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Modifiers of liver-related manifestation in the course of NAFLD

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List of abbreviations: AAT, alpha-1 antitrypsin; AATD, AAT deficiency; ARLD, alcohol related liver disease; AUDIT, alcohol use disorder identification test; BMI, body mass index; CDT, carbohydrate deficient transferrin; CI, confidence interval; CK, cytokeratin; CVD, cardiovascular disease; FLIP, fatty liver inhibition of progression; GWAS, genome-wide association studies; HC, hepatocellular; HCC, hepatocellular carcinoma; HFE-gene, human homeostatic iron regulator gene; HSD17B13, 17 β -hydroxysteroid dehydrogenase 13; aHR, adjusted hazard ratio; MARC1, mitochondrial amidoxime-reducing component 1; MBOAT7, membrane bound O-acyltransferase domain-containing 7; MRI-PDFF, magnetic resonance imaging – proton density fat fraction; MRS, magnetic resonance spectroscopy; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; aOR, adjusted hazard ratio, PEth, phosphatidylethanol; Pi, proteinase inhibitor; PNPLA3, patatin-like phospholipase domain-containing 3; RES, reticuloendothelial system; SAF, steatosis, activity, fibrosis; SERPINA1, serine proteinase inhibitor 1; SNP, single-nucleotide polymorphism; T2DM, type 2 diabetes mellitus; TM6SF2,

transmembrane 6 superfamily 2; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; ULN, upper limit of normal.

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1. Introduction

Hepatic steatosis was once considered an innocent bystander of minimal importance for clinicians and patients. Today, the progressive potential of non-alcoholic fatty liver disease (NAFLD) is indisputable, and NAFLD is rising as a major indication for liver transplant.[1, 2] The incidence of NAFLD mirrors the global epidemic of obesity worldwide.[3] The global prevalence of NAFLD is estimated to 25%, with the highest prevalence in the Middle East and South America, and the lowest prevalence in Africa.[4]

NAFLD entails a spectrum of histological features that ranges from non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH) with or without fibrosis.[5, 6] There is a strong association between the severity of NAFLD and the components of the metabolic syndrome.[7-9] NAFLD is also independently associated with cardiovascular disease and type 2 diabetes mellitus (T2DM).[10-13]

With a prevalence ranging between 20-33% in most countries, NAFLD will become a significant health care issue for patients and health care systems.[4, 14] Luckily, only a minority of NAFLD-patients will progress to cirrhosis with development of decompensation and liver related death.[15] NAFLD is a dynamic disease state with considerable fluctuation (*i.e.* progression and regression) of inflammation and fibrosis, often described as a *seesaw* effect.[16-18] Particularly, the inflammatory grade, *i.e.* lobular inflammation and ballooning, is highly dynamic, partly attributed to lifestyle factors that are difficult to completely account and control for in clinical trials. These include weight change, dietary composition and alcohol consumption. It is also important to remember that lobular inflammation and ballooning have high inter- and intraobserver variability with significant sampling variability.[19-26] NASH, *i.e.* the presence of steatosis, lobular inflammation and ballooning,[27, 28] is regarded as the

progressive disease state, as NASH-patients have higher fibrosis stage compared to NAFL-patients and higher all-cause and liver-related mortality.[29, 30]

Fibrosis stage is, not surprisingly, a strong predictor of outcome in patients with NAFLD.[31-33] Therefore, patients with high risk of fibrosis progression are the ones that should be targeted with lifestyle and pharmacological interventions.[34-36] This review will focus on factors that has been shown to affect fibrosis progression and the development of liver cirrhosis, decompensation and liver-related mortality in NAFLD-patients.

2. Metabolic Syndrome

2.1 Type 2 Diabetes Mellitus and Insulin Resistance

Since 1980 the age-standardized prevalence of T2DM in adults has doubled in men (from 4.3% to 9.0%) and increased in women (from 5.0% to 7.9%).[37] NAFLD is highly intertwined with T2DM, showing a bidirectional interaction.[9, 30, 38-40] The prevalence of T2DM in NAFLD-patients ranges from 45% to 75% in hospital based studies and from 30% to 60% in population based studies.[41] Furthermore, the overall prevalence of NAFLD in individuals with T2DM is estimated at 55%.[42] Nonetheless, whether NAFLD precedes or succeeds T2DM is still unclear.[43, 44]

In a systematic review and meta-analysis by Bellestri *et al*, patients with NAFLD had a twofold increase in the risk of incident T2DM.[45] Similarly, Chen *et al* showed that NAFLD patients (diagnosed with ultrasonography) had more than a twofold increase for T2DM (aHR 2.08, 95%CI 1.93-2.33 for men and aHR 2.65, 2.43-2.88 for women), in a study with 132,377 adults, followed over a period of 6 years.[46]

There are few papers studying the relationship between patients with biopsy proven NAFLD and the risk of developing T2DM. In a seminal paper by Ekstedt *et al*, 129 well defined biopsy-

proven NAFLD patients were included and followed prospectively and longitudinally.[47] At inclusion 11 out of 129 patients (8.5%) had T2DM. After a mean follow-up time of 13.7 years, 69 out of 129 patients (53%) had T2DM or impaired glucose tolerance. In an extended follow-up of the same cohort, 71 out of 129 had T2DM or impaired glucose tolerance (55%) after a mean follow-up of 19.8 years.[48] Similarly, McPherson *et al*, showed an increase in T2DM in 108 patients with biopsy-proven NAFLD.[49] At baseline 48% had T2DM, which increased to 65% after a median follow-up of 6.6 years.

