# Liver-related complications and metabolic comorbidities during long-term follow-up of patients with Non-Alcoholic Fatty Liver Disease

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# Liver-related complications and metabolic comorbidities during long-term follow-up of patients with Non-Alcoholic Fatty Liver Disease

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Liver-related complications and metabolic comorbidities during long-term follow-up of patients with Non-Alcoholic Fatty Liver Disease


Abstract

Background: Non-alcoholic fatty liver disease (NAFLD) is highly associated with the metabolic syndrome, and due to increasing prevalence of for example obesity it is now the most common liver disease in the world. A minority progress to advanced fibrosis/cirrhosis, which is associated with increased mortality, but it is not entirely clear which patients who have an increased risk of fibrosis.

General aim:To describe the long-term clinical development and prognosis of biopsy-proven NAFLD, focusing on liver-related morbidity, metabolic comorbidities and mortality.

Methods: In Paper 1, patients with long-term insulin resistance, a risk factor for developing NAFLD, were invited to assessment of liver function tests and if elevated patients were further examined for a diagnosis of NAFLD. In Paper 2-4, all patients with biopsy-proven NAFLD in Malmö, Sweden 1978-2006 were identified, and further assessed with an extensive review of patients' medical files regarding long-term risk of cirrhosis development, liver-related events, metabolic comorbidities, chronic kidney disease and mortality, and the use of non-invasive fibrosis scoring system in early indentification of these risk patients. Follow-up time in all four papers were between 17-27 years.

Results: Only 15% (n=25) of patients with long-term insulin resistance in Paper 1 had elevated liver function tests at long-term follow-up, and of these only 23.8% had NAFLD diagnosed with imaging. Patients with NAFLD had significantly higher prevalence of the metabolic syndrome and progressive insulin resistance (type 2 diabetes mellitus (T2DM) or impaired fasting glucose). Of all patients with biopsy-proven NAFLD included in Paper 2-4 survival was significantly lower than a reference population. The prevalence of cirrhosis at follow-up was 17%, and 13.8% developed liver-related events. Hepatocellular cancer (HCC) was diagnosed in nearly 6% of patients. The most common metabolic comorbidity at follow-up was hypertension in 66% af patients, and T2DM in 53%. NAFLD patients with advanced fibrosis (stage 3-4) had significantly higher prevalence of T2DM. Chronic kidney disease (CKD) was prevalent in 12.5% at inclusion, but only significantly higher in the highest age group (> 55 years). At follow-up 37.5% had developed CKD, however not significantly different to the reference group. NAFLD patients with long-term CKD had significantly higher mortality, which was explained by an increased prevalence of metabolic comorbidities including T2DM, not CKD per se. When calculating simple non-invasive fibrosis scoring systems (inclucing NAFLD fibrosis score and FIB-4 index) from the time of biopsy, these could with acceptable accuracy identify NAFLD patients with an increased risk of overall mortality, future liver-related events, T2DM, cardiovascular disease and CKD.

Conclusions: NAFLD development in patients with long-term insulin resistance is associated with a progress of metabolic comorbidities. Of all patients with biopsy-proven NAFLD 17% developed cirrhosis and 6 % HCC at long-term follow-up. Overall mortality is significantly higher in NAFLD than in a reference population. Long-term CKD in NAFLD is associated with increased overall mortality, which is explained by metabolic comorbidities. Simple non-invasive fibrosis scoring systems can be used for early identification of NAFLD patients with increased risk of future liver-related events and overall mortality, but also of future metabolic comorbidities and CKD.



Key words Chronic kidney disease, CKD, Epidemiology, Fibrosis, Hepatocellular cancer, Insulin resistance, Liver cirrhosis, Metabolic syndrome, NAFLD; NASH; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis

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# List of papers

The thesis is based on the following original papers:

1. **Önnerhag K**, Nilsson PM, Lindgren S. Insulin resistance with impaired fasting glucose increases the risk of NAFLD. Open Journal of Gastroenterology. 2013; 3:170-176.

2. **Önnerhag K**, Nilsson PM, Lindgren S. Increased risk of cirrhosis and hepatocellular cancer during long-term follow-up of patients with biopsy-proven NAFLD. Scand J Gastroenterol. 2014; 49(9):1111-8.

3. **Önnerhag K**, Dreja K, Nilsson PM, Lindgren S. Increased mortality in non-alcoholic fatty liver disease with chronic kidney disease is explained by metabolic comorbidities. Clin Res Hepatol Gastroenterol. 2019. PMID: 30827925. Article in Press.

4. **Önnerhag K**, Hartman H, Nilsson PM, Lindgren S. Non-invasive fibrosis scoring systems can predict future metabolic complications and overall mortality in Non-Alcoholic Fatty Liver Disease (NAFLD). Scand J Gastroenterol. 2019. Accepted manuscript.

# Abbreviations

| | |
|---|---|
| AFLD | Alcoholic Fatty Liver Disease |
| ALT | Alanine Aminotransferase |
| AST | Aspartate Aminotransferase |
| AUROC | Area-under-the ROC-curve |
| BMI | Body Mass Index |
| CI | Confidence Interval |
| CKD | Chronic Kidney Disease |
| CKD-EPI | Chronic Kidney Disease Epidemiology Collaboration |
| CT | Computer Tomography |
| CVD | Cardiovascular Disease |
| DNL | De Novo Lipogenesis |
| eGFR | Estimated Glomerular Filtration Rate |
| FIB-4 | Fibrosis-4 Index |
| γ-GT | Gamma Glutamyl Transferase |
| HCC | Hepatocellular Cancer |
| HOMA-IR | Homeostatic Model Assessment of Insulin Resistance |
| HR | Hazard Ratio |
| HT | Hypertension |
| ICD | WHO International Classification of Diseases |
| IFG | Impaired Fasting Glucose |
| IQR | Interquartile Range |
| IR | Insulin Resistance |
| LFTs | Liver Function Tests |

| | |
|---|---|
| MDCS | Malmö Diet and Cancer Study |
| MPP | Malmö Preventive Project |
| MRI | Magnetic Resonance Imaging |
| MRS | Magnetic Resonance Spectroscopy |
| MetS | Metabolic Syndrome |
| NAFL | Non-Alcoholic Fatty Liver |
| NAFLD | Non-Alcoholic Fatty Liver Disease |
| NASH | Non-Alcoholic Steatohepatitis |
| NAS | NAFLD Activity Score |
| NEFA | Non-Esterified Fatty Acids |
| NFS | NAFLD Fibrosis Score |
| OR | Odds Ratio |
| PEth | Phosphatidyl Ethanol |
| ROS | Reactive Oxygen Species |
| SAF score | Steatosis Activity Fibrosis Score |
| SD | Standard Deviation |
| SREBP-1 | Sterol Regulatory Element Binding Protein 1 |
| T2DM | Type 2 Diabetes Mellitus |
| TG | Triglycerides |
| US | Ultrasonography |
| VLDL | Very Low-Density Lipoprotein |

# Introduction

## Background

> Fat people have fat livers [1].

The above statement has been known for many decades. Accumulation of hepatic fat in patients with various metabolic risk factors, such as obesity, and without obvious alcohol overconsumption, was first described in the mid-1900s [2-4]. It was not until 1980 it became recognized as a significant disease when Ludwig et al described histopathological changes including steatosis, inflammation and even cirrhosis in patients with obesity and type 2 diabetes mellitus (T2DM) [5]. The condition was then named Non-Alcoholic Steatohepatitis (NASH).

Over the years it has become clear that NASH is a specific histopathological disease which requires liver biopsy to separate it from non-NASH. This has led to a change in nomenclature. Today we refer to the condition as Non-Alcoholic Fatty Liver Disease (NAFLD), which encompasses the entire spectrum of disease.

In the past few years there has been a tremendous increase in research articles involving NAFLD. In March 2019 there were over 16,000 PubMed citations for the search "NAFLD", of which more than two thirds of the publications are from the past five years.

There is a strong association between NAFLD and the metabolic syndrome, and due to the increasing prevalence of obesity and T2DM worldwide NAFLD is now the most common chronic liver disease globally [6]. Patients with NAFLD are at increased risk of developing cirrhosis, hepatocellular cancer, end-stage liver disease, and developing and progressing in metabolic comorbidities including cardiovascular disease. So how can the story of the fatty liver be heart-breaking?

## Definition

NAFLD is defined as the accumulation of excess fat in hepatocytes, i.e. more than 5% of liver weight. It is typically associated with metabolic risk factors and requires the exclusion of secondary causes of hepatic steatosis, the most common being

alcohol overconsumption (Table 1) [7-10]. The level 5% corresponds to the hepatic triglyceride content measured with magnetic resonance spectroscopy (MRS) in the Dallas Heart Study, where the 95$^{th}$ percentile was 5.56% in subjects without obvious risk factors for liver steatosis [11].

**Table 1.** Examples of secondary causes of liver steatosis.

| Drugs | Genetic causes | Nutritional | Miscellanous |
|---|---|---|---|
| Amiodarone<br>Corticosteroids<br>Methotrexate<br>Tamoxifen<br>Valproate<br>Etc. | Celiac disease<br>Wilson disease<br>Familial combined hyperlipidemia<br>Abetalipoproteinemia<br>Glycogen storage disease<br>Lipodystrophy<br>Etc. | Total parental nutrition<br>Severe weight loss<br>Starvation<br>Etc. | Alcohol<br>Hepatitis C<br>HIV<br>Acute fatty liver of pregnancy<br>HELLP<br>Toxic (for example amanita phalloides)<br>Etc. |

The term NAFLD refers to the entire spectrum of disease [12]. Non-alcoholic fatty liver (NAFL) is defined as simple steatosis, without inflammation, hepatocyte injury or fibrosis. In NASH, inflammation and signs of hepatocellular injury with ballooning are present, with or without fibrosis. NAFLD-associated fibrosis and cirrhosis refers to the development of fibrosis in cases with present or previous steatosis. Cryptogenic cirrhosis, i.e. cirrhosis without known aetiology despite extensive evaluation, is clearly associated with obesity and T2DM. Therefore, NAFLD is believed to be the underlying cause of cryptogenic cirrhosis in the majority of cases [13].

# Histology

Histological findings in NAFLD are similar, but not entirely identical, to the findings in alcoholic liver disease [14]. For a diagnosis of NAFLD histologically the accumulation of hepatic fat, mainly in the form of triglycerides, as macrovesicles in the cytoplasm of more than 5% of hepatocytes, not weight, must be present [15]. Steatosis is most intensive around the central veins, in the acinar zone 3, and the periportal area is only involved in late stages or in extensive disease (Figure 1). To diagnose NASH, lobular inflammation (which includes mostly lymphocytes and macrophages) and hepatocyte injury (ballooning) in acinar zone 3 must be present, in addition to steatosis. Fibrosis also develops initially around the central veins in acinar zone 3. Cirrhosis is typically macronodular or mixed. Additional histological findings are for example mild hepatic siderosis, mild chronic portal inflammation, mega-mitochondria, apoptotic bodies and lipogranulom formation [14, 16]. The presence of fibrosis is not required for a diagnosis of NASH. NASH can progress to fibrosis, but fibrosis might also develop in a small proportion without previous

NASH [17]. Advanced fibrosis is defined as bridging fibrosis or cirrhosis, i.e. stage 3-4 (Table 2). When cirrhosis develops typically features of NAFLD including steatosis may diminish or even disappear [18].

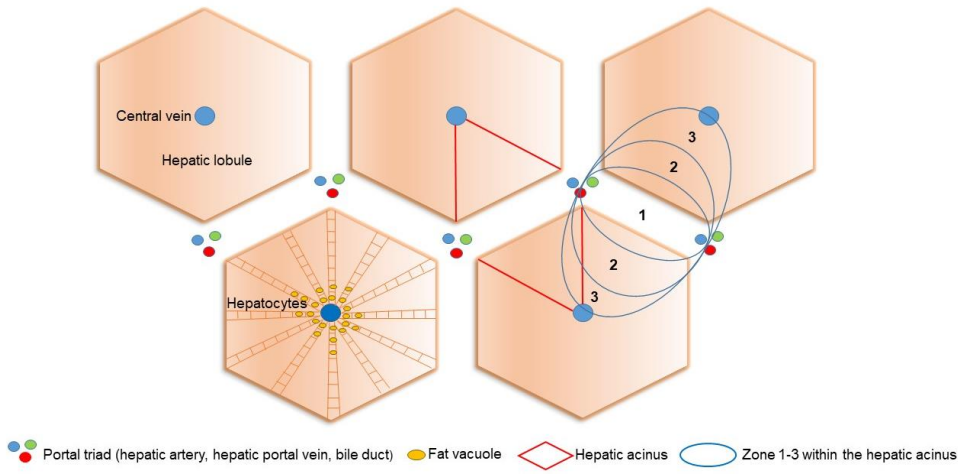


**Figure 1.** Hepatic lobules, with steatosis in hepatocytes in acinar zone 3.

Traditionally, the purpose of a liver biopsy is to differentiate between diagnoses and for grading and staging of a disease, according to various scoring systems [19, 20]. Over the years there has been a substantial intra- and inter-observer variability in diagnosing NASH in liver biopsy specimens [14].

In 1999 a system for grading inflammatory activity and fibrosis staging in NASH was presented for the first time [21]. A new algorithm including not only NASH, but the entire spectrum of NAFLD, was later constructed by the multicentre Clinical Research Network for NASH (NASH-CRN) [16]. A NAFLD Activity Score (NAS) was constructed from 14 major histological variables. A NAS $\geq$5 correlated with a diagnosis of NASH. This threshold has then been used for diagnosing NASH in many clinical studies. However, NAFLD Activity Score was not constructed to diagnose NASH, only grading of the activity, and it is not entirely consistent with a definite diagnosis of NASH.

In 2012 another algorithm, Steatosis Activity Fibrosis Score (SAF score), was constructed for grading and staging of the disease (Table 2) [22]. A simplified diagnostics of NASH, the Fatty Liver Inhibition of Progression (FLIP) algorithm, was developed from the SAF score (Figure 2) [23].

**Table 2.** Steatosis Activity Fibrosis score (SAF score).

| | **Steatosis (S0-3)** | **Activity (A0-4)** | | **Fibrosis (F0-4)** |
|---|---|---|---|---|
| | *Large and mediumsized droplets (acinar zone 3)* | *Ballooning of hepatocytes* | *Lobular inflammation, 2 or more inflammatory cells, in 20x magnification* | |
| **Grade/stage 0** | <5% | Normal hepatocytes | No inflammation | No fibrosis |
| **Grade/stage 1** | 5-33% | Clusters of hepatocytes with rounded shape and pale cytoplasm | Mild, <2 foci per lobule | 1a-b: mild/ moderate peri-sinusoidal zone 3<br>1c: periportal/ portal |
| **Grade/stage 2** | 34-66% | See above, plus at least one enlarged ballooned hepatocyte | Moderate, >2 foci per lobule | Perisinusoidal and periportal/ portal without bridging |
| **Grade/stage 3** | >67% | | | Bridging fibrosis |
| **Grade/stage 4** | | | | Cirrhosis |

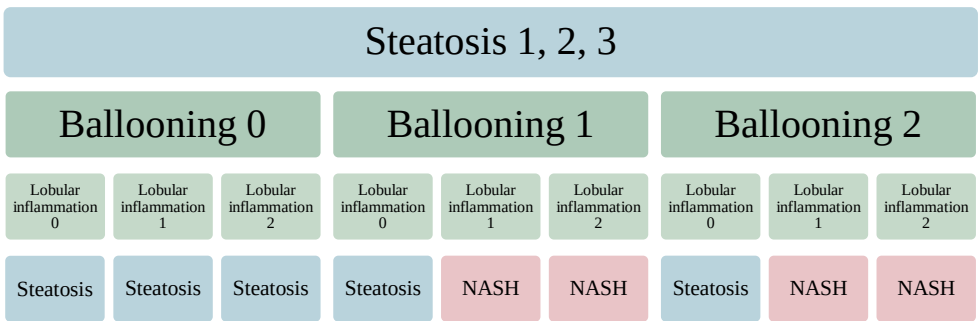


**Figure 2.** SAF score (according to Table 2) and the FLIP algorithm for diagnosing NASH.

# Diagnosis

Does the patient have NAFLD? Does the patient with NAFLD have NASH? Does the patient with NAFLD have fibrosis? These diagnostic questions have clinical implications concerning morbidity and mortality. Guidelines recommend the assessment of NAFLD with ultrasonography, liver enzymes and/or non-invasive biomarker tests in patients with metabolic risk factors [10]. In many cases steatosis

is an incidental finding in for example a radiological examination. All diagnostic modalities have more or less debatable use in NAFLD. Liver biopsy is invasive and with potential harmful complications and should be restricted to certain patients (see *Diagnosis, Liver Biopsy*).

