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THE IMPORTANCE OF FAT AND ALCOHOL FOR PROGRESSION AND PROGNOSIS IN CHRONIC LIVER DISEASE

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**Karolinska
Institutet**

Stockholm 2016

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Published by Karolinska Institutet.

Printed by EPrint AB 2016

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ISBN 978-91-7676-271-4

Anybody who has been seriously engaged in scientific work of any kind realizes that over the entrance to the temple of science are written the words: 'Ye must have faith.'

- Max Planck

The importance of fat and alcohol for progression and prognosis in chronic liver disease

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Chronic liver disease is an increasing cause of global morbidity and mortality. The popular belief is that liver disease is caused mainly by alcoholic liver disease or viral hepatitis. However, the most common cause of chronic liver disease today is non-alcoholic fatty liver disease (NAFLD), which is associated with obesity and insulin resistance rather than alcohol. NAFLD is considered to become the most common cause for need of liver transplantation in the coming years. Today, the most common cause of liver transplantation in Sweden is primary sclerosing cholangitis (PSC) - a rare but very serious disease of the bile ducts that become inflamed and obliterated, and is associated with a high risk of development of cholangiocarcinoma.

The role of concurrent use of alcohol in NAFLD and PSC is controversial. Part of this thesis explores the effect of alcohol on the degree of liver damage in these two diseases. We found that a low consumption of alcohol, around one unit per day, is not associated with a higher stage of fibrosis in the liver in PSC and should be safe in these patients. For NAFLD, we found that a low to moderate consumption of alcohol was associated with a lower risk for a higher fibrosis stage, up to thirteen units of alcohol per week. However, patients who had biochemical evidence of high alcohol consumption had a higher risk of more severe liver damage. This is well in line with other studies and indicates a J-formed risk profile for alcohol consumption in NAFLD.

In another part of the thesis we studied the long-term risk of having fat accumulation in the liver and if overweight per se can predict development of severe liver disease. We found that the strongest histological marker for disease-specific mortality in NAFLD after a follow-up of in mean 26 years was the stage of fibrosis, and found no excess mortality in patients with signs of inflammation in the liver after adjustment for the stage of fibrosis. The risk of being overweight was studied in close to 45.000 men in their late adolescence who were conscribed to military service in 1969-1970 after adjustment of potential confounders, such as alcohol consumption. Body mass index (BMI) was found to be an independent predictor of development of severe liver disease after a mean follow-up of 39 years.

Taken together, this thesis indicates that a low to moderate consumption of alcohol is safe in PSC and possibly protective in NAFLD. Furthermore, we found that the strongest predictor of disease-specific mortality in NAFLD is the stage of fibrosis, which can have implications for the design of endpoints in future clinical studies. Also, the finding that overweight per se is a predictor for development of severe liver disease is important for public health decision-making.

LIST OF SCIENTIFIC PAPERS

- I. Hannes Hagström, Per Stål, Knut Stokkeland, Annika Bergquist.
Alcohol consumption in patients with primary sclerosing cholangitis.
World J Gastroenterol. 2012 Jun 28; 18(24):3105-11
- II. Mattias Ekstedt, Hannes Hagström, Patrik Nasr, Mats Fredrikson, Per Stål, Stergios Kechagias, Rolf Hultcrantz.
Fibrosis is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up.
Hepatology. 2015 May;61(5):1547-54
- III. Hannes Hagström, Patrik Nasr, Mattias Ekstedt, Stergios Kechagias, Kristina Önerhag, Emma Nilsson, Fredrik Rorsman, Reza Sheiki, Hanns-Ulrich Marschall, Rolf Hultcrantz, Per Stål
Low to moderate lifetime alcohol consumption is associated with lower stages of fibrosis in non-alcoholic fatty liver disease
Manuscript
- IV. Hannes Hagström, Per Stål, Rolf Hultcrantz, Tomas Hemmingsson, Anna Andreasson.
Overweight in late adolescence predicts development of severe liver disease later in life after 39 years of follow-up.
Manuscript accepted for publication in Journal of Hepatology

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LIST OF ABBREVIATIONS

ALT	alanine aminotransferase
AUDIT	alcohol use disorders identification test
AST	aspartate aminotransferase
BMI	body mass index
CRP	c-reactive protein
CDT	carbohydrate-deficient transferrin
CVD	cardiovascular disease
CDR	causes of death register
CI	confidence interval
GGT	gamma-glutamyltransferase
HR	hazard ratio
HE	hepatic encephalopathy
HCV	hepatitis c virus
HCC	hepatocellular carcinoma
IBD	inflammatory bowel disease
ICD	international classification of diseases
kPa	kilopascal
LDH	lifetime drinking history
MCV	mean corpuscular volume
NAS	NAFLD activity score
NHANES	national health and nutrition examination survey
NPR	national patient register
NAFLD	non-alcoholic fatty liver disease
NAFL	non-alcoholic fatty liver
NASH	non-alcoholic steatohepatitis
OR	odds ratio
PEth	phosphatidyl ethanol
PIN	personal identification number

PBC	primary biliary cirrhosis / cholangitis
PPAR	peroxisome proliferator-activated receptor
PSC	primary sclerosing cholangitis
SAF	steatosis activity fibrosis
SD	standard deviation
SREBP	sterol regulatory element response-binding protein
TE	transient elastography
TNF	tumour necrosis factor

1 INTRODUCTION

While there has been a tremendous success for modern medicine in the latest 30 years, with for instance a reduction of mortality in cardiovascular disease (CVD) of more than 25 % (1), there has been little progress in mortality in liver disease. For instance, recent data from the UK indicates that liver disease is the only increasing cause of death with a 500 % increase in the standardized mortality ratio since 1970, depicted in figure 1 (2).

Liver diseases are common causes of mortality and morbidity worldwide. There are many different diseases that can lead to liver damage. Damage to the liver generally results in formation of scar tissue, fibrosis. Fibrosis can be progressive over the years, and the end result is cirrhosis. In rural settings viral hepatitis dominates the disease spectrum, with for instance a prevalence of chronic hepatitis C virus (HCV) in Egypt of up to 15 % while the prevalence in Sweden is estimated at around 0.3 % (3, 4). In North America and Europe, alcohol is often considered the most common cause of chronic liver disease. However, non-alcoholic fatty liver disease (NAFLD) is highly more prevalent than alcohol induced liver disease even on a global scale. The prevalence of NAFLD is estimated to be as high as 46% in some populations and is more closely discussed below (5, 6).

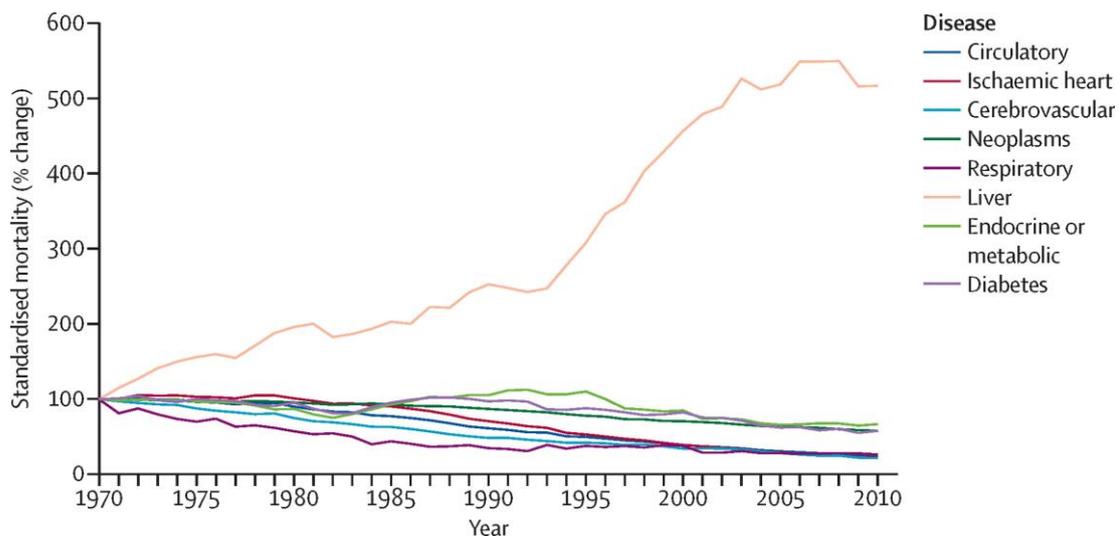


Figure 1. Standardized mortality ratios for causes of death in the UK. From Williams et al, Lancet 2014;384:1953-1997. Published with permission.

This indicates that liver disease is one of the major challenges for modern medicine as well as for health care politicians in the years ahead.

1.1 NON-ALCOHOLIC FATTY LIVER DISEASE

The prevalence of overweight and obesity has risen dramatically in the world during the last 30 years. For instance, the prevalence of obesity in the US has increased from 13% in 1972 to almost 35% in 2012 and is estimated to account for up to 10% of total health expenditure (2). A figure of the increasing trend of obesity in OECD countries is presented in figure 2 (7).

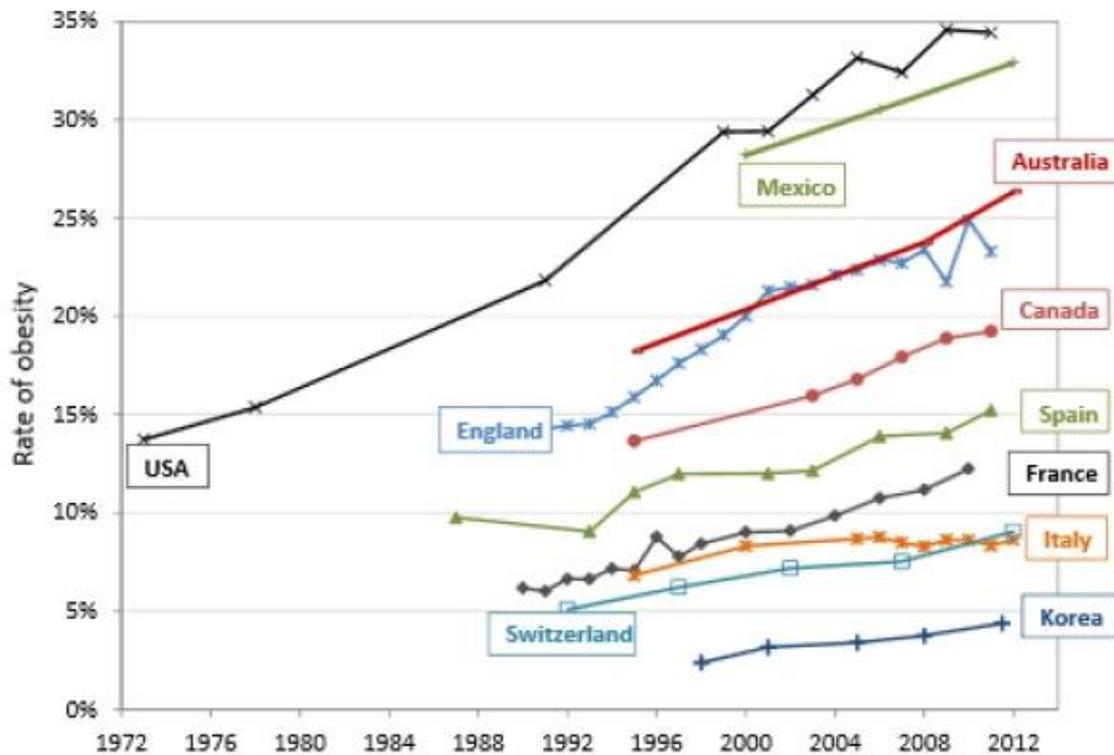


Figure 2. Prevalence of obesity in selected OECD countries from 1972 – 2012. Obesity update © OECD 2014 (7).

Coupled to this epidemic of overweight and obesity, NAFLD has emerged as the most prevalent liver disease in the world (5, 8). This disease is now considered to be the hepatic manifestation of the metabolic syndrome, and is heavily associated with obesity and insulin resistance.