To date there are 14 dual biopsy studies in patients with NAFLD, including 740 individuals with an overall T2DM prevalence of approximately 43% (Table 1).[47, 49-61] In these studies, none show that T2DM predicts fibrosis progression, however, Adams *et al*, showed that T2DM was a predictor of fibrosis progression rate. Nevertheless, patients with NAFLD and concomitant T2DM portend an increased risk of mortality[33, 62] and an increased risk of liver related morbidity.[62] Also, patients with T2DM seem to have increased mortality in the presence of concomitant NAFLD.[63]

In long-term follow-up studies of patients with biopsy-proven NAFLD the majority of studies have not shown T2DM to be a significant risk factor for liver-related outcomes. However, in a retrospective study with 148 patients undergoing transjugular liver biopsy, diabetes was more prevalent in patients with liver-related clinical outcomes (including all-cause mortality) compared to patients without diagnosis of T2DM (62.5% vs. 27.4%).[64] Moreover, in a recent study by Vilar-Gomez *et al*, T2DM was proven to be a robust negative predictor of transplantation free survival (HR 3.33, 95%CI 1.69–6.54) and liver-related outcome (sHR 2.82, 95%CI 1.54–5.15 for decompensation and sHR 4.72, 95%CI 2.13–10.45 for hepatocellular carcinoma [HCC]).[65]

An estimated 8.4% of all deaths are attributed to T2DM with an approximate reduction in lifetime of about 6 years compared to non-T2DM individuals.[66, 67] T2DM, and especially insulin-dependent T2DM, increases death of all causes.[68-70] In a study by the Emerging Risk Factor Collaboration group, the adjusted hazard ratio among patients with T2DM compared with persons without diabetes was 1.80 (95%CI 1.71-1.90) for death from any cause and 1.25 (95%CI 1.19-1.31) for death from cancer - with liver cancer (*e.g.* HCC) having the highest risk (aHR 2.16, 95%CI 1.62-2.88).[67] Moreover, in T2DM-patients that died from other causes other than cancer and nonvascular causes, the risk of death secondary to liver disease was 2.28 (95%CI 1.90-2.74). These data were corroborated in a recent study by Campbell *et al*, were T2DM-patients had more than a twofold relative risk increase in dying of liver related causes.[71]

The relationship between T2DM and HCC is well established.[72-74] In an important article from the United Kingdom, Dyson *et al* showed an increase in HCC mortality from the year 2000 to 2010 (1.8-fold increase, rising from 2.0 to 3.7 per 100,000), mainly attributing to NAFLD – now the most common chronic liver disease associated with HCC (35% of all cases).[75] In 2004, El-Serag *et al*, showed that patients with T2DM, without viral hepatitis infection or alcohol overconsumption, had a twofold increase of developing HCC compared to patients without diabetes (aHR 2.13, 95%CI 1.99-2.28).[76] Moreover, T2DM seems to be an independent risk factor for developing HCC, mainly attributed to NAFLD.[76, 77] Similarly, a more than twofold increase in the risk of developing HCC amongst patients with T2DM have been observed in two meta-analyses.[78, 79]

Before the diagnosis of NAFLD was broadly accepted, early case-control studies showed cryptogenic cirrhosis to be related with the development of HCC.[80-82] Similarly, NAFLD is related to HCC. In most studies, there is an approximately twofold risk of developing HCC in patients with T2DM or NAFLD. However, because of the strong association between NAFLD

and T2DM, it is hard to know if the increased risk of HCC is secondary to T2DM or its hepatic manifestation (*i.e.* NAFLD). Therefore, more studies are needed, depicting whether NAFLD patients with T2DM have an increased risk of HCC compared to NAFLD patients without T2DM.

2.2 Overweight, Obesity and Weight Change

Individuals with a body mass index (BMI) ≥ 30 kg/m² (*i.e.* obesity) have increased sixfold since 1980, affecting over 600 million individuals in 2016, with an additional 1.3 billion overweight (BMI 25.0-29.9 kg/m²) individuals.[3] Overweight has previously been seen as a culprit in all-cause mortality, especially in death from cardiovascular disease and malignancy. However, there is an ongoing debate on the relationship between overweight and mortality.[83] Nonetheless, there is a clear consensus on obesity and increased all-cause mortality.[84-86]

The prevalence of NAFLD is highly related to body weight, with increasing prevalence in overweight and obese individuals. However, it is important to acknowledge that NAFLD is not uncommon in lean individuals.[87] In a recent study by Lazo *et al*, the prevalence of NAFLD increased exponentially in individuals with higher BMI; with a prevalence of 57% in men and 44% in women with a BMI >35 kg/m². [88] However, this estimated prevalence is probably an underestimation, since all patients were diagnosed with ultrasonography – a method with low sensitivity in patients with low grade steatosis. The gold standard for diagnosing NAFLD is magnetic resonance spectroscopy (MRS), [89, 90] where the commonly used cut-off of 5% or 5.56% is applied. [91, 92] Using the cut-off of 5.56%, the prevalence of NAFLD among 2,287 individuals included in the Dallas Heart Study was 31%. [93] However, the cut-off of 5% is questioned [94-96] with some studies recommending a lower cut-off of 3%. [97, 98]

There exists a high correlation between overweight/obesity and NAFLD. However, the causal relationship between the two is not clear.[99, 100] In the Coronary Artery Risk Development in Young Adults study, future development of NAFLD was related to weight gain during young adulthood.[101] Furthermore, weight loss, either by lifestyle intervention or bariatric surgery, seem to resolve NAFLD (and insulin resistance).[102-106] Moreover, in a recent study by Vilar-Gomez *et al*, 239 biopsy-proven NAFLD patients underwent lifestyle changes to reduce their body weight.[107] Patients were followed for 52 weeks, after which a repeat liver biopsy was performed. Weight loss >5% showed a significant reduction in steatosis, inflammation, ballooning and fibrosis. The resolution of these histological parameters increased in patients with higher percentage weight reduction.

Obesity and visceral adiposity seems to predict the development of severe liver disease in the general population.[108-110] In a prospective study by Calle *et al*, an increased risk of mortality from cancer showed a linear association with increasing BMI, in both men and women.[111] Moreover, they showed an exponential increase in the risk of liver cancer in male subjects for every 5 unit increase in BMI. Similar findings were reported by Hagström *et al*, where 1.2 million men enlisted for military conscription in Sweden, were followed for a mean period of 28.5 years.[112] At the end of follow-up, 5281 cases of severe liver disease and 251 cases of HCC were identified. Individuals who were overweight and obese had an increased hazard ratio for HCC of 1.68 (95%CI 1.09-2.57) and 4.28 (95%CI 2.25-8.15), respectively.

3. Alcohol

In the Western world, alcohol overconsumption is the leading cause of advanced decompensated liver disease.[113] Thus, a potentially important factor for the course of NAFLD is the impact of the quantity, pattern, and duration of alcohol consumption. Weekly alcohol consumption in excess of 210 g for men and 140 g for women exclude subjects from NAFLD research studies.[114] However, these arbitrary thresholds are based on levels above

which the risk of cirrhosis is higher and has not been specifically shown to influence NAFLD.[115] On the other hand, the most common cause of mortality and morbidity in NAFLD patients is cardiovascular disease (CVD)[47, 116] and NAFLD and CVD share many common risk factors. There is evidence for beneficial effects of modest alcohol consumption on risk of metabolic syndrome and insulin resistance,[117] which are important components of the NAFLD disease process.

