the treatment of NASH.

Obeticholic acid (OCA) functions as a nuclear receptor agonist, targeting the farnesoid X receptor, and has been studied as an antifibrotic agent for NASH. After 18 months of treatment with 25 mg OCA, 22.4% of the patients achieved at least one-stage improvement in fibrosis stage (with no worsening of NASH), while in the placebo group, nearly 10% (9.6%) achieved similar results. A common side effect of this drug is pruritus and elevated LDL cholesterol levels.\textsuperscript{168,169}

Resmetirom is a drug that targets the thyroid hormone receptor β (THR-β) that is highly expressed in the liver. Animal studies have shown its potential to regulate various metabolic pathways, including improving insulin sensitivity, reducing cholesterol and triglycerides, and mitigating apoptosis. Resmetirom is a receptor agonist with specific uptake in the liver. So far, data from the phase II trial have been published, demonstrating that treatment of resmetirom for 36 weeks reduced liver steatosis (assessed by MRI-PDFF) by 8.5% compared to placebo, which showed 2.3% absolute reduction in liver fat. At baseline, both the treatment group and the placebo group predominantly had F1 fibrosis (56% and 46%, respectively). The phase III trial is ongoing, and the long-term effects of resmetirom treatment are yet to be determined.\textsuperscript{170}

Semaglutide, a GLP-1 receptor agonist administered subcutaneously, is currently approved for treating T2DM and obesity and is undergoing testing for NASH. The phase II trial revealed that a weekly dose of 0.4 mg resulted in NASH resolution in 59% of patients, compared to 17% in the placebo group. Fibrosis improvement by at least one stage was similar in both the placebo and treatment groups.\textsuperscript{171}

Pharmaceutical trials encounter several challenges. Histological endpoints are typically assessed after a relatively short period, usually 1–1.5 years. It is noteworthy that up to 10–20% of patients in the placebo group achieve primary endpoints related to histological improvement or resolution. This is likely a combination of the natural history of the disease and histological sampling variability. Another factor is the interpretation of histology by the pathologists, where both intra- and interrater agreement vary greatly.
Inclusion of participants (Linköping cohort) (Paper I–IV)

All participants were initially referred to the Department of Gastroenterology and Hepatology, Linköping University Hospital, Sweden between 1988 and 2018. Participants are part of an existing cohort with NAFLD consisting of 289 participants (as of April 2023). The original inclusion of patients took place from 1988 to 1993, followed by a subsequent follow-up study where they were invited back for clinical assessment and liver biopsy. A total of 129 participants were included in the first cohort, of whom 88 took part in the first follow-up (during 2003–2005), and 59 accepted a second follow-up (between 2013–2015). Over the years, additional participants (n=160) have been included in the cohort, but not all have participated in a follow-up, see Figure 6.
All participants received a diagnosis of NAFLD after excluding other possible causes of liver disease, such as viral or autoimmune hepatitis, medications, or drugs. All participants reported alcohol consumption below 140 grams per week (for both males and females).

**Selection of participants in cross-sectional study (Paper I)**
For this paper, only participants with available whole blood stored at -80°C were considered for inclusion in the study. A total of 131 participants underwent review for potential inclusion.

Inclusion criteria were:
- Available whole blood for analysis of PEth
- Liver biopsy
- AUDIT-C

Included participants had not previously received any advice regarding alcohol consumption. Individuals with pre-existing or suspected cirrhosis who had been advised to reduce or abstain from alcohol consumption were excluded from this study.

**Selection of participants in cohort study (Paper II)**
For this study, only participants with at least two liver biopsies or a clinical diagnosis of cirrhosis at follow-up were considered for inclusion. All participants with liver biopsy at baseline were reviewed for inclusion in the cohort study.

The inclusion criteria were as follows:
- Liver biopsy performed at follow-up or a previous diagnosis of cirrhosis
- Available whole blood for analysis of PEth
- AUDIT-C from the follow-up visit

**Selection of participants in study of PNPLA3 (Paper III)**
Participants in this study were part of the first follow-up in 2003–2004, during which liver biopsies were performed, and extracted DNA samples were saved. A total of 68 participants were reviewed for potential inclusion. Only participants with wildtype PNPLA3 (CC) or that were homozygous for variant PNPLA3 (GG) were included in the study.
Selection of participants in validation cohort (Linköping cohort) (Paper IV)

Only participants with a liver biopsy at the first or second follow-up visit were considered for inclusion. Eligible participants needed to have stored plasma samples at -80ºC for the proteomic analysis. A total of 88 participants were reviewed for potential inclusion.

Inclusion and selection of patients in discovery cohort (Uppsala cohort) (Paper IV)

Participants in the discovery cohort were recruited from the Department of Gastroenterology and Hepatology, Akademiska University Hospital, Uppsala, Sweden or from participating in the SCAPIS (Swedish CardioPulmonary bioImage Study) study. All participants exhibited clinical suspicion of NAFLD and met at least one of the following criteria: elevated alanine aminotransferase (more than 1.5 times the upper limit of normal), evidence of steatosis on imaging, biopsy confirming NAFLD (conducted within three months prior to the screening visit), or a concentration of caspase-cleaved fragment of cytokeratin 18 (CK18 M30) exceeding 180 U/L. The diagnosis of NAFLD was established after ruling out other potential liver diseases and excluding alcohol consumption surpassing 210 grams in males and 140 grams in females. Informed consent was obtained from all participants before their inclusion. Unless a liver biopsy had been performed within three months prior to inclusion, all participants underwent a liver biopsy. Plasma samples were collected four to eight weeks after the liver biopsy. A total of 134 participants were screened for inclusion in the discovery cohort, see Figure 6.

The inclusion criteria were identical for participants in both the discovery and validation cohorts and were as follows:

- Liver biopsy
- Available plasma for proteomic analysis

Data collection

Clinical evaluation of participants (Linköping cohort) (Paper I–IV)

A standardized case report form was completed for each participant, covering their previous and current medical history, including medication usage. Each participant also underwent regular clinical examination, measurement of blood pressure and anthropometric measurements of height, weight, and waist circumference. Blood tests were drawn after an overnight fast. Blood samples (whole blood, plasma, and serum) for storage at -80ºC.
were collected from 2003 and onwards (i.e., participants included in 1988–1993 did not have any stored blood samples from baseline).