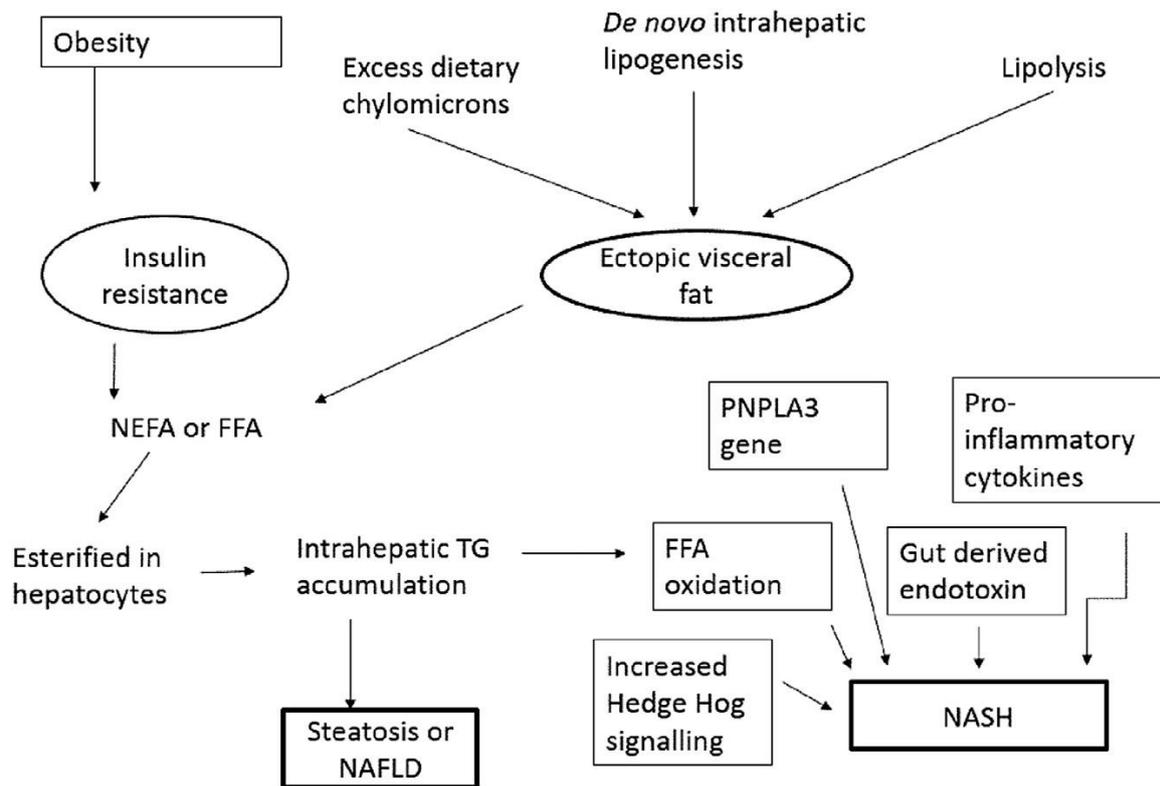
The prevalence of NAFLD depends partly on the studied population and the prevalence of overweight and obesity, as well as on the diagnostic method used. In the U.S., prevalence has been estimated to 46% using liver biopsy (6) but only 11% by using levels of serum liver enzymes as marker for NAFLD (9). Also, genetic predisposition to NAFLD is likely. For instance, persons with an Asian background develop NAFLD at lower BMI levels than Caucasians do (5). The prevalence of NAFLD in Europe is estimated to around 2-44% depending on the studied population (10). The global prevalence of NAFLD has recently been estimated to around 25% (11).

A subgroup of around 10-20% of patients with NAFLD develops inflammation in the liver, named non-alcoholic steatohepatitis (NASH) (12, 13). This aspect of the NAFLD disease spectrum can at present only be diagnosed using liver biopsy. Patients without NASH are considered to have non-alcoholic fatty liver (NAFL). The traditional hypothesis was that only patients with NASH are at risk for progressive fibrosis, with 10-25% of NASH patients at risk for development of cirrhosis and/or hepatocellular carcinoma (HCC) (14-17). However, it is only logical that patients without NASH at early stages of the NAFLD disease spectrum can later go on to develop NASH and progressive fibrosis, which has recently been proven

(18-20). This puts even more persons at risk for potential liver damage and makes it harder to define which part of the population that should be screened for more advanced stages of disease or followed clinically.

The pathophysiology behind NAFLD and NASH is highly complex, and why some persons develop NASH is in particular poorly understood. In the setting of insulin resistance, lipolysis of adipose tissue is increased, leading to an increase in free fatty acid flow through the portal vein to the liver. Also, hepatic de novo lipogenesis is up-regulated in insulin resistance, and patients with NAFLD often have an increased dietary intake of lipids. These combined effects all contribute to hepatic steatosis when free fatty acids are esterified into triglycerides (21-23). Interestingly, steatosis per se is not necessarily dangerous. In an elegant experiment, Yamaguchi et al blocked the final step in triglyceride formation in mice by blocking diacylglycerol acyltransferase 2 (DGAT2) through antisense nucleotide treatment. The result was a reduction in hepatic steatosis, but a marked increase in inflammation and fibrosis (24), indicating that hepatic steatosis might actually be a mechanism protecting hepatocytes from lipotoxicity induced by oxidation of free fatty acids. However, the exact pathophysiology behind development of NASH remains shrouded. The two-hit hypothesis was established by Day in 1998 (25). According to this, hepatic steatosis is the first “hit”, sensitizing hepatocytes to further injury by inflammation, oxidative stress and mitochondrial dysfunction. This theory has since then evolved, and currently, a “multiple-hit” hypothesis has been perceived as more likely (22, 23). This includes contribution from genetic susceptibility, gut-derived endotoxins, pro-inflammatory cytokines and oxidative stress, and is beyond the scope of the introduction in this thesis. A brief summary of the pathophysiology of NAFLD and NASH is presented in figure 3.

To date, there is no effective medical treatment for either NAFLD or NASH. Lifestyle changes, if successful, on the other hand have a dramatic positive effect on almost all aspects of the disease (26). In a recent study from Cuba of the impact of weight loss on NASH, 97% of patients who lost more than ten percent of total body weight during one year cleared all the fat from the liver. A reduction in inflammation and even in fibrosis was also seen. However, a weight loss of more than ten percent could only be seen in about ten percent of the included patients (27). Other lifestyle changes that have some level of evidence is a reduction in intake of fructose, usually derived from consumption soft drinks that are sweetened with high-fructose corn syrup, which has been implicated as a risk factor for accumulation of fat and fibrosis in the liver (28, 29). Also, coffee intake has been suggested to be protective both in NAFLD and in other liver diseases (30, 31), possibly by inhibiting the activation of hepatic stellate cells (32). A reduction in the risk of development of HCC has also been observed (33-35). Provided that no relative contraindication to coffee consumption such as an uncontrolled high blood pressure exists, some researchers advocate an intake of 2-3 cups per day.



NEFA: Non esterified fatty acids, FFA: Free fatty acid, PNPLA: Patatin-like phospholipase domain-containing protein 3, NAFLD: Non-alcoholic fatty liver disease, NASH: Non-alcoholic steatohepatitis

Figure 3. Pathophysiology of NAFLD and NASH. From Than et al. A concise review of non-alcoholic fatty liver disease. *Atherosclerosis* 2015;239:192-202. Published with permission.

1.1.1 NASH diagnosis

As stated above, the clinical entity of NASH can currently only be diagnosed through liver biopsy. The hallmark of NAFLD is obviously hepatic steatosis, and at least 5% of hepatocytes have to be steatotic for the pathologist to be able to set the NAFLD diagnosis (36). Other hallmarks of NAFLD include swelling of apoptotic hepatocytes, called ballooning, and lobular inflammation as well as fibrosis. Typical histopathological findings are presented in figure 4.

During the years, several scoring systems for the histological severity of NAFLD have been proposed. Brunt et al in 1999 proposed a scoring system for the severity of NASH, which is presented in table 1. However, this system was not intended to separate cases with NASH from cases without, and is currently less used (37).

Grade of steatohepatitis					Staging fibrosis	
Grade	Steatosis	Ballooning	Lobular inflammation	Portal inflammation	Stage	Fibrosis
Mild	Involves up to 2/3rds	Occasional, zone 3	Scattered, mild acute and chronic	None or mild	0	None
Moderate	Any degree	Obvious, zone 3	Mild, associated with ballooning	Mild to moderate	1	Zone 3 perisinusoidal fibrosis only
Severe	Typically more than 2/3rds	Marked, mainly zone 3	Mild to moderate	Mild to moderate	2	Zone 3 perisinusoidal fibrosis and periportal fibrosis
					3	Bridging fibrosis
					4	Cirrhosis

Table 1. Brunt scoring system for NASH severity.

In 2005, the American NASH clinical research network (NASH CRN) proposed a new grading system for the histological severity of NAFLD, which was named the NAFLD activity score (NAS). In this score, steatosis is graded on a semi-quantitative scale as 0-3, lobular inflammation as 0-3 and ballooning as 0-2, yielding a total score of 0-8 (38). The NAS was constructed as being an endpoint in clinical trials, but was quickly adopted as a mean to diagnose NASH and differentiate it from NAFL. This was however not intended, and the authors have clearly defined this (39). The NAS has also been criticized for not including fibrosis in the score, as well as overstating the importance of steatosis in the algorithm.

A more recent scoring system is the SAF score, for Steatosis Activity Fibrosis (40, 41). In this system, steatosis is required for inclusion in the algorithm at all and is scored as 0-3. Biopsies are then graded for ballooning on a 0-2 scale and lobular inflammation on a 0-2 scale. A score of at least one for both steatosis, ballooning and lobular inflammation is required for NASH diagnosis. A figure of the diagnostic algorithm of NASH according to the SAF scoring system is presented in figure 5.

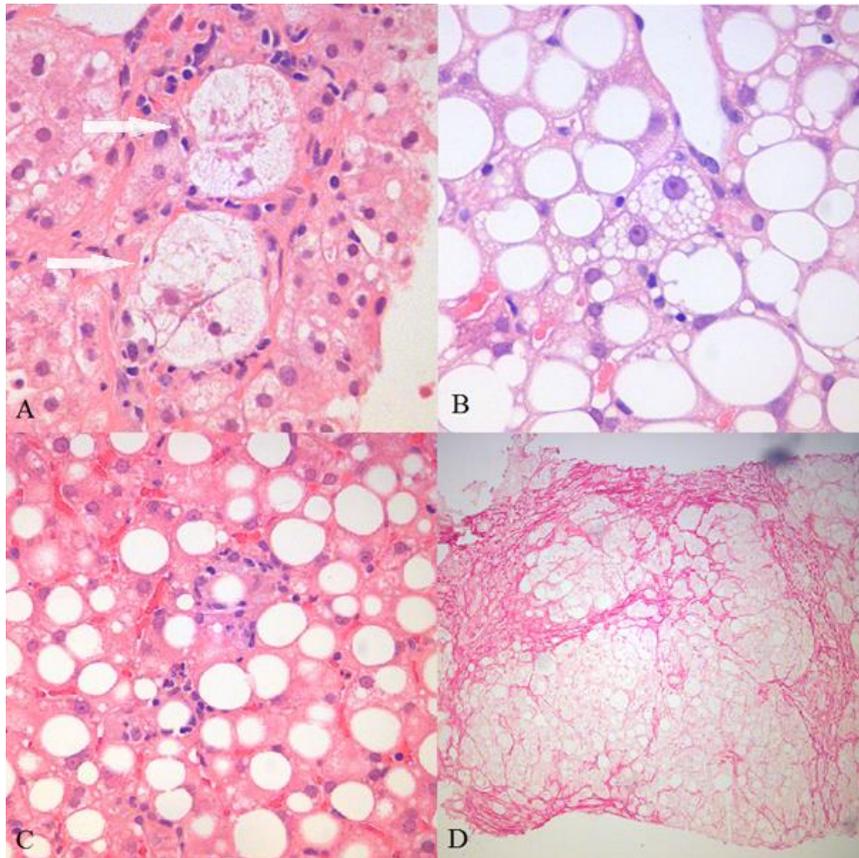


Figure 4. Histopathology of NAFLD. The picture depicts some hallmarks of the NAFLD entity. In A is seen the classical ballooning of two hepatocytes (arrows). In B, macro- and microvesicular steatosis can easily be seen. In C, small infiltrates of inflammatory cells are seen in the liver lobules, and in D, the end-stage of cirrhosis is visible (Sirius red staining). Photo by Kajsa Villiamsson, Karolinska Institutet.

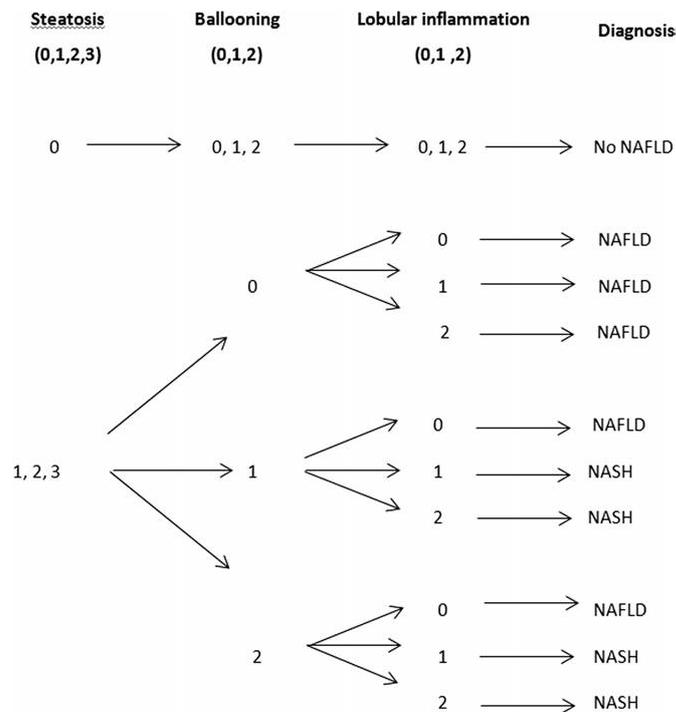


Figure 5. Diagnostic algorithm of NASH according to the SAF scoring system. From Bedossa et al, Hepatology 2014;60:565-575. Published with permission.

1.1.2 Prognosis in non-alcoholic fatty liver disease

The increase in the prevalence of NAFLD (13, 42) together with the astonishing success of new direct acting antiviral drugs to cure HCV (43) indicates that NAFLD will likely become the major cause of advanced liver disease in the near future. Indeed, in the US NAFLD is already the number two cause for need for liver transplantation, and is projected to be number one after 2020 (44).

Patients with NAFLD have an increase in overall as well as cardiovascular and liver-specific mortality (15, 45-48), but making an individual prognosis in NAFLD is challenging. Firstly, the prevalence of NAFLD is staggering, making the potential numbers needed to screen to detect cases with or at risk for advanced liver disease enormous. Secondly, only a subpopulation of patients with NAFLD goes on to develop NASH, progressive fibrosis or cirrhosis. Thirdly, the rate at which NAFLD patients develop progressive fibrosis is varying, with some data to support a rate of 7-14 years for progression of one fibrosis stage (49). Thus, the need for tools to be able to identify patients at risk for adverse outcomes is huge.