**Table 3.** The spectrum of NAFLD, including diagnostic methods and epidemiology.

| | Definition | Diagnosis | Epidemiology | Clinical features |
|---|---|---|---|---|
| **NAFLD** | The entire spectrum of disease | Imaging<br>Biopsy<br>Non-invasive scores | Prevalence 17-46% [10], 23.7% in Europe [24]. | Associated with MetS and IR |
| **NAFL** | Presence of >5% steatosis without inflammation and fibrosis | Imaging<br>Biopsy<br>Non-invasive scores | 75 % of NAFL does not progress to NASH or fibrosis | No increased mortality |
| **NASH** | Presence of >5% steatosis with inflammation and hepatocyte ballooning, with or without fibrosis | Biopsy | Prevalence 1.5-6.45% [8].<br>future risk in up to 25 % of NAFL patients [25] | No increased mortality |
| **NAFLD fibrosis** | Presence of >5% steatosis and fibrosis, initially in zone 3 | (Imaging)<br>Biopsy<br>Elastography<br>Non-invasive scores | 14 years for progessing one fibrosis stage in NAFL, 7 years in NASH [26] | Increased mortality if advanced fibrosis |
| **NAFLD cirrhosis** | Cirrhosis (fibrosis stage 4) with past or present histological evidence of steatosis | Imaging<br>Biopsy<br>Elastography<br>Non-invasive scores | Up to 25% of NASH patients progress to cirrhosis [25] | Increased mortality |
| **Cryptogenic cirrhosis** | Cirrhosis (fibrosis stage 4) with unknown aetiology, in many cases associated with the metabolic syndrome | Imaging<br>Biopsy<br>Elastography<br>Non-invasive scores | Incidence 1.2/100 000/year in Sweden [27] | Increased mortality |

## Liver function tests

Typically patients with NAFLD have mildly to moderately elevated liver function tests, including AST (aspartate aminotransferase), ALT (alanine aminotransferase) and γ-GT (Gamma Glutamyl Transferase), with an AST to ALT ratio < 1 [28, 29] . Up to 80% of NAFLD patients have normal ALT, and the entire spectrum of disease can occur with normal liver function tests [30, 31]. The most common cause of elevated liver function tests in Sweden is NAFLD [32]. In recent years it has become clear that using liver function tests to diagnose NAFLD is not a sensitive method compared to ultrasonography [33]. However, elevated liver function tests in NAFLD is associated with more advanced disease with progression of fibrosis [34].

Diagnosing NAFLD also includes laboratory assessment to exclude chronic liver disease of other causes including of overconsumption of alcohol and viral hepatitis.

## Imaging

Ultrasonography (US) and Computer Tomography (CT) can diagnose NAFLD with good accuracy if the level of steatosis is at least 20-33% [35, 36]. With US the steatotic liver appears brighter. US is easily available, but operator-dependent and the sensitivity in detecting steatosis in obesity is lower. With CT one has to take the exposure of ionizing radiation into account.

Steatosis can also be detected with magnetic resonance imaging (MRI), with specific signalling for fat. With magnetic resonance spectroscopy (MRS) the proton density fat fraction (PDFF) is a sensitive measure of triglyceride levels, even in mild steatosis, i.e. grade 1 (Table 2). MRS is not routinely available on MR scanners. MRI methods have therefore been developed to use PDFF on routine machines [36].

Fibrosis has no specific MR signal and can only in severe cases be diagnosed using the different imaging methods. There is no imaging method to diagnose NASH (Table 3).

## Elastography

As stated above, only severe fibrosis and cirrhosis can be seen using imaging methods. If fibrosis develops the liver parenchyma becomes stiffer. Liver stiffness or elasticity can be measured non-invasively using either transient elastography or methods that can be combined with imaging, for example magnetic resonance elastography (MRE), or shear wave elastography and acoustic radiation force imaging with ultrasonography [36].

Transient elastography with controlled attenuation parameter (CAP) can be used without imaging to diagnose steatosis, but is less sensitive than MRI-PDFF [37].

## Non-invasive scoring systems and biomarkers

There is an increasing need to diagnose NAFLD, including steatosis, NASH and fibrosis with simple non-invasive methods. Several panels and equations have been developed and extensively studied and evaluated [38].

For diagnosing steatosis “SteatoTest” (including α-2-macroglobulin, haptoglobin, apolipoprotein A1, total bilirubin, γ-GT, fasting glucose, triglycerides, cholesterol, ALT, age, gender, BMI) [39], “Fatty Liver Index” (triglycerides, BMI, γ-GT, waist circumference) [40], “NAFLD Liver Fat Score” (metabolic syndrome, type 2 diabetes mellitus, fasting insulin, AST, ALT) [41] among others can be used with reasonably accuracy. The sensitivity of the above mentioned tests is 87-95% and the specificity 70-95%.

For diagnosing fibrosis several simple, and also more complex models, have been developed. “NAFLD fibrosis score” (NFS: age, glucose, BMI, platelet count, albumin, AST/ALT ratio) [42], “Fibrosis-4 index” (FIB-4: age, AST, ALT, platelet count) [43], “BARD score” (BMI, AST/ALT ratio, diabetes) [44], “FibroTest” (age, α-2-macroglobulin, total bilirubin, γ-GT, apolipoprotein A1) [45] and “Enhanced liver fibrosis test” (ELF: hyaluronic acid, type III collagen, TIMP-1) [46] among others have good diagnostic accuracy with a sensitivity of 77-85% and specificity 65-98%.

As with imaging methods there is also a problem in diagnosing NASH with non-invasive models. Circulating cytokines and adipokines, for example lower levels of adiponectin (see *Pathogenesis and Pathophysiology*, *Adipose tissue*) and higher levels of TNF-α, can be associated with NASH, but the results are inconsistent [38]. Cytokeratin 18 fragments, markers of cell death, possibly correlates with the severity of NASH, and Cytokeratin 18-M65 (uncleaved CK18), can possibly predict NASH [47, 48]. Diagnostic panels and equations to diagnose NASH exist, but require further validation.

### Liver biopsy

Liver biopsy has been considered gold standard in diagnosing all aspects of NAFLD, despite sampling variability and intra-observer variability in assessment, and it is still the only method to diagnose NASH [49]. It is an invasive method with a low, but potentially life-threatening, risk of complications including intra-peritoneal bleeding. It should be carried out in selected cases with intermediate or high values in non-invasive fibrosis scoring systems and/or elastography, in cases with a suspected high risk of advanced liver disease and to exclude other chronic liver disease.

## Clinical features

### Metabolic risk factors

*Metabolic syndrome*

NAFLD is strongly associated with metabolic risk factors, especially insulin resistance, but also established T2DM, obesity, hyperlipidaemia and hypertension. If several simultaneous metabolic risk factors are present there is an association with more progressive liver disease [50, 51].

The metabolic syndrome (MetS) is a constellation and co-occurrence of the above mentioned metabolic risk factors, and it is used to identify individuals with a high risk of associated complications [52]. There are several international definitions of the MetS, and all definitions include for example insulin resistance or T2DM, obesity and hypertension. The prevalence of the MetS in Europe is 26% of adult women and 41% of adult men. There is a strong association between NAFLD and the MetS. The prevalence of the MetS in NAFLD is around 43% and in NASH around 71% (Table 4) [24]. The association might be bi-directional, where one influence the development and progression of the other [53]. Insulin resistance is the common pathophysiological component linking NAFLD and the MetS. NAFLD is therefore often considered as the hepatic component of the MetS.

*Insulin resistance*

Insulin resistance plays a major pathogenic role in the development of NAFLD and is prevalent in the vast majority of NAFLD patients [54]. It is defined as a reduced sensitivity of insulin to act not only in adipose tissue and liver, but the entire body, either as high insulin levels achieving a normal metabolic response or normal levels with an inadequate response. It can be caused by for example obesity and by genetic factors, and precede T2DM with several years. The prevalence of insulin resistance in the general population is unknown, since it is asymptomatic and with difficulties regarding diagnostic methods [55]. Insulin resistance can be diagnosed with the euglycemic insulin clamp method, which is not practical in large population-based studies. Instead simple equations as the non-invasive Homeostatic model assessment of insulin resistance (HOMA-IR: f-glucose x f-insulin/22.5) are often used [56]. In a clinical setting there are several problems in diagnosing insulin resistance using HOMA-IR since there are several immune assays internationally to measure insulin and a reference value for defining insulin resistance is missing. The prevalence of insulin resistance in the general population is unknown. A previous study from a population-based prospective cohort using HOMA-IR validated with euglycemic clamp reported a prevalence of insulin resistance in 9.6% of subjects without metabolic diseases (including impaired glucose tolerance, hypertension, and dyslipidaemia) [57]. The prevalence of insulin resistance in co-existing metabolic diseases was between 54-95%.

*Diabetes Mellitus*

Insulin resistance can progress to T2DM. The global pooled overall prevalence of T2DM in NAFLD is 23% and in NASH 44% [24]. On the other hand, approximately 70% of patients with T2DM have NAFLD [58]. There is a 2-5 fold increase in the risk of developing T2DM in NAFLD, possibly higher in NASH and fibrosis, when adjusting for metabolic confounders, and it is mandatory to screen for T2DM in NAFLD patients [10, 59, 60]. The presence of T2DM is also associated with progression of liver disease [61]. An increased prevalence of NAFLD in type 1

diabetes mellitus is described, i.e. in patients with low levels of insulin, although the risk is lower than in patients with hyperinsulinemia [62].

*Obesity*

Together with insulin resistance and T2DM, overweight/obesity is a major risk factor for developing NAFLD. Parallel with an increasing prevalence of obesity, predominately central obesity, there is an increasing prevalence of NAFLD in the general population [63]. The prevalence of obesity, defined as a BMI >30 kg/m$^2$, is in the general population in individuals over 15 years of age in Sweden around 12% [64]. The global pooled overall prevalence of obesity in NAFLD is 51%. It is slighter lower in Europe, around 37% in patients with NAFLD diagnosed with ultrasonography, and 89% in patients with biopsy-proven NASH [24]. On the other hand, an increasing BMI results in a higher prevalence of NAFLD. Up to 90% of obese patients have NAFLD, compared to 67% in overweight (defined as a BMI >25 kg/m$^2$) patients [65]. Waist-hip ratio, taking central obesity in account, seems more strongly associated with NAFLD than Body Mass Index (BMI) and can also predict future liver-related events [66, 67].

*Hyperlipidaemia*

Hyperlipidaemia/dyslipidaemia is frequent, with a global pooled overall prevalence of 69% in NAFLD patients and 72% in NASH. In studies specifically reporting hypertriglyceridemia the pooled overall prevalence is 41% in NAFLD and 83% in NASH [24].

*Hypertension*

Hypertension (HT) is a major component of the MetS and an important risk factor for cardiovascular disease (CVD). There is limited data investigating the pathogenic role of hypertension in NAFLD. Globally the pooled overall prevalence of hypertension in NAFLD is 39% and in NASH 68% [24]. Approximately 50% of patients with hypertension have NAFLD. The effect seems bidirectional where NAFLD influence the development of hypertension and hypertension can lead to more severe liver disease [68].

**Table 4.** Associated metabolic comorbidities in NAFLD patients.

| | **Prevalence** |
|---|---|
| **Obesity** | 51% in NAFLD globally,<br>37% in NAFLD in Europe, 82% in NASH [1] |
| **Type 2 Diabetes Mellitus** | 23% in NAFLD, 44% in NASH[1] |
| **Hyperlipidaemia/Dyslipidaemia** | 69% in NAFLD, 72% in NASH[1] |
| **Hypertriglyceridaemia** | 41% in NAFLD, 83% in NASH[1] |
| **Hypertension** | 39% in NAFLD, 68% in NASH[1] |
| **Metabolic Syndrome** | 43% in NAFLD, 71% in NASH[1] |
| **Chronic Kidney Disease** | 20-50% in NAFLD[2] |

[1]Global pooled overall prevalence [24]. [2]According to reference [10].

## Patient demographics

NAFLD is usually diagnosed in middle-aged patients. Previous studies have shown conflicting results regarding sex distribution, but it is now regarded as a disease without sex predilection [69, 70].

The association between NAFLD and ethnicity have mostly been studied in the United States, where the risk is highest among Hispanics, lowest in blacks and intermediate in whites [71]. Worldwide the prevalence of NAFLD is highest in the Middle East and South America, followed by Asia, Europe and North America, and lowest in Africa. [24] The increasing prevalence of metabolic risk factors in some countries, and genetics, (see *Pathogenesis and Pathophysiology, Genetics*) are possible explanatory factors to the differences worldwide.

## Symptoms

Many patients are asymptomatic [70]. Hepatomegaly is a common finding on physical examination, and it is associated with dull abdominal pain in the upper right quadrant and malaise. In patients with cirrhosis typical stigmata including ascites and spider angiomas can be present. If NAFLD is associated with extrahepatic disease (see *Clinical features, Extrahepatic conditions*) specific symptoms, including fatigue and cognitive impairment, are seen. NAFLD is also associated with a lower quality of life compared to the general population [72].

## Alcohol

Diagnosing NAFLD requires the exclusion of alcohol overconsumption, which is usually defined as >20 gram alcohol/day for women and >30 gram/day for men. In many NAFLD studies 20 gram/day is the maximum allowed level to differentiate between alcoholic fatty liver disease (AFLD) and NAFLD [8, 10]. Previous studies have shown that an alcohol intake above this threshold can result in hepatic steatosis, and in susceptible individuals, especially women, be hepatotoxic [73]. Moderate alcohol consumption below 20 grams/day might have a beneficial effect on liver histology in NAFLD, but the results are conflicting [74, 75]. Alcohol overconsumption in combination with metabolic risk factors including T2DM have a synergistic negative effect on liver histology through common pathogenic mechanisms [76]. In many cases there is a combination between both NAFLD and AFLD that explains the metabolic liver disease.

### Extrahepatic conditions

Apart from liver disease and associated metabolic complications there are several conditions associated with NAFLD [8, 77, 78]. There is as a strong association between NAFLD and obstructive sleep apnoea syndrome, independent of BMI, causing fatigue in many patients. There is also an association with osteoporosis, psoriasis, hypothyroidism and other endocrinopathies, and extra-hepatic malignancies including colorectal cancer. Recently several studies have reported an independent association with sarcopenia, a progressive loss of skeletal muscle mass (See *Pathogenesis and Pathophysiology, Diet and lifestyle*) [79].