An important confounder when investigating the role of alcohol in the progression or improvement of NAFLD is the assessment of alcohol consumption. The recommended tool for excluding excessive alcohol consumption when diagnosing NAFLD is the Alcohol Use Disorders Identification Test (AUDIT), in which specific questions explore consumption, dependence, and alcohol related problems.[118, 119] However, people consuming alcohol may be prone to inaccurately report that they do not have a problem, particularly when meeting physicians evaluating their liver. This creates a need for more objective methods to investigate a person's drinking habits. Serum levels of the specific alcohol marker carbohydrate deficient transferrin (CDT) can be used when heavy drinkers are investigated but for social drinkers and risk drinkers CDT lacks adequate sensitivity.[120] Analysis of phosphatidylethanol (PEth) has emerged as a more sensitive and specific method[121] but has hitherto been used only in few NAFLD studies thus making it hard to assess its utility in the NAFLD setting.

Studies on effects of alcohol in NAFLD have evaluated four different aspects: 1) effects of alcohol on prevalence or incidence of NAFLD, 2) effects of alcohol on the severity of established NAFLD, 3) association of alcohol consumption with hepatocellular carcinoma in NAFLD, and 4) association of alcohol consumption with mortality in NAFLD patients.

A recent meta-analysis of mostly cross-sectional studies concluded that moderate alcohol consumption was associated with a 23% reduction in the prevalence of fatty liver disease.[122] In a prospective Japanese study of subjects without liver disease at baseline drinking alcohol

was associated with decreased incidence of fatty liver diagnosed by ultrasonography.[123] Moreover, moderate alcohol consumption did not induce hepatic steatosis in healthy individuals when hepatic triglyceride content was measured prospectively with proton magnetic resonance spectroscopy in a randomized study.[124]

The largest study assessing the second aspect was recently reported by Chang *et al.*[125] They studied the effect of moderate alcohol consumption on non-invasive liver fibrosis indices in 58,927 Korean adults with NAFLD and low fibrosis scores who were followed for a median of 8.3 years. They concluded that moderate alcohol consumption was significantly and independently associated with worsening of non-invasive markers of fibrosis. The rationale for the study is relevant, since fibrosis stage is the best predictor of future liver-related morbidity and overall mortality in NAFLD.[33, 126] Thus, their study may indicate that modest alcohol consumption is harmful in subjects with NAFLD. However, a major weakness of using non-invasive fibrosis markers is that, although they are excellent in ruling out significant fibrosis, their ability to confirm advanced fibrosis is limited when liver biopsy is used as the reference method. Thus, worsening of fibrosis indices does not necessarily imply that liver fibrosis has progressed during follow-up.

Liver biopsy is still considered the gold standard for assessing the severity of NAFLD. In a cross-sectional study of adult patients with biopsy-proven NAFLD, after exclusion of heavy and binge drinkers, modest alcohol consumption was associated with 34% less hepatocellular ballooning and 44% lower risk of liver fibrosis compared with nondrinkers.[127] Similar results were shown in a Swedish study of 120 NAFLD patients with biopsy-proven NAFLD in which a maximum of 13 drinks per week was associated with lower fibrosis stage.[128] However, increased levels of PEth in blood was associated with higher stages of fibrosis. This may indicate that more pronounced alcohol consumption, contrary to modest consumption, is harmful in NAFLD or that assessment of alcohol consumption through questionnaires is prone

to error. In another histopathological study from Sweden,[129] 71 NAFLD patients were followed for an average of almost 14 years and it was shown that heavy episodic drinking was associated with increased risk of progression of fibrosis. Further evidence for a potentially harmful effect of moderate alcohol consumption on the progression of NAFLD comes from a recently published longitudinal study,[130] in which it was concluded that NAFLD patients with moderate alcohol consumption were less likely to experience spontaneous improvement in liver histology.

Currently, twelve studies have assessed the impact of alcohol on histopathology in NAFLD (Table 2). Robust conclusions cannot be drawn since study design varies and particularly since the definition of moderate alcohol consumption is not consistent. However, type of alcohol and pattern of consumption seem to affect the histopathological course of NAFLD. Generally, consumption of moderate amounts of alcohol (< 70 g/week) is associated with a lower rate of NASH and fibrosis, especially if wine is consumed in a non-binge pattern. However, this is not a consistent finding. In some studies, moderate alcohol consumption is associated with a more advanced histopathological stage. Binge drinking (occasional consumption of > 60 g ethanol in males and > 48 g in females) may be harmful since it is associated with higher fibrosis stages.

There is increasing evidence to suggest an additive, or even a synergistic, effect between alcohol consumption and BMI for the development of HCC.[131] In a recent Japanese study of 301 patients with biopsy-proven NAFLD, patients with modest drinking had a significantly higher risk of developing HCC compared with nondrinkers.[132]

Results regarding the effect of alcohol consumption on survival in NAFLD patients have been conflicting.[123, 127] Recently, 4,568 subjects with NAFLD from the National Health and Nutrition Examination Survey were evaluated. Consumption of 7 g to 21 g alcohol per day decreased the risk of overall mortality by 41% compared with not drinking.[133] Since NAFLD patients are more likely to die from CVD than liver disease these results are in accordance with

previous studies showing that modest alcohol consumption is associated with decreased risk of cardiovascular disease mortality.[134] However, a major weakness of the aforementioned study[133] is that the diagnosis of NAFLD was based on a biochemical model and not on imaging or histology.

In summary, most studies indicate that modest alcohol consumption is associated with decreased risk for development of fatty liver disease and moderate drinking may be associated with increased survival in NAFLD patients. Emerging evidence indicates an additive risk of BMI and alcohol for the development of HCC in NAFLD. There are conflicting results regarding the role of alcohol for fibrosis progression in established NAFLD. Further studies are needed before well founded advice can be given to NAFLD patients regarding modest alcohol consumption.