There has been considerable debate on if NAFLD is a disease entity in its own right, or if it is simply an epiphenomenon of the metabolic syndrome. However, in recent years the current paradigm has shifted toward a recognition that patients with NAFLD are at increased risk for overall and disease-specific mortality, as well as morbidity (50, 51). Adams et al produced the first major study in 2005 of the natural history of NAFLD patients, and showed that NAFLD patients had a roughly 30% increased risk for death, and that death in liver disease was the third leading cause of death, compared to being the thirteenth most common cause of death in the reference population (15). Since then, a number of studies have addressed the prognosis and natural history of NAFLD, of which two major studies are Swedish. Ekstedt et al performed an elegant study of 129 NAFLD patients, where 71 had undergone two liver biopsies during a mean follow-up of 13.8 years, and showed that primarily patients with NASH had an increased risk of death. Progressive fibrosis between the two biopsies was associated with weight gain and development of diabetes (47). From Stockholm, Söderberg et al confirmed this finding in 118 NAFLD patients who were followed for up to 28 years, again showing that liver disease was the third most common cause of death (46). Also, patients with NAFLD has been shown to have an increased risk for carotid intima plaque formation independent of confounders (52), is extremely common in patients with myocardial infarction, where a higher degree of steatosis has been linked a more severe cardiac artery disease, although that particular study was based on ultrasound only (53).

1.2 PRIMARY SCLEROSING CHOLANGITIS

Primary sclerosing cholangitis (PSC) is a rare, chronic and progressive disease of the large and medium sized bile ducts (54). PSC is highly linked to presence of inflammatory bowel disease, with around 80% of PSC-patients having signs of inflammatory bowel disease, and PSC is present in roughly 4% of patients with ulcerative colitis (55). The disease is more common in the Nordic countries than the rest of the world, with an estimated prevalence in

Sweden of around 16 per 100.000 (56), and occurs more often in men than in women (55). For unknown reasons, the bile ducts become inflamed and fibrotic, leading to cholestasis and increasing the risk for bacterial infections of the bile tree (cholangitis). PSC leads to cirrhosis and liver failure in most, but not all cases. Also, the risk for development of cholangiocarcinoma in PSC patients compared to healthy controls is dramatically increased by some 161 times (57). Mean time from diagnosis to death or need for liver transplantation has been estimated to between 12-18 years (58, 59). There is currently no approved therapy for PSC (54), and many patients experience a fear for future complications.

1.3 CIRRHOSIS

Cirrhosis is the end stage of all chronic liver diseases. Over time, chronic damage to the liver results in activation of stellate cells, which resides in the perisinusoidal space. Stellate cells have multiple functions, including production of collagen fibres in response to on-going liver damage (60, 61). Collagen fibres are deposited in the extracellular matrix of the liver, and accumulation of these results in fibrosis. Fibrosis usually starts to form in the portal tracts of the liver and can spread between portal tracts and central veins to form a web-like pattern, or cirrhosis. However, if the on-going liver damage is removed, such as if an alcoholic patient stops drinking or if a viral hepatitis is cured, fibrosis and even cirrhosis can regress (62). There are a number of classification systems to stage the amount of fibrosis in the liver on a liver biopsy, but most stage fibrosis on a 0-4 scale where 0 means no fibrosis and 4 means cirrhosis (38, 63, 64).

Progression of fibrosis is usually a silent process, and few patients experience any symptoms during the course of the disease, and even early stages of cirrhosis are symptom-free. This is called compensated cirrhosis. However, with the development of cirrhosis the liver's function starts to fail. The liver has a multitude of functions, including being essential for energy homeostasis, production of glucose, many essential proteins and degradation of toxic substances from the blood. When these functions start to fail, symptoms do arise and many patients experience fatigue, anorexia, bruises easily and can develop liver decompensation.

1.3.1 Decompensated cirrhosis

Classical decompensation is the manifestation of bleeding esophageal varices, ascites, jaundice or hepatic encephalopathy, all described below. The pathogenesis of these symptoms is complicated, but a common denominator is portal hypertension. Cirrhosis leads to a higher resistance in the liver to blood from the vena portae and the hepatic artery. Furthermore, the oncotic pressure in the blood is reduced in cirrhosis as the production of proteins such as albumin is diminished. This combined effect leads to a rise in the pressure in the portal vein, which has a number of consequences, described below. These symptoms are serious, and in almost all cases lead to hospitalization.

The prognosis for patients who develop decompensated cirrhosis is dismal. One way to predict mortality is the often-used Child-Pugh scoring system (65), which grades cirrhosis in three stages, A, B and C depending on the stage of hepatic encephalopathy, ascites and levels

of albumin, PK-INR and bilirubin. Patients with Child-Pugh A have a relatively benign prognosis, with a one year survival of 100%, while patients with Child-Pugh B have a 80% and patients with Child-Pugh C have a 45% one year survival (65).

1.3.1.1 Ascites

Ascites is the formation of fluid in the abdominal cavity. With the increase in portal pressure, fluid is extruded into the abdominal cavity, forming a transudate which may build up to 15-20 litres of ascites in the abdomen. Large amounts of ascites can compress the diaphragm and the lungs, leading to dyspnoea. Also, bacteria from the gut can translocate into the ascites and cause an infection, named spontaneous bacterial peritonitis, which is the most common infection in cirrhotics (66). Ascites can be treated with diuretics and/or repeated laparocentesis.

1.3.1.2 Esophageal varices

With the increase in portal pressure, blood from the splanchnic vessels, which drains through the portal vein, must take alternate routes. The most common shunting pathway is through small veins in the esophagus, which then can become dilated and form varicose veins, called varices. These varices are usually asymptomatic, but can start to bleed, which can lead to exsanguination. Esophageal varices can be treated with non-specific beta-blockers as a bleeding prophylaxis, which reduce the portal pressure, or repeated band ligation of the varices (67). Bleeding varices are treated with vasoactive drugs that reduce portal pressure in combination with antibiotics and band ligation (67).

1.3.1.3 Hepatic encephalopathy

Hepatic encephalopathy (HE) is a state of confusion related to cirrhosis. Briefly, toxic substances from the gut, in particular ammonia, that are usually detoxified by the liver bypasses this through shunting via for example esophageal varices. These substances later reach the brain and are thought to lead to astrocyte swelling, causing several neurological symptoms (68). More recent research has also found that cerebral blood flow is reduced in episodes of HE, possibly by inhibition of cerebral energy metabolism related to increased ammonia concentrations (69).

1.3.1.4 Jaundice

Jaundice is the yellowing of the skin and eyes, caused by increasing amounts of bilirubin in the blood. This symptom is not unique to cirrhosis, as bilirubin can increase due to a number of other diseases, such as haemolysis or extrahepatic obstruction of the bile ducts. However, in cirrhosis bilirubin is increased, as the liver cannot longer keep up with the degradation of bilirubin from degraded erythrocytes.

1.3.1.5 Hepatorenal syndrome

Hepatorenal syndrome (HRS) is a severe syndrome which can be seen in patients with advanced cirrhosis. It is characterized by a rapid deterioration in kidney function in the

absence of kidney-specific diseases, and is associated with a dismal prognosis. Hepatorenal syndrome is thought to be an effect of vasoconstriction in the renal arteries secondary to splanchnic vasodilation seen in advanced cirrhosis. Treatment with albumin and vasoconstrictors is commonly applied, which can improve short-term mortality, and patients with HRS should be evaluated for liver transplantation (70, 71).

1.4 ALCOHOL CONSUMPTION IN SYNCHRONOUS LIVER DISEASE

It is well known that alcohol consumption can lead to serious liver damage (72), but this is the case only in a subset of drinkers (73, 74). Despite this, the exact pathophysiology behind alcohol-induced liver damage is not entirely clear. Some 90-95% of heavy drinkers develop hepatic steatosis (74), which is thought to occur due to up-regulation of key transcription factors involved in lipid metabolism, such as up-regulation of sterol regulatory element response-binding protein 1c (SREBP-1c), leading to reduced beta-oxidation and increased de novo lipogenesis (74). Alcohol can also induce down-regulation of the nuclear transcription factor peroxisome proliferator-activated receptor (PPAR)-alpha, leading to reduced hepatic beta-oxidation (75, 76).

Alcohol is primarily metabolized in hepatocytes to acetaldehyde by cytosolic alcohol dehydrogenase, but also by cytochrome P4502E1 in microsomes and catalase in peroxisomes. Acetaldehyde is then metabolized by aldehyde dehydrogenase to acetate that is released into the blood and used as energy in muscles and by the brain. The intermediary metabolite in this pathway, acetaldehyde, is highly reactive and can lead to formation of reactive oxygen species and subsequent mitochondrial damage (77, 78). This is thought to lead to activation of hepatic stellate cells, but other pathways such as activation of TLR4 and the innate and adaptive immune system is also possible (74).

The traditional way to counsel patients with a concurrent liver disease has been to advise complete abstinence from alcohol consumption. The evidence for this however has been scarce. Alcohol consumption in low to moderate amounts has been associated with several positive as well as negative endpoints. For instance, persons consuming any amount of alcohol had a 25% reduction compared to non-drinkers for cardiovascular mortality in a large meta-analysis, with the largest benefit in persons who consumed 1-2 drinks per day (79). Another large study found a protective effect in moderate drinkers compared to non-drinkers for development of rheumatoid arthritis (80). On the other hand, an increased risk for development of a number of malignancies has been reported, for example breast and colon cancer (81, 82). It can therefore be argued that an individual risk profile should be obtained in each individual, with respect to heredity and other risk factors.

Regarding alcohol consumption in patients with a concurrent liver disease, most evidence points to an increased risk for accelerated liver damage in persons consuming high amounts of alcohol. For instance, in persons with hepatitis C who consume more than 210 grams of alcohol per week, the risk for cirrhosis is increased by 130% compared to non-drinkers (83), and one study found an increase in the risk for fibrosis progression even in persons consuming low to moderate amounts of alcohol (84). Furthermore, in hereditary hemochromatosis, the risk for cirrhosis is increased approximately nine times by drinking more than 60 gram of alcohol per day (85).

The role of alcohol consumption in fibrosis progression in PSC was prior to this thesis not explored. However, one study found a possible increase in risk for development of

cholangiocarcinoma in PSC-patients who currently consumed any amount of alcohol compared to persons who reported never to have consumed any amount of alcohol (86).

Regarding NAFLD, this is currently a diagnosis of exclusion, meaning that all other liver diseases or causes of fat accumulation into the liver must be excluded. This is particularly difficult regarding alcoholic liver disease, since the histological picture of NAFLD and alcoholic liver disease is almost identical. Thus, significant alcohol consumption must be ruled out before the NAFLD diagnosis can be set, which is usually done through a careful patient history coupled with analysis of biomarkers. The limit of alcohol consumption that is allowed is different in different countries, but is in Sweden set to 30 grams of alcohol per day in men and 20 grams in women. One often cited study found that the risk threshold for cirrhosis development is around 30 grams of alcohol per day (87). However, a recent Swedish study found that consumption of 32 grams of alcohol per day in men and 16 grams per day in women for three months did not cause hepatic steatosis (88).

Binge drinking, usually defined as drinking more than five units of alcohol per occasion, have in one study been associated with an increased risk for fibrosis progression in NAFLD (89). More recent data, on the other hand, points to a possibly reduced risk for NAFLD in persons consuming any amounts of alcohol compared to persons not drinking alcohol (90). Also, in subjects with NAFLD, alcohol consumption in moderate amounts has been associated with a reduced risk for NASH as well as fibrosis (91). This is in contrast to a number of larger studies indicating a possibly synergistic effect of obesity and alcohol consumption on a number of biomarkers for liver damage, such as transaminases. In an analysis of 13 580 persons included in the third US National Health and Nutrition Examination Survey (NHANES), alcohol consumption was associated with higher transaminases only in overweight and obese participants, suggesting an interaction (92). The same effect has been seen in other large studies (93, 94), but all of these studies has used liver transaminases as the outcome variable, contrasting to the much more detailed studies with liver biopsies where a protective effect of alcohol was seen (91).

When studying lifestyle related parameters, such as alcohol consumption, one must always take into account the possibility of confounding factors. In regard to alcohol, there are several potential confounders, including smoking, physical activity and other dietary factors such as consumption of fructose and coffee that could partly explain the association between liver damage and alcohol consumption (28, 31), which has either been imperfectly or not at all been addressed in previous studies.

1.5 THE IMPACT OF OVERWEIGHT AND OBESITY ON THE LIVER

Apart from being associated with NAFLD, overweight and obesity has been implicated as risk factors in liver disease in a wider sense. In hepatitis C, the presence of hepatic steatosis has been independently associated with a faster progression of liver damage (95), and a high body mass index has also been linked to a lack of response to antiviral therapy (96) as well as to an increased risk for development of HCC (97). In primary biliary cirrhosis (PBC), the role

of overweight and obesity is less explored, but one study found that steatosis and a higher BMI was each associated with a higher disease stage (98). Another study found that BMI > 25 and a NAS over five each were associated with more severe bile duct damage (99).

In more advanced stages of liver disease, an increased risk of around 83% has been found for development of primary liver cancer in obese individuals even after adjustment for alcohol consumption and viral hepatitis (100). Also, in patients with cirrhosis an increased risk for liver decompensation has been found for obese patients, 43% in obese cirrhotics compared to 15% in patients with cirrhosis and a BMI of <25, after a median follow-up of 59 months (101).

2 AIMS

General aims of this thesis were to increase our knowledge of the role of alcohol in synchronous liver disease, and to study the prognosis of NAFLD as well as the association between overweight and development of liver disease.