Numerous studies have shown an increased risk of chronic kidney disease (CKD), with a reported prevalence of 20-50% in NAFLD patients [10]. The association is possibly independent of multiple shared risk factors including T2DM. A meta-analysis concluded that the risk of both prevalent and incident CKD is increased, with an odds ratio around 2 in NAFLD patients, but with a higher prevalence and incidence in NASH and advanced fibrosis compared to NAFL [80]. There is today no general recommendation to assess kidney function in NAFLD patients.

## Epidemiology

### Prevalence

Parallel with the increasing prevalence of obesity and other metabolic risk factors the prevalence of NAFLD is increasing and it is now the most common chronic liver disease worldwide [8, 10, 63]. The exact prevalence in the general population is not entirely known and prevalence figures in previous studies depend on the diagnostic modality and study population. Several large scale population-based cohort studies have been conducted throughout the years, several of these in Europe. Among these were the Dionysos Study from Italy using US as diagnostic method, in participants with a median age of 59 years, and found NAFLD in 23% [81]. In two population-based cohort studies from Finland one used elevated liver function tests as diagnostic method in participants with a median age of 61 years and found a prevalence of NAFLD of 25%. In the other Finnish study Fatty Liver Index was used as diagnostic method, in participants with a median age 62 years, resulting in a higher prevalence of 41% [82, 83]. A meta-analysis recently summarized several of the prevalence studies and estimated the prevalence of NAFLD to 25% globally, with a slightly lower prevalence in Europe [24]. The prevalence of NASH in the general population is between 1.5-6.45%, i.e. in up to 25% of NAFLD patients [8].

### Incidence

The incidence of NAFLD has only been investigated in a few studies, most of these from Asia. In a follow-up of the Italian Dionysos study mentioned above, the incidence of NAFLD diagnosed with US was 18.5 per 1,000 person-years [65]. Studies from Asia have shown a high pooled overall incidence of around 52 per 1,000 person-years (95% CI, 28.31-96.77), whereas studies from Western countries have reported an incidence around 28 per 1,000 person-years (95% CI, 19.34-40.57) [8, 24].

### Liver transplantation

NAFLD, either because of end-stage liver disease or HCC, is now the second leading aetiology for liver transplantation in the United States, with a prognosis of becoming the most common cause in the near future [84]. In the Nordic countries NAFLD is the second most rapidly increasing indication for liver transplantation [85]. Despite older age, metabolic comorbidities and more severe liver disease at the time of transplantation the survival seems comparable to other diagnoses of chronic liver disease. The increasing prevalence of obesity and NAFLD have a high economic impact. Since NAFLD recipients are older and have more metabolic risk factors than others on the transplantation waiting-list, the risk of post-surgery complications might increase. However, increasing prevalence of NAFLD in the population could also mean less available liver donors.

## Pathogenesis and Pathophysiology

### Insulin resistance

We know that insulin resistance is prevalent in almost all patients with NAFLD and that it plays a crucial part in the pathogenesis of NAFLD, but how?

Insulin resistance is a common feature in obesity, especially abdominal, but it is also associated with other causes such as genetic factors and medications. The mechanism of how obesity can cause systemic insulin resistance is not fully understood. The liver, together with adipose tissue and skeletal muscle, are the main sites of insulin action [86]. Our bodies store energy that is needed in the fasting state; lipids in the adipose tissue as triglycerides (TG), and glucose in the liver as glycogen. Normally in the fasting state insulin controls hepatic glucose output from glycogen to maintain a normal plasma glucose. In NAFLD the inhibition of insulin on hepatic glucose output is impaired, leading to an increase in plasma glucose,

which stimulates β-cells in the pancreas, leading to compensatory higher insulin levels, and in many cases eventually T2DM. High insulin levels stimulate de novo lipogenesis (DNL), by inducing enzymes such as Sterol Regulatory Element Binding Protein 1 (SREBP-1) in the liver, leading to further steatosis, although this only accounts for a smaller fraction of hepatic fat (Figure 3) [87]. A deleterious metabolic circle develops, where NAFLD aggravates insulin resistance, and insulin resistance leads to increased steatosis.

Insulin also normally restrains very low–density lipoprotein (VLDL) production in the liver and inhibits adipose tissue lipolysis. In an insulin resistant state this leads to an over-production of triglyceride-rich VLDL particles in the liver and increased levels of circulating lipids, non-esterified free fatty acids (NEFA), from the adipose tissue [60, 86]. The liver can normally extract NEFA efficiently, but the increased levels of NEFA in systemic insulin resistance provides extra energy for gluconeogenesis. This leads to an increased rate of hepatic glucose production, a major cause of hepatic insulin resistance. Lipid supply to the liver comes from three different sources; *NEFA* from lipolysis in adipose tissue (increases in obesity or rapid weight loss for example), *triglycerides* (TG) from de novo lipogenesis in the liver and *chylomicron remnants* from food intake [87, 88]. The uptake of fatty acids in the liver leads to a production of diacylglycerol (DAG) and TG. If the rate of hepatic TG synthesis exceeds the rate of catabolism (i.e. oxidation of fatty acids and export of TG in the form of VLDL) accumulation of lipids mainly in the form of TG will result in NAFLD [60]. Increased production of DAG can also inhibit insulin signalling in hepatocytes, causing further hepatic insulin resistance.

Insulin resistance seems to be the primary driving force in the pathogenesis of NAFLD. The inter-relationship between NAFLD, hepatic insulin resistance and systemic insulin resistance is strong but complex, where NAFLD is both a consequence and a promoter of insulin resistance.

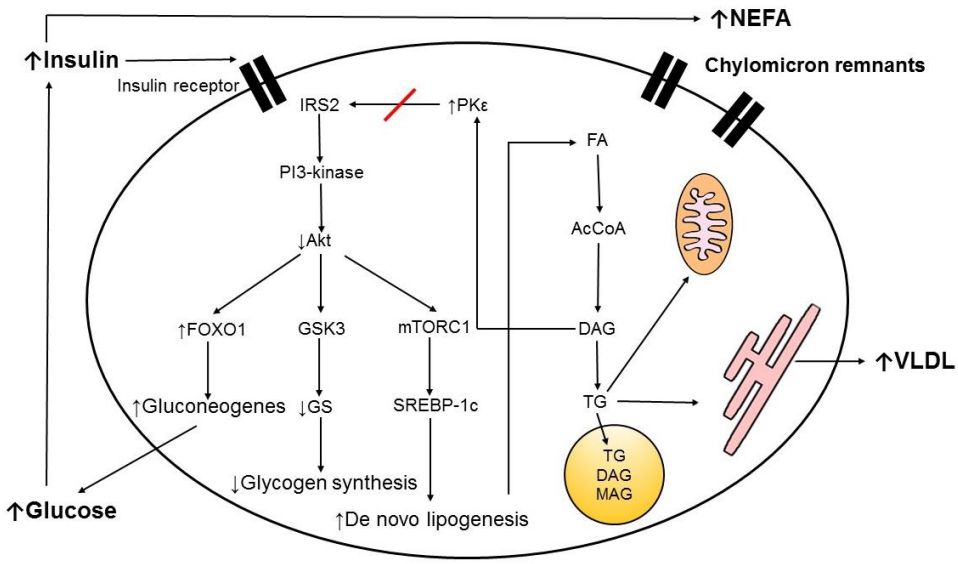


**Figure 3.** Pathogenic mechanisms of steatosis in the hepatocyte.
AcCoA, Acetyl coenzyme A; DAG, Diacyl glycerol; FOXO1, Forkhead box O transcription factor; GSK3, Glycogen synthase kinase 3; GS, Glycogen synthase; FA, Fatty acids; MAG, Monoacyl glycerol; mTORC1, Mammalian target of rapamycin; NEFA, Non-esterified fatty acids; PI3-kinase, Phosphatidylinositol 3-kinase; PK, Protein kinase; SREBP-1c, Sterol regulatory element bindning protein 1c; TG, Triglycerides. Figure adapted from [60, 61, 87].

## Adipose tissue

There is a strong association between obesity, and insulin resistance, NAFLD and cardiovascular disease (CVD). Obesity is a heterogeneous condition and between 10-30% of obese individuals are considered metabolically healthy [89]. The main function of adipose tissue is to store fatty acids and release these through lipolysis when needed in the fasting state. Lipids are mainly stored in subcutaneous fat depots, but when the capacity of storage has reached its limits the excess can be stored for example intra-abdominally (in visceral adipose tissue) and in the liver [88]. An increase in visceral fat, not subcutaneous, is of particular importance in the pathogenesis of insulin resistance, NAFLD, NASH and CVD [89-92]. Visceral fat is associated with an increase in hepatic steatosis, and with the extent of liver inflammation and myocardial fat. As explained above, insulin inhibits lipolysis and in insulin resistance this function is impaired resulting in increased release of fatty acids both from subcutaneous and visceral fat. Fatty acids from the visceral adipose tissue are released directly in the portal vein. However, the release of fatty acids from visceral adipose tissue only represents a fraction of NEFA delivered to the liver [88]. More importantly, the expanded visceral adipose tissue is also a major endocrine organ which produces different hormones and cytokines responsible for

the pathogenesis of insulin resistance and chronic inflammation seen in obesity and NAFLD [89].

Leptin and adiponectin are the two adipokines most extensively studied in NAFLD [93]. Adipose tissue consist of 50% adipocytes and 50% other cells including immune cells [89]. Adipokines are peptides secreted mainly by adipocytes. Other peptides from the adipose tissue, including interleukin- 6 and TNF-α, are mainly secreted by immune cells.

Leptin has an important role in energy homeostasis and in appetite regulation [93]. Circulating levels of leptin are higher in NAFLD compared to controls, and higher in NASH compared to NAFL, and the level is proportional to the amount of adipose tissue. The effect of leptin on histological end-points are controversial. Animal studies have shown a possible protective effect of leptin against liver steatosis in early stages, but it can also be a promotor of inflammation and fibrosis.

Adiponectin levels, on the other hand, are contrary to other adipokines lower in NAFLD and NASH than controls [93]. It acts against steatosis by stimulating oxidation of fatty acids in hepatocytes, and by decreasing gluconeogenesis and de novo lipogenesis. Adiponectin also suppresses pro-inflammatory cytokines (for example IL-6 and TNF-α) and anti-fibrotic pathways. The hepatic effect of adiponectin is partly because of activation of the PPARs (perixsome proliferator-activator receptors).

The PPARs belong to the nuclear receptor superfamily and regulate the transcription of various target genes. They consist of several isoforms with unique functions in different tissues including adipose tissue (predominately PPAR-γ) and liver (predominately PPAR-α) [89]. They have a role in fatty acid oxidation, glucose metabolism and inflammation. In one study of overweight and obese NAFLD patients with paired biopsies hepatic PPAR-α-expression negatively correlated with insulin resistance, visceral fat and the presence and severity of steatosis, NASH and fibrosis [94]. There was a positive correlation with the level of adiponectin. An increase in PPAR-α significantly correlated with histological improvement at follow-up.

## Diet and lifestyle

A hypercaloric diet, which exceeds the rate of caloric expenditure, is the most common cause of NAFLD in developed countries (Figure 4) [60]. Apart from leading to expansion of adipose tissue, causing insulin resistance and lipolysis, specific diets can also influence the development and progression of NAFLD. A diet rich in saturated fatty acids can upregulate the hepatic enzyme SREBP-1 leading to increased de novo lipogenesis in the liver [95]. Certain fatty acids are also considered lipotoxic. Long-chain saturated fatty acids, abundant in animal fat, affect

mitochondrial metabolism, and inflammatory and fibrotic pathways, which promotes liver injury, inflammation and apoptosis. Increased amounts of dietary carbohydrates leads to increased amount of substrates for de novo lipogenesis. Simple sugars can also be converted to fatty acids more easily than complex carbohydrates. Fructose, a sweetener in soft drinks, can stimulate de novo lipogenesis and inhibit mitochondrial beta-oxidation in the liver, and is associated with NAFLD and higher fibrosis stage. Choline is an essential nutrient required for VLDL synthesis and lipid export from the liver, and a choline-deficient diet is linked to NAFLD.

A sedentary lifestyle with physical inactivity is associated with NAFLD, and can lead to both obesity and sarcopenia, i.e. a loss of skeletal muscle and strength. Sarcopenia is associated with an increased risk of NAFLD and progressive liver disease, which seems to be independent of insulin resistance and obesity [79]. Insulin resistance, on the other hand, can lead to a loss of muscle mass and increased amounts of muscle fat, which correlates to the amount of liver fat. Skeletal muscle is also an endocrine organ secreting myokines, possibly participating in pathophysiology of NAFLD.

## Microbiota

Approximately 800-1000 different bacterial species, or one hundred trillion organisms, are found in the human gastrointestinal tract. They are grouped into phyla, the largest being Gram-negative Bacteroides and Gram-positive Firmicutes [96]. The composition of the gut microbiota is highly individual and it can influence various metabolic functions, and the development and severity of NAFLD. Small intestinal bacterial overgrowth (SIBO) is found more frequently in NAFLD, and seems to correlate with the severity of disease. There are several mechanisms of how microbiota can contribute to the development of NAFLD, including diet, intestinal permeability and altered bile acids [95].

The gut microbiota is strongly linked to diet. Saturated fatty acids and fructose can alter the gut microbiota to a composition which favours the development of obesity and NAFLD. Dietary choline can be converted to an inflammatory metabolite by gut microbiota, leading not only to choline deficiency contributing to the pathogenesis of NAFLD (see *Pathogenesis and pathophysiology, Diet and lifestyle*), but also toxic effects by metabolites. Changes in gut microbiota can lead to increased gut permeability, with the uptake of bacterial by-products including endotoxins that can trigger inflammation, as in the development of NASH. Bacteria can also chemically modify bile acids. Bile acids, apart from regulating bile acid synthesis, can bind and activate the farnesoid X receptor (FXR), which regulates lipid and carbohydrate metabolism (see *Pathogenesis and Pathophysiology, Miscellaneous pathogenic causes*).

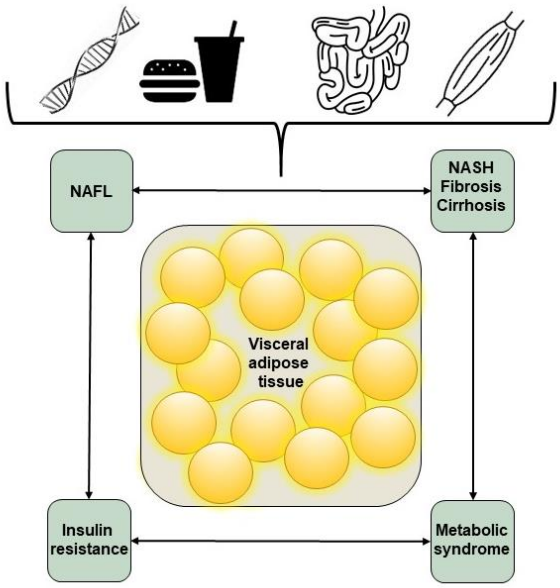


**Figure 4.** Pathogenenesis of NAFLD, where genetics, microbiota and a sedentary lifestyle with a hypercaloric diet, physical inactivity and sarcopenia contribute to the disease. Figure adapted from [92].

## Genetics

The presence of NAFLD is mainly explained by environmental factors, but genetic factors can determine how we respond to excess caloric intake and influence the progression of disease [97]. Twin studies have revealed a strong hereditary component of around 50%, in steatosis and the level of fibrosis [98]. Genetic variants can also lead to NAFLD without insulin resistance and obesity [99]. Genome-wide association studies have not identified a single gene, but numerous genes, associated with the disease. Only two genes have been repeatedly reported in several studies: PNPLA3 and TM6SF2.