**Clinical evaluation of participants (Uppsala cohort) (Paper IV)**
Each participant in the Uppsala cohort underwent a screening visit with clinical evaluation, covering previous and current medical history, measurement of blood pressure and anthropometric measurements of height and weight. Clinical blood panels were conducted during the screening visit. Additionally, blood tests for storage at -80°C were obtained after an overnight fast, within 4–8 weeks following the liver biopsy procedure.

**Liver biopsy (Paper I–IV)**
All participants in the original Linköping cohort underwent liver biopsy (n=129) and all participating at follow-up were offered a second or third liver biopsy. Participants with a previous diagnosis of cirrhosis were not offered a liver biopsy. Other participants in the cohort underwent liver biopsy if clinically relevant (such as elevated values from transient elastography or clinically suspected advanced fibrosis). Liver biopsies were performed within 3 months of clinical evaluation.

All participants in the Uppsala cohort underwent liver biopsy as part of the NAFLD diagnosis. For participants in the Uppsala cohort, liver biopsy was performed 1–4 weeks after the screening visit.

Liver biopsies were performed after an overnight fast using ultrasound guidance and a 1.6 mm Biopince® needle. Liver biopsies were performed either at Linköping University Hospital or Akademiska University Hospital, Uppsala, depending on which cohort the participant belonged to. Biopsy method did not differ between the centers.

The obtained biopsy samples were fixed in formalin for subsequent histological analysis by the pathologist.

**Genetic analysis (Paper III, IV)**
Genotyping of PNPLA3 for participants in Paper III and the validation cohort of Paper IV was performed using pre-prepared DNA from the included participants. Gene analysis of rs738409 was conducted using polymerase chain reaction (PCR) with the primer pair: AGTTTGTGCCCCTGCTCACT/TGTGAGCACAATCTCAGGGC and the following program: 96°C 5'[96°C 15”-60°C 15”-72°C 30”]30, followed by restriction enzyme analysis. The 220 base pair amplicons were treated in separate reactions with NlaIII (ThermoFisher Scientific; ER1831) which specifically cleaves C alleles, and FokI (ThermoFisher Scientific; FD2144) which cleaves G alleles, and analyzed using agarose gel electrophoresis.
Genetic analysis of PNPLA3 in the discovery cohort (Paper IV) was conducted by Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany.

Cell experiments (Paper III)
Human HepG2 cells were used for cell experiments. Experiments were performed at least three times. Cells were grown under basal, fed (for 48 hours), or starved (16 hours, after being fed for 33 hours) conditions. Basal conditions consisted of low-glucose DMEM (1000 mg/L) and 10% FBS. Fed conditions were during high-glucose DMEM (4500 mg/L), 10% FBS and albumin-conjugated oleate. The cells were transfected with PNPLA3 vectors, either wildtype I148 or variant M148, and studied under energy-deprived conditions. For detailed analysis of autophagy, the cells were also transfected with mRFP-GFP-LC3 plasmid. Lysosomal inhibition was induced by treatment with Bafilomycin A1 (Sigma).

Cells were lysed in cracking buffer, heated, and run on SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, blocked, and probed with specific antibodies (PNPLA3 [Abcam, ab81874]; PLIN2 [Abcam, ab37516]; PLIN3 [Abcam, ab47638]; GADPH [Abcam, ab9485]; LC3B [Novus, NB100-2220], LAMP1 [Abcam, ab25630]; 6x-His [Abcam, ab18184]; actin [Sigma, A5441]; tubulin [Sigma, T9026]). Images were acquired with Bio-Rad Molecular Imager Chemidoc XRS + device and quantified using BioRad Image Lab Software 5.2.1.

For confocal imaging, cells were incubated with lipid dye BODIPY (Invitrogen, D3922) or Lysotracker Green (Invitrogen, L7526), fixed with 4% formaldehyde, blocked with blocking buffer (1% FBS and 0.1% Triton X-100 [Sigma] in PBS), and incubated with primary antibodies, and subsequently AlexaFluor-labelled antibodies as well as nucleus-stained with Hoechst (Invitrogen, H3569) or lipid-stained using monodansylpentane (Abgent, SM1000A). Primary antibodies were 6x-His (Abcam, ab18184); LC3B (Novus, NB100-2220) and AlexaFluor-antibodies were Invitrogen, A11031 and A31573. After mounting, cells were studied using Nikon A1 Plus confocal microscope and images were acquired and analyzed with NIS-elements, version 4.50.

Liver biopsy slides (Paper III)
The biopsy slides used in Paper III were dewaxed by using xylene (VWR), rehydrated with ethanol, and heated at 95°C in antigen retrieval solution (10 mM trisodium citrate, 0.05% Tween-20; Sigma). They were washed and blocked with immunofluorescence buffer (IFF, 2% fetal calf serum and 1% BSA in PBS) and incubated with primary antibodies (PNPLA3 [sc-49594; Santa-Cruz Biotechnology] and LC3B [NB100-2220, Novus])
overnight at 4°C. Then subsequently washed and incubated with secondary antibodies (Alexa-Fluor labelled antibodies; A-11057 and R37118, Invitrogen), washed, and mounted using Vectashield mounting medium (Vector laboratories, H-1400).

Data analysis

**Histological assessment of liver biopsies (Paper I–IV)**

All liver biopsies were reviewed by an experienced liver pathologist. Liver biopsies performed on participants in the discovery cohort (Paper IV) were also reviewed by a second pathologist. Histological parameters were graded according to Kleiner et al.\(^34\) The diagnosis of NASH was defined using the fatty liver inhibition progression (FLIP) algorithm.\(^39\) Stereological point counting (SPC) used in Paper III was previously performed by ME. In SPC, 10 images of each biopsy were reviewed using a point grid and subsequently counting hits on lipid droplets and normal hepatocytes. This results in a percentage of the biopsy area affected by steatosis.

**Assessment of alcohol consumption (Paper I, II)**

All participants were interviewed about their alcohol consumption during the last month, and it was converted into average weekly consumption of standard units. The participants independently filled out the AUDIT-C questionnaire without any interference from the investigators (JB, SK, ME, and PN). The participants were provided with an image illustrating standard units. Later, JB reviewed the questionnaires and translated the information into average weekly ethanol consumption as well as the frequency of binge drinking. Binge drinking was defined as consumption of ≥5 units (for males) or ≥4 units (for females) of alcohol per occasion, at least once per month. The data from the clinical interviews was also reviewed and translated into average weekly ethanol consumption. One standard unit was considered to contain 12 grams of ethanol.\(^{14}\)

AUDIT-C consists of three questions: 1) How often do you have a drink containing alcohol? 2) How many drinks containing alcohol do you have on a typical day when you are drinking? 3) How often do you consume six or more drinks on a single occasion?