The specific aims of this thesis was to

1. To study the impact of lifetime alcohol consumption on the severity of liver disease in PSC and NAFLD
2. To study the prognostic impact of several histological parameters on disease-specific mortality in NAFLD
3. To study the prognostic impact of overweight and obesity on the development of severe liver disease after an extended follow-up period

3 METHODS AND MATERIALS

3.1 STUDY PARTICIPANTS (STUDY 1, 2, 3 AND 4)

In study 1, we examined the role of lifetime alcohol consumption on the severity of fibrosis in patients with PSC. All patients with PSC at the Karolinska University Hospital are recorded at a local register. We used 141 patients that at the time of the study were living in the Stockholm area and attended regular follow-up visits at our clinic. In total, 45 patients were excluded from the study due to either not being able to give informed consent or missing data regarding exposure or outcome variables, leaving 96 patients for the final analysis.

In study 2, we investigated the role of different histological scoring systems on the risk of mortality in NAFLD, the NAS and the fibrosis stage (see paragraphs 3.3.1.1 and 3.3.1.2). We pooled data from two previously studied cohorts regarding the long-term prognosis in NAFLD (46, 47), which used patients with confirmed NAFLD investigated at the Karolinska University Hospital or the Linköping University Hospital for elevated transaminases between 1980-1993.

Study 3 examined the role of lifetime alcohol consumption in patients with NAFLD. We performed a prospective multi-centre study using the “Svensk Internmedicinsk LeverKlubb” (SILK) network. This group includes research-focused clinicians from all university hospitals in Sweden. For this study, participants from the Karolinska University Hospital (data coordinating centre), Linköping University Hospital, Sahlgrenska University Hospital, Akademiska University Hospital (Uppsala) and Skåne University Hospital contributed to the study. Between 2011 and 2015, 139 subjects underwent a liver biopsy during a liver disease workup, and were diagnosed with NAFLD. In total, 19 patients were excluded from the study due to missing data regarding exposure or outcome data, leaving 120 patients for the final analysis.

In study 4, we examined the association between BMI in late adolescence and future risk for severe liver disease. We used historical data from men who were enlisted for conscription between 1969 and 1970. Conscription was mandatory at this time, and only persons with severe handicaps were excluded. We obtained data on 49 321 men, of which there were complete data regarding all covariates on 44 248 men.

3.2 ASSESSMENT OF ALCOHOL CONSUMPTION (STUDY 1, 3 AND 4)

As part of a standard liver disease workup, the question of past and present alcohol consumption is always addressed. However, this can be difficult to examine in detail due to a number of factors. Firstly, the patient might not want to disclose his or her true alcohol habits, since this is perceived as private and high alcohol consumption can be stigmatizing. Secondly, recall bias regarding past alcohol consumption might be

present. There are a number of methods available to try to quantify an individual patient's past and present alcohol consumption, including questionnaires, techniques in taking patient history as well as biomarkers. Some of these take lifetime consumption of alcohol into account, but some do not. Only looking at current alcohol consumption infers the risk of misclassifying a patient who has had past overconsumption of alcohol, but has recently stopped drinking, as a non-drinker.

In study one and three, we used detailed questionnaires regarding current and lifetime alcohol consumption, as well as and specific biomarkers. In study four, data was gathered from a register of men who were enlisted for conscription. Data on alcohol consumption came from questionnaires describing alcohol consumption at that time, and was categorized as 0 grams per week, 1-100 grams per week, 101-250 grams per week or more than 250 grams per week.

3.2.1 Lifetime drinking history questionnaire

The lifetime drinking history (LDH) is a very detailed questionnaire regarding the current and lifetime drinking habits of an individual patient (102). The LDH has been validated and has high test-retest correlation (103, 104). It allows for the calculation of the total number of units of alcohol during a person's lifetime, with the possibility of calculating changes in drinking habits during life. It also allows measurement of total number of binge drinking episodes, defined as drinking five or more units of alcohol at one occasion. One unit of alcohol is equivalent to twelve grams of alcohol. In study one and three, we used the LDH to assess current and lifetime alcohol intake. Patients were thoroughly informed about the questionnaire and later filled it out at home. When data were missing, the patient was contacted by telephone and information was supplemented through a telephone interview.

Alcohol consumption was calculated as total units of alcohol intake during a subject's lifetime, and was then divided into units per week. Number of binge-drinking episodes was likewise calculated as per drinking week. Drinks consumed during the last week before filling in the questionnaire was separately recorded.

3.2.2 Biomarkers of alcohol consumption

A number of biomarkers are available to identify excess alcohol consumption. However, most biomarkers have low sensitivity and / or specificity and are of minor use to clinicians trying to distinguish alcohol-related liver damage from e.g. NAFLD. Analysis of transaminases (ALT, AST) is a common first-line step in identifying liver damage per se, but carries the risk of misclassification since primarily AST can be increased in a number of other scenarios, most commonly muscular damage. Other markers of recent alcohol consumption includes gamma-glutamyltransferase (GGT) and

mean corpuscular volume (MCV), which are both non-specific and can be increased in other diseases than alcohol-related liver disease (105).

More specific markers of recent alcohol consumption include carbohydrate-deficient transferrin (CDT) and phosphatidyl ethanol (PEth). The CDT test is often used internationally, while PEth is more common in Sweden. An increase in CDT is evident first after consumption of 50-80 grams of alcohol per day during at least one week (106), which is much higher than the normally recommended safe intake of 20-30 grams per day (73), meaning that subjects consuming between 30-50 grams per day will not be identified by the CDT test. Phosphatidyl ethanol is relatively new biomarker, of which the 16:0/18:0 variant has been identified as the most sensitive biomarker for alcohol consumption during the preceding 2-3 weeks (107, 108). The correlation between PEth and alcohol intake is roughly linear, and there has been no reports of false-positive increases in PEth in subjects who abstain from alcohol.

In study 1 and 3, we used CDT and PEth as markers of recent alcohol consumption in the respective cohorts.

3.3 ASSESSMENT OF LIVER DAMAGE

As most liver diseases are asymptomatic at first, assessment of the grade of liver damage can be challenging. Non-invasive biomarkers, such as liver transaminases are usually a first-line test to identify subjects with an on-going liver injury. However, these biomarkers are not perfect, and for instance in NAFLD, the entire spectrum of liver disease from benign liver histology to cirrhosis can be seen in NAFLD patients with normal transaminases (109).

As discussed above, with manifest cirrhosis the function of the liver starts to fail, and this can be seen in laboratory parameters such as albumin, bilirubin and PK-INR. However, this indicates already manifest severe liver disease, and there is a need to be able to pick up these cases earlier in the disease continuum, when the disease in question is potentially treatable and before cirrhosis appear.

In study two and three, all subjects were examined with a liver biopsy, and in study one all subjects were examined using transient elastography (described below). In study two and four, outcome data were obtained from national, population-based registers.

3.3.1 Liver biopsy (study 2 and 3)

The gold standard for diagnosis of a number of liver diseases is to perform a liver biopsy. Liver biopsies have been performed since the 1920:s, and allow a pathologist to evaluate the biopsy for a number of different characteristics, including steatosis, fibrosis, inflammation and bile duct damage (110). However, being an invasive procedure liver biopsy is associated with a number of risks. The mortality associated

with liver biopsy has been estimated to 1/1000 – 1/10000, and patients with cirrhosis are at an increased risk for mortality (110).

In study two, 149 biopsies from the 229 patients with NAFLD were re-examined by a single expert liver pathologist (R.H.), blinded to patient characteristics and scored for the NAS and for fibrosis scores. Six biopsies were of poor quality and were not used for analysis. Seventy-four liver biopsies were not available for re-examination, but had previously been reassessed by an experienced liver pathologist as part of one of the prior follow-up studies (47). There was a low reproducibility ($\kappa = 0.062$) of hepatocellular ballooning and lobular inflammation between the two pathologists. Therefore, these 74 patients were excluded from the analyses of NAS. However, they were still included in the analyses of fibrosis stage, as the agreement on fibrosis stage between the two pathologists was substantially higher ($\kappa = 0.73$)

In study three, the same expert liver pathologist examined all biopsies centrally and scored these for NAS and fibrosis scores.

3.3.1.1 NAFLD activity score (NAS)

As described in the introduction, the NAS is a semi-quantitative scoring system of biopsies from NAFLD patients. The score incorporates fat, ballooning and lobular inflammation. Fat is scored on a scale of 0-3, where 0 indicate that between 0-5% of the surface area of the biopsy is steatotic, 1 indicate that 5-33% is steatotic, 2 indicate that 33-66% is steatotic and 3 indicate that > 66% is steatotic. Ballooning is scored on a 0-2 scale, where 0 indicate no ballooning, 1 indicate few ballooned cells and 2 indicate many ballooned cells or prominent ballooning. Lobular inflammation is scored on a 0-3 scale, where 0 indicate no lobular inflammation, 1 indicate <2 foci per 200 x field, 2 indicate 2-4 foci per 200 x field and 3 indicate more than 4 foci per 200 x field. This yields a total score of 0-8 (38).

3.3.1.2 Fibrosis score

Fibrosis was scored according to the Kleiner fibrosis score (38) on a 0-4 scale, where 0 indicate no fibrosis, 1 indicates periportal or perisinusoidal fibrosis, 2 indicate perisinusoidal and periportal or portal fibrosis, 3 indicate bridging fibrosis and 4 indicate cirrhosis.

3.3.2 Transient elastography (study 1)

In study one, we did not consider it ethical to perform a liver biopsy in roughly 100 PSC patients with known disease and where a liver biopsy should add very little, if anything, to the individual prognosis. Instead, we performed transient elastography on all participants.

Transient elastography (TE) is a relatively new ultrasound-based technique to estimate the stiffness of the liver, and uses both ultrasound and low-frequency elastic waves formed by an air compressor probe, and whose propagation velocity is related to liver elasticity (111). This allows the estimation of liver fibrosis in a non-invasive manner. Results are expressed as kilopascals (kPa). The TE technique is most validated in patients with hepatitis C (112), but had at the time of study one also been tested in patients with PSC, where a cut-off of 17.3 kPa for detection of cirrhosis was found (113).

We divided the population into two subgroups: patients with significant and non-significant fibrosis. Significant fibrosis was defined as either elastography values ≥ 17.3 kPa, based on the available evidence (113), or a clinical diagnosis of cirrhosis diagnosed with histology or typical radiological and biochemical findings of cirrhosis (such as irregular hepatic parenchyma, splenomegaly, oesophageal varices, presence of intraabdominal collaterals) or previous manifestation of liver decompensation. In nine patients elastography failed, most often due to overweight or obesity. In six of these, presence of significant fibrosis was evident from clinical data and they were included into the “significant fibrosis” group. The three patients with no available information on fibrosis from either elastography or clinical data were excluded.

3.3.3 Swedish population-based registers (study 2 and 4)

The Nordic countries including Sweden are unique in regard to having extensive, detailed and population-based registers of its populations. This allows for high-quality epidemiological research with very low loss to follow-up. There are a number of registers, of which the causes of death register and the patient registers were used for study two and four. All of these registers are based upon the unique personal identification number.

3.3.3.1 The personal identification number

All Swedish citizens are given a unique, ten-digit personal identification number (PIN) after birth or immigration. This allows for linkage to and between registers (114).

3.3.3.2 Causes of death register

The Causes of Death Register (CDR) contains data from 1961 regarding the causes of death of all Swedish citizens, including if the person died abroad. It is mandatory for the responsible physician to report the underlying cause of death (e.g. stroke) and any disease that could have contributed to the death of the individual (e.g. atrial fibrillation).

3.3.3.3 Patient register

The National Patient Register (NPR) was established in 1964, and includes information on dates of hospital admissions, discharges, and diagnoses classified according to International Classification of Diseases (ICD) codes, 7-10. The register also includes information on hospital-based outpatient visits since 2001. The coverage of the register is approximately 99% of all somatic discharge diagnoses, and the validity of hospital discharge diagnoses is between 85-95% depending on diagnosis (115).

3.3.3.4 Total population register

The total population register contains data from the Swedish tax agency, and contains data on PIN, sex, age and living location as well as on emigration from Sweden. For study two, we used this register to create a matched control population. For each case with NAFLD, ten controls were selected and matched for sex, age and living location at the time of the initial liver biopsy.

3.3.4 Outcome variables

For study two, we examined causes of death in the cases that had died, with data drawn from the CDR. Cases with NAFLD and matched controls were cross-checked against the CDR, and disease-specific causes of death according to the ICD 8, 9 or 10 versions were obtained.

For study four, we used diagnoses of liver cirrhosis, decompensated liver disease (ascites, esophageal varices [bleeding or not bleeding], hepatorenal syndrome or hepatic encephalopathy), hepatocellular carcinoma or liver failure from the NPR, or death from any of the above in the CDR as our primary end point variable *severe liver disease*. This endpoint was chosen to identify as many true cases as possible, as all of these diagnoses usually lead to hospitalization and thus entry into the NPR, or death and thus entry in the CDR.

3.4 STATISTICS

3.4.1 Study 1

Continuous variables were analysed using the Mann-Whitney *U*-test or the Wilcoxon Signed Rank Test where appropriate. For comparison of categorical data the χ^2 analysis was used or, in the case of small expected frequencies, the Fisher exact test. For correlation tests of linear data, the Pearson *r* test was used. We controlled the results for duration of disease using co-variance analysis of variance. Statistical analyses were done using the Statistica® 9.1 software (StatSoft Inc., Tulsa OK) and SAS 9.2 software (SAS Institute Inc., Cary NC).