The PNPLA3 gene, expressed in the liver and adipose tissue, encodes a protein, also called adiponutrin. It is structurally related to Triacylglycerol lipase, the enzyme hydrolysing triglycerides to diacylglycerol (DAG). The variant PNPLA3 I148M is a genetic polymorphism, where isoleucine has been substituted to methionine at position 148 [100]. The I148M variant is associated with increased hepatic steatosis, lower hepatic VLDL secretion and lower levels of adiponectin which has anti-inflammatory and anti-fibrotic effects (see *Pathogenesis and Pathophysiology, Adipose tissue*), but the exact mechanism is not completely understood. Carriers of the PNPLA3 I148M have an increased risk of advanced liver disease and HCC [99]. Interestingly the prevalence of the I148M variant in the general population, which

is around 30-50%, is highest among Hispanics and lowest in Africa, corresponding to the ethnical differences in NAFLD prevalence [99, 100].

TM6F2, transmembrane 6 superfamily 2, is a protein highly expressed in the liver, but with unknown biological function. Different allele variants can modify hepatic lipid secretion and is associated with NAFLD, dyslipidaemia and cardiovascular risk.

In a future perspective, analyses of genetic variants might be used in the evaluation and risk stratification of NAFLD [99].

## Miscellaneous pathogenic causes

In recent years bile acids receptors, including farnesoid X receptor (FXR) and Takeda G-protein-coupled receptor 5 (TGR), have emerged as important regulators of lipid and glucose metabolism, and in the pathogenesis of NAFLD [101]. Changes in the amount and composition of the bile acid pool, by for example medication or microbiota composition, can affect bile acid receptors, leading to multiple metabolic changes and the development and progression of NAFLD. FXR is highly expressed in the liver and distal small intestine. Activation of FXR by bile acids in the liver leads to inhibition of bile acids synthesis, and also inhibition of lipogenesis and gluconeogenesis in the normal state. Activation of TGR in adipose tissue regulates energy expenditure, and activation of TGR in the intestine promotes Glucagon-like peptide 1 release (GLP-1), which stimulates the release of insulin from the pancreas.

GLP-1, an incretin, is a hormone secreted from the gastrointestinal tract after food intake. It stimulates insulin secretion, a function that is impaired in T2DM. A previous study have shown a reduced incretin effect in NAFLD patients compared to obese controls, independently of glucose tolerance [102].

Iron overload has been associated with both insulin resistance and NAFLD [103]. Elevated ferritin is linked to an increased risk of T2DM and the MetS. It is also linked to prevalent NAFLD and is a strong predictor of the incidence of NAFLD. In other words, iron overload is possibly contributing to the pathogenesis of NAFLD, but high levels of ferritin can also be explained by simultaneous insulin resistance in NAFLD. There are conflicting results regarding the association between hepatic iron concentration and liver injury including fibrosis. Excess iron is associated with oxidative stress, and the production of reactive oxygen species (ROS), pro-inflammatory and pro-fibrotic cytokines, which can potentiate NASH development. HFE gene mutations seems not to be associated with NAFLD.

## Disease progression

NAFLD is a complex disease with multiple interactions between environmental factors, metabolic imbalances including insulin resistance, and genetics. NAFL with simple steatosis is a common disease, but a small proportion can progress to life-threatening cirrhosis and HCC. Which NAFLD patients and why?

Life-style factors leading to caloric excess expand the adipose tissue, which becomes inflamed and insulin resistant, resulting in increased lipolysis which is crucial for the development of NAFLD. Triglycerides are not hepatotoxic, and the presence of NASH does not correlate to the degree of steatosis [25]. Multiple insults can act together on the steatotic liver in genetically predisposed subjects to promote the progression to NASH, for example lipotoxicity from saturated fatty acids, endotoxins from gut microbiota, pro-inflammatory adipokines, altered activation of nuclear receptors, and the production of ROS from β-oxidation in peroxisomes because of mitochondrial inhibition, which results in oxidative stress [104]. When mechanisms that cope with these insults are overwhelmed, hepatocytes become injured and die. Injured hepatocytes release cytokines promoting further inflammation and stimulation of hepatic stellate cells promoting fibrosis [25]. Whether hepatocyte injury in NASH is a cause or consequence of the inflammation is not clear.

Risk factors for progressive disease include age >50 years, obesity, insulin resistance, T2DM and PNPLA3. Obesity, T2DM and PNPLA3 also increase the risk of HCC [61, 99]. Insulin resistance cause NAFLD, and NAFLD aggravates insulin resistance, eventually leading to the development of T2DM. The pro-inflammatory milieu in NAFLD with increased CRP, IL-6, TNF-α and ROS, together with for example atherogenic dyslipidaemia contributes to CVD and CKD (Figure 5). Metabolic risk factors are associated with the progression of NAFLD, and a futile metabolic circle develops where metabolic comorbidities aggravates NAFLD, and NAFLD aggravates metabolic comorbidities. Understanding the pathogenesis and pathophysiology of NAFLD and progressive liver disease have resulted in numerous treatment studies. However, due to the complex interplay between NAFLD and metabolic pathways, lifestyle interventions and treatment of comorbidities including T2DM still remain the cornerstone of NAFLD management. The futile circle needs to be interrupted to avoid progression of disease.

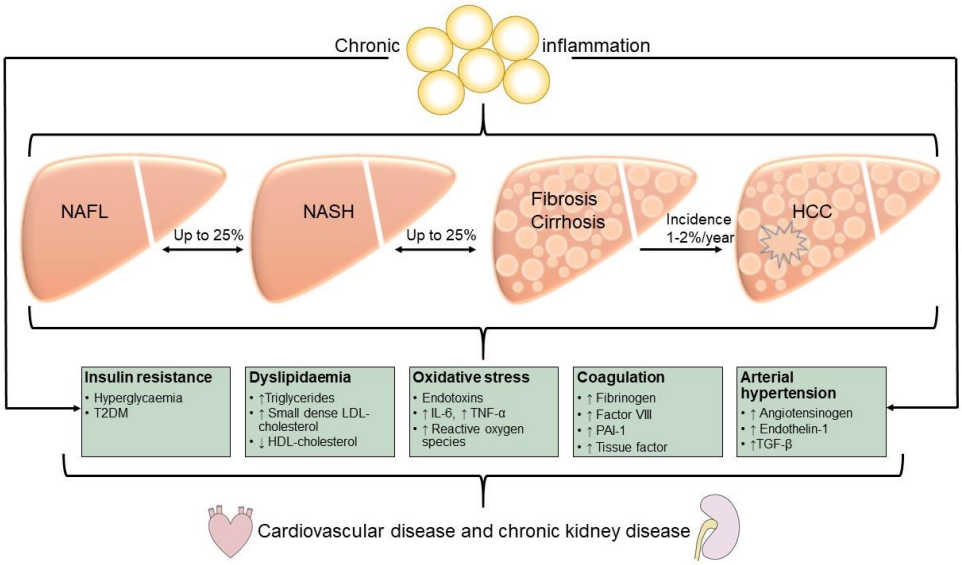


**Figure 5.** Natural history of NAFLD, and progression to CKD and CVD. Figure adapted from [25, 61, 105].

# Natural history

## Progression of liver disease

NAFL can progress to NASH, and to fibrosis, possibly in some cases without previous NASH (Figure 5). The rate of progression is generally slow. Few studies have included serial biopsies to evaluate the rate of fibrosis progression. A meta-analysis consisting of 11 studies with a total of 411 patients concluded that 34% had fibrosis progression (at least 1 stage), 43% stable fibrosis and 23 % regression after 2145.5 person-years, corresponding to a mean follow-up time of 5.2 years per person [26]. Patients with NASH had a faster progression rate than patients with NAFL, 7 years for progressing one fibrosis stage compared to 14 years. A subgroup of both NAFL and NASH has been identified as fast progressors, and can rapidly develop advanced fibrosis. The results are conflicting whether a higher steatosis grade is associated with progressive fibrosis. No association has been found between the severity of inflammation in NASH and risk of progressive fibrosis [106]. Steatosis, inflammation and even fibrosis are reversible by reducing metabolic risk factors including weight loss and physical exercise [107, 108].

Few longitudinal studies investigating risk factors associated with progression of fibrosis exist. Hypertension and low AST/ALT ratio at the time of the baseline

biopsy was in the above mentioned meta-analysis associated with fibrosis progression. In cross-sectional studies age, BMI, insulin resistance, T2DM and the MetS are associated with advanced fibrosis [109] Genetic factors including PNPLA3 is associated with fibrosis.

NAFL can progress to NASH in up to 25% of patients [8, 25]. The overall risk of developing cirrhosis is not entirely known, but has been reported to approximately up to 25% of NASH patients [42]. Advanced fibrosis/cirrhosis is prevalent in 12-17% of NAFLD patients undergoing liver biopsy and in 3-4% of community-dwelling patients [110]. Since NAFLD is a disease with a slow progression rate and most commonly diagnosed in middle-aged patients, many will never develop cirrhosis and liver-related events. In two studies with biopsy-proven NAFLD, with a mean follow-up of 13.7 and 12.6 years, 5.4% and 4.2% respectively developed liver-related events including for example ascites and variceal haemorrhage [34, 111]. In another study, including NAFLD patients with stage 3-4 fibrosis with stable disease, 19.4% developed liver-related complications over 7 years, however a lower cumulative incidence compared to patients with hepatitis C [112].

As in cirrhosis of all causes there is a risk of developing HCC, a risk which is also lower than in cirrhosis due to viral hepatitis. Obesity increases the all cancer risk, but with an especially high relative risk for HCC [113]. A meta-analysis showed that in study populations with NASH and cirrhosis the cumulative incidence of HCC ranged from 2.4% in 7 years to 12.8% in 3 years [114]. More worryingly several studies have also reported an increased risk in NAFL without fibrosis/cirrhosis. In the above mentioned meta-analysis the cumulative incidence of HCC in non-cirrhotic patients was 0.3-2% in studies up to 20 years [114]. Patients with NAFLD and HCC are older, have more metabolic comorbidities and shorter survival than other patients with HCC [8]. In line with other causes of cirrhosis, patients with NAFLD cirrhosis should be considered for HCC screening.

## Cardiovascular disease

The presence of NAFLD predicts future cardiovascular disease (CVD). Metabolic comorbidities in NAFLD, including insulin resistance, T2DM, obesity, dyslipidaemia and hypertension are all known risk factors for CVD. The emerging fact that chronic kidney disease (CKD) seems to have a higher prevalence in NAFLD adds another risk factor to CVD. Numerous previous studies have reported an association with atherosclerosis, cardiac dysfunction, and atrial fibrillation and thromboembolic events [105, 115]. NAFLD is associated with subclinical atherosclerosis, such as carotid artery intima-media thickness [116]. It is also independently associated with the severity of coronary artery disease. Altered cardiac structure with diastolic dysfunction and cardial ectopic fat depots are associated with increased visceral adipose tissue and NAFLD [115, 117]. Only a

few studies exist regarding the association with ischemic stroke, but the risk appears to be increased [118].

Few prospective studies have investigated the incidence of CVD in NAFLD, and most of the population-based studies have used ultrasonography to diagnose NAFLD. In one study the 10-year CVD risk was 11%, which corresponded to the calculated Framingham risk score of 10.5%, significantly higher than an age- and sex-matched control group from the general population [119]. In a recent retrospective cohort of biopsy-proven NAFLD 28% experienced a CVD event after a mean of 18.6 years (Hazard ratio (HR) 1.54, 95% CI: 1.30-1.83, $p<0.001$) compared to age and sex-matched controls [120]. No histological feature was associated with this increased risk.

A meta-analysis with over 30,000 individuals, of whom 36% had NAFLD with a median follow-up time of 6.4 years, recently concluded that the presence of NAFLD is associated with a 64% increase in the risk of fatal and/or non-fatal CVD (odds ratio (OR) 1.64, 95% CI: 1.26-2.13) [121]. More "severe" NAFLD, in this meta-analysis a heterogeneous definition, had an even higher risk (OR 2.58, 95% CI: 1.78-3.75).

Still, it is not clear whether NAFLD is a risk marker for CVD or an independent risk factor that adds to the risk of developing CVD. If so, the presence of NAFLD could be used in risk stratification for CVD besides established risk factors.

## Mortality

Overall mortality rate is significantly increased in NAFLD patients compared to the age- and sex-matched general population [122]. It is the development and severity of fibrosis that is the strongest predictor for the increased mortality in NAFLD [111, 123, 124]. NAFL with simple steatosis is a benign condition with good survival at long-term follow-up [125]. The presence of NASH or the level of SAF score is not associated with increased mortality [126, 127] .

The pooled overall mortality rate ratio was in a meta-analysis 1.58 (95% CI 1.19-2.11) for stage 1 fibrosis and 6.40 (95% CI 4.11-9.95) for cirrhosis, compared to NAFLD patients without fibrosis [124]. For liver-related death the pooled mortality rate ratio increased exponentially; 1.41 (95% CI 0.17-11.95) for fibrosis stage 1 and 42.30 (95% CI 3.51-510.34) for cirrhosis.

CVD is the most common cause of death in NAFLD, followed by extra-hepatic malignancy and liver-related mortality including end-stage liver disease and HCC [128].

To be able to identify patients at risk of future fibrosis, which increases the risk of liver-related and metabolic morbidity and mortality, especially cardiovascular, is

urgently needed for the risk stratification and care of NAFLD patients. The story of how a fatty liver can break a heart is still partly a draft.

# Aims

## Overall aims

To describe the long-term clinical development and prognosis of biopsy-proven NAFLD, focusing on liver-related morbidity, metabolic comorbidities and mortality.

## Specific aims

**Paper 1**: To investigate the prevalence of elevated liver function tests and NAFLD in a high risk population with long-term insulin resistance without type 2 diabetes mellitus.

**Paper 2**: To describe the natural history, including mortality, liver-related morbidity and the development of metabolic comorbidities, in biopsy-proven NAFLD.

**Paper 3**: To evaluate the association between NAFLD and chronic kidney disease, including prevalence, long-term future risk and effect on mortality.

**Paper 4**: To determine whether non-invasive fibrosis scoring systems can be used for early identification of NAFLD patients with increased risk of metabolic comorbidities, liver-related events and overall mortality.

# Material and methods

## Study population

### Malmö Liver Biopsy Register

In paper 2, 3 and 4 the study population was selected from a local liver biopsy register, including all liver biopsies undertaken for medical reasons at the University Hospital in Malmö, Sweden between 1978 and 2006. A total of 1683 biopsies were included in the register. All biopsies were routinely stained and evaluated by a pathologist for a histological diagnosis. Thereafter, they were evaluated by a hepatologist, including review of patients' medical files, laboratory tests and histology, for a clinical diagnosis. Results of relevant laboratory tests at the time of biopsy were included in the register. Since the register started before the availability of reagents for detecting Hepatitis C (HCV) antibodies, HCV was later retrospectively excluded in all subjects who underwent biopsy between 1978 and 1989 [129].

Patients with a histological diagnosis of "steatosis only", "steatosis plus lobular cell necrosis" or "cirrhosis", and with a clinical diagnosis of "non-alcoholic", "obesity- or diabetes-related" or "cryptogenic" were included for further assessment. After further extensive review of patients' medical files, including assessment of alcohol overconsumption, 155 patients were classified as primary NAFLD. Cryptogenic cirrhosis was in our studies only classified as NAFLD if there was simultaneous steatosis and metabolic risk factors.