For Paper II, alcohol consumption was quantified at follow-up. Participants reported alcohol consumption of less than 140 grams per week at baseline but did not fill out questionnaires, nor were blood samples saved. Depending on the year of the follow-up, some patients had an additional question attached to the AUDIT-C questionnaire related to if and how the participant’s alcohol consumption had changed since inclusion.
Analysis of phosphatidylethanol (Paper I, II)
All participants had available whole blood for the analysis of phosphatidylethanol. Blood samples were collected during the first or second follow-up, stored at -80°C and later thawed for analysis. PEth 16:0-18:1 was measured using the liquid chromatography with tandem mass spectrometry (LC-MS/MS) method. In short, samples are prepared by adding solvents and separated through chromatography. The sample is then passed through a mass analyzer, separated by specific ions’ mass-to-charge ratio, and hit a detector that quantifies the different ions. The limit of quantification (LOQ) for PEth 16:0-18:1 was 0.025 μmol/L (equivalent to 18 ng/mL). Analysis of PEth was made blinded to patient data.

Image analysis of biopsy slides (Paper III)
Image analyses of previously prepared liver biopsy slides and HepG2 cells were done using NIS-elements version 4.50. For analysis of LDs in the liver biopsies, LC3-II signal at 488 nm was used to detect lipídated LC3 (fluorescence intensity threshold 500–4905). Lipid-containing vesicles were identified by applying inverted thresholding with fluorescence intensity in the range of 0–10 of objects with a minimal size of 10 µm and circularity of 0.57. A doughnut structure (3 µm wide) was created using the dilate function to generate a region of interest around the perimeter of lipid vesicles. LC3-II-positive lipid vesicles were defined as doughnuts positive for LC3-II peaks above 500 in intensity. LD count was calculated by subtracting the number of doughnuts with LC3-II-positive lipid vesicles.

Analysis of proteomics (Paper IV)
Proteomic analysis was performed on plasma previously stored at -80 °C. Plasma samples in the discovery cohort were drawn four to eight weeks after the liver biopsy was performed. Plasma samples from the validation cohort were collected during the study visit, within three months before the liver biopsy. For plasma samples from both cohorts, the levels of 276 (3×92) proteins were measured from three different Olink® panels (Olink® Inflammation, Cardiovascular II, and Cardiovascular III) using Proximity Extension Assay (PEA) technology. PEA is a specific type of immunoassay where two matched DNA-labelled antibodies bind to a specific target protein. The DNA labels hybridize after binding and serve as a template for PCR and are proportional to the concentration of the target protein. An advantage of PEA is the minimal risk of cross-binding, which is common during traditional immunoassays. Analyses in the discovery cohort were performed at SciLifeLab Affinity Proteomics, Uppsala, and at Olink®, Uppsala, Sweden for the validation cohort. Protein levels were reported as Normalized Protein eXpression (NPX) on a log2-scale.
Ethical considerations (Paper I–IV)

All participants signed informed consent. All studies were conducted in full accordance with the ethical guidelines of the 1975 Declaration of Helsinki. The study designs were approved by the regional ethics committees in Linköping and Uppsala (Dnr 02-454, amendments: 2011-468-32, 2012-229-32, 2013/72-32; dnr 2016/250, amendments 2016/250/1-4).

Statistical analysis

Paper I, II

Continuous variables are presented as mean ± standard deviation (SD) and binominal variables as percentages. Categorical variables are as percentages (Paper I) or median with interquartile range (Paper II). Differences between two groups were compared using student t–test or Mann–Whitney U–test. ANOVA was used for comparisons of three or more groups. Differences between baseline and follow-up characteristics in the cohort were evaluated using paired t–test. Categorical variables were compared using Fisher exact test. Correlations were calculated using Pearson r or Spearman rho (ρ) when applicable. Univariable and multivariable logistic regression models were used for evaluating fibrosis and different methods of assessing alcohol consumption.

In the cross-sectional study, binary logistic regression was used for evaluating the risk of advanced fibrosis using alcohol consumption data from AUDIT-C, clinical interview, and PEth-value. Alcohol consumption was examined using both continuous and interval data. Alcohol consumption, as evaluated by AUDIT-C, was categorized into three groups: 1) 0–2.99 g/week; 2) 3–66 g/week; 3) >66 g/week. Consumption assessed through clinical interview was divided into three groups: 1) 0–2.99 g/week; 2) 3–96 g/week; 3) >96 g/week. Consumption assessed by PEth was divided into two groups: 1) PEth <50 ng/mL and 2) PEth ≥50 ng/mL.

In the multivariable models, adjustments were made for age, sex, BMI, and T2DM. When analyzing the association between T2DM and alcohol consumption, all alcohol assessments were transformed into dichotomous variables: 1) consumption of <66 g/week or >66 g/week (for assessment by AUDIT-C), 2) consumption of <96 g/week or >96 g/week (for clinical interview assessments), 3) PEth <50 ng/mL or PEth ≥50 ng/mL.

In the cohort study, three different models of logistic regression were performed. In the first model, (i.e., Model 1), the variables age, sex, BMI, weight change (weight difference between baseline and follow-up), diagnosis of T2DM, NASH at baseline biopsy, and alcohol consumption (assessed by AUDIT-C, interview, and PEth) were all tested separately in binary
logistic regression for assessing the risk of developing significant fibrosis progression (i.e., progressing ≥2 stages of fibrosis or developing cirrhosis-related complications). Cirrhosis-related complications were defined as development of ascites, hepatocellular carcinoma, moderate or large varices or variceal hemorrhage, or hepatic encephalopathy. Alcohol consumption was tested, both using continuous data and interval data. Different intervals of alcohol consumption were attained from the logistic regression model. Consumption assessed by AUDIT-C was separated into three groups: 1) 0–2.99 g/w; 2) 3–65.99 g/w; 3) ≥66 g/w. Consumption assessed by interview was also divided into three groups: 1) 0–2.99 g/w; 2) 3–96 g/w; 3) >96 g/w. Optimal cut-off for PEth was derived from receiver operating characteristic analysis, and was divided into two groups: 1) <48 ng/mL; 2) ≥48 ng/mL. In the second model (i.e., Model 2), adjustments were made for any significant covariables with a p<0.10 as a threshold of significance. Possible covariables were age, sex, weight change, BMI, T2DM, and NASH. In the third model (i.e., Model 3), clinically relevant parameters were used for adjustment, irrespective of their significance level. Variables adjusted for were age, sex, weight change, T2DM, and NASH.