4. Genetics

4. 1 Genome-wide Association Studies

The large differences in NAFLD prevalence between regions and ethnicity are multifactorial, but genetic factors are clearly one explanation for the variation observed. Genome-wide association studies (GWAS) have identified several gene loci associated with NAFLD. The non-synonymous chromosome 22 single-nucleotide polymorphism (SNP) in the patatin-like phospholipase domain-containing 3 (PNPLA3, rs738409 c.444 C>G, p.Ile148Met) and the non-synonymous chromosome 19 SNP in the transmembrane 6 superfamily 2 (TM6SF2) has repeatedly been associated with hepatic steatosis as well as inflammation and fibrosis.[135-138] The development of NAFLD-related HCC has been associated with the PNPLA3 genotype.[139-141] Interestingly, recently gene loci such as the mitochondrial amidoxime-

reducing component 1 (MARC1) and the 17 β -hydroxysteroid dehydrogenase 13 (HSD17B13) has shown to be protective against fatty liver and fibrosis.[142-144]

The gene locus rs641738 at the membrane bound O-acyltransferase domain-containing 7 (MBOAT7) has been associated with NAFLD,[145] but this association has recently been disputed.[146] There is a number of additional genes that is associated with NAFLD such as the LYPLAL1, GCKR and PP1R3B.[147, 148]

By studying the functional role of each gene associated with NAFLD has given many interesting openings to study the pathogenesis of the disease. Although, the era of personalized medicine is yet to start. Genetic testing for at least PNPLA3 and TM6SF2 will be important in future clinical trials.

4.2 Hemochromatosis and Iron dysregulation

Many patients with NAFLD have manifest iron dysregulation with 58% having hyperferritinemia,[149] >34% having stainable hepatic iron[150, 151] and several having mutations in the HFE gene.[151, 152]

The relationship between iron and NAFLD was first described by George *et al*, who showed that hepatic iron (Perl's stain or hepatic iron concentration) had the strongest association with fibrosis stage in 51 patients with NASH.[153] In a seminal article by Bugianesi *et al*, 167 patients with biopsy-proven NAFLD were evaluated.[154] Higher level of ferritin was associated with an increased risk of present higher fibrosis stage.

The relationship of serum ferritin with severity of NAFLD has been examined in several studies.[154-157] In a study by the NASH Clinical Research Network (CRN), 628 patients with biopsy proven NAFLD were included.[157] Patients with ferritin higher than 1.5 times and 2.5 times upper limit of normal had a 1.67 and 2.46-fold increased risk of advanced fibrosis.

Moreover, Hagström *et al* showed that biopsy-proven NAFLD patients with higher levels of ferritin had a long-term increased risk of death.[158] Although the association between ferritin and advanced fibrosis has been corroborated by several study groups[156, 157, 159], the use of ferritin for predicting presence of advanced fibrosis in NAFLD is low (ferritin >1.5 x ULN: AUROC 0.56, 95%CI 0.52-0.60) with a sensitivity and specificity of 27% and 84%, respectively.[159]

Early case studies of iron depletion through phlebotomy showed decreased insulin resistance,[160] improvement of steatosis grade,[161] and liver enzymes.[161, 162] However, in a phase 2 clinical trial[163] and a randomized controlled trial[164], phlebotomy had no effect on liver enzymes, hepatic fat (measured with MRI), insulin resistance or histological features of NAFLD. It is notable that the endpoint in these two studies was not fibrosis progression, decompensation or liver-related mortality.

In a study by Nelson *et al*, 849 biopsy-proven NAFLD patients were enrolled.[150] Approximately one third (34.5%) had hepatic iron deposits, divided into a hepatocellular (HC) pattern (7.4%), a reticuloendothelial system (RES) cell pattern (10.7%) or mixed pattern (16.4%). The pathogenic effect of iron deposit depended on the cellular location in the liver, where patients with RES iron-staining pattern were more likely to have features of any stage of fibrosis and advanced fibrosis, portal inflammation and ballooning compared to patients with HC iron-staining pattern. Furthermore, patients with RES iron deposits had an increased level of TUNEL positive cells (a marker of apoptosis) in the liver and increased levels of malondialdehyde (a marker of oxidative stress) as well as CK-18 (a marker of apoptosis) in serum. [165] Also, in an Italian study by Valenti *et al*, 587 biopsy-proven NAFLD patients were enrolled to investigate the effects of (serological and histological) iron and genetic hemochromatosis in NAFLD.[166] They reported that hepatocellular iron accumulation was associated with a higher risk of fibrosis stage >1 (aOR 1.7, 95%CI 1.2-2.3) compared to patients

without siderosis. Although, there was no significant association between presence of genetic hemochromatosis (or specific HFE-genotypes) and the severity of fibrosis, one third of patients with HFE-mutations had hepatocellular iron deposits.

Elevated ferritin is commonly seen in patients with NAFLD and could indicate more advanced disease. But, significant elevation of hepatic iron content in individuals without genetic predisposition is uncommon.[167] Phlebotomy of NAFLD patients with elevated ferritin is probably unnecessary in a clinical setting. Nevertheless, patients with increased stainable iron in liver biopsies could still benefit from iron depletion regarding fibrosis progression, which warrants further investigation.

4.3 Alpha-1 Antitrypsin Deficiency

Alpha-1 antitrypsin (AAT) is a serum protein produced predominantly in liver hepatocytes.[168] It is coded by the serine proteinase inhibitor, SERPINA1 gene (previously known as the protein inhibitor, or Pi locus), and variants of AAT mutations typically lead to misfolding of AAT in the endoplasmic reticulum and decreased serum AAT concentrations, resulting in AAT deficiency (AATD).[168] Typically, severe forms of AATD results in low levels of AAT (~15% of normal) and is a “common rare disease”, being the third most common genetic disorder leading to death globally.[168-170] There are more than 150 SERPINA1 alleles described, with the normal allele referred to as “M”. However, the most frequent and well investigated diseases associated with SERPINA1 mutations are the “Z” and “S” alleles with the lung and the liver being the most commonly affected organs.[168]

AATD is most prevalent in Scandinavia and North America, and in a meta-analysis by Serres *et al*, the global prevalence for heterozygotic SERPINA1 mutations (PiMS and PiMZ) is 3.4% and for that of homozygotic mutations (PiZZ, PiZS and PiSS) is 0.8%.[171] The two largest population-based studies performed, investigating the prevalence of Z and S allele, were in

newborn infants in Sweden and Oregon, USA, with a prevalence of 1:1639 and 1:5097, respectively.[172, 173]

While the presence of PiZZ genotype portend a high risk of future liver disease, the role of PiMZ in liver disease remains controversial.[174-177] In a study by Regev *et al*, 651 patients with known chronic liver disease, of whom 26% had NAFLD, were tested for AAT phenotypes.[178] Although they did not find any association between the heterozygous PiZ state of AATD and the presence of chronic liver disease, the presence of PiMZ was more common in NAFLD patients with decompensated liver disease. Similarly, approximately 20-30% of NAFLD patients awaiting liver transplant have the PiMZ phenotype.[176, 179]