### Malmö Diet and Cancer Study

Paper 1 included patients from the Malmö Diet and Cancer Study (MDCS). The MDCS is a population-based prospective cohort study, enrolling individuals from the city of Malmö, Sweden between 1991 and 1996. The main aim was to investigate the impact of diet on cancer. Men born 1923-1945 and women born 1923-1950 were recruited via personal letters and public advertisements [130, 131]. The complete cohort consisted of 28,098 individuals, of whom 11,063 were men (39%) with a mean age of 59.3 years, and 17,035 women (61%) with a mean age of 57.5 years,

corresponding to approximately 40% of the eligible population. Baseline examination comprised assessment of past and present medical history, medication, life-style factors and dietary habits with a self-reported questionnaire, and a physical examination and laboratory tests. To evaluate the representability of the MDCS cohort it was compared against age-matched non-participants in Malmö with similar socio-demographic structure [131]. The MDCS cohort had lower cancer and cardiovascular mortality, and higher self-reported good health.

A random sample of 50% of the original MDCS cohort was invited to a subsequent study, called the MDSC-cardiovascular arm (MDCS-CV, Figure 6) [132]. In all, 6103 individuals participated, and 5540 of these returned for fasting blood samples at a separate visit. Insulin resistance was calculated as HOMA-IR and participants were stratified in quartiles according to the level of HOMA-IR. HOMA-IR above the gender-specific 75$^{th}$ percentile was classified as insulin resistance, i.e. 1.80 for women and 2.12 for men.

A stratified sample of the MDSC-CV including 909 individuals were re-examined in 1999-2000 [133]. This cohort, MDSC-HOMA, consisted of 40% of insulin resistant participants according to baseline HOMA-IR. Subjects with T2DM were excluded.

Insulin resistant participants from the MDSC-HOMA cohort were invited to the study in Paper 1.

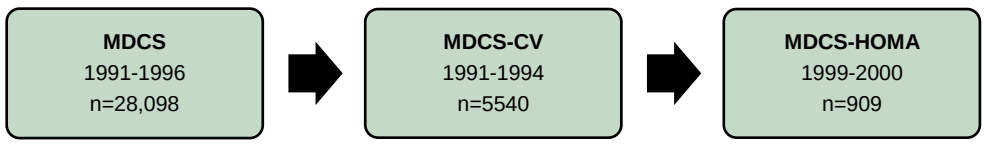


**Figure 6.** Malmö Diet and Cancer Study (MDCS).

## Malmö Preventive Project

Paper 2 and 3 included participants from the Malmö Preventive Project (MPP), a population-based prospective cohort, enrolling individuals between 1974 and 1992. Birth cohorts were invited to participate. A total of 33,346 individuals, of whom 22,444 were men (67.3%) born 1921-1949 with a mean age 46 years, and 10,902 women (32.7%) born 1926-1949 with a mean age of 49 years participated, corresponding to an attendance rate of 71% [134]. The aim of MPP was to start a large-scale screening program to identify high-risk individuals regarding for example alcohol abuse, cardiovascular risk factors including hypertension, hyperlipidaemia and impaired glucose tolerance. Baseline examination included a self-administered questionnaire on life-style and medical history, a physical examination and laboratory tests.

Information on overall mortality and causes of death are regularly obtained from the Causes of Death register from the Swedish National Board of Health and Welfare, a register of date and cause of death since 1961. Inpatient diagnoses are regularly obtained from National Patient Register from the Swedish National Board of Health and Welfare, a register founded in 1964. It contains all diagnoses on hospitalized patients according to WHO International Classification of Disease (ICD 8, 9 and 10), with complete national coverage from 1987.

In 2002-2006 the Malmö Preventive Project Re-examination Study (MPP-RES) was conducted (Figure 7). From the target population of approximately 25,000 individuals, still alive and living in the Malmö area, 18,238 (63% men) participated in the MPP-RES, which included a questionnaire, anthropometric measures and laboratory tests [135].

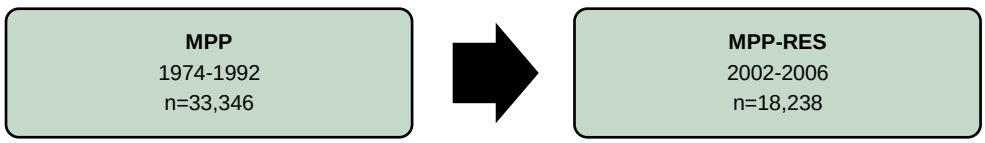


**Figure 7.** The Malmö Preventive Project cohort.

# Definitions

Hypertension was defined as a systolic blood pressure of ≥140 mm Hg, a diastolic blood pressure of ≥90 mm Hg, pharmacological therapy or a physician's diagnosis. T2DM was defined as fasting plasma glucose of ≥7.0 mmol/L, pharmacological therapy or a physician's diagnosis. Impaired fasting glucose (IFG) was defined as fasting plasma glucose of 6.1-7 mmol/L. BMI >25 kg/m$^2$ was defined as overweight and a BMI >30 kg/m$^2$ as obesity.

# Paper-specific methods

## Paper 1

From the MDCS HOMA-cohort insulin-resistant individuals, i.e. the gender-specific 75$^{th}$ percentile of HOMA-IR (1.80 for women and 2.12 for men), were invited by a personal letter to blood sampling for liver function tests (ALT, AST, γ-GT, bilirubin and PK-INR). Liver function tests were not included in the original analyses in the MDCS cohort. 305 individuals were identified from the MDCS

HOMA-cohort, 20 excluded due to relocation or death, and 285 invited to the study (Figure 8). Individuals with elevated liver function tests, a surrogate marker for NAFLD, were enrolled. Baseline examination included a complete medical history including past and present alcohol intake, a physical examination, imaging of the liver, biochemical analyses, for example fasting insulin, glucose, cholesterol and triglycerides, and laboratory tests to exclude other causes of chronic liver disease including carbohydrate deficient transferrin (CDT) for alcohol overconsumption. Insulin resistance was re-calculated at baseline as HOMA-IR. Alcohol intake >20 gram/day was defined as alcohol overconsumption. The metabolic syndrome was defined using the WHO criteria [52]. NAFLD was defined as the presence of steatosis on US or CT examination in subjects without alcohol overconsumption and other chronic liver disease.

## Paper 2

From the entire MPP cohort, participants with alcohol overconsumption or other chronic liver disease were first excluded by using in-patient ICD diagnoses indicating alcoholic liver disease or alcohol dependency from the National Patient Register (Figure 9). Individuals with alcohol overconsumption were also excluded according to 10 questions in the MPP questionnaire, based on the brief Michigan Alcohol Screening Test (MAST), the validated Malmö modification of the brief MAST. A previous study classified the questions either as moderate or heavy alcohol habits [136]. The questions representing moderate alcohol habits were classified as 1 point each in our study, i.e. “Do you drink before going to a party?”, “Do you usually drink a bottle of wine or corresponding amounts of alcohol over the weekend?” and “Do you drink a couple of drinks (or beers) a day to relax?”. The questions representing heavy alcohol habits were classified as 3 points each, i.e. “Do you tolerate more alcohol now than you did ten years ago?”, “Do you fall asleep after moderate drinking without knowing how you got to bed?”, “Do you have a bad conscious after drinking?”, “Do you take a drink (or beer) the day after a party?”, “Do you try to avoid alcoholic beverages for a determined period of time, e.g. a week?” and “Have you difficulties not drinking more than your friends?”. The final question “Are you a teetotaller” was classified as 1 point. Individuals with an alcohol score ≥3 points were excluded from further analyses. The remaining participants with an in-patient ICD diagnosis of chronic liver disease including NAFLD were identified. The remaining individuals in the MPP cohort were then also matched with the local liver biopsy register, and patients with biopsy-proven NAFLD were identified. Hospital medical records were studied from inclusion in the MPP cohort to the end of 2011 or death. The national Causes of Death register was used for mortality. The entire remaining MPP, without biopsy-proven NAFLD, was used as a control group. Steatosis, fibrosis and inflammation were classified in

a semi-quantitative manner according to pathology records, since the biopsy specimens were not re-evaluated for established NAFLD classification systems.

## Paper 3

All patients with biopsy-proven primary NAFLD between 1978 and 2006 were identified from the local liver biopsy register. Stage of fibrosis was recorded according to the Batts-Ludwig scoring system. Stage 2-4 fibrosis was classified as significant fibrosis. Patients' hospital medical records were studied for anthropometrics, laboratory tests and diagnoses, from inclusion to endpoint (death or end of 2016). Secondary causes of NAFLD were excluded. Evaluation of kidney function as estimated glomerular filtration rate (eGFR) according to the CKD-EPI equation, which either incorporates serum creatinine or Cystatin C, was calculated at baseline and last follow-up [137]. According to international practical guidelines for chronic kidney disease CKD 1 (normal function) equals >90 mL/min/1.73m$^2$, CKD 2 (mildly decreased kidney function) 60-89 mL/min/1.73m$^{2,}$ CKD 3a (mildly-moderately decreased) 44-59 mL/min/1.73m$^2$, CKD 3b (moderately-severely decreased) 30-44 mL/min/1.73m$^2$, CKD 4 (severely decreased) 15-29 mL/min/1.73m$^2$ and CKD 5 (kidney failure) less than 15 mL/min/1.73m$^2$. CKD 3-5 is defined as CKD i.e. an eGFR of <60 mL/min/1.73m$^2$ [138]. Patients with NAFLD were stratified into sub-groups according to CKD development from inclusion to latest follow-up. *Group 1*: CKD 1-2 at baseline and follow-up. *Group 2*: CKD 1-2 at baseline and CKD 3-5 at follow-up. *Group 3*: CKD 3-5 at baseline and CKD 3-5 at follow-up. For prevalence of CKD and for correlation analyses between NAFLD and CKD, the MPP-RES cohort was used as a control group, where creatinine was analysed at baseline and Cystatin C at follow-up in a subgroup. The national Causes of Death register was used for mortality. Patients were lost to follow-up if medical records could not be retrieved, if follow-up time was less than two years, and if emigration or unknown vital status.

## Paper 4

Patients with biopsy-proven primary NAFLD, identified in Paper 3, were included. Stage of fibrosis was recorded according to the Batts-Ludwig scoring system. Fibrosis stage 3-4 was classified as advanced fibrosis. Laboratory tests at the time of biopsy were used to calculate four different non-invasive fibrosis scores. *NFS*: -1.675 + 0.037 x age (years) + 0.094 x BMI (kg/m$^2$) + 1.13 x impaired fasting glucose or diabetes (yes = 1, no = 0) + 0.99 x AST/ALT ratio – 0.013 x platelets (x10$^9$/L) – 0.66 x albumin (g/dL) [42]. *FIB-4 index*: age (years) x AST (U/L) /platelets (x10$^9$/L) x √ALT (U/L) [43]. *BARD:* Scale 0-4, BMI ≥28 kg/m$^2$ = 1 point, AST to ALT ratio ≥ 0.8 = 2 points, Diabetes Mellitus = 1 point [44]. *APRI:* ((AST (U/L) / upper limit

of normal) / platelets ($x10^9$/L)) x 100 [139]. The NFS, FIB-4 index and APRI scores were classified into three risk categories (low, intermediate and high risk of advanced fibrosis) according to cut-points in the original publications. These are -1.455 and 0.676 for NFS, 1.30 and 2.67 for FIB-4 index, and 0.5 and 1.5 for APRI. Since the original BARD only included two risk categories (low 0-1 points, high risk 2-4 points), we classified the BARD score into three risk categories (low 0-1 points, intermediate 2 points, high risk 3-4 points) to compare against the other scoring systems. CKD was diagnosed according to the CKD-EPI equation (see *Paper-specific methods*, *Paper 3*). Patients' medical records were scrutinized in detail for metabolic diagnoses (T2DM and CVD, including ischemic heart disease and ischemic stroke) and liver-related events (hepatocellular cancer (HCC), ascites, encephalopathy and variceal bleeding) from inclusion (time of liver biopsy) to endpoint (death or end of 2016). Patients were lost to follow-up if medical records could not be retrieved, if follow-up time was less than one year, and if emigration or unknown vital status.

# Statistical methods

Statistical analyses were performed using IBM SPSS Statistics version 19.0-24.0. Mann-Whitney U-test was used to calculate differences between groups for categorical variables, skewed variables and small samples in Paper 1-3. The independent samples t-test calculated differences between normally distributed continuous variables in Paper 2 and 3. For non-parametric measure of association in Paper 1-3 Spearman's rank correlation coefficient was used. Wilcoxon signed-rank test was used in Paper 2 to analyse significant differences between tests at inclusion and endpoint. The Chi-square test calculated differences in categorical variables between groups in Paper 2-4, for example in total mortality and metabolic diseases. For categorical paired variables the non-parametric Kruskal-Wallis test calculated differences between three groups, as in the different fibrosis groups in Paper 4. The Kaplan-Meier method was used in Paper 2-4 to construct unadjusted survival curves, with log-rank test for comparison between the groups. Cox regression models (univariate and multivariate) calculated the association between CKD and mortality in Paper 3, and between fibrosis scores and outcome in Paper 4, with estimates presented as hazard ratio (HR). In Paper 4, ROC curves were created separately for the different fibrosis scoring systems, and Area-under-ROC-curve (AUROC) calculated for the included outcomes. A p-value $<0.05$ was considered statistically significant in all papers.

# Ethical considerations

Written informed consent was obtained from all the participating patients (Paper 1). The local ethics committee at Lund University, Sweden approved of the study designs (Paper 1-4). All studies were in compliance with the declaration of Helsinki.

# Results

## Paper 1

Of the 285 individuals with long-term insulin resistance (IR) identified from the MDCS HOMA-cohort, 165 accepted analyses of liver function tests, a response rate of 57.9% (Figure 8). Only 25 (15%) of these had elevated liver function tests. After exclusion of other chronic liver disease or other co-existing illness, 21 (12.7%) individuals remained. All 21 individuals underwent baseline examination, imaging of the liver and further laboratory tests. Of these individuals, only 5 (23.8%) had moderate or severe steatosis on imaging. The presence of steatosis correlated significantly with the MetS, HOMA-IR and ALT. There were significant differences in the prevalence of the MetS, in T2DM plus impaired fasting glucose (IFG), HOMA-IR and ALT between patients with or without steatosis (Table 5). 80% of the NAFLD patients fulfilled the criteria for the MetS, and 80% had either IFG or T2DM.

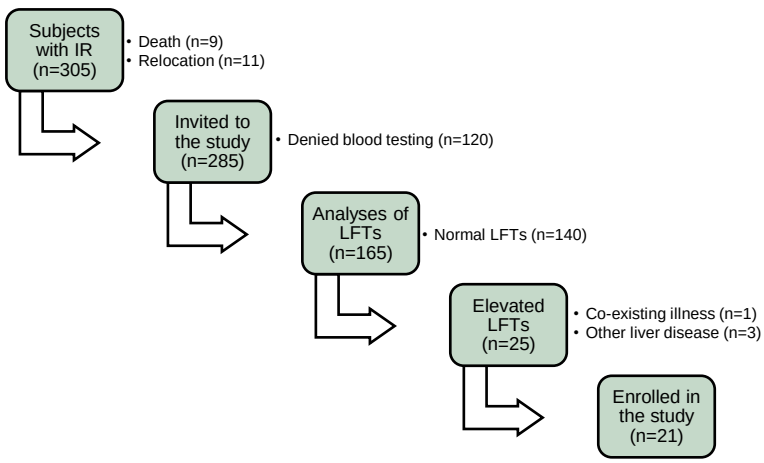


**Figure 8.** Flowchart for participant inclusion.

Follow-up time from the diagnosis of insulin resistance, i.e. the inclusion in the MDCS cohort, until the diagnosis of NAFLD was up to 17 years. Individuals who

were no longer insulin resistant in the present study (n=5), i.e. below the gender-specific 75$^{th}$ percentile of HOMA-IR in the MDCS cohort, had no signs of steatosis.