A p-value of <0.05 was considered statistically significant. All statistical analyses were made using IBM SPSS Statistics for Windows version 25.0 (Paper I) or version 26.0 (Paper II), (IBM Corp., Armonk, NY).

**Paper III**
Results are shown as mean value ± standard error of the mean (SEM). Statistical analysis was performed by using student t–test in Microsoft Excel (Microsoft Corporation, 2016). Differences were considered statistically significant if p<0.05.

**Paper IV**
Continuous variables are presented as mean ±SD, and categorical variables as percentages. Student t–tests or Mann–Whitney U–tests were performed for between-group comparisons (F0–1 vs. F2–4). Comparison of categorical variables was made by Chi-square or Fischer’s exact test. The significance level was set at p<0.05. Association between the ordinal outcome fibrosis stage and each biomarker was analyzed using univariate and multiple proportional odds regression models. The multiple models were adjusted for age, BMI, and PNPLA3. The proportional odds assumption for the top-ranking biomarkers in the ordinal regression models was assessed by visual inspection of graphs comparing means of X|Y with and without the assumption of proportional odds.\(^\text{17}\) The generalized c statistic, calculated as (Somers’ D+1)/2, was used to describe the predictive ability of the proportional odds models. The generalized c estimates the probability of
No Alcoholic Fatty Liver Disease

52 correctly discriminating between two cases from different categories. It equals 0.5 for a model with random discrimination and 1 for a model with perfect discrimination. The significance level was set at the Bonferroni threshold of $p < 0.05 / \text{number of biomarkers}$. In addition to the proportional odds regression models, we applied machine learning in terms of random forests (RF) and Boruta analyses. RF analysis was used to assess associations between biomarkers and fibrosis stage by ordering biomarker’s prognostic importance. Then, Boruta analysis was performed to confirm which biomarkers had larger than random association with fibrosis stage in the RF model. Boruta adds shuffled copies of the original biomarkers in the RF model and iteratively compares the importance of the variables. Biomarkers with importance higher than the maximum random variable are classified as confirmed, biomarkers with lower importance are rejected, and if not confirmed nor rejected, biomarkers are classified as tentative. Analyses of ordinal logistic regression, RF, and Boruta were repeated in the validation cohort. Biomarkers passing the Bonferroni threshold in both cohorts were seen as markers with strong evidence of effect.

Analyses of participants’ characteristics in the two cohorts were performed using IBM SPSS Statistics for Windows, version 26.0 (IBM Corp., Armonk, NY). Analyses of biomarkers and fibrosis stage were performed using R v4.2.3 and the R-packages “rms”, “ranger” and “Boruta”.

Non-Alcoholic Fatty Liver Disease
RESULTS

Moderate alcohol consumption (Paper I, II)

Moderate alcohol consumption in NAFLD was studied in a cross-sectional and cohort study. The cross-sectional study included a total of 86 participants, while the cohort study included 82 participants. There was an 85% (n=70) overlap of participants between the two studies.

Only participants who had not received prior healthcare advice on alcohol consumption were included in the cross-sectional study. The majority of participants were male, approximately 60 years old, with a mean BMI of 30.2 kg/m². They were categorized into two groups based on fibrosis stage, where 17.4% (n=15) had advanced fibrosis (F3–4) and 82.6% (n=71) with F0–2. Participants with advanced fibrosis were significantly more likely to be diagnosed with T2DM (73.3% vs. 43.7%, p<0.05).

The cohort study had a mean follow-up time of 17.2 years (range 3.3–32.8 years). The majority of participants were overweight males. On average, they gained 2 kgs of weight between baseline and follow-up, and the prevalence of T2DM increased (60% at follow-up vs. 12% at baseline). Additionally, most participants had hypertension. The cohort was divided based on fibrosis progression, considering a progression of 2 or more fibrosis stages or the development of cirrhosis-related complications as significant fibrosis progression. Out of the 82 participants, 26.8% (n=22) showed significant fibrosis progression, while 73.2% (n=60) showed no progression. Baseline characteristics did not differ between the two groups.

Alcohol consumption and T2DM

Regarding alcohol consumption and T2DM, there was a significant difference in the diagnosis of T2DM between the two groups in the cross-sectional study. To explore a possible association with advanced fibrosis, participants were categorized into four groups based on alcohol consumption and T2DM diagnosis: 1) low alcohol consumption and no T2DM, 2) low alcohol consumption and T2DM, 3) moderate alcohol consumption and no T2DM, 4) moderate alcohol consumption and T2DM. The definition of moderate alcohol consumption was determined through multivariable logistic regression models. Three different cut-offs were used: A) >66 grams/week (AUDIT-C), B) >96 grams/week (clinical interview), and C) PETH ≥50 ng/mL.
Figure 7. Alcohol consumption and T2DM. Advanced fibrosis and alcohol consumption divided by different cut-offs and separated by T2DM. * = p<0.05, ** = p<0.01.

Abbreviations: AUDIT-C, alcohol use disorder identification test-consumption; T2DM, type 2 diabetes mellitus; PEth, phosphatidylethanol; g/wk, grams/week; F, fibrosis stage. Metabolism Clinical and Experimental 2021. Published with permission.

Similar results were seen regardless of the assessment method used for evaluating alcohol consumption, see Figure 7. Participants with both T2DM and moderate alcohol consumption displayed significantly more advanced fibrosis compared to individuals with low consumption, irrespective of T2DM status.
Moderate alcohol consumption and histology

In the cross-sectional study, the group with advanced fibrosis exhibited higher average weekly consumption and PEth-value. Similar findings were noted in the cohort, as participants with significant fibrosis progression displayed higher weekly consumption and significantly greater PEth-values in comparison to non-progressors. The cohort was also assessed on binge drinking, which was significantly more frequent in the significant fibrosis progression group, see Table 8.

Table 8. Alcohol consumption in Paper I, II.