3.4.2 Study 2

Continuous variables are presented as mean (standard deviation), and categorical variables are presented as number (percentage). Histopathological agreement was analysed by the kappa (κ) coefficient. Analyses of mortality risks were done with the proportional hazard model (Cox regression), stratified on matching number. The model was tested for proportionality with Schoenfeld residuals. Survival curves were made according to the Kaplan-Meier method. All statistical analyses were performed with Stata v. 12.1 (StataCorp, College Station, Texas, US). $P < 0.05$ was considered statistically significant.

3.4.3 Study 3

Differences between continuous variables were analysed using the Mann-Whitney U-test, and between categorical variables using Fisher's exact test. We applied an ordinal logistic regression model to estimate the effect of alcohol consumption on the stage of fibrosis after checking for the assumption of proportionality, and a logistic regression model to estimate the effect of alcohol consumption on the presence of NASH. In a separate model, we divided the cohort into two groups, stratified on the median weekly alcohol consumption. We used a stepwise forward approach to identify any potential parameter associated with the studied outcome, using a p-value of ≤ 0.1 as significant and constructed one crude univariate model per parameter and one multivariate model including all significant parameters. The multivariate models included lifetime alcohol consumption per drinking week as the primary independent variable, and the full model was adjusted for age at biopsy, diabetes mellitus type 2, arterial hypertension, BMI and smoking status, dichotomized as ever or never. Data are presented as odds ratios (OR) with 95% confidence intervals (CI), with a significance level of 0.05. All analyses were performed using STATA version 12.1 (StataCorp, College Station, Texas, US).

3.4.4 Study 4

After exclusion of missing data, a final sample of 44 248 men was available. The association with BMI as a continuous variable was calculated using Mann-Whitney rank sum test for dichotomous variables and Spearman rank correlation for categorical and continuous variables. A multivariate Cox regression model was used to assess the effect of BMI on the outcome of severe liver disease. We tested BMI both as a continuous and as a categorical variable. We used a stepwise forward approach to identify any potential parameter associated with the primary outcome variable of severe liver disease, using a p-value of ≤ 0.1 as significant. The final models with BMI as a continuous variable and BMI as a categorical variable were consequently adjusted for alcohol consumption, use of narcotics, smoking, cognitive ability and high blood pressure at conscription. Estimates of the final models are presented as Hazard ratios (HR). The men were followed until the first registered diagnosis of severe liver disease,

death of any cause, emigration or the end of the follow-up period. After emigration the men were considered lost to follow-up but contributed with the time until emigration to the analysis. All analyses were performed in STATA 13.0 (StataCorp, College Station, Texas, USA) and a two-sided alpha value of 0.05 was used to test for statistical significance.

3.5 ETHICAL CONSIDERATIONS

The local ethical committee at Karolinska Institutet approved all studies in this thesis. For study one and three, individual written and oral informed consent was obtained in all cases.

4 RESULTS

4.1 STUDY 1

We identified 96 patients with PSC and complete data on exposure and outcome. There were 66% men, mean age was 47 ± 13 years (range: 22-75 years) and 73 patients (76%) were diagnosed with concomitant inflammatory bowel disease (IBD). Mean elastography value was 11.1 ± 8.2 kPa (range: 2.8-48 kPa). Seven patients (7.3%) had been diagnosed with PSC before they first started drinking alcohol. There were no cases of patients with Child-Pugh score of 10 (i.e., class C) or higher.

Mean lifetime alcohol intake was 3882 units (median: 2275 units, range: 0-20 270 units), giving a mean weekly consumption of 2.6 units per week. Only nine percent (9/96) drank equal to or more than one unit per day, and only one person had a mean consumption of more than two units per day. We classified 26 patients as having significant fibrosis, and 70 patients as not having significant fibrosis.

There were no significant differences in mean units of alcohol consumed per year between patients with significant and non-significant fibrosis. There was no correlation between yearly alcohol intake and elastography values. A scatterplot of mean drinks per year versus elastography values is presented in figure 6. To further evaluate if the drinking habits changed after PSC diagnosis, the LDH data were compared before and after PSC diagnosis. Among patients with non-significant fibrosis, we found an increase in total alcohol consumption after PSC diagnosis (111 units per year vs. 151 units per year, $P = 0.07$) whereas a decrease in total alcohol consumption after PSC diagnosis (103 units per year vs. 88 units per year, $P = 0.59$) was found in the significant fibrosis group. Binge-drinking before and after PSC diagnosis was 14.9 binges per year vs. 9.6 binges per year ($P = 0.24$) in the non-significant fibrosis group and 4.3 binges per year vs. 3.6 binges per year ($P = 0.5$) in the significant fibrosis group. The significant fibrosis group had lower CDT values (0.88% vs. 1.06%, $P = 0.02$) and lower PEth values (0.1 vs. 0.33, $P = 0.0016$) than the non-significant fibrosis group.

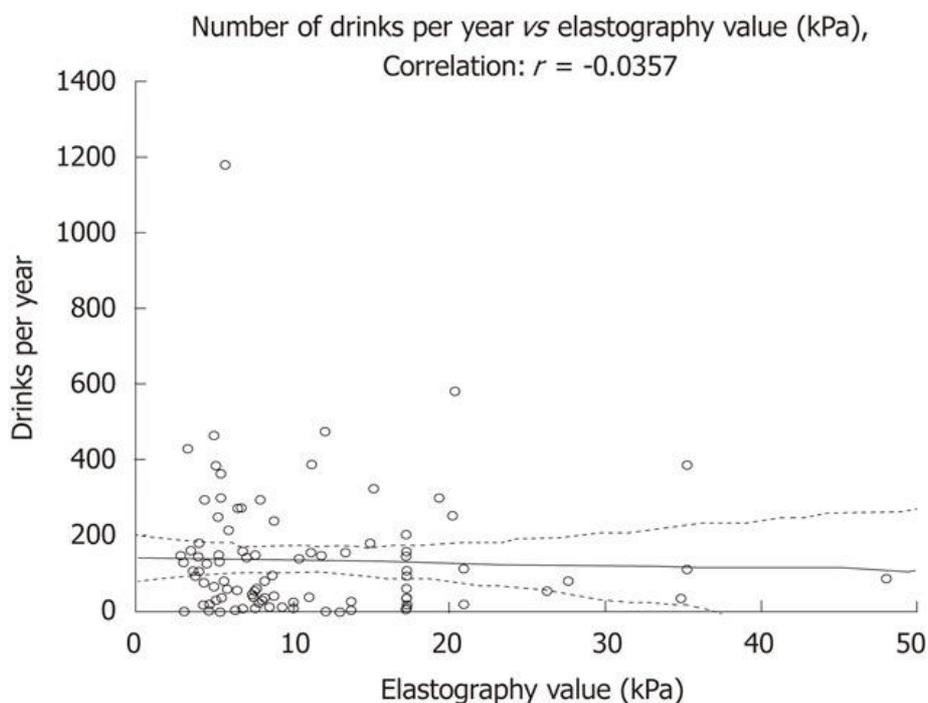


Figure 6. Scatterplot of drinks per year versus elastography value, expressed in kPa, from study 1. World Journal of Gastroenterology 2012. Published with permission.

4.2 STUDY 2

The original histology reports were available on all 229 patients from the two previous studies. Of the 149 patients with complete histological data, 76 patients (49%) had NAS 0-4 and fibrosis stage 0-2, 57 patients (37%) had NAS 5-8 and fibrosis stage 0-2, eight patients (5%) had NAS 0-4 and fibrosis stage 3-4, and finally eight patients (5%) had NAS 5-8 and fibrosis stage 3-4. Of the 74 patients in whom histology were missing for re-evaluation, 65 (86%) had fibrosis stage 0-2 and 11 (14%) had fibrosis stage 3-4.

The cohort was followed for a mean of 26.4 years (± 5.6 , range 6-33), or 5.400 person years. During follow-up, 96 patients and 786 individuals from the reference population died. In six patients and 40 reference individuals no cause of death was provided from the Registry of Causes of Death, which is either due to death in recent time or missing data entry from the reporting clinician. These patients were included in the analysis of overall mortality, but left out in the analysis of disease-specific mortality. Four patients emigrated during the study period and were lost to follow-up. These patients still contributed with 31 years of follow-up time to the analysis. Overall mortality in the entire cohort was significantly increased, with a hazard ratio (HR) of 1.29 (95% confidence interval [CI] 1.04-1.59, $P = 0.020$).

In the subgroup of patients with NAS 0-4 and fibrosis stage 0-2, overall mortality was not increased compared to the reference population (HR 1.13, 95% CI 0.79-1.60, $P=0.51$). There were no cases of HCC in this group. Death from events related to cirrhosis was significantly increased (HR 4.86, 95% CI 1.08-22.0, $P=0.04$). This was due to death of two patients with cirrhosis, with fibrosis stage 2 at baseline. Neither death from cardiovascular disease, non-gastrointestinal malignancy, or other diseases was significantly increased compared to the reference population.

In the subgroup of patients with NAS 5-8 and fibrosis 0-2, overall mortality was not increased (HR 1.41, 95% CI 0.97-2.06, $P=0.07$). Death from HCC was significantly increased (HR 15.67, 95% CI 4.1-59.86, $P < 0.001$). Mortality from cardiovascular disease (CVD) was not different from that of the reference population (HR 1.38, 95% CI 0.72-2.65, $P=0.34$). There was also an increased risk of death from respiratory diseases, e.g., chronic obstructive pulmonary disease or asthma (HR 3.95, 95% CI 1.2-13.0, $P=0.024$).

Apart from three deaths from HCC, there were no cases of death from events linked to cirrhosis in this group. All patients with HCC in this group had developed cirrhosis during follow-up, and the fibrosis stages at baseline were 0, 2, and 2, respectively, in these patients. Mortality from other diseases was not different compared to the reference population (data not shown).

There were no significant differences regarding any cause of death between the groups NAS 0-4 / fibrosis stage 3-4 and NAS 5-8 / fibrosis stage 3-4. Therefore, the data in this group were pooled and analysed together. The overall mortality in this group (NAS 0-8, fibrosis stage 3-4) was significantly increased (HR 3.3, 95% CI 2.27-4.76, $P < 0.001$).

A table of hazard ratios for disease-specific causes of death in the respective subgroups is presented in table 2, and Kaplan-Meier curves for the entire cohort and the respective subgroups is presented in figure 7.

Cause of Death	Entire Cohort (n = 229)	<i>P</i>	NAS 0-4, F0-2 (n = 76)	<i>P</i>	NAS 5-8, F0-2 (n = 57)	<i>P</i>	NAS 0-8, F3-4 (n = 16)	<i>P</i>
Overall mortality	1.29 (1.04-1.59)	0.020	1.13 (0.79-1.60)	0.511	1.41 (0.97-2.06)	0.072	3.28 (2.27-4.76)	<0.001
Cardiovascular disease	1.55 (1.11-2.15)	0.01	1.19 (0.65-2.20)	0.557	1.38 (0.72-2.65)	0.335	4.36 (2.29-8.29)	<0.001
Hepatocellular carcinoma	6.55 (2.14-20.0)	0.001	No outcome	—	15.7 (4.1-59.9)	<0.001	16.9 (1.95-146)	0.01
Cirrhosis	3.2 (1.05-9.81)	0.041	4.86 (1.08-22.0)	0.04	No outcome	—	10.8 (1.38-83.9)	0.023
Gastrointestinal malignancy	0.60 (0.22-1.64)	0.322	1.26 (0.60-2.65)	0.546	0.54 (0.075-3.96)	0.548	No outcome	—
Nongastrointestinal malignancy	1.18 (0.70-1.98)	0.545	1.24 (0.55-2.76)	0.602	0.85 (0.27-2.65)	0.778	No outcome	—
Infectious disease	2.71 (1.02-7.26)	0.046	3.12 (0.72-13.5)	0.129	2.22 (0.31-16.4)	0.435	13.0 (3.13-54.5)	<0.001
Respiratory disease	1.01 (0.31-3.32)	0.979	No outcome	—	3.95 (1.22-13.0)	0.024	No outcome	—

Table 2. Hazard ratios for disease-specific causes of death in the respective histopathological subgroups in study 2.