Of the 140 individuals who were excluded due to normal liver function tests, there were no significant differences in HOMA-IR, BMI, systolic and diastolic blood pressure, total cholesterol and triglycerides from the reinvestigation in 1999-2000, indicating that the included patients in Paper 1 were not previously more severely affected by metabolic comorbidities. None of the 284 insulin resistant individuals from the MDCS-HOMA cohort who did not participate in the present study had an inpatient diagnosis of NAFLD.

**Table 5.** Clinical and biochemical characteristics of indivuals with and without NAFLD on imaging.

| | **NAFLD (n=5)** | | **Non-NAFLD (n=16)** | | |
|---|---|---|---|---|---|
| | **Mean±SD n (%)** | **Median (IQR)** | **Mean±SD n (%)** | **Median (IQR)** | **p-value** |
| **Age** (years) | 70.20±6.98 | 73.00 (63.0-76.0) | 73.56±4.53 | 74.50 (71.25-77.0) | 0.36 |
| **Sex** (female) | 3 (60%) | n/a | 13 (81%) | n/a | 0.34 |
| **BMI** (kg/m$^2$) | 30.79±6.49 | 29.60 (24.95-37.23) | 27.97±3.96 | 27.55 (24.30-30.70) | 0.46 |
| **AST** (U/L) | 28.31±13.86 | 26.51 (15.06-42.77) | 20.48±8.43 | 15.66 (15.06-23.49) | 0.21 |
| **ALT** (U/L) | 61.45±33.73 | 72.29 (27.11-90.36) | 27.11±12.65 | 21.69 (16.27-38.55) | **0.04*** |
| **γ-GT** (U/L) | 108.43±99.40 | 81.93 (45.78-185.54) | 59.04±46.99 | 37.35 (18.67-100.60) | 0.14 |
| **HOMA-IR** | 4.49±1.56 | 5.28 (2.80-5.79) | 2.24±0.99 | 2.13 (1.74-2.35) | **<0.01*** |
| **Cholesterol** (mmol/L) | 6.02±1.14 | 5.50 (5.15-7.15) | 5.31±1.38 | 5.05 (4.43-5.92) | 0.15 |
| **Triglycerides** (mmol/L**)** | 2.14±0.98 | 1.90 (1.25-3.15) | 1.65±0.91 | 1.40 (0.93-2.05) | 0.32 |
| **T2DM** | 2 (40%) | n/a | 2 (12.5%) | n/a | 0.18 |
| **IFG** | 2 (40%) | n/a | 1 (6.3%) | n/a | 0.07 |
| **T2DM + IFG** | 4 (80%) | n/a | 3 (18.8%) | n/a | **0.01*** |
| **MetS** | 4 (80%) | n/a | 4 (25%) | n/a | **0.03*** |

# Paper 2

After the exclusion of chronic liver disease other than NAFLD and/or suspected alcohol overconsumption, 36 patients with biopsy-proven NAFLD were identified from the MPP cohort (Figure 9). A total of 11,726 (35.2%) individuals had an

alcohol score ≥3 points (see *Paper-specific methods, Paper 2*) or an ICD diagnosis indicating alcohol overconsumption.

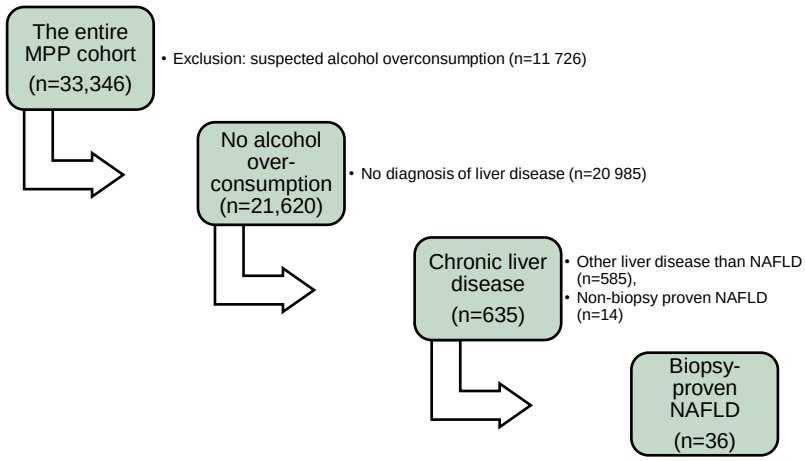


**Figure 9.** Flowchart for participant inclusion.

Inclusion of the NAFLD patients in the MPP cohort was between 1975 and 1989, and with an end-point until the end of 2011 or death, follow-up time was 27 years (Table 6). Using the entire remaining MPP cohort (n=33,310) as a control group, which included patients with alcohol overconsumption and other chronic liver disease, there were significant differences in liver function tests, HOMA-IR, BMI at inclusion in the MPP cohort between NAFLD patients and controls (Table 6).

Of all the patient with biopsy-proven NAFLD 55.6% (n=20) had simple steatosis, without inflammation and fibrosis. Cirrhosis was present in the biopsy in 13.9% (n=5). At follow-up, 63.9% (n=23) had been diagnosed with hypertension, 55.6% (n=20) with T2DM, 41.7% (n=15) with dyslipidaemia and 36.1% with CVD (n=13). Cirrhosis was present in 25% (n=9) at follow-up and 13.9% (n=5) with HCC. All patients with HCC had underlying cirrhosis. Patients with cirrhosis and HCC at follow-up had a trend of higher prevalence of metabolic comorbidities compared to the remaining patients with NAFLD, however only the prevalence of hypertension was significantly higher in HCC patients (p=0.02).

At end-point, 58.3% (n=21) had died compared to 32.8% (n=10,932) in the remaining MPP cohort (Chi-square test, p=0.004). Overall crude survival rate revealed a significantly higher mortality in the NAFLD group compared to the remaining MPP cohort (Figure 10). Primary causes of death in the NAFLD group was CVD (47.6%), HCC (23.8%), infection (14.3%), end-stage liver disease (4.8%), extra-hepatic malignancy (4.8%) and CKD (4.8%).

**Table 6.** Clinical and biochemical data at inclusion in the MPP cohort.

| | NAFLD (n=36) | | Control group (n=33,310) | | |
|---|---|---|---|---|---|
| | **Mean±SD, %** | **Median (IQR)** | **Mean±SD, %** | **Median (IQR)** | **p-value** |
| **Sex F/M** | 27.8/72.2 | n/a | 32.7/67.3 | n/a | 0.53 |
| **Age inclusion** (years) | 47.48±7.30 | 47.65 (46.08-53.44) | 45.67±7.41 | 46.88 (39.80-49.13) | 0.15 |
| **Follow-up time** (years) | 27.03±6.86 | 28.70 (23.84-32.02) | 27.02±7.00 | 29.78 (23.47-32.24) | 0.99 |
| **Smoking no/yes** | 61.1/38.9 | n/a | 53.6/44.5 | n/a | 0.43 |
| **BMI** (kg/m$^2$) | 25.91±3.85 | 25.63 (23.23-27.23) | 24.57±3.62 | 24.14 (22.12-26.48) | **0.045*** |
| **SBP** (mm Hg) | 128.53±14.66 | 127.50, (120.00-139.25) | 126.26±15.55 | 125.00 (115.00-135.00) | 0.36 |
| **DBP** (mm Hg) | 86.72±13.43 | 85.00 (80.00-90.00) | 84.21±9.67 | 85.00 (80.00-90.00) | 0.27 |
| **AST** (U/L) | 34.34±21.08 | 28.92 (22.29-39.76) | 22.89±11.45 | 21.08 (18.01-25.30) | **<0.001**** |
| **ALT** (U/L) | 51.81±41.57 | 33.73 (24.70-71.08) | 23.49±16.87 | 19.28 (14.46-26.51) | **<0.001**** |
| **γ-GT** (U/L) | 77.10±62.05 | 51.20 (27.71-128.92) | 36.14±51.20 | 24.7 (17.47-37.95) | **<0.001**** |
| **HOMA-IR** | 4.04±4.22 | 2.72 (1.47-5.33) | 2.31±2.45 | 1.68 (0.77-2.92) | **0.011*** |
| **Cholesterol** (mmol/L) | 5.82±1.18 | 5.86 (5.09-6.64) | 5.67±1.10 | 5.59 (4.92-6.32) | 0.46 |
| **Triglycerides** (mmol/L) | 1.54±0.74 | 1.47 (1.01-1.88) | 1.38±0.95 | 1.16 (0.85-1.63) | 0.20 |

DBP=diastolic blood pressure. SBP=Systolic blood pressure.

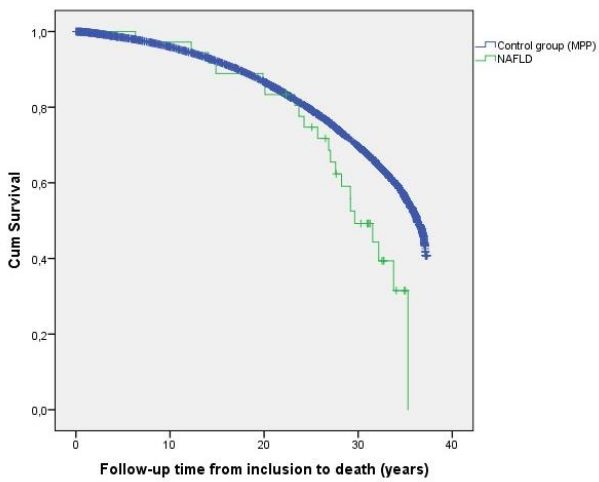


**Figure 10.** Overall survival of NAFLD patients compared to the remaining MPP cohort (p=0.005).

# Paper 3

From the local liver biopsy register 191 patients with steatosis and no alcohol overconsumption were identified. After review of patients' medical files 36 patients were excluded due to secondary causes of NAFLD. Of the remaining 155 patients with primary NAFLD, 120 had evaluation of kidney function at the time of biopsy and at follow-up (Figure 11). Age at inclusion of NAFLD patients was 52.5±13.1 years (mean±SD), follow-up time 19.5±9.0 years and 39.2% were women. Histologically, 73.3% (n=88) had no or minimal fibrosis (stage 0-1 according to the Batts-Ludwig classification) and 26.7% (n=32) significant fibrosis (stage 2-4).

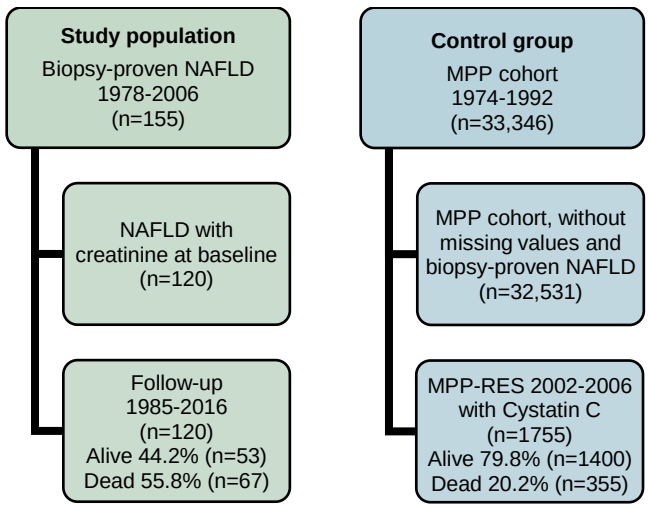


**Figure 11.** Flowchart for participant inclusion.

At baseline, 12.5% (n=15) of NAFLD patients fulfilled the criteria for CKD, compared to 2.1% (n=36) in the control group (p<0.001). Due to significant differences in age at baseline between the groups, NAFLD patients and controls were stratified into age groups (at baseline <40 years, 50-55 years and >55 years, and at follow-up <60 years, 60-75 years and >75 years). CKD prevalence was only significantly higher in the highest age group (>55 years) at baseline (25% vs. 9.4%, p=0.03). At follow-up overall CKD prevalence was 37.5% in the NAFLD group and 30.8% in the control group (p=0.124), with no significant differences when stratified into age groups.

Although there was a significant association between NAFLD and CKD in the entire study population (Spearman´s correlation coefficient $r_s$ 0.157, p<0.01), the strongest correlation to CKD was age ($r_s$ 0.194, p<0.01).

NAFLD patients who had developed CKD at follow-up (28.3%, n=34) had significantly higher prevalence of T2DM at baseline (p=0.005), hypertension at baseline (p=0.022) and at follow-up (p=0.020), and CVD at follow-up (p=0.047) compared to NAFLD patients with preserved kidney function throughout the entire study period (59.2%, n=71).

At follow-up, a total of 55.8% (n=67) had died. Overall crude survival rate among NAFLD patients stratified according to CKD development revealed a significantly higher mortality among patients with long-term CKD, i.e. *group 3* who had CKD both at inclusion and follow-up (Figure 12).

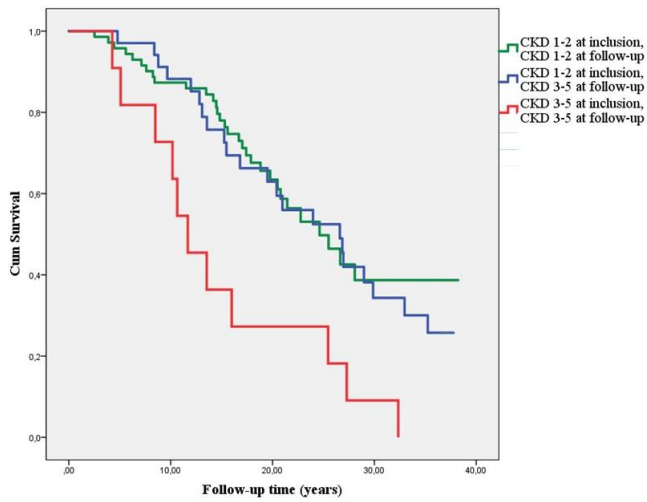


**Figure 12.** Crude survival of NAFLD patients according to CKD development. Log-rank test p<0.001 between CKD 1-2/1-2 and 3-5/3-5. Log rank test p<0.003 between CKD 1-2/3-5 and CKD 3-5/3-5.

Unadjusted Cox regression analysis revealed a hazard ratio (HR) of 2.60 (95% CI 1.44-4.71, p=0.002) for long-term CKD in predicting overall mortality, and an adjusted HR of 2.07 (95% CI 1.03-4.14, p=0.041) when adjusting for age and sex (Table 7). However, when adding T2DM and hypertension (Table 7, Model 3), or T2DM, hypertension and fibrosis stage (Table 7, Model 4), the result was not significant. Long-term CKD per se could not explain the increase in mortality, but metabolic comorbidities and fibrosis did.

**Table 7.** CKD as a predictor of overall mortality in the NAFLD group.

| | HR (95% CI) | p-value |
|---|---|---|
| **Model 1 (unadjusted)** | | |
| CKD at baseline | 2.60 (1.44-4.71) | 0.002 |
| **Model 2 (adjusted for age and sex)** | | |
| CKD at baseline | 2.07 (1.03-4.14) | 0.041 |
| **Model 3 (adjusted for age, sex, T2DM and HT)** | | |
| CKD at baseline | 1.68 (0.83-3.38) | 0.150 |
| **Model 4 (adjusted for age, sex, T2DM, HT and fibrosis stage)** | | |
| CKD at baseline | 0.79 (0.39-1.61) | 0.516 |

# Paper 4

As explained under *Results*, *Paper 3*, 155 patients with biopsy-proven primary NAFLD were identified from the local liver biopsy register. After initial exclusion, due to short follow-up time or emigration, 144 patients remained. Mean follow-up time was 18.8 years±9.2 years, age at biopsy 53.2±13.4 years, and 42.4% (n=61) were women.