In an important multi-center study by Strnad *et al*, 1148 patients with biopsy proven NAFLD and 2462 with biopsy proven alcohol related liver disease (ARLD) were enrolled, with both cohorts comprising cases with cirrhosis and controls.[180] In patient with NAFLD, 13.8% of patients with cirrhosis (9/68) had PiZ variant present, compared to 2.4% of those without any stage of fibrosis (9/362). The PiZ variant increased the risk of developing cirrhosis in patients with NAFLD (aOR 7.3, 95%CI 2.2-24.8). Similarly, patients with cirrhosis secondary to ARLD had an increased prevalence of PiZ compared to controls with ARLD and no fibrosis (6.2% vs. 2.2%) and an increased risk of developing cirrhosis if carrying the PiZ variant (aOR 5.8, 95%CI 2.9-11.7).

5. Histology

5.1 Liver fibrosis

Advanced fibrosis stage is the strongest independent predictor of all-cause mortality, liver-related mortality and decompensation in NAFLD patients. In two recent systematic reviews and meta-analyses by Singh *et al* and Dulai *et al*, increased mortality was observed for every fibrosis stage.[31, 181] Singh *et al* showed that 33.6% had fibrosis progression.[181] The overall annual

fibrosis progression was found to be 0.07 stages for NAFL and 0.14 stages for NASH, corresponding to one stage of fibrosis progression over a median of 14.3 years and 7.1 years for NAFL and NASH, respectively.

There are 14 studies with paired biopsies in NAFLD-patients, including in total 740 patients with a median follow-up time between biopsies ranging from 2 to 13.8 years (Table 1).[47, 49-61] In 10 of the studies, including 416 patients, fibrosis stage at baseline and follow-up are present.[47, 49, 50, 52-54, 56-59] Equal to the meta-analysis by Singh et al[181], 37% show fibrosis progression (153/416) and 12% show progression from low stage fibrosis (F0-F2) to advanced fibrosis (F3-F4) (Table 1). However, with an alternating definition of NASH over time, comparisons are difficult to make in between studies. Nonetheless, in the present serial biopsy studies, few parameters predict fibrosis progression. Interestingly, presence of NASH or NAS at baseline does not correlate with fibrosis progression in these studies.

In an interesting article by Sanyal *et al*, 475 NAFLD-patients from the Simtuzumab trials, with NASH and bridging fibrosis or compensated cirrhosis, were followed for 96 weeks with some undergoing repeat liver biopsy.[61] Albeit fibrosis stage (according to Ishak) did not predict fibrosis progression from bridging to cirrhosis or from cirrhosis to liver-related clinical events, serum fibrosis markers and hepatic collagen content (per 5% increase) did. The importance of histological fibrosis[31-33, 182] and biochemical fibrosis markers[183, 184] in predicting disease progression is repeatedly underlined. However, not all patients with fibrosis stage 3 progress to cirrhosis or end-stage liver disease. Given the significant variability and sampling error in utilizing liver biopsy, it is difficult to know if the relationship between fibrosis stage/hepatic collagen content/fibrosis biomarkers with fibrosis progression is a true association or merely a misclassification of baseline fibrosis stage.

5.2 Non-Alcoholic Steatohepatitis

Patients with NASH have increased mortality as shown in a previous meta-analysis by Musso *et al* where subjects with NASH had an almost two-fold increase in overall mortality and six-fold increase in liver-related mortality.[29] There is a strong association between NASH and fibrosis making it hard to differentiate between the effect of NASH, per se, on prognosis.

In the landmark paper by Brunt *et al* they characterized the histopathological hallmarks of NASH. It unified the lesions of steatosis and inflammation into a grade (0-3; ranging from none to mild, moderate and severe) and those of fibrosis, into a stage (ranging from 0-4).[185] Although this scoring system was appealing, it was developed for NASH and did not encompass the entire spectrum of NAFLD. Therefore, a multicenter cooperative named NASH Clinical Research Network (NASH-CRN) was formed.[186] The NASH-CRN developed a scoring protocol to include the entire spectrum of NAFLD.[27] The developed scoring system was coined NAFLD Activity Score (NAS). In NAS the unweighted sum of grades of steatosis, lobular inflammation, and hepatocellular ballooning presented the severity of NAFLD. Absence of NASH was defined as $NAS \leq 2$, “borderline NASH” as a NAS of 3 or 4 and definite NASH as $NAS \geq 5$. However, in that study, the authors clearly stated that NAS is not intended to replace the pathologist’s diagnostic determination of NASH. In 2011, Younossi *et al*, studied the kappa (κ) agreement between Kleiner and Brunt’s classification of NASH, which yielded a slight agreement ($\kappa=0.178$).[187] Nonetheless, NAS is recommended to be used to define, quantify, and show progressions or regression of disease in clinical trials.[188]

In 2014, a new score named SAF (Steatosis, Activity, Fibrosis) was developed with a very good interobserver agreement for NASH ($\kappa=0.80$).[28] In SAF score, steatosis and fibrosis are defined similarly to that of NAS, but with disease activity defined as the sum of ballooning and inflammation. The SAF score classifies NAFLD-patients as having mild (activity <2 and fibrosis <2) or significant (activity >2 or fibrosis >2) disease severity. In the same study, the Fatty Liver Inhibition of Progression (FLIP) algorithm was presented. With the FLIP algorithm,

all NAFLD-patients with ≥ 1 point in steatosis, ballooning and lobular inflammation each, are defined as having NASH. The FLIP algorithm differs from NAS so as when a NAFLD-patient with steatosis grade 3 and lobular inflammation grade 2 (NAS=5) is evaluated according to NAS – the definition of definite NASH is fulfilled. However, when evaluated according to the FLIP-algorithm, the same patient would be defined as “not-NASH”, though the FLIP-algorithm depends on inflammation and ballooning (except the main trait of steatosis) for the definition of NASH.