<table>
<thead>
<tr>
<th></th>
<th>Alcohol consumption (AUDIT-C) (g/week)</th>
<th>Alcohol consumption (interview) (g/week)</th>
<th>PEth-value (ng/mL)</th>
<th>Binge drinking (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-sectional</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=86)</td>
<td>31.0 (±34.4)</td>
<td>35.6 (±40.3)</td>
<td>43.3 (±84.7)</td>
<td>NA</td>
</tr>
<tr>
<td>F0–2 (n=71)</td>
<td>27.6 (±31.9)</td>
<td>31.2 (±36.8)</td>
<td>38.4 (±83.1)</td>
<td>NA</td>
</tr>
<tr>
<td>F3–4 (15)</td>
<td>47.5 (±42.0)</td>
<td>56.3 (±50.7)</td>
<td>66.5 (±91.6)</td>
<td>NA</td>
</tr>
<tr>
<td>Cohort (n=82)</td>
<td>28.6 (32.2)</td>
<td>32.1 (±38.8)</td>
<td>55.0 (±86.9)</td>
<td>25.6</td>
</tr>
<tr>
<td>No progression</td>
<td>25.8 (±31.9)</td>
<td>27.5 (±34.5)</td>
<td>43.6 (±74.1)</td>
<td>18.3</td>
</tr>
<tr>
<td>(n=60)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Significant fibrosis</td>
<td>36.2 (±34.9)</td>
<td>44.5 (±47.3)</td>
<td><strong>86.3 (±111.1)</strong></td>
<td><strong>45.5</strong></td>
</tr>
<tr>
<td>progression (n=22)</td>
<td></td>
<td></td>
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</tr>
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</table>

Alcohol consumption assessed by AUDIT-C, clinical interview, PEth-value, and binge drinking in both the cross-sectional and cohort study. Values in bold indicate p-value <0.05 compared with the F0–2 group (Paper I) or the no progression group (Paper II). Abbreviations: AUDIT-C, alcohol use disorder identification test-consumption; PEth, phosphatidylethanol; NA, not applicable.

To further examine the impact of moderate alcohol consumption on fibrosis stage, logistic regression models were performed in both the cross-sectional and cohort study. In the cross-sectional study, the risk of advanced fibrosis increased by 1.4–1.6% for every gram of ethanol consumed per week. After adjusting for age, sex, BMI, and T2DM, the risk further increased to 1.8–1.9%. PEth-value as a continuous variable did not reach statistical significance in the cross-sectional population. Logistic regression was also used to assess consumption intervals and potential associations with advanced fibrosis. For alcohol consumption evaluated by AUDIT-C, the highest risk of advanced fibrosis was observed with
consumption exceeding 66 grams per week (aOR 9.7, 95% CI 1.4–68.9). However, this threshold was not applicable when assessing consumption through a clinical interview. Instead, a threshold of more than 96 grams per week showed a significant association with advanced fibrosis (aOR 8.5, 95% CI 1.05–69.6) compared to the low-no consumption group (consuming 0–2.99 grams/week). No significant associations were found when comparing the low-no-consumption group with those consuming up to 66 grams per week or up to 96 grams per week, respectively. A PEth-value ≥50 ng/mL was also linked to advanced fibrosis, with a cOR of 3.3 (95% CI 1.02–10.5). After adjustment, the risk increased to an aOR of 5.5 (95% CI 1.4–22.1).

Similar results were observed in the cohort study. In this case, three different models were constructed to investigate potential predictors of significant fibrosis progression. Another variable considered when assessing alcohol consumption was binge drinking, defined as ≥5 units per occasion for males and ≥4 units for females, at least once per month.

Table 9. Predictors of fibrosis progression.

<table>
<thead>
<tr>
<th></th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cOR</td>
<td>p-value</td>
<td>aOR</td>
</tr>
<tr>
<td>AUDIT-C (≥66 g/week)</td>
<td>5.4</td>
<td><strong>0.04</strong></td>
<td>4.8</td>
</tr>
<tr>
<td>Interview</td>
<td>8.0</td>
<td><strong>0.04</strong></td>
<td>8.6</td>
</tr>
<tr>
<td>PEth (≥48 ng/mL)</td>
<td>4.2</td>
<td><strong>0.01</strong></td>
<td>4.1</td>
</tr>
<tr>
<td>Binge drinking (≥66 g/week)</td>
<td>3.7</td>
<td><strong>0.02</strong></td>
<td>3.3</td>
</tr>
</tbody>
</table>

Univariable and multivariable logistic regression models for significant fibrosis progression or development of cirrhosis-related complications in the cohort (Paper II). Bold values indicate statistical significance at the p<0.05 level. Model 1: unadjusted estimates. Model 2: adjusted for weight change. Model 3: adjusted for weight change, sex, T2DM, age, and NASH. Abbreviations: AUDIT-C, alcohol use disorder identification test-consumption; PEth, phosphatidylethanol. Adapted from Hepatology Communications 2023. Published with permission.

In the cohort, the association between fibrosis progression and continuous variables of consumption and PEth-value did not reach statistical significance. However, applying different thresholds for consumption and PEth-
value revealed an increased risk of significant fibrosis progression, see Table 9. The strongest association was observed with a PEth-value ≥48 ng/mL and binge drinking. In the fully adjusted model, the aOR for significant fibrosis progression with a PEth-value ≥48 ng/mL was 5.9 (95% CI 1.6–21.4), and 5.1 (95% CI 1.4–18.1) for binge drinking. Conversely, the progression of other histological parameters, such as steatosis, lobular inflammation, and ballooning, did not exhibit a significant association with alcohol consumption or PEth-value.

PNPLA3 (Paper III)

In the HepG2 cell experiments, M148 cells exhibited significantly larger total area of LDs compared to I148. Additionally, M148 cells displayed a greater number of LDs coated with PNPLA3. Following starvation and BODIPY staining, I148 cells demonstrated a reduced intensity of BODIPY indicating a decrease in lipid content. Conversely, the intensity remained unchanged in M148 cells, suggesting that starvation does not induce autophagy to the same extent in M148.

When comparing the autophagic flux between I148 and M148 cells using LC3-II-turnover, a distinct difference was observed between the two genetic variants. In starved conditions, the ratio of autophagic flux (measured as the ratio of LC3-II levels in the presence and absence of Bafilomycin A1, a lysosomal inhibitor) increased by 2.5-fold in I148 cells, while it was approximately 1 in M148 cells. The process of autophagy was further investigated and differentiated by the formation of autophagosomes and autolysosomes using a tandem mRFP-GFP-LC3 plasmid. In acidic environments, such as lysosomes, the GFP tag becomes weaker. Thus, GFP·RFP·LC3 puncta represent autophagosomes and isolation membranes, while GFP·RFP·LC3 puncta characterize autolysosomes. Comparing I148 and M148 cells, it was found that I148 cells produced 50% more autolysosomes, with no difference in the creation of autophagosomes or isolation membranes between the two genetic variants. Immunofluorescence staining of cells during starved conditions revealed that PNPLA3 co-localized with LC3-II on LDs, and no differences were observed between I148 and M148.