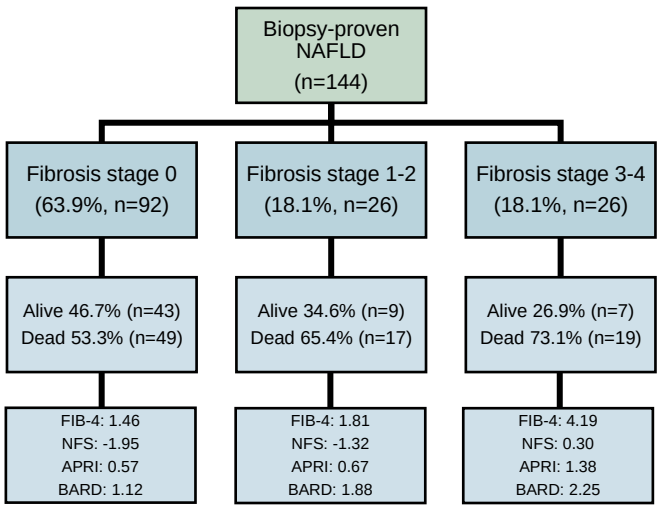


**Figure 13.** Mean values of non-invasive fibrosis scoring systems according to fibrosis stage, p<0.001 for FIB-4, NFS and APRI, and p=0.001 for BARD.

At inclusion, 63.9% (n=92) had NAFL and 18.1% (n=26) had advanced fibrosis stage 3-4 (Figure 13). All four fibrosis scoring systems showed significant differences between fibrosis stage 0, 1-2 and 3-4 (Figure 13).

At follow-up, 16.7% (n=24) had been diagnosed with cirrhosis and 5.6% (n=8) with HCC. In all, 13.9% (n=20) had liver-related events, with ascites being the most common complication (9.0%, n=13). At inclusion, the most common metabolic comorbidity was overweight (Table 8). Patients with advanced fibrosis had significantly higher prevalence of T2DM at inclusion, compared to those without advanced fibrosis (Chi-square test, p=0.007). At follow-up the most common metabolic comorbidity was hypertension, followed by CVD and T2DM. CKD was present in 12.5% (n=18) of patients at inclusion, and 32.6% (n=47) at follow-up. Total overall mortality was 59.0% (n=85), with CVD being the most common primary cause of death.

**Table 8.** Patients' characteristics at baseline and follow-up.

| | Mean±SD, n(%) | Median (IQR) | Missing (n) |
|---|---|---|---|
| **Age** (years) | 53.2±13.4 | 54.4 (43.0-63.3) | 0 |
| **Follow-up time** (years) | 18.8±9.2 | 17.7 (12.1-25.7) | 0 |
| **Age at death** (years) | 75.6±9.4 | 75.5 (70.6-82.9) | 0 |
| **BMI** (kg/m2) | 28.0±4.6 | 27.7 (24.9-30.7) | 12 |
| **AST** (U/L) | 51.9±41.8 | 41.2 (29.4-64.6) | 0 |
| **ALT** (U/L) | 79.1±64.5 | 56.5 (36.6-101.8) | 0 |
| **Platelets** ($x10^9$) | 210.8±77.0 | 207.0 (158.5-258.5) | 3 |
| **Albumin** (g/L) | 41.0±5.9 | 42.0 (38.8-45.0) | 10 |
| **Creatinine** (µmol/L) | 86.2±18.5 | 84.0 (75.0-98.9) | 6 |
| **T2DM at inclusion** | 32 (22.2) | n/a | 0 |
| **T2DM at follow-up** | 77 (53.5) | n/a | 5 |
| **Overweight at inclusion** | 106 (73.6) | n/a | 5 |
| **Overweight at follow-up** | 76 (52.8) | n/a | 25 |
| **Hypertension at inclusion** | 66 (45.8) | n/a | 0 |
| **Hypertension at follow-up** | 95 (66.0) | n/a | 4 |
| **CVD at inclusion** | 17 (11.8) | n/a | 1 |
| **CVD at follow-up** | 77 (53.5) | n/a | 6 |
| **CKD at inclusion** | 18 (12.5) | n/a | 6 |
| **CKD at follow-up** | 47 (32.6) | n/a | 19 |

After calculating AUROC, the FIB-4 index, NFS and APRI significantly predicted liver-related events, and FIB-4 index, NFS and BARD predicted overall mortality, with moderately good accuracy (Table 9). NFS was the only score that significantly predicted all non-hepatic complications (T2DM, CVD and CKD).

Overall crude survival rate (Kaplan-Meier method with log-rank test) showed that the high-risk group of all four non-invasive scoring systems significantly predicted overall mortality ($p<0.001$ for all scoring systems compared to the low-risk group).

**Table 9.** Area under the ROC-curve for non-invasive fibrosis scoring systems in predicting outcomes at follow-up (AUROC±SE(95% CI)).

| | FIB-4 index | NFS | APRI | BARD |
|---|---|---|---|---|
| **Overall mortality** | 0.82±0.04<br>(0.75-0.90)<br>**p<0.001** | 0.82±0.04<br>(0.74-0.90)<br>**p<0.001** | 0.59±0.05<br>(0.50-0.68)<br>p=0.070 | 0.75±0.04<br>(0.66-0.83)<br>**p<0.001** |
| **Liver-related events** | 0.81±0.06<br>(0.69-0.93)<br>**p<0.001** | 0.77±0.06<br>(0.64-0.89)<br>**p<0.001** | 0.82±0.05<br>(0.72-0.92)<br>**p<0.001** | 0.61±0.08<br>(0.50-0.75)<br>p<0.151 |
| **T2DM** | 0.55±0.05<br>(0.45-0.65)<br>p=0.288 | 0.61±0.05<br>(0.51-0.72)<br>**p=0.031** | 0.63±0.05<br>(0.54-0.73)<br>**p=0.006** | 0.57±0.05<br>(0.47-0.67)<br>p=0.173 |
| **CVD** | 0.74±0.05<br>(0.65-0.83)<br>**p<0.001** | 0.76±0.05<br>(0.67-0.85)<br>**p<0.001** | 0.53±0.05<br>(0.43-0.63)<br>p=0.505 | 0.69±0.05<br>(0.60-0.79)<br>**p<0.001** |
| **CKD** | 0.64±0.05<br>(0.54-0.74)<br>**p=0.009** | 0.63±0.06<br>(0.52-0.74)<br>**p=0.025** | 0.56±0.06<br>(0.45-0.66)<br>p=0.316 | 0.64±0.05<br>(0.54-0.74)<br>**p=0.013** |

Multivariate Cox regression analyses, in models adjusted for parameters not included in the equations including fibrosis stage, showed that the high-risk category of FIB-4 index, NFS and APRI significantly predicted future liver-related events (Table 10). All risk categories of FIB-4 index and NFS significantly predicted overall mortality and non-hepatic metabolic complications. The high risk category of APRI predicted overall mortality, and all non-hepatic metabolic complications. None of the risk categories of BARD could significantly predict the included future outcomes.

In summary, FIB-4 index and NFS performed similarly, and better than BARD and APRI, in the predictive capacity of the included outcomes. However, although significant results were obtained, these scoring systems only predicted metabolic outcomes with fairly good accuracy, and overall mortality and liver-related events with moderately good accuracy. Despite this, the results indicate that patients in the intermediate and high risk category, despite fibrosis stage, should regularly be examined with the intention to find metabolic complications and chronic kidney disease.

**Table 10.** Multivariate adjusted Hazard ratios (HR) for risk categories (low, intermediate and high risk) of non-invasive fibrosis scoring systems in predicting future outcomes.

| | | FIB-4[1] | NFS[2] | APRI[3] | BARD[4] |
|---|---|---|---|---|---|
| **Overall mortality** | Low | 1.0 | 1.0 | 1.0 | 1.0 |
| | Intermediate | 3.09 (1.66-5.73) **p<0.001** | 3.13 (1.77-5.54), **p<0.001** | 1.17 (0.70-1.98) p=0.55 | 1.09 (0.59-0.98) p=0.80 |
| | High | 6.46 (3.17-13.15) **p<0.001** | 11.61 (4.54-29.71) **p<0.001** | 2.75 (1.24-6.12) **p=0.01** | 1.82 (0.98-3.39) p=0.06 |
| **Liver-related events** | Low | 1.0 | 1.0 | 1.0 | 1.0 |
| | Intermediate | 0.48 (0.07-3.38) p=0.464 | 2.38 (0.49-11.49) p=0.282 | 2.57 (0.47-15.20) p=0.297 | 0.80 (0.23-2.75) p=0.718 |
| | High | 5.88 (1.25-27.80) **p=0.025** | 12.72 (1.67-97.00) **p=0.014** | 9.36 (1.34-65.31) **p=0.024** | 0.67 (0.16-2.74) p=0.574 |
| **T2DM** | Low | 1.0 | 1.0 | 1.0 | 1.0 |
| | Intermediate | 2.14 (1.11-4.14) **p=0.024** | 2.20 (1.17-4.14) **p=0.015** | 1.54 (0.90-2.66) p=0.118 | 0.91 (0.48-1.73) p=0.770 |
| | High | 4.18 (1.96-8.92) **p<0.001** | 20.74 (6.90-62.38) **p<0.001** | 2.70 (1.16-6.28) **p=0.021** | 1.91 (0.98-3.72) p=0.056 |
| **CVD** | Low | 1.0 | 1.0 | 1.0 | 1.0 |
| | Intermediate | 2.67 (1.40-5.09) **p=0.003** | 4.39 (2.39-8.07) **p<0.001** | 1.05 (0.60-1.82) p=0.872 | 1.32 (0.67-2.57) p=0.423 |
| | High | 6.52 (3.07-13.86) **p<0.001** | 16.88 (5.68-50.23) **p<0.001** | 3.21 (1.40-7.37) **p=0.006** | 1.92 (0.98-3.77) p=0.057 |
| **CKD** | Low | 1.0 | 1.0 | 1.0 | 1.0 |
| | Intermediate | 4.77 (1.95-11.64) **p=0.001** | 3.31 (1.41-7.74) **p=0.006** | 1.49 (0.71-3.11) p=0.288 | 1.32 (0.58-3.02) p=0.512 |
| | High | 7.25 (2.51-20.94) **p<0.001** | 31.38 (7.92-124.38) **p<0.001** | 4.31 (1.46-12.69) **p=0.008** | 1.89 (0.77-4.65) p=0.165 |

[1]Adjusted for sex, BMI>25, CVD, T2DM, hypertension, fibrosis stage.
[2]Adjusted for sex, CVD, hypertension, fibrosis stage.
[3]Adjusted for age, sex, BMI>25, CVD, T2DM, hypertension, fibrosis stage.
[4]Adjusted for age, sex CVD, hypertension, fibrosis stage.

## Hepatic complications

In Paper 2, the prevalence of cirrhosis was 25% at follow-up. Of these, 14% were diagnosed with cirrhosis at inclusion and 11% developed cirrhosis during a follow-up time of 27 years, i.e. a cumulative incidence of cirrhosis among NAFLD patients of 12.9%. In Paper 4, which included patients from Paper 2, the prevalence of cirrhosis was 11% at inclusion, and 16.7% at follow-up (mean 18.8 years), i.e. a cumulative incidence of cirrhosis of 6.25% .

The prevalence of HCC was 13.8% at end-point in Paper 2, and 5.6% in Paper 4, all with a previous diagnosis of cirrhosis.

In Paper 4, 13.9% developed liver-related complications, with ascites being the most common complication.

## Metabolic complications

Only a minority of patients with long-term insulin resistance had elevated liver function tests, and steatosis as a cause of this, as shown in Paper 1. Patients with NAFLD in Paper 1 had significantly higher prevalence of the MetS, and T2DM and impaired fasting glucose, and were more insulin resistant than patients without NAFLD.

At long-term follow-up, hypertension was the most common metabolic complication (63.9% in Paper 2 and 66% in Paper 4) among NAFLD patients. The prevalence of T2DM was 55.6% in Paper 2 and 53.5% in Paper 4. In Paper 2, 36.1% had been diagnosed with CVD (i.e. angina pectoris and myocardial infarction), and 53.5% (also including ischemic stroke) in Paper 4.

NAFLD patients with advanced fibrosis had significantly higher prevalence of T2DM, as shown in Paper 4**.**

The prevalence of CKD at follow-up was 37.5% in Paper 3 and 32.6% in Paper 4 at follow-up.

## Mortality

The most common cause of death was CVD in both Paper 2 and 4 (47.6% and 39% respectively). In Paper 2, end-stage liver disease including HCC was the second most common cause of death, whereas in Paper 4 it was non-hepatic malignancies. Crude mortality in Paper 2 was significantly higher among NAFLD patients

compared to a control group, including patients with other chronic liver diseases and over-consumption of alcohol. NAFLD patients with long-term CKD, as shown in Paper 3, had significantly higher mortality than NAFLD patients with preserved kidney function. The increased mortality could not be explained by CKD, but a higher prevalence of significant liver fibrosis and metabolic comorbidities. The intermediate and high-risk group of FIB-4 index and NFS predicted overall mortality in biopsy-proven NAFLD.

# Discussion

To conclude the main findings in this thesis, progression of metabolic comorbidities can lead to an increased risk of developing NAFLD, and in biopsy-proven NAFLD an increased risk of more advanced liver disease, of CKD, and of overall mortality. Using non-invasive fibrosis scoring systems seems to identify NAFLD patients with increased risk of future metabolic and hepatic morbidity, and overall mortality.

The strengths of the studies are a long-term follow-up, and the ability to include laboratory tests and diagnoses from an extensive review of patients' medical files, information that cannot be found in national registers. All cases of NAFLD were in **Paper 2-4** biopsy-proven.

Studies in NAFLD are heterogeneous, with different modalities including imaging, non-invasive tests and histology to diagnose NAFLD. This makes it difficult to compare results between different studies. The decision to perform a liver biopsy in the included patients from the Malmö Liver Biopsy Register was based on clinical grounds, in most cases because of elevated liver function tests and in some cases because of a pathological finding on imaging. This might lead to a bias, where patients with more severe NAFLD will be included in the present studies, and the result difficult to extrapolate to the vast majority of NAFLD patients.

In **Paper 1**, elevated liver function tests were used as a surrogate marker of suspected NAFLD. We know today that the majority of NAFLD patients have normal liver function tests, and a majority of patients with long-term insulin resistance and normal liver function tests might therefore also have NAFLD. The reference values of liver function tests have changes during the years [140]. Reference values are set on the basis that 95% of healthy individuals are included within the cut-off limits. The upper reference values of liver function tests (AST ALT and γ-GT) are nowadays higher compared to 15 years ago, possibly because of an increased prevalence of overweight and obesity in the general population, and a change in alcohol consumption. This could mean that a subgroup of patients with subclinical liver disease and normal liver function tests will now be undiagnosed. Of the 155 patients with primary NAFLD in the Malmö Liver Biopsy Register, 66% of the men and 76% of the women had elevated liver function tests (data not published). In **Paper 2**, patients with biopsy-proven NAFLD had significantly higher liver function tests at inclusion in the MPP cohort compared to the remaining MPP cohort, in some cases many years before they were diagnosed with NAFLD.