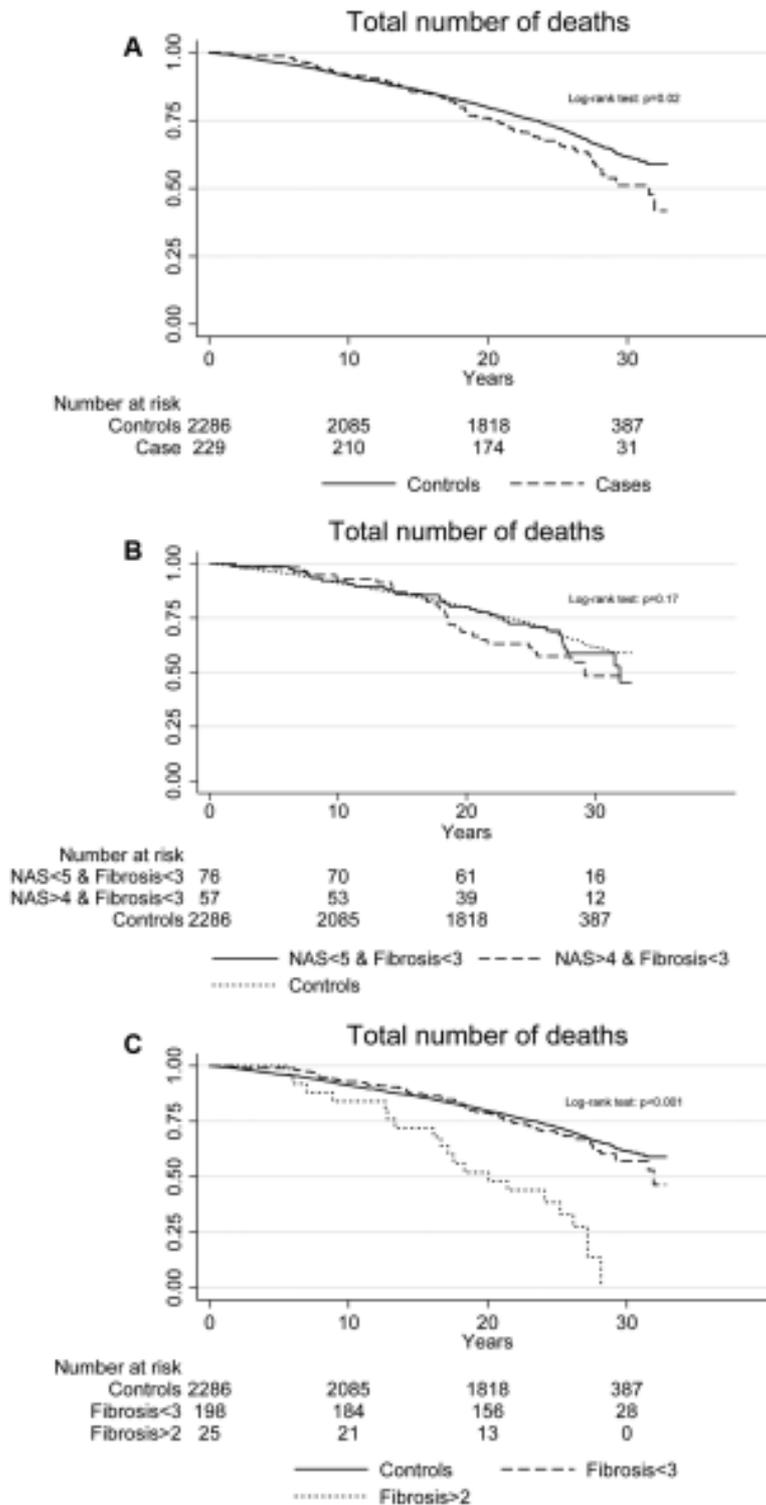


Figure 7. Kaplan-Meier curves for the entire cohort and the respective subgroups in study 2. Hepatology 2015. Published with permission.

4.3 STUDY 3

Between 2011 and 2015, 139 subjects were identified and gave informed consent. Of these, 7 had < 5% of fat in the biopsy and were excluded as were another eleven subjects who did not return the questionnaires despite reminder. In one case, the biopsy was unavailable for analysis. This left 120 patients for analysis. Furthermore, 13 subjects had a PEth value of ≥ 0.3 $\mu\text{mol/L}$ (median 0.52, range 0.31-1.05), indicating more pronounced recent alcohol consumption than reported at the visit or in the questionnaires; these 13 subjects were evaluated separately and not included in the primary statistical models.

4.3.1 Alcohol consumption

Lifetime consumption of alcohol in the cohort was reported at a median of 1.1 units per week (range 0-13.2). Binge-drinking occurred at a median of 1.2 times per year (range 0-45). There were no statistically significant differences between persons who consumed less versus more than the median weekly consumption of alcohol (1.1 units / week) in regard to any of the studied potential confounders.

4.3.2 Impact of alcohol on liver histology

Up to a maximum of 13 reported drinks per week, each additional unit in alcohol consumption was associated with decreasing odds ratios (OR) for having a higher fibrosis stage, both in the univariate model (OR, 0.86; 95% confidence interval [CI], 0.77-0.97; $p=0.016$) and in the multivariate model (adjusted [a] OR, 0.86; 95% CI, 0.76-0.97; $p=0.017$). Association between tested variables, including alcohol measurements, are presented in table 3.

When dividing the cohort in two groups, more or less than the median weekly lifetime alcohol consumption of 1.1 units/week, the 50 subjects who consumed more had lower odds ratios for a higher fibrosis stage than the 57 subjects who consumed less than 1.1 units/week, both in the univariate (OR 0.37; 95% CI, 0.18-0.75; $p=0.006$) and in the multivariate (aOR 0.38; 95% CI 0.18-0.80; $p=0.01$) analyses. Binge drinking was not associated with fibrosis stage (OR 0.97; 95% CI 0.93-1.01; $p=0.11$). When dividing binge drinking into quartiles (0, 0.1-0.9, 0.9-4.0, 4.0-45), the highest quartile (>4 episodes per year, $N=27$) did not have a higher fibrosis stage than the three lower quartiles (1.5 vs. 1.8, $p=0.38$).

Variable	OR, 95% CI	P-value	aOR, 95% CI	P-value
Units of alcohol per week ¹	0.86, 0.77-0.97	0.016	0.86, 0.76-0.97	0.017
Diabetes mellitus type 2	2.91, 1.40-6.05	0.004	2.63, 1.13-6.14	0.025
Hypertension	2.21, 1.10-4.47	0.027	1.35, 0.55-3.30	0.51
BMI ¹	1.06, 0.98-1.14	0.15	1.04, 0.97-1.13	0.28
Smoking, ever	1.46, 1.01-2.09	0.042	1.26, 0.84-1.89	0.27
Age at biopsy ¹	1.02, 1.00-1.04	0.042	1.00, 0.97-1.03	0.84
Drinks per drinking day ¹	0.95, 0.82-1.10	0.46	-	-
Number of binges per year ¹	0.97, 0.93-1.01	0.11	-	-
AUDIT score ¹	1.01, 0.88-1.17	0.86	-	-
Coffee consumption ²	1.01, 0.98-1.03	0.67	-	-
Soft drink consumption ²	1.00, 0.96-1.03	0.81	-	-
Physical activity ³	0.87, 0.64-1.20	0.40	-	-

Table 3. Crude and adjusted odds ratios for increasing fibrosis stage in study 3. ¹

For each unit increase. ²For each unit increase per week. ³For each step increase on a 6-digit scale. Abbreviations: OR, odds ratio. CI, confidence interval. aOR, adjusted odds ratio. BMI, body mass index. AUDIT, alcohol use disorders identification test.

The thirteen subjects with PEth values ≥ 0.3 $\mu\text{mol/L}$ had almost three-times increased odds ratios for having higher more advanced stages of fibrosis than subjects with PEth < 0.3 $\mu\text{mol/L}$, both in the univariate (OR 2.76; 95% CI 1.06-7.21; $p=0.038$) and in the multivariate models (aOR 2.77; 95% CI 1.01-7.59; $p=0.047$). Subjects with intermediate PEth values (0.05-0.3 $\mu\text{mol/L}$) did not have an increased risk for a higher fibrosis stage, compared to subjects with PEth < 0.05 $\mu\text{mol/L}$ (OR 0.80; 95% CI 0.30-2.10; $p=0.65$).

Crude odds ratios for associations of the study variables with the presence of NASH, as defined by the FLIP algorithm, are presented in Table 4. No association for any studied lifetime alcohol consumption parameter with a NASH diagnosis was found. Only smoking was identified as a potential confounder on NASH diagnosis in the univariate

analysis (OR 0.66; 95% CI 0.43-1.00; p=0.05) and a trend was noted for BMI (OR 1.08 for each unit increase in BMI, 95% CI 0.99-1.18; p=0.09). Soft drink or coffee consumption did not differ between subjects with and without NASH (mean 3.8 vs. 3.6 per week, p=0.67; and mean 13.4 vs. 15.5 cups per week, p=0.82, respectively).

Subjects with PEth \geq 0.3 $\mu\text{mol/L}$ had overall higher NAS (5.6 vs. 4.2, p=0.004) and a substantially increased risk of NAS \geq 5, both in the univariate (OR 7.58; 95% CI 1.60-35.87 p=0.01) and in the multivariate analysis (aOR 17.09; 95% CI 2.03-143.56, p=0.009).

4.3.3 Blood parameters

Subjects who consumed more than the median amount of 1.1 units of alcohol per week had lower values of inflammatory markers, including TNF-alpha (8.6 vs. 10.5 ng/L, p=0.05) and highly sensitive CRP (2.4 vs. 5.3 mg/L, p=0.004). There were no significant differences in lipid profiles or transaminases (data not shown).

Variable	OR, 95% CI	P-value
Units of alcohol per week ¹	0.98, 0.86-1.11	0.71
Diabetes mellitus type 2	1.01, 0.46-2.22	0.99
Hypertension	1.06, 0.49-2.32	0.88
BMI ¹	1.08, 0.99-1.18	0.09
Smoking, ever	0.66, 0.43-1.00	0.05
Age at biopsy ¹	1.01, 0.98-1.04	0.48
Drinks per drinking day ¹	0.99, 0.83-1.17	0.9
Number of binges per year ¹	0.99, 0.95-1.04	0.79
PEth \geq 0.3 $\mu\text{mol/L}$	3.55, 0.75-16.84	0.11

Table 4. Crude odds ratios in association with NASH defined as per the FLIP algorithm in study 3. ¹For each step increase. Abbreviations: OR, odds ratio. CI, confidence interval. BMI, body mass index. PEth, phosphatidyl ethanol.

4.4 STUDY 4

Out of the 44 248 men included in the univariate analyses, 2 935 (6.6%) were overweight with a BMI equal to or greater than 25 (mean BMI: 21.0, range: 12.9-44.6). Of these, 352 (0.8%) were obese with a BMI equal to or greater than 30. Baseline data per BMI category are presented in Table 5. All the covariates were significantly associated with level of BMI although the sizes of the difference were rather small. For example, level of alcohol consumption did not differ much between BMI categories (Table 5). Smoking was common at conscription with 58.4% reporting being a smoker. Although the proportion of non-smokers was similar across the BMI-categories, the proportion of men smoking more than 10 cigarettes a day increased with increasing BMI. Overall, the categories below a BMI of 25 displayed the lowest rate of poor health behaviours and highest ranks of health markers, except for use of narcotics that decreased with an increasing BMI.

The association for development of severe liver disease per covariate is presented as crude hazard ratios in Table 6. Alcohol consumption was associated to development of severe liver disease later in life in a dose-dependent fashion. Compared to drinking 1-100 grams of alcohol per week, the relative risk increased among those consuming 101-250 grams of alcohol per week (HR 1.82, 95%CI 1.48-2.30, $p < 0.001$) or more than 250 grams per week (HR 5.38, 95%CI 4.06-7.13, $p < 0.001$). Similarly, smoking was associated with development of severe liver disease later in life comparing to non-smokers in a dose-dependent fashion from 6-10 cigarettes per day (HR 1.43, 95%CI 1.09-1.89, $p < 0.001$) to more than 20 cigarettes per day (HR 4.74, 95%CI 3.38-6.64, $p < 0.001$). Likewise, use of narcotics, high blood pressure, poor self-rated health, low cardiovascular fitness and low cognitive ability were associated with development of severe liver disease later in life.

The men were followed for a period of in mean 37.8 years ($SD \pm 5.0$, range 0.1-39) or 1 674 527 person-years. During this time, 3 101 men died and 424 men, 0.96%, emigrated and were considered lost to follow-up. A total of 393 men were diagnosed with severe liver disease. Of these, 174 cases were first diagnosed with decompensated liver disease and 165 cases were first diagnosed with liver failure or cirrhosis as first diagnosis. In addition, 54 men died with liver disease reported as main or underlying cause of death. A total of 213 out of the 393 cases of severe liver disease died during the follow-up period. Mean time to the first diagnosis of severe liver disease was 24.7 years ($SD \pm 11.4$, range 3-39).

4.4.1 Body mass index as a predictor of severe liver disease

BMI as a continuous variable was significantly associated with an increased risk of severe liver disease, both in the univariate (HR 1.06 per each unit increase in BMI, 95%CI 1.02-1.09, $p = 0.002$) and the multivariate models (HR 1.05 per each unit

increase in BMI, 95%CI 1.01-1.09, p=0.008) (Table 7). When analysing BMI as a categorical variable, BMI 25-30 was associated with an increased risk for severe liver disease in the univariate model (HR 1.72, 95%CI 1.21-2.45, p=0.002) as well as in the multivariate model (HR 1.64, 95%CI 1.16-2.32, p=0.006) compared to participants with BMI 18.5-22.5 (Table 7). A Kaplan-Meier curve for the development of severe liver disease, stratified on overweight status is presented in figure 8.

BMI	<18.5	18.5-22.5	22.5-25.0	25.0-30	≥30	p-value
N	6119	28450	6744	2583	352	
Alcohol						<.001
<i>0 g/week (%)</i>	5.5	6.9	6.5	6.8	7.9	
<i>1-100g/week (%)</i>	70.7	71.5	69.1	69.9	67.9	
<i>100-250 g/week (%)</i>	20.3	18.8	20.3	19.3	19.9	
<i>>250 g/week (%)</i>	3.5	2.8	4.1	4.1	4.3	
Smoking						<.001
<i>Non-smoking (%)</i>	41.5	36.5	45.5	43.1	40.1	
<i>1-5 cig/day (%)</i>	11.6	11.5	10.4	8.4	8.2	
<i>6-10 cig/day (%)</i>	21.1	24.3	17.4	17.8	15.6	
<i>11-20 cig/day (%)</i>	22.6	24.3	22.9	25.7	31.3	
<i>>20 cig/day (%)</i>	3.3	3.4	3.9	5.1	4.8	
Use of narcotics (%)	13.7	12.0	10.0	7.1	6.8	<.001
High BP (%)	6.6	8.0	10.9	18.4	38.6	<.001
Cognitive ability (mean)	5.4	5.5	5.3	5.0	4.8	<.001
Self-rated health (mean)	1.9	1.8	1.8	1.9	1.9	<.001
Cardiovascular fitness (mean)	4.7	6.1	6.9	6.7	6.1	<.001

Table 5. Mean values per covariate, stratified on BMI-category in study 4. Associations between variables and BMI have been tested using Mann-Whitney rank sum test for dichotomous variables and Spearman rank correlation for ordinal and continuous variables. Abbreviations: BMI: Body Mass Index. BP: Blood Pressure. Cig: Cigarettes.