A total of 35 liver biopsies were analyzed (M148, n=8; I148, n=27) in Paper III. When examining lipid droplets in detail, the average area of LDs was similar in M148 and I148 (240 vs. 218 µm²). However, the amount of lipid content was significantly higher in M148 (155 vs. 94 per mm²). Moreover, lipids positive for PNPLA3 and LC3-II were also significantly larger in M148 (280 vs. 217 µm²), and there were significantly more LDs positive for PNPLA3 and LC3-II in M148 (43 vs. 15 per mm²), see Figure 8. The colocalization between LC3-II and PNPLA3 was comparable between I148 and M148, consistent with the findings observed in HepG2 cells.
Figure 8. A) PNPLA3 and LC3 immunofluorescence staining in liver samples I/I (n=27) and M/M (n=8). PNPLA3 (red), LC3 (green), BF (bright field). B) scale bar = 10 µm. C) shows total number of LDs and LDs negative/positive for LC3-II and PNPLA3, bars represent mean ±SEM. D) shows LD average area of total LDs and LDs negative/positive for LC3-II and PNPLA3, bars represent mean ±SEM. * indicate statistical significance at the p<0.05 level. Abbreviations: LC3, microtubule-associated protein 1 light chain 3; PNPLA3, patatin-like phospholipase domain-containing 3; LD, lipid droplet; BF, bright field. Adapted from Journal of Cellular Biochemistry 2019. Published with permission.

The suggested role of PNPLA3 in hepatocytes is to potentially initiate autophagosome formation by interacting with LC3-II during lipophagy. However, in the context of M148, it appears to impede the elongation of the isolation membrane and hinder the formation of the autolysosome.
Consequently, lipophagy is impaired, contributing to an accumulation of stored lipids (i.e., steatosis), see Figure 9.

Figure 9. Suggested function of lipophagy in I148 and M148 hepatocytes. Abbreviations: I148, wildtype gene of PNPLA3; PNPLA3, patatin-like phospholipase domain-containing 3; LC3, microtubule-associated protein 1 light chain 3; M148, variant gene of PNLP3; LD, lipid droplet. Journal of Cellular Biochemistry 2019. Published with permission.

Proteomics (Paper IV)

A total of sixty participants were included in the discovery cohort, with thirty-six participants (60%) having fibrosis stage F0–1, and twenty-four participants having F2–4 (40%). Age, BMI, PNPLA3 genotype, and T2DM were evenly distributed between the two groups.

In the validation cohort, fifty-nine participants were included, with thirty-five participants (59.3%) having fibrosis stage F0–1, and twenty-four participants (40.7%) having F2–4. Age, BMI, T2DM, and PNPLA3 genotype were evenly distributed between the two groups.

A total of 276 proteins were analyzed using three different Olink panels, with 92 proteins per panel. Ten proteins overlapped, resulting in 266 proteins used for further analysis. All proteins were individually analyzed using an ordinal logistic regression model, and after applying Bonferroni correction (0.05/266), five proteins showed significant association with fibrosis stage in the discovery cohort (ACE2, GDF-15, HGF, IGFBP-7, and SELE). Random forest analysis and Boruta algorithm were also applied to the biomarkers to assess their importance. All five biomarkers ranked
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among the top eleven proteins according to random forest analysis and were confirmed as candidate biomarkers using the Boruta algorithm.

The same analyses were conducted on the proteomics data in the validation cohort. Ordinal logistic regression analysis revealed 26 biomarkers associated with fibrosis stage after Bonferroni correction. Among these biomarkers, four of the five previously identified in the discovery cohort showed significant association with fibrosis stage in ordinal logistic regression analysis (ACE2, GDF-15, HGF, and IGFBP-7, but not SELE). All four biomarkers ranked within the top 21 biomarkers in the random forest analysis, and their significance was further confirmed through the Boruta algorithm.

**Figure 10.** Combination of p-values from unadjusted ordinal regression in the discovery and validation cohort. The bold lines represent the Bonferroni corrected significance cut-off value (0.05/266).
Thus, four biomarkers that significantly associated with fibrosis stage were found in the two cohorts, see Figure 10. These proteins were individually tested as well as in various combinations to assess their ability to distinguish between F0–1 and F2–4. The prediction model combining ACE2, HGF, and IGFBP-7 demonstrated the best performance, with c-statistics of 0.83 and 0.82 in the discovery and validation cohorts, respectively (see Table 10).

Table 10. Biomarker models and their discriminatory function to classify F0–1 and F2–4.

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Discovery cohort (n=60)</th>
<th>Validation cohort (n=59)</th>
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<tr>
<td></td>
<td>Correctly classified</td>
<td>Correctly classified</td>
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<tr>
<td></td>
<td>C-statistic (n=36) F0-1</td>
<td>F2-4 (n=24)</td>
</tr>
<tr>
<td></td>
<td>C-statistic (n=35) F0-1</td>
<td>F2-4 (n=24)</td>
</tr>
<tr>
<td>ACE2</td>
<td>0.73</td>
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</tr>
<tr>
<td></td>
<td>(86.1%) (50.0%)</td>
<td>(85.7%) (62.5%)</td>
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<tr>
<td>GDF-15</td>
<td>0.72</td>
<td>0.77</td>
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<tr>
<td></td>
<td>(77.8%) (54.2%)</td>
<td>(80.0%) (50.0%)</td>
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<tr>
<td>HGF</td>
<td>0.78</td>
<td>0.74</td>
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<tr>
<td></td>
<td>(77.8%) (62.5%)</td>
<td>(71.4%) (58.3%)</td>
</tr>
<tr>
<td>IGFBP-7</td>
<td>0.78</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>(91.7%) (45.8%)</td>
<td>(82.9%) (58.3%)</td>
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<tr>
<td>ACE2 + HGF +</td>
<td>0.83</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>ACE2 + HGF + IGFBP-7</td>
<td>(91.7%) (66.7%)</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>(91.7%) (66.7%)</td>
<td>(77.1%) (70.8%)</td>
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</tbody>
</table>

Non-Alcoholic Fatty Liver Disease
DISCUSSION

This thesis provides insights into different parts of NAFLD diagnosis and pathophysiology. The genetic variant PNPLA3 is linked to NAFLD and more severe disease. In our experimental study, we observed the interaction of PNPLA3 with LC3-II, suggesting its potential contribution to defective lipophagy in the M148 variant. Additionally, there is a need for more advanced methods of non-invasive staging of the disease, where a proteomic biomarker profile could assist in identifying patients with a more advanced liver disease. Furthermore, we demonstrated that moderate alcohol consumption and PETH-value as low as 48 ng/mL are associated with fibrosis progression.