Recently, two articles, by Ekstedt *et al* and Angulo *et al*, showed that liver fibrosis, and no other histological features predicted disease specific and all-cause mortality in NAFLD-patients.[33, 126] The impact of NAS, did not have any effect on disease specific or all-cause mortality when adjusting for fibrosis. However, Ekstedt *et al*, showed that patients with NAS 5-8 and fibrosis stage 0-2 had a risk of developing HCC (HR 15.7, 95%CI 4.1-59.9), but no increased risk of overall mortality (HR 1.41, 95%CI 0.97-2.06).[126] SAF was recently evaluated in a Scandinavian study including 139 biopsy proven NAFLD-patients, with a follow-up of 25.3 years. After adjusting for fibrosis, SAF score did not predict all-cause mortality.[189]

The mechanisms driving fibrosis progression in NAFLD are multifactorial[190] with inflammation being the catalyst driving activation of stellate cells and matrix turnover and deposition.[191] The high inflammatory disease state in NAFLD is commonly defined as NASH according to NAS, SAF or FLIP. The idea that inflammation surpasses fibrosis in NAFLD is not contested. However, the notion that the presence of NASH correlates with presence of fibrosis does not mean that NASH equals the prediction of fibrosis progression. To date, there is no objective evidence that the presence of NASH at baseline, by any of the mentioned definitions, correlate with progression of fibrosis. And therefore, resolution of NASH is not likely to be synonymous with regression of fibrosis. Hence, caution should be

taken when NASH is used as a surrogate for disease progression in observational and pharmacological trials.

5.3 Ballooning Degeneration

Ballooning degeneration is a form of hepatocyte apoptosis, histologically resulting in hepatocyte swelling, nuclei shrinkage and fragmentation. In a meta-analysis by Argo *et al*, lobular, portal or necroinflammatory (*i.e.* ballooning degeneration) inflammation predicted development of advanced fibrosis.[193] Moreover, Singh *et al* showed a more rapid fibrosis progression rate in patients with NASH compared to NAFL.[181] Also, Angulo *et al*, showed that NASH, defined by NAS, did not associate with long term outcomes in NAFLD when adjusting for fibrosis. However, patients with portal inflammation and ballooning degeneration showed an increased risk of end-stage liver disease.[33] Furthermore, a non-significant trend of ballooning degeneration as a predictor of fibrosis progression was seen in the study by McPherson *et al* ($p=0.08$).[49] This was later corroborated by Sanyal *et al* in the Simtuzumab trials study, where baseline levels of severe ballooning degeneration (grade 2 vs. 0) were associated with disease progression (HR 4.83, 95%CI 1.45-16.07).[61]

5.4 Steatosis Grade

In the presence of hepatic steatosis (grade ≥ 1) and absence of lobular inflammation, ballooning and fibrosis, the term isolated steatosis is used. However, when isolated steatosis is accompanied by inflammation, the term non-alcoholic fatty liver (NAFL) is used.

Isolated steatosis is considered a benign condition and therefore few studies have focused on the natural history of this entity. In a serial biopsy study by Teli *et al* from 1995, 12 patients with isolated steatosis were included and followed for a median of 10.3 years.[52] Only one patient progressed, and did so from F0 to F1. Moreover, in a British serial biopsy study by

McPherson et al, 17 were diagnosed with isolated steatosis of whom 4 had fibrosis progression.[49]

Further, recent studies have demonstrated fibrosis progression in a significant proportion of NAFL-patients.[49, 59, 60] In a prospective study by Wong *et al*, 29 patients with repeat liver biopsies were diagnosed with NAFL.[59] After a follow-up of 3 years, 28% showed fibrosis progression. Moreover, Pais et al presented fibrosis progression in 6 out of 25 patients (24%).[60] And in the study by McPherson *et al*, 10 out of 27 patients (37%) showed fibrosis progression.[49]

In an abstract by McPherson, 321 NAFLD-patients with serial biopsies were followed for 4.1 years.[194] Out of the 321 patients, 35% showed evidence of fibrosis progression, with no difference between NASH and NAFL. However, steatosis grade 2-3 was associated with fibrosis progression ($p < 0.001$). Similarly, in a study by Ajmera et al, 95 patients with paired biopsies also underwent MRI-proton density fat fraction (PDFF).[194] Among the 38 patients without fibrosis (i.e. stage 0) at baseline, patients with higher liver fat (defined as MRI-PDFF $\geq 15.7\%$) had a higher rate, albeit nonsignificant, of fibrosis progression (38.1% vs 11.8%, $p = 0.067$). Moreover, after adjusting for age, sex, ethnicity and BMI, patients with higher liver fat at baseline had an increased risk of fibrosis progression (aOR 6.67, 95%CI 1.01-44.1). It is interesting that the amount of steatosis is associated with a progressive disease state in NAFLD. The majority of lipids in steatosis is triglycerides. The accumulation of triglycerides within the hepatocytes is considered protective with respect to cell toxicity.[195] So the association between steatosis grade and progressive disease is most likely driven by other lipid classes, such as free fatty acids (*e.g.* palmitic acid), cholesterol, lysophosphatidylcholine, and ceramides.[196]

6. Conclusion

Disease progression in NAFLD (*i.e.* worsening of fibrosis stage, decompensation, liver-related and all-cause mortality) is highly related to traits of the metabolic syndrome, in particular T2DM/insulin resistance and overweight/obesity. Given the rising incidence of obesity and T2DM globally, we have only seen the beginning of the NAFLD epidemic. From the available evidence, 5-10% of NAFLD-patients will develop cirrhosis and cirrhosis related complications.

Screening for NAFLD with liver enzymes and/or ultrasound in subjects with obesity or the metabolic syndrome is recommended in the EASL-EASD-EASO clinical practice guidelines for NAFLD.[114] This recommendation is a challenge for the health care system given the high prevalence of obesity and T2DM in the general population. A recent survey of the public health response to NAFLD in 29 European countries found a general lack of awareness and national policies.[197] On the other hand, a community-based screening and risk stratification pathway could be cost-effective.[198]

The most important, often underestimated, confounder in NAFLD research is unrecognized or underreported high alcohol consumption. Most NAFLD studies rely on patient self-estimation of alcohol consumption using AUDIT(-C). Although available, very few studies have used objective direct alcohol markers to validate self-estimated consumption. There is increasing evidence that there is an additive effect of metabolic risk factors and alcohol consumption in progressive NAFLD. It would be very interesting to see a retrospective analysis of objective direct alcohol markers' effect on treatment outcome and fibrosis progression in the available large, finalized treatment studies of NAFLD.

As we have outlined in this review, there are several modifiers of disease progression in NAFLD. Some are traditional metabolic risk factors – others are related to genes and lifestyle. Given that there is a strong strong association between NAFLD, metabolic profile, alcohol

consumption and disease state it is difficult to determine what factors are driving progression or simply mirroring it.