Variable	HR (95% CI)	p-value
Alcohol		
<i>0 g/week</i>	<i>.71 (.41-1.25)</i>	<i>.24</i>
<i>1-100 g/week</i>	<i>Ref</i>	
<i>100-250 g/week</i>	<i>1.85 (1.47-2.33)</i>	<i><.001</i>
<i>>250 g/week</i>	<i>5.37 (3.99-7.22)</i>	<i><.001</i>
Smoking		
<i>Non-smoking</i>	<i>Ref</i>	
<i>1-5 cig/day</i>	<i>1.15 (.77-1.72)</i>	<i>.50</i>
<i>6-10 cig/day</i>	<i>1.53 (1.13-2.06)</i>	<i>.005</i>
<i>11-20 cig/day</i>	<i>2.61 (2.02-3.37)</i>	<i><.001</i>
<i>>20 cig/day</i>	<i>5.17 (3.61-7.41)</i>	<i><.001</i>
Use of narcotics	3.17 (2.55-3.95)	<.001
High BP	1.29 (0.95-1.77)	.10
Cognitive ability ¹	.88 (.85-.93)	<.001
Self-rated health ²	1.24 (1.12-1.37)	<.001
Cardiovascular fitness ¹	.90 (.86-.95)	<.001

Table 6. Crude HRs for development of severe liver disease per covariate in study 4.

¹Hazard ratios for each step increase on the 9-point scales. ²Hazard ratios for each step increase on the 5-point scale. Abbreviations: SD: Standard Deviation. HR: Hazard Ratio. CI: Confidence Interval. BP: Blood Pressure. Cig: Cigarettes.

BMI	Crude	p-value	Adjusted ¹	p-value
Continuous ²	1.06 (1.02-1.09)	0.002	1.05 (1.01-1.09)	0.008
Categorical				
<18.5	1.12 (0.84-1.50)	0.45	1.11 (0.83-1.49)	0.47
18.5 – 22.5	1.0	Ref	1.0	Ref
22.5 – 25	1.15 (0.87-1.51)	0.34	1.17 (0.89-1.55)	0.26
25-30	1.72 (1.21-2.45)	0.002	1.64 (1.16-2.32)	0.006
> 30	1.82 (0.75-4.40)	0.19	1.59 (0.64-3.95)	0.31

Table 7. Crude and adjusted hazard ratios with 95% confidence intervals for development of severe liver disease depending on BMI at time of conscription in study 4.

¹Adjusted for alcohol consumption, use of narcotics, smoking, high blood pressure and cognitive ability at conscription. ²HR corresponds to increased risk for severe liver disease per each unit increase in BMI.

Abbreviation: BMI: Body Mass Index.

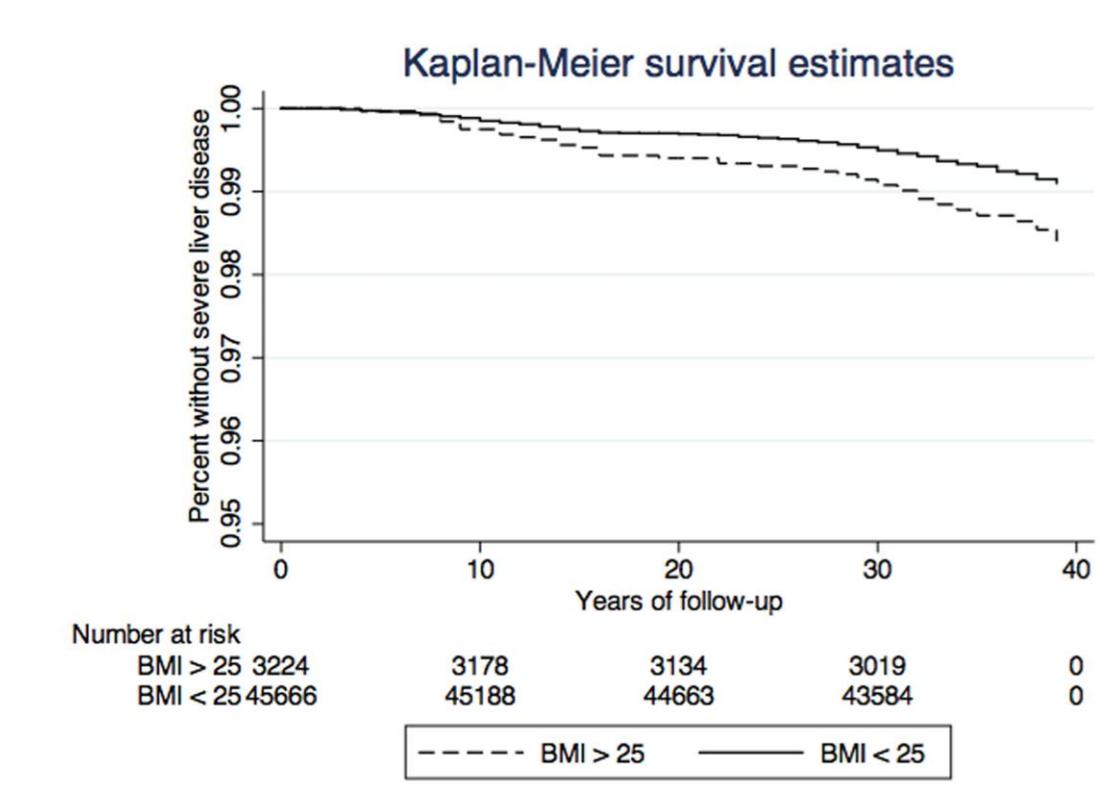


Figure 8. Kaplan-Meier curve for the development of severe liver disease, stratified on overweight status in study 4.

5 DISCUSSION

5.1 METHODOLOGICAL CONSIDERATIONS

5.1.1 Study design

When conducting medical research, one can basically perform either an experimental or an observational study. All studies in this thesis are examples of observational studies, using different methodologies. Study one and three are cross-sectional studies, where one group is compared to another, with no consideration of a temporal aspect. In contrast, study two and four are examples of cohort studies, where time is used as a parameter. In a cohort study, subjects that are or are not exposed to a potential harmful or beneficial factor (the exposure) are followed over time, and the outcome of interest is studied along the path. Differences between the exposed and unexposed groups are then calculated.

5.1.2 Bias

All research carries the risk of error, and medical research is no different. One differs between random errors, which can usually be handled by increasing the sample size of a studied population, and systematic errors, or bias (116). Bias must be dealt with either in the design of the study, or in its interpretation.

5.1.2.1 Selection bias

A selection bias is a danger to most medical studies. This can occur when there are differences between the exposed and the unexposed groups in a study in regard to how they were selected for the study, or if they stayed in the study for the entire study period or not. For example, if study subjects for a screening study are recruited by newspaper ads, individuals that are either at a higher risk for the studied outcome, or more concerned with their health might be over-represented, and thus introducing a bias to the study. Another example is the “healthy worker effect” (117). If studying the effect of an exposure on a specific outcome in workers, for instance the incidence of lower back pain in construction site workers and comparing this to the general public, a selection bias is introduced, as the general public is partly composed of persons that are too weak to work, and might have a higher risk of having lower back pain.

5.1.2.2 Information bias / misclassification

Misclassification can occur if there is an error in how study participants are classified as exposed or unexposed, or if they reach they study outcome or not. For example, persons drinking large amounts of alcohol might underreport their alcohol consumption, and be classified as non-drinkers. A specific form of misclassification is recall bias, where persons with a specific outcome, for instance myocardial infarction might recall their studied exposure, for instance past smoking history, different than persons without that specific outcome.

5.1.2.3 Confounding

Confounding is very common in epidemiological studies, and must almost always be dealt with. Per definition, a confounder is a parameter that is both associated with the exposure and the outcome variable, but not a link in the causal pathway. A classic example of this is the risk of alcohol on development of lung cancer. Persons who drink alcohol often smoke, so if not taking smoking into account when studying this would most likely lead to inflated estimates on the risk of lung cancer in persons who drink alcohol. A schematic of confounding is presented in figure 9.

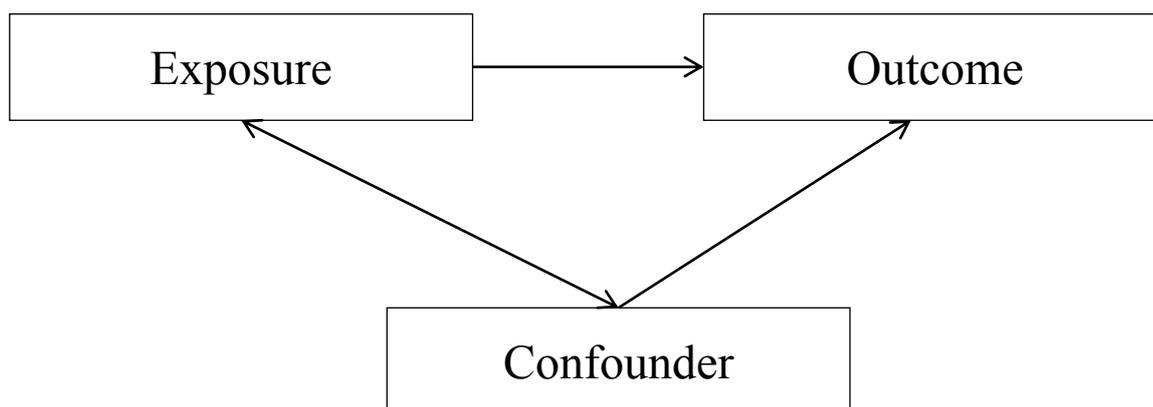


Figure 9. Schematic of the confounding bias principle. The confounding variable is associated both with the exposure and the outcome, but is not a part of the causal pathway.

5.1.3 Potential biases in the thesis

There are several potential biases in the current thesis, which have been dealt with through different means. In study one and three, the main risk of bias is the recall bias of the LDH questionnaire. Study subjects might, knowingly or unknowingly, under- or over-report their past or current alcohol consumption. However, the LDH questionnaire has been validated and displays high test-retest reliability (103, 104). Also, removing subjects in study three with high PEth values should have reduced this type of bias. Selection bias should be minimal in study one, as PSC patients are rare, and nearly all patients in the Stockholm area were attending regular visits at our clinic at the time of the study. There is on the other hand a risk for selection bias in study three, as these patients were recruited from tertiary setting hospitals, and thus we might have selected cases with a higher disease activity than the common NAFLD patient. However, the results of the study should be generalizable to at least other university hospital patient groups.

The same type of bias could be present in study two, that was also based on NAFLD patients that underwent liver biopsy. However, in that case all biopsies were performed due to increases in liver enzymes, and not of suspicion of manifest liver damage.

Study four uses a different methodology than the previous three studies in that study participants were not selected from a larger group of potential study subjects, but instead we

had access to around 97% of the total Swedish male population in the relevant age categories at the time. This heavily reduces the risk for selection bias. Outcomes were selected to reduce the risk for detection bias as much as possible, by using only cases with severe liver disease that almost always leads to hospitalization or death and thus capture in the relevant registers.

One limitation in study 4 is that it only includes men and the results may not be generalizable to women. There are previous studies indicating that a high BMI is a risk factor for future liver disease also in women. For example, Liu and co-workers found a high BMI a significant attributable factor for development of liver cirrhosis in women in the British Million Women Study, (118) but studies on young women are lacking.

5.2 FINDINGS AND IMPLEMENTATIONS

The role of low-grade alcohol consumption in patients with liver disease has not been studied extensively. It has, despite the lack of evidence, been the general consensus between clinicians that patients with any liver disease should not consume alcohol at all. Depending on the specific liver disease and the stage of disease, this may or may not be correct. For instance, low-grade alcohol consumption has been associated with a reduced risk of cardiovascular mortality (79). Patients with NAFLD have an increased risk for death in cardiovascular disease, so for instance consuming 1-2 drinks per day could actually be beneficial. On the other hand, alcohol has also been associated with increased risk for a number of malignancies, for instance breast and colon cancer (81, 82). Thus, an individualized approach regarding alcohol consumption should be sought when evaluating a patient with any liver disease.

In study one, we evaluated a large cohort of patients with the rare disease PSC. Our main finding was that alcohol consumption of around one drink per day was not associated with higher stages of fibrosis, as evaluated by transient elastography or clinically evident cirrhosis. The use of the LDH questionnaire allowed us to track lifetime changes of alcohol consumption, and we found that PSC-patients with significant fibrosis had reduced their alcohol intake after the diagnosis of PSC, compared to patients with no significant fibrosis. This could reflect that patients with more severe disease feel less well and might try to reduce any additional damage to the liver. We found no difference in BMI between patients with and without significant fibrosis.

PSC is an incurable, chronic liver disease, which is also associated with a reduced quality of life (119). Low-grade alcohol consumption, for instance a glass of wine per day, in patients with PSC should therefore be considered safe in patients who feel that this could improve their quality of life.

The link between alcohol and liver disease has been known for a long time. As alcohol consumption almost invariably leads to fat accumulation in the liver through stimulation of de novo lipogenesis and reduced beta-oxidation (120), it was long thought that all patients with fatty liver were consuming large amounts of alcohol, and were not telling clinicians the truth about their alcohol habits. The first finding that patients who very credibly were not

alcoholics came from the Mayo clinic in 1980, where Ludwig et al showed a remarkably similar histological image to alcoholic steatohepatitis in obese patients with in many cases the metabolic syndrome, and named it non-alcoholic steatohepatitis (NASH) (121). Since then, the field of NAFLD research has exploded, with an exponential growth of published articles to match the increase in prevalence of obesity and NAFLD. The prognosis and natural history of NAFLD is heterogeneous and still poorly understood, with many potential factors able to influence the progression of the disease.