Multiple genes have been linked to disease severity in NAFLD, with PNPLA3 being the most significant genetic risk factor for advanced liver disease. While the precise function of this protein is not clearly established, our findings indicate that the PNPLA3 M148 variant leads to impaired autophagic flux and reduced lipophagy, resulting in the accumulation of large lipid droplets. This was seen both in the Hep G2 cell line and in the human liver biopsies.

Despite the discovery of the association between NAFLD and the PNPLA3 variant 15 years ago, genetic testing is still not routinely performed in clinical practice. This can be largely attributed to the fact that the PNPLA3 variant accounts for approximately 5% of disease variance in NAFLD and the lack of effective pharmacological treatment. Interestingly, when attempting to predict liver fat using a score consisting of T2DM, metabolic syndrome, serum insulin, AST, and AST/ALT-ratio, the addition of PNPLA3 genotype resulted in less than a 1% improvement in score accuracy.

The biomarker model discovered in our study not only distinguishes between significant and low-stage fibrosis but also holds possible biological applications. All three biomarkers (ACE2, HGF, and IGFBP-7) included in the model are connected to various cellular processes that could be linked to NAFLD pathophysiology. ACE2 convert angiotensin II into angiotensin 1–7. Angiotensin 1–7 have anti-inflammatory and anti-fibrotic effects whilst angiotensin II can stimulate HSCs proliferation. A high level of ACE2 may correspond to active fibrogenesis. HGF has been observed at elevated levels in patients with NASH and fibrosis and is linked to antioxidation and tissue regeneration. IGFBP-7 has been shown to induce steatosis and fibrosis in rodents, with elevated levels being linked to advanced fibrosis in humans with NAFLD.
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We systematically analyzed 266 biomarkers across two separate cohorts in Paper IV. Employing several statistical analyses, our primary focus was to identify the most robust biomarker candidates capable of discriminating between different fibrosis stages. Our intention was not to uncover every potentially associated protein but rather to enhance the prospects of creating and validating a model in an independent sample – our validation cohort.

We developed a three-protein model that accurately classified 78% of the participants into either the F0–1 or F2–4 fibrosis categories. Patients from both the discovery and validation cohort were deliberately divided into two groups based on their fibrosis stage. This stratification aimed not only to ensure evenly distributed groups (with 35–36 participants in stages F0–1 and 24 participants in stages F2–4 across the two cohorts) but also to hold clinical relevance. Naturally, stage 3 fibrosis is closer to cirrhosis than stage 2, and in some instances, a biopsy displaying F3 may even indicate cirrhosis due to potential sampling errors. However, patients with stage 2 fibrosis exhibit increased mortality rates and are clinically relevant to diagnose, as are patients with advanced fibrosis.

It is worth noting that as clinical practices are increasingly moving away from invasive techniques like liver biopsy and towards non-invasive methods such as VCTE and blood-based testing, the development of specific non-invasive biomarker models holds promise for aiding in the diagnosis and staging of NAFLD, possibly addressing the limitations of current approaches.

The cross-sectional study (Paper I) revealed an interesting association between moderate alcohol consumption (defined as PEth ≥50 ng/mL or alcohol consumption >66 g/week) and advanced fibrosis, serving as the basis for conducting the cohort study (Paper II). To accurately capture true fibrosis progression, we chose to define it as progression of two or more stages. If we had chosen only one stage progression, half of the participants would have progressed between baseline and follow-up. However, as Ratziu et al. have shown, there is a risk of sampling bias and overestimation when differentiating between only one stage of progression. In the cohort study, alcohol consumption was assessed thoroughly at follow-up. Unfortunately, we lack whole blood or questionnaires from baseline. However, we assumed that participants’ alcohol consumption remained relatively constant between baseline and follow-up. A subset of patients responded to an additional question on the AUDIT-C questionnaire, inquiring whether they had changed (reduced/increased) their alcohol consumption since their first study visit. The majority of patients stated that their alcohol consumption had remained unchanged, allowing us to consider consumption as a constant variable. A strength of the cohort study is the long follow-up time, namely 17.2 years, giving the different
**Discussion**

Histological features enough time to develop and progress. Interestingly, moderate alcohol consumption was only associated with fibrosis progression, not with the progression of any other histological features.

Our findings in both Paper I and Paper II are in line with other biopsy-verified studies on cohorts of NAFLD patients with moderate alcohol consumption. As previously stated, they have associated moderate alcohol consumption with more severe histology, cirrhosis, and development of HCC. Notably, the updated 2023 AASLD clinical practice guidelines for managing NAFLD patients now recommend complete abstinence from alcohol for patients with fibrosis stage 2 or higher.

In our fully adjusted model (Model 3, Paper II), we also discovered that binge drinking and PEth ≥48 ng/mL were associated with fibrosis progression or development of cirrhosis-related complications. Undoubtedly, including the analysis of a direct alcohol biomarker should be a component of the diagnostic process for patients with liver disease. It is not only instrumental as a decisive diagnostic tool but also serves as an aid in patient discussion and the prediction of clinical outcomes. Our study has compellingly demonstrated that a PEth level of ≥48 ng/mL is linked to the progression of fibrosis. Interestingly, Hagström and colleagues conducted a cross-sectional study involving 120 NAFLD patients to investigate their fibrosis stage in relation to lifetime alcohol consumption, while also measuring PEth-levels. In their study, the average weekly alcohol consumption was categorized into quartiles, with the consumption in the fourth quartile showing a wide range from 3.1 to 13.3 standard drinks. While their findings indicated that moderate alcohol consumption was associated with a lower fibrosis stage, a PEth-value ≥0.3 µmol/L (≥210 ng/mL) was linked to an increased risk of advanced fibrosis. This highlights the importance of incorporating alcohol biomarker measurement into clinical practice.