In the clinical setting, the aforementioned risk factors, together with several accurate non-invasive techniques can be adequately utilized to find NAFLD patients with advanced fibrosis. However, in patients with less advanced disease state, it is difficult to identify those with rapid disease progression. These patients, with a more dismal disease trajectory, should be the ones targeted with surveillance and pharmacological intervention.

7. References

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8. Tables

Table 1. Serial biopsy studies in patients with NAFLD.

Authors, year (ref.)	Study design, Cohort selection NASH prevalence (%) NASH definition Sample size Follow-up time (median (range) years)	Age (median (range or SD) years) BMI kg/m ² or Weight kg or Obesity % T2DM	Fibrosis, baseline	Fibrosis, follow-up	Fibrosis progression, stable or regression	Baseline predictors of progression
Lee 1989[50]	Retrospective, Pathology records NASH 100% Definition NASH: N/A N=13 Follow-up: 3.3 (1.2-6.9)	Age: 55.4 (±10.7) Obese: 61% T2DM: 39%	F0 0(0%), F1 3(23%), F2 6(46%), F3 3(23%), F4 1(8%)	F0 0(0%), F1 1(8%), F2 6(46%), F3 3(23%), F4 3(23%)	P 5/13 S 8/13 R 0/13	None
Powell et al. 1990[51]	Retrospective Tertiary center NASH 100% Definition NASH: Fat and lobular inflammation w/wo fibrosis N=13 Follow-up: 3.0 (2.0-8.5)	Age: 48.9 (±11.7) Weight: 83.0 (±14.6) T2DM: 46%	F0 5(38%) F1-3 7(54%) F4 1(8%)	F0 2(15%) F1-3 9(70%) F4 2(15%)	P 4/13 S 8/13 R 1/13	None
Teli et al. 1995[52]	Retrospective, Pathology records NASH 0% (100% isolated steatosis) Definition NASH: N/A N=12 Follow-up: 10.3 (7.6-16)*	Age: 48.2 (±9.8) Weight: 73 (±18) T2DM: N/A	F0 12(100%) F1 0(0%) F2 0(0%) F3(0%) F40(0%)	F0 11(92%) F1 1(8%) F2 0(0%) F3(0%) F4 0(0%)	P 1/1 S 11/12 R 0/12	None
Ratzliff et al. 2000[54]	Retrospective, Tertiary referral NASH: 29% Definition NASH: Necroinflammation (lobular necrosis and/or piecemeal necrosis) N=14 Follow-up: 5 (1.5-15)	Age: 49 (20-79)† BMI: 29 (25-47)† T2DM: 16%†	F0 4(29%) F1 10(71%) F2 0(0%) F3 0(0%) F4 0(0%)	F0 8(57%) F1 4(29%) F2 1(7%) F3 0(0%) F4 1(7%)	P 2/14 S 8/14 R 4/18	None
Evans et al. 2002[53]	Retrospective, Pathology records NASH 100% Definition NASH: Brunt et al N=7 Follow-up: 7 (5.5-14)	Age: 57.5 (±8.4) BMI: 32.4 (±4.7) T2DM: 43%	F0 4(57%) F1 1(14%) F2 2(29%) F3 0(0%) F4 0(0%)	F0 2(29%) F1 1(14%) F2 3(43%) F3 1(14%) F4 0(0%)	P 4/7 S 3/7 R 0/7	None

Harrison et al. 2003[55]	Retrospective, Pathology records/tertiary center NASH: 41% Definition NASH: Brunt et al N=22 Follow-up: 5.7 (1.4-15.7)	Age: 50.6 (33–64) BMI: 33.8 (26.5-48.6) T2DM: 41%	F0-2 20(91%) F3-4 2(9%)	F0-2 18(82%) F3-4 4(18%)	P 7/22 S 11/22 R 4/22	AST
Fassio et al. 2004[56]	Prospective, tertiary referral NASH: 100% Definition NASH: Macrovesicular steatosis (> 10%) + lobular inflammation + ballooning, Mallory hyaline fibrosis, sinusoidal fibrosis or a combination thereof. N=22 Follow-up: 4.3 (3-14.3)	Age: 45 (20-69) BMI: 30 (24-38) T2DM 36%	F0 3(14%) F1 11(50%) F2 4(18%) F3 4(18%) F4 0(0%)	F0 3(14%) F1 11(50%) F2 4(18%) F3 4(18%) F4 0(0%)	P 7/22 S 11/22 R 4/22	Obesity and BMI
Adams et al. 2005[57]	Retrospective, Pathology records NASH: 93% Definition NASH: Steatosis + lobular inflammation + ballooning OR steatosis + any stage of fibrosis N=103 Follow-up: 3.2 (0.7-21.3)	Age: 45 (19-65) Obese: 67% T2DM: 42%	F0 25(25%) F1 21(20%) F2 23(22%) F3 18(18%) F4 16(16%)	F0 26(25%) F1 12(12%) F2 23(22%) F3 23(22%) F4 19(18%)	P 38/103 S 35/103 R 30/103	BMI, T2DM and Fibrosis stage
Hui et al. 2005[58]	Retrospective, tertiary referral, NASH: N/A Definition NASH: Graded biopsy according to Brunt. 6 patients had NAS4-6 (35%) N=17 Follow-up: 6.1 (3.8-8.0)	Age: 41.8 ($\pm$ 10.3) BMI: 28.6 ($\pm$ 3.4) T2DM: 24%	F0 11(65%) F1 5(29%) F2 1(6%) F3 0(0%) F4 0(0%)	F0 5(29%) F1 8(47%) F2 3(18%) F3 0(0%) F4 1(6%)	P 9/17 S 8/17 R 0/17	None
Ekstedt et al. 2006[47]	Prospective, Tertiary referral NASH 49% Definition NASH: Steatosis + lobular inflammation + ballooning OR steatosis + any stage of fibrosis N=70 Follow-up: 13.8 (10.3-16.3)	Age: 48 (24-66) BMI: 27 (21-43) T2DM: 9%	F0 36(51%) F1 19(27%) F2 11(16%) F3 4(6%) F4 0(0%)	F0 24(34%) F1 22(31%) F2 11(16%) F3 7(10%) F4 6(9%)	P 29/70 S 30/70 R 11/70	Δ Weight >5kg, ALT, AST, Platelet count, IR _{HOMA} , SPC
Wong et al. 2010[59]	Prospective, tertiary referral NASH: 33% Definition NASH: steatosis + hepatocytes ballooning or intralobular hepatocyte necrosis. N=52 Follow-up: 3	Age: 44 ($\pm$ 9) BMI: 27.4 ($\pm$ 3.7) T2DM: 50%	F0 26(50%) F1 17(33%) F2 7(13%) F3 1(2%) F4 1(2%)	F0 28(54%) F1 15(29%) F2 2(4%) F3 4(8%) F4 3(6%)	P 14/52 S 25/52 R 13/52	LDL, Δ Weight, Δ BMI
Pais et al. 2013[60]	Retrospective, tertiary referral NASH: 64% Definition NASH: Steatosis + lobular inflammation + ballooning OR steatosis + lobular inflammation + advanced fibrosis N=70 Follow-up: 3.4 (1-12)	Age: 52 ($\pm$ 10.5) BMI: 29 ($\pm$ 3.6) T2DM: 35%	F0 17(24%) F1 18(26%) F2 13(19%) F3 19(27%) F4 3(4%)	N/A	P 20/70 S 30/70 R 20/70	N/A