In study two, we performed a study of markers for overall mortality in patients with NAFLD with the hitherto longest duration of follow-up ever presented. We show that the most important factor when evaluating future risk for mortality in a patient with NAFLD is the presence of significant fibrosis in the liver. We found no significant impact on mortality for the commonly used NAS scoring system, although a trend was noted for overall mortality and an increase in the risk of death due to HCC was found (table 2). Our findings were recently corroborated in another large follow-up study by the late Paul Angulo et al. This study showed no excess mortality in patients with a high NAS, but indicated that fibrosis stage was highly associated with mortality (122).

The NAS is currently used as an endpoint in on-going phase III studies with thousands of patients, but if it cannot accurately predict mortality, it could be argued that other endpoints should be used instead, as a valid endpoint should measure how a patient feels, functions or survives either directly or through a proxy variable (123). Indeed, most of the current large clinical trials also use reduction of fibrosis stage as an endpoint, but it remains to be seen if this effect also has an impact on mortality. Apart from the extended follow-up in this study, there were several strengths. All patients were diagnosed with gold standard liver biopsy, there were very low loss to follow-up through population based registers, and even though the study was relatively small with 229 patients, this still represents one of the largest biopsy-proven NAFLD cohorts worldwide. Nevertheless, results should be interpreted with the relatively small sample size in mind.

Study three was similar to study one, in that we used the LDH questionnaire to examine the effect of lifetime alcohol consumption on disease severity in a chronic liver disease, this time looking at NAFLD. Our main finding was that an increase in alcohol intake, up to 13 units per week, was associated with a lower risk for having liver fibrosis, using an ordinal regression model. Conversely, subjects with PEth values over the established cut-off of 0.3 $\mu\text{mol/L}$ had an increased risk for liver fibrosis. This indicates that the risk profile for alcohol consumption on the risk of liver fibrosis has a J-like shape, with the lowest risk for persons drinking 1-2 units of alcohol per day. This is interestingly similar to the risk profile for alcohol consumption on the risk for cardiovascular disease. A number of studies have showed that regular alcohol consumption of around 1 unit per day in women and 1-2 per day in men seem to reduce cardiovascular morbidity and mortality (124-127). Patients with NAFLD and low stages of fibrosis (0-1) who consume alcohol in low to moderate amounts, i.e. below 13 units per week, should not be advised to stop doing so. However, individuals with NAFLD

with concomitant risk factors for alcohol-associated malignancies should be given an individual risk profile.

In study four, we found that overweight and a high BMI in late adolescence were associated with a higher risk for development of severe liver disease up to 39 years later. This risk was not affected by alcohol consumption, smoking or other potential confounders at baseline. Overweight persons had a 64% increased risk for development of severe liver disease, and a one unit increase in BMI (kg/m^2) was associated with a 5% increased risk for this outcome. Overweight and obesity have been implicated as risk factors for liver disease in previous studies. For example, Ioannou et al showed that in persons without cirrhosis who drank little alcohol, obesity was associated with a 4.1 times increased risk of death in or hospitalization for liver disease after a mean of 12.9 years follow-up. That study was composed of older individuals (25-74 years of age), with a lower number of participants (N=11 465) and a shorter follow-up duration. Thus, our study is larger and we display a longer follow-up time, why our estimates should be more accurate. However, we had very few individuals with obesity in this study (0.8%), which is most likely why we see no significantly increased risk in this subpopulation.

The strengths of this study are the large population-based cohort (n=44 248), very long follow-up time (39 years) and low (1%) loss to follow-up, which minimizes the risk of selection bias. Also, investigating men of a relatively low age as in the present study minimizes the risk for reverse causality regarding alcohol, since a long-term use of alcohol can also lead to overweight and obesity. We had access to detailed and credible baseline data regarding exposure status (height and weight) on almost the entire male population from the study period, as well as on a multitude of possible confounders such as alcohol consumption and use of narcotics. The national, population-based registers used for ascertaining outcome status are validated and a source of very high quality data. The use of liver decompensation and cirrhosis, which in almost all cases leads to hospitalization at some point, and liver-related death as a joint outcome variable for severe liver disease, allowed us to minimize bias regarding the outcome status. However, there were generally too few disease-specific outcomes, such as HCC for disease-specific analyses, which would require a larger cohort. For instance, overweight and obesity in early childhood has been shown to increase the risk of development of HCC in a large Danish cohort study with 285 884 subjects (128).

The prevalence of overweight and obesity has risen sharply since the inclusion period in this study (129), although the high prevalence of obesity seems to have flattened out during the last decade, at least in the US (130). The mean time from conscription to development of severe liver disease in this study was long, on average 25 years. It is highly likely that there will be more cases of severe liver disease in the future, which could affect health policy decisions. The current study suggests that the increased risk of a high BMI for the development of severe liver disease later in life is present already from an early age. It is possible that this increased risk is caused by a longer exposure to being overweight, compared to becoming overweight or obese later in life, and that individuals with a longer

history of being overweight have an increased risk of severe liver disease. This could have implications in the care of for example patients with NAFLD with a short versus a long duration of being overweight and should be explored in future studies.

6 CONCLUSIONS

- Low grade alcohol consumption is not associated with significant fibrosis in PSC.
- Low to moderate alcohol consumption, up to a maximum of 13 units per week, is associated with a lower fibrosis stage in NAFLD.
- The most robust marker for overall and disease-specific mortality in NAFLD is fibrosis stage. Future clinical trials should use reduction of fibrosis stage as an endpoint.
- A high BMI and overweight in late adolescence is associated with an increased risk for development of severe liver disease. Lifestyle modification in early adulthood including weight loss should be advised by health policies to reduce this risk.

7 FUTURE RESEARCH

One previous study has suggested that alcohol consumption could be a risk factor for development of cholangiocarcinoma (CCC) in patients with PSC. Study subjects with PSC in this thesis consumed low amounts of alcohol, and the cross-sectional methodology did not allow us to study the impact of alcohol on the risk of CCC development. This should be explored in future studies, and we are planning to follow up this very well-defined cohort in a subsequent study in the near future, specifically looking at the risk of alcohol consumption on CCC development.

The main finding of one of the studies in this thesis is that fibrosis is the strongest predictor for disease-specific mortality in NAFLD. However, this still requires liver biopsy, which is impractical to perform in the large group of subjects with possible NAFLD. Studies are needed to identify subjects at risk for development of fibrosis, preferably using non-invasive methodologies. Also, larger studies of subjects with NAFLD are required to identify which patients that have an increased risk for morbidity, such as development of diabetes mellitus type 2 and cardiovascular disease. The “magic bullet” in NAFLD research at the moment is identification of reliable, inexpensive and non-invasive biomarkers to identify subjects with NASH and fibrosis, respectively. This would allow researchers and industry to perform large clinical trials without the need for liver biopsy. We are currently investigating the effect of insulin-like binding protein 1 (IGF-BP1) as a marker of fibrosis in NAFLD, as well as participating in a study looking at performance of the Enhanced Liver Fibrosis (ELF) score in NAFLD.

The finding that a high BMI in late adolescence is a risk factor for development of severe liver disease suggests that a longer duration of being overweight or obese is more harmful to the liver than becoming overweight later in life. This should be explored in future studies. Also, even with almost 45 000 study subjects, examining the effect of a high BMI on specific rare outcomes such as hepatocellular carcinoma was not possible in the current study. Future studies should include more study subjects, or a case-control methodology could be applied.

In study three, we found that alcohol consumption is associated with lower stages of fibrosis in NAFLD, and that alcohol was associated with lower markers of inflammation, including TNF-alpha. Even though we had access to detailed data on possible confounders, due to the cross-sectional methodology, there could be residual confounding as well as other potential biases. A very interesting study would be to perform a randomized controlled study looking at low alcohol consumption versus abstinence in patients with established NAFLD.

There is currently no established treatment for NAFLD, but there is considerable interest from the pharmacology industry, as the global revenue for a potential drug against NASH is estimated to 20-30 billion USD annually. At the moment, two large phase III studies are ongoing, but in phase II these drug candidates have either not shown impressive results or have caused problematic side-effects. We are currently exploring the possibility to perform a proof-of-concept study of a diet intervention to treat NAFLD, basically free of side-effects

and very inexpensive. This would allow patients with NAFLD to remain free of medications and most likely reduce the economic burden on society.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Leversjukdom är en allt vanligare orsak till död i världen. Traditionellt förknippar man framför allt leversjukdom med alkoholberoende, och många patienter med leversjukdom av annan anledning känner sig stigmatiserade på grund av detta. Tvärtemot denna myt så är den vanligaste leversjukdomen idag fettlever. Detta är nära kopplat till övervikt och höga nivåer av insulin i blodet, och innebär att fett inlagras i levern. Fettlever finns i nuläget hos 20-30% av den amerikanska befolkningen. Det är oklart vilka faktorer hos denna stora grupp som bäst förutsäger vilka som har en ökad risk att avlida. Man uppskattar att fettlever kommer vara den vanligaste orsaken till behov av levertransplantation inom en nära framtid. I Sverige är i nuläget en annan, ovanlig leversjukdom den vanligaste orsaken till behov av levertransplantation. Vid primär skleroserande kolangit (PSC) drabbas gallgångarna i och utanför levern av oklar anledning av sjukdom och skrupnar ihop. Detta leder i slutändan till skrumplever och patienterna har en starkt ökad risk för cancer i lever och gallgångar.

Det är oklart om en viss alkoholkonsumtion vid fettlever och PSC är säkert. Vi studerade därför detta hos två grupper av patienter med dessa sjukdomar. Vi fann att låggradig konsumtion av alkohol vid PSC inte var förenat med en svårare leversjukdom, och att låggradig alkohol konsumtion motsvarande max sju enheter per vecka är säkert vid PSC. För fettlever fann vi att alkoholkonsumtion, upp till max tretton enheter per vecka hade en skyddande effekt på utveckling av skrumplever. Däremot hade patienter som hade tecken till en högre alkoholkonsumtion än så också en högre risk för skrumplever.

Vi undersökte i en studie vilka faktorer i levern som bäst kan förutsäga sjukdomsspecifik dödlighet. Vi använde oss av en unik grupp med 229 patienter med fettlever som hade följts över i medel 26 år och kopplade denna grupp till befintliga register över dödsfall och sjukhuskontakter. Vi fann att den viktigaste prognostiska faktorn för sjukdomsspecifik dödlighet var mängden av bindväv som fanns i levern.

Övervikt och fetma har kopplats till en ökad risk att utveckla leversjukdom. Det är dock oklart om övervikt i sig självt är kopplat till utvecklingen av leversjukdom, eller om överviktiga t.ex. dricker mer alkohol än normalviktiga. För att studera detta analyserade vi data från cirka 45 000 män som genomgick mönstring under perioden 1969-70. Under mönstringen fick männen svara på detaljerade frågor om sin alkoholkonsumtion samt andra livsstilsmönster. Längd och vikt registrerades. Vi kopplade dessa data till samma register som i den föregående studien och fann att övervikt i sig, även efter att ha tagit alkoholkonsumtion i beaktande, var en oberoende riskfaktor för utveckling av svår leversjukdom upp till 39 år senare i livet.

Sammanfattningsvis är resultaten från denna avhandling att det är säkert för patienter med PSC att dricka upp till en enhet alkohol per dag och att låggradig alkoholkonsumtion kan vara skyddande vid NAFLD. Vidare är den viktigaste riskfaktorn för död i NAFLD hur mycket bindväv det finns i levern, och övervikt är en oberoende riskfaktor för utveckling av svår leversjukdom.

9 ACKNOWLEDGEMENTS

All patients that contributed to these studies – without you the work could never have been accomplished.

Per Stål, my main supervisor for your infinite patience, deep knowledge in hepatology and your support for all my crazy ideas.

Rolf Hultcrantz, my co-supervisor for your street-smartness, answers to all my questions, teaching me liver histology and access to your vast international research network.

Annika Bergquist, my co-supervisor and head of the Centre for Digestive Diseases, for being a role model both as a clinician, leader and a scientist. Enthusiastic, empathic and experienced.

Jan Bolinder, head of the Department of Medicine, Huddinge for creating a good research environment.

Anna Andreasson, for your expertise in epidemiology, research methodology and putting up with my lack of patience. And all the cakes! I hope we can do some nice studies in the future.

Patrik Nasr, Mattias Ekstedt and Stergios Kechagias, my co-authors and kindred NAFLD nerds. See you at the next conference!

Knut Stokkeland, for introducing me to the LDH tool and always answering my questions.

Mattias Carlsten, my mentor for advice in all aspects of research and life. And wine!

Tomas Hemmingsson, for allowing me to use the conscription cohort in study 4.

Pia Loqvist, for assistance with patients and logistics during study 1 and 3.

Annika Karlsson, for always answering my logistical questions with the same enthusiasm.

Everybody in the SILK network.

Maria Altman, my teacher in epidemiology, for opening my eyes to this field of research.

Matteo Bottai, my teacher in biostatistics.

Erik, Lina, Pia, Laura, Ghazaleh, Martin & Åsa. My residence colleagues at the Centre for Digestive Diseases, for all the advice, laughs and discussions.

Niklas, Jonas, Gösta. Söder bröder glöder.

My family. Anna, Martin, Sally & Valter. For getting my mind off research, and all the food! Maud, my dear mother for always being there, no matter what. I love you. Ingrid, my beloved daughter for the insight that there are better things in life than work.

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