We acknowledge that the scope of our studies is constrained by a limited number of participants. We were also unable to perform gender-based analyses due to the small sample size. In all four studies, participants were recruited from a tertiary setting, which introduces the potential for selection bias towards a population with more severe liver disease.

In Paper I, we observed a significant association between moderate alcohol consumption and advanced fibrosis when compared to participants with minimal to no consumption. Nevertheless, our investigation in Paper II did not reveal any discernible disparity between light alcohol consumption and minimal to no alcohol intake (defined as 0-2.99 g/week versus 3-65.9 g/week). Consequently, it is possible that alcohol consumption up to 66 g/week might influence histological outcomes in NAFLD differently than in those with low to no consumption.
Assessing alcohol consumption is inherently challenging and carries a substantial risk of recall bias. To aid in the interpretation of the participants consumption pattern, we used three different methods of alcohol consumption in both Paper I and II. Furthermore, in the second paper, we expanded our analysis to encompass the prevalence of binge drinking and its potential correlation with fibrosis progression.

The third study (Paper III) was conducted with the purpose of exploring the specific effect of variant PNPLA3 on lipophagy, focusing on hepatocytes and humans with either the wildtype or homozygote M148 genotype. The lipophagy process in the heterozygote genotype remains unclear. More detailed analysis of autophagy, including the assessment of autophagic flux and lysosomal activity, was only performed using the HepG2 cell line.

Our proteomic study (Paper IV) is also not without its limitations. Firstly, it is based on cross-sectional data, which leaves us with uncertainty regarding the precise timeframe to which our findings are applicable. These findings might be part of either early or late stages in the fibrogenic process. Discovered biomarkers could reflect ongoing damage, potential healing processes, or a combination of both. However, investigation and quantification of biological processes in the pathophysiology of NAFLD help aid our understanding of the disease and might provide keys to future pharmacological interventions.

The ongoing debate surrounding the potential harm or benefit of moderate alcohol consumption is primarily based on previous epidemiological studies that have demonstrated a J-shaped curve of mortality in relation to alcohol intake. In other words, abstainers exhibit higher mortality rates compared to moderate consumers, while excessive alcohol consumption is associated with increased mortality, following a J-shaped pattern. Similar patterns have been observed when assessing cardiovascular disease and cardiovascular-related mortality in the general population in relation to alcohol consumption.

However, a crucial question arises: Are the individuals in the abstention group comparable to moderate consumers in terms of their health and level of physical activity? It has been previously established that abstainers, when compared to moderate drinkers (defined as up to 1 or 2 drinks per day for women and men, respectively), tend to have more cardiovascular risk factors, such as lower socioeconomic background, reduced physical activity, and higher BMI. A comprehensive meta-analysis by Stockwell et al. further explored all-cause mortality and potential biases associated with the abstainer reference group. The analysis revealed that half of the included studies had abstention groups consisting of both former and occasional drinkers. Interestingly, when pooling the studies without controlling for factors such as follow-up years, race, smoking, and inclusion/exclusion of ill subjects, they were able to replicate the
aforementioned J-shaped curve (i.e., low-moderate consumers exhibited lower all-cause mortality compared to abstainers). However, after controlling for these factors, they found a significantly increased risk of mortality among former drinkers and high-volume drinkers (45–≥65 grams/day) compared to abstainers. In the other consumption groups (<1.30 grams/day; 1.30–<25 grams/day; 25–<45 grams/day), no significant difference was found compared to abstainers, and the relative risk (RR) approached 1.0. This suggests that not only bias within the abstention group can influence the result but also that the overall methodology of the studies may have a greater impact on the findings than alcohol consumption itself.

Recently, a new definition of NAFLD has been proposed. It now falls under the broader term of SLD (Steatotic Liver Disease), wherein NAFLD is to be replaced by MASLD (Metabolic dysfunction-Associated Steatotic Liver Disease). Additionally, in cases where both MASLD and high alcohol consumption are present (ranging from 140–350 g/week for females and 210-420 g/week for males), a new term, MetALD (MASLD and increased alcohol intake), is proposed.

MASLD is characterized by the presence of steatosis on imaging or biopsy, along with at least one of five cardiometabolic criteria. The cardiometabolic criteria entail being overweight, impaired glucose tolerance or T2DM, high blood pressure, and hyperlipidemia (either elevated triglycerides, low HDL, or lipid-lowering treatment). It is worth noting that up to 99.5% of NAFLD patients can be reclassified under these new criteria.

In our studies, all participants in the cross-sectional studies (i.e., Paper I, III, and both cohorts in Paper IV) met the newly proposed MASLD criteria. In Paper II, 4 out of 82 participants did not meet the criteria at baseline; however, all four (as well as all other participants) met the criteria at follow-up. This suggests that the results from Paper I-IV can be applied to the new MASLD definition.

Twenty-five per cent of the population suffer from NAFLD and at least 1–2% are deemed “at risk” of developing severe liver-related disease. Conducting research on the pathophysiology of the disease and identifying risk factors is crucial for effective treatment, classification, and advising these patients in the future.
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CONCLUSIONS

Moderate alcohol consumption in NAFLD
Phosphatidylethanol should be measured and used in the clinical evaluation of NAFLD patients. Patients with an elevated PEth (≥48 ng/mL) or alcohol consumption of >66 grams/week should be advised to refrain from alcohol. The current European guidelines for NAFLD include a much too generous accepted amount of alcohol consumption, which should be revised to align with the evidence supporting a reduced alcohol consumption in NAFLD.

PNPLA3 gene variant in NAFLD
PNPLA3 is undisputedly involved in disease development in NAFLD. We have presented a potential mechanism involving lipophagy and its defective counterpart in the PNPLA3 variant. While its practical application in clinical practice is yet to be determined, incorporating genetic profiling for risk assessment of NAFLD severity may become part of future non-invasive assessments.

Proteomics in NAFLD
Proteomics holds significant potential for non-invasive assessment of NAFLD patients. By combining ACE2, HGF, and IGFBP-7 biomarkers, we were able to differentiate significant fibrosis in two independent study populations.
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Non-Alcoholic Fatty Liver Disease
Papers

The papers associated with this thesis have been removed for copyright reasons. For more details about these see:

https://doi.org/10.3384/9789180752923
Non-Alcoholic Fatty Liver Disease
Insights into Alcohol Consumption, Genetics, and Proteomics

Julia Blomdahl