McPherson et al. 2015[49]	Retrospective, tertiary referral NASH: 75% Definition NASH: NASH: steatosis + ballooning + inflammation +/- fibrosis. Also 3 patients defined as NASH; 1 because of steatosis + fibrosis 3 and 2 because of steatosis + lobular inflammation + fibrosis stage 2 N=108 Follow-up: 6.6 (1.3-22.6)	Age: 48 ($\pm$ 12) BMI: 34 ($\pm$ 5) T2DM: 48%	F0 23(21%) F1 29(27%) F2 33(31%) F3 23(21%) F4 0(0%)	F0 23(21%) F1 19(18%) F2 19(18%) F3 33(31%) F4 13(12%)	P 45/108 S 43/108 R 20/108	AST/ALT ratio, Platelet count, Fib4
Sanyal et al. 2019[61]	Prospective, clinical trial data NASH: 82% Definition NASH: NASH CRN (NAS $\geq$ 4) N=217† Follow-up: 2.4 (0.03-3.9)	Age: 55 (48-59) BMI: 33.7 (30.3-38.4) T2DM: 67%	F0 0(0%) F1 0(0%) F2 0(0%) F3 217(100%) F4 0(0%)	N/A	P 48/217 S 127/217 R 42/217	INR, Platelet count, Hepatic collagen content, α -SMA expression, ELF score, FibroTest, FIB-4, APRI, sLOXL2

*In a subset of patients (n=26) who did not have cancer or biochemical signs of autoimmune disease and who did not die during follow-up.

†For entire cohort of 93 patients

‡Only patients with fibrosis stage 3 included in this table.

Abbreviations: ALT, alanine transaminase; APRI, AST to platelet ratio index; AST, aspartate transaminase; BMI, body mass index; ELF, enhanced liver fibrosis; F0-4, fibrosis stage 0-4; FIB-4, fibrosis-4; INR, international normalized ratio; IR_{HOMA}, homeostatic model assessment for insulin resistance; LDL, low-density lipoprotein; NA, not applicable; NASH, non-alcoholic steatohepatitis; NASH-CRN, NASH-Clinical Research Network; P, progression; R, regression; S, stable; sLOXL2, serum lysyl oxidase like 2; SMA, smooth muscle antibody; SPC, stereological point counting; T2DM, type 2 diabetes mellitus.

Table 2. Studies assessing the impact of alcohol on histopathology in NAFLD.

Author (year)	Sample size	Diagnosis of NAFLD	Study design	Definition of moderate alcohol consumption	Assessment of alcohol consumption	Focus/highlight	Outcome
Dixon et al. 2001[200]	105	Liver biopsy	Cross-sectional	<200 g/week	Clinical interview + questionnaire	Liver histology	No significant difference in NASH after adjusting for insulin resistance and diabetes
Cotrim et al. 2009[201]	132	Liver biopsy	Cross-sectional	<280 g/week	Clinical interview	Liver histology	No difference in liver histology
Ekstedt et al. 2009[129]	71	Liver biopsy	Cohort	< 140 g/week	Clinical interview + questionnaire	Fibrosis progression	Binge drinking was associated with higher fibrosis stage
Ascha et al. 2010[202]	195	Cirrhosis (liver biopsy or symptoms of portal hypertension)	Cohort	<168 g/week	Not stated	HCC	Alcohol consumption as a risk factor for HCC
Dunn et al. 2012[127]	582	Liver biopsy	Cross-sectional	<140 g/week	Questionnaire	Liver histology, steatohepatitis	Less steatohepatitis and fibrosis in moderate consumers
Kwon et al. 2014[203]	77	Liver biopsy	Cross-sectional	<40 g/week	Questionnaire	Liver histology, lifetime consumption	Higher rate of fibrosis F3/F4 in low/no consumption group
Sookoian et al. 2016[204]	266	Liver biopsy	Cross-sectional/mendelian randomization	210 g/week (male) 140 g/week (female) + gene carriers	Clinical interview	Genetic carriers as a measure of alcohol consumption, no protective association of moderate	Higher rate of steatosis and inflammatory changes in non-carriers (i.e. drinkers)

						consumption on histology	
Hagström et al. 2017[128]	120	Liver biopsy	Cross-sectional	168 g/week	Questionnaire + PEth	Liver histology	Reduced risk of fibrosis in moderate consumers. Elevated PEth levels increase risk of significant fibrosis
Ajmera et al. 2018[130]	285	Liver biopsy	Cohort	< 140 g/week	Clinical interview + questionnaire	Liver histology	Greater reduction in steatosis and NASH in non-drinkers
Yamada et al. 2018[205]	178	Liver biopsy	Cross-sectional	≤140 g/week	Questionnaire	Liver histology	Lower fibrosis score in moderate consumers
Mitchell et al. 2018[206]	187	Liver biopsy	Cross-sectional	210 g/week (male) 140 g/week (female)	Clinical interview + questionnaire	Fibrosis, binge-drinking, type of alcohol	Less fibrosis among subjects consuming wine <70 g/w, and in non-binge drinkers
Kimura et al. 2018[132]	301	Liver biopsy	Cohort	<140 g/week	Clinical interview + questionnaire	HCC	Higher incidence of HCC plus prevalence of cirrhosis in moderate consumers

Abbreviations: F0-4, fibrosis stage 0-4; HCC, hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis; PEth, phosphatidylethanol.