Previous studies have also shown that elevated liver function tests are associated with more advanced liver disease in NAFLD [34]. Including only patients with elevated liver function tests in **Paper 1** and patients with biopsy-proven NAFLD in **Paper 2-4** could mean a selection of more severely affected patients, both regarding liver disease and metabolic comorbidities.

One limitation in **Paper 1** is the lack of a control group. In retrospect, a possibility would have been to invite all patients with insulin resistance (fourth quartile of HOMA-IR in the MDSC-CV/MDSC-HOMA cohort) despite the level of liver function tests and compare these against a control group consisting of non-insulin resistant individuals (for example the first quartile of HOMA-IR in the MDSC-CV/MDSC-HOMA cohort), which would have resulted in a much larger sample size, less skewed data, smaller standard error and possibly achieving more significant results. In **Paper 2**, the control group consisted of the remaining individuals without biopsy-proven NAFLD from the MPP cohort, which represented the general population. There were no significant differences in sex, age and follow-up time between cases and controls in **Paper 2**. In **Paper 3**, the control group was a subgroup from the MDCS-RES cohort, with evaluation of CKD both at baseline and follow-up. In some aspects in **Paper 2** and especially in **Paper 3** the control group was significantly different from the study population. In **Paper 4**, the control group, or reference population, was NAFLD patients with low risk of fibrosis according to non-invasive fibrosis scoring systems, compared to NAFLD patients with intermediate and high risk of fibrosis. Using control groups from national registers, matched in age, sex and municipality, enable us to compare for example mortality rate and ICD diagnoses, but not specific laboratory tests since these are not included in registers.

Using national registers is a possibility because of unique personal identity numbers assigned to all residents in Sweden [141]. The coverage of the national registers are 100% regarding date of birth and death within Sweden [142]. The National Patient Register from the Swedish National Board of Health and Welfare includes almost 100% of hospital discharge (inpatient ICD-diagnoses), but only 80% of out-patient diagnoses [143]. The Patient Register was founded in 1964, but before 1987 the coverage was not complete. Out-patient diagnoses from hospitals were added to the register in 2001. Out-patient visits from general practitioners in primary care are not included. Since NAFLD in most cases is diagnosed at outpatient visits in primary care there is no possibility to use national registers for complete identification of NAFLD patients. Hypertension and T2DM are also in most cases treated in primary care. Reviewing the medical files of NAFLD patients in **Paper 2-4**, in order to find metabolic comorbidities, is therefore a considerable strength. Also, since several biopsies were undertaken years before complete coverage of the National Patient Register, using registers instead of reviewing medical files would mean underdiagnosing the included diagnoses.

Over the years the method of diagnosing of T2DM has changed, from blood glucose to plasma glucose, which gives 11% higher glucose values. The diagnosis of hypertension is nowadays stricter. This makes it difficult to compare the prevalence of metabolic comorbidities over time. What was not regarded as hypertension 30 years ago might today fulfil the criteria. In **Paper 2-4**, we used modern criteria for diagnosing hypertension and T2DM, and in cases with blood glucose this was re-calculated to plasma glucose. By reviewing medical files we could therefore diagnose metabolic comorbidities with the same criteria throughout the entire study period in **Paper 2-4**.

In early studies it seemed that NAFLD affected more women than men. Further studies have now confirmed that NAFLD has no sex predilection [5, 70]. In **Paper 1-4** the proportion of women were between 27.8% and 60%. In **Paper 1** patients were recruited if they were above the sex-specific upper reference values of liver function tests, which are higher in men. Due to small sample size further analyses of the included patients were not stratified according to sex. In **Paper 2**, there were no significant differences in sex distribution, age and follow-up time between NAFLD patients and the MPP cohort, why no stratification was done in calculating crude mortality between cases and controls. In **Paper 3**, there were however significant differences between NAFLD patients and controls in sex distribution and age. Mortality was therefore calculated both in total, and stratified according to sex, both analyses showing the same results, i.e. a significantly higher mortality in NAFLD patients with long-term CKD. In **Paper 4**, we did not stratify any of the analyses according to sex distribution, but adjusted the regression analyses for sex in both **Paper 3** and **4**.

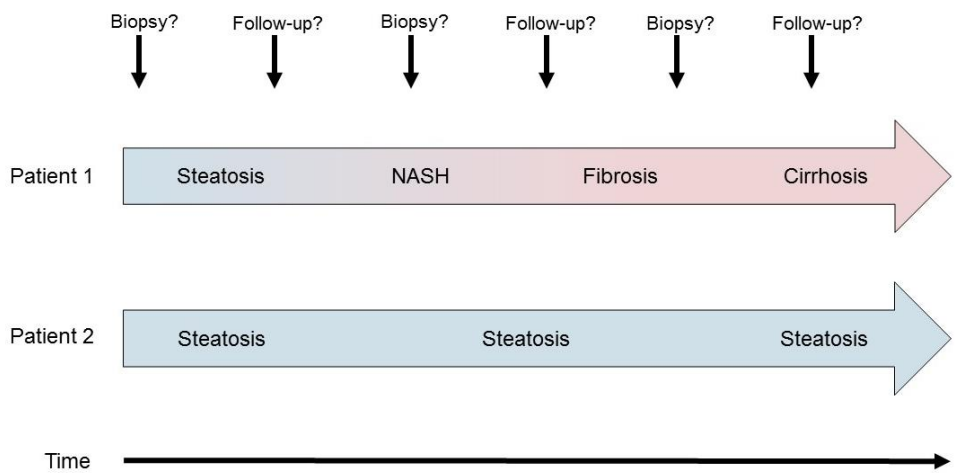


**Figure 14.** Natural history of NAFLD – when to perform a biopsy and when to follow-up?

In **Paper 2-4**, NAFLD was diagnosed only at baseline, with liver biopsy. Follow-up time was different between the patients. Depending on when the biopsy was performed and when the follow-up was done, it is difficult to draw conclusions about the natural history of the disease (Figure 14). Some patients had obvious liver disease at follow-up, with cirrhosis, HCC and decompensation. There is a possibility that we have included several patients without NAFLD at follow-up, since we did not include serial biopsies. A previous meta-analysis concluded that 23% had regression of fibrosis at follow-up [26]. T2DM, among other risk factors, is associated with fibrosis progression [109]. NAFLD patients develop a vicious metabolic circle with increasing prevalence of metabolic comorbidities over time including T2DM. In our studies 53-56% of the included patients had developed T2DM at follow-up (**Paper 2-4**). The global pooled overall prevalence of T2DM in NAFLD is 23% [24]. Considering the high prevalence of T2DM in our studies one can assume that a high percentage still had NAFLD at follow-up.

To exclude alcohol overconsumption in NAFLD studies is challenging. In **Paper 1**, we excluded patients with alcohol overconsumption with Carbohydrate Deficient Transferrin (CDT) and anamnestic questions. In **Paper 2**, over one third of patients in the entire MPP cohort were excluded due to suspected alcohol overconsumption. The National Board of Health and Welfare in Sweden classifies hazardous drinking as more than 14 standard glasses/week for men and 9 standard glasses/week for women, where one standard glass equals 12 grams of alcohol. This means a maximum of 24 grams of alcohol/day for men and 15 grams/day for women. The Public Health Agency of Sweden estimates that 17% of adults between 16-84 years have a hazardous alcohol consumption [144]. Excluding one third of the individuals in the MPP cohort by using the questions in the Malmö-MAST is possibly too strict. A great deal of effort was put in to exclude alcohol overconsumption in the Malmö Liver Biopsy Register in **Paper 2-4**, both at inclusion in the register and for the present studies, by an extensive review of patients' medical files over the entire study period. In future studies specific laboratory tests including for example phosphatidyl-ethanol (PEth) should be mandatory for a NAFLD diagnosis [145]. NAFLD and AFLD share similar histological features, but a moderate alcohol consumption may have a beneficial effect on the histology in NAFLD [75]. Both metabolic risk factors and alcohol overconsumption can lead to steatohepatitis, fibrosis and cirrhosis, but the progression rate is slower in NAFLD. Alcohol overconsumption can have a synergistic effect on the liver damage in NAFLD [76]. In the absence of alcohol, saturated fatty acids, compared to unsaturated fatty acids, can promote lipotoxicity and apoptosis. Saturated fatty acids seems protective in AFLD, which is one possible explanation why a moderate alcohol intake, in contrast to alcohol over consumption, might be protective [95]. Previous studies have also shown that Escherichia Coli in the gut microbiome can produce ethanol and possibly induce liver damage [95, 146]. The enzyme alcohol dehydrogenase (ADH) also

seems impaired in the liver, perhaps due to insulin impairment, which theoretically also can contribute to liver damage in NAFLD. The name non-alcoholic fatty liver disease clearly indicates that alcohol overconsumption must be excluded, however it might instead be referred to as "endogenous alcoholic liver disease" [146].

Re-assessment of the liver biopsies in **Paper 2-4** to classify histological findings according to more modern scoring systems, including SAF score, was not performed in our studies. All included biopsies had present steatosis (mild, moderate or severe), and fibrosis was classified according to the Batts-Ludwig scoring system. Since the definition of NASH has changed during the years, there was no possibility to diagnose NASH in any of the biopsies. Also, only a handful patients had serial biopsies why we can´t draw any conclusions regarding histological natural history.

The risk of developing cirrhosis in NAFLD is estimated to be around 6% [25]. With a prevalence of NAFLD of 25% this could mean a risk of 1.5% in the general population. Many NAFLD patients will never develop liver-related complications because of the slow progression rate, older age and increased mortality mainly due to CVD. In **Paper 1**, no patients with insulin resistance and elevated liver function tests had suspected cirrhosis. In **Paper 2**, 25% had cirrhosis at follow-up. In **Paper 4**, which included patients from **Paper 2**, 16.7% had been diagnosed with cirrhosis at follow-up. The prevalence of cirrhosis in our studies was higher than previously reported, probably because of selection bias. The prevalence of HCC in our studies was 13.9% in **Paper 2** and 5.6% in **Paper 4**, and all cases had underlying cirrhosis. In **Paper 4**, 13.9% developed liver-related events including HCC, over a mean follow-up of 18.8 years. Excluding HCC from liver-related events resulted in a prevalence of 7.6%, which seems comparable to previous studies [34, 111]. As shown in **Paper 4**, non-invasive fibrosis scoring systems seem to early identify these risk individuals with moderately good accuracy.

In line with previous studies, CVD was the most common primary cause of death in **Paper 2** and **4**. HCC and extra-hepatic malignancies respectively were the second most common cause. Overall crude mortality in **Paper 2** was significantly higher in NAFLD compared to the control group. Previous studies have shown that fibrosis and no other histologic feature can explain this increased risk of mortality [111, 123]. In **Paper 3**, significant fibrosis (stage 2-4) was indeed associated with an increased risk of mortality with a HR of 2.44 (1.45-4.08, p=0.001, adjusted for age and sex). Patients with long-term CKD had significantly higher overall crude mortality, which was explained by metabolic comorbidities, such as T2DM, and by fibrosis stage. It is therefore of utmost importance to prevent the progression to fibrosis, and the progression of metabolic risk factors in NAFLD.

Insulin resistance is present in the vast majority of NAFLD patients. Measuring insulin resistance with HOMA-IR has several limitations. Since insulin secretion is pulsatile one measurement of insulin is not as reliable as a mean of several

measurements [56]. Reference values for HOMA-IR are missing and there is no standardisation of insulin assays which results in significant inter-laboratory variability [147]. In one study, defining the 95$^{th}$ percentile of HOMA-IR in individuals resulted in a cut-off of 1.9 in patients with ≥5.56% liver fat on magnetic resonance spectroscopy (MRS) [147]. All patients invited in **Paper 1** were considered to be insulin resistant in a previous study, i.e. the 75$^{th}$ percentile of HOMA-IR (1.80 for women and 2.12 for men). Mean HOMA-IR at inclusion in **Paper 1** was 2.70 for women and 3.02 for men. However, only a minority of these had steatosis on imaging. None of the patients who were now below the previous sex-specific 75$^{th}$ percentile of HOMA-IR had steatosis on imaging, despite all of them having at least one metabolic risk factor. Improving insulin resistance in early stages seems to be of importance to avoid the development of significant steatosis. This is in line with a previous study showing that individuals with high levels of insulin both at baseline and follow-up had higher risk of developing NAFLD, compared to those with low levels of insulin at follow-up, even if the level was high at baseline [148]. In **Paper 2**, there were significant differences in HOMA-IR between patients with biopsy-proven NAFLD and the remaining MPP cohort (mean±SD: 4.04±4.22 vs. 2.31±2.45, p=0.011) at inclusion in the MPP cohort. In **Paper 3-4** there was no analysis of HOMA-IR.

Insulin resistance can progress to T2DM. As mentioned above 53-56% of patients with biopsy-proven NAFLD in our studies had been diagnosed with T2DM at follow-up, a high percentage compared to previous studies. The prevalence of T2DM in Sweden is 3.5-4% [149]. In **Paper 1**, due to small sample size, combining the prevalence of T2DM and impaired fasting glucose (IFG) resulted in significantly higher prevalence of this metabolic comorbidity in patients with NAFLD on imaging, compared to those without NAFLD. In **Paper 2**, there was a tendency of higher prevalence of T2DM in patients with cirrhosis and HCC compared to the remaining patients with NAFLD, however also in this study the sample size was small. In **Paper 3**, NAFLD patients had significantly higher prevalence of T2DM at baseline compared to the control group MDCS-RES. The increased risk of mortality in long-term CKD in **Paper 3** could be explained by metabolic comorbidities including T2DM. In **Paper 4**, patients with advanced fibrosis (stage 3-4) had significantly higher prevalence of T2DM.

One of the most common metabolic comorbidities in NAFLD is obesity, a major risk factor for insulin resistance. However, NAFLD can be diagnosed in lean patients and in an recent meta-analysis 22% of the included NAFLD patients were not overweight or obese [150]. Lean patients in this meta-analysis had a lower risk for fibrosis and NASH. A Swedish study of 646 NAFLD patients, where 19% were lean, found no increased risk of mortality in these patients [151]. In the Malmö Liver Biopsy Register 24% of the 155 NAFLD patients were lean (data not published). There were no differences in fibrosis stage between lean and overweight patients.

However, there were significant differences in metabolic comorbidities, with a higher prevalence of hypertension at baseline and follow-up, and T2DM at follow-up in the overweight group. There were no differences in liver function tests between the groups, but estimated GFR was significantly lower in overweight/obesity patients. Mortality in the overweight/obese group was higher, but not significantly.

In The Global Burden of Disease Study, a global collaboration in studying risk factors that affects morbidity and mortality in the world, the leading five risk factors in high-income countries in Europe were smoking, high blood pressure, high fasting glucose, overweight and alcohol [152]. These risk factors not only shortens life, but also cause considerable morbidity. Several of these are the same risk factors that cause NAFLD. It is unknown whether NAFLD contributes to the increased morbidity and mortality in patients with these risk factors in the Global Burden of Disease report, but theoretically it does. The obvious, but difficult, solution is prevention of these risk factors. Still today we have no specific treatment to offer patients with advanced NAFLD, only life-style modifications and treatment of underlying metabolic comorbidities, where some medication might have an additional effect on liver histology [107, 108].

Despite having been described for decades and with a high prevalence in the general population the awareness of NAFLD is fairly high among specialists, but only moderately high among general practitioners in primary care, and low among patients with metabolic risk factors [153-155]. Although the risk of severity is low, the increasing prevalence in the general population can lead to an increasing amount of patients with liver-related and metabolic morbidity and mortality. The awareness must be raised that NAFLD is a not negligible hepatic component of the metabolic syndrome. To prevent the progression of the vicious metabolic circles in NAFLD, and the development of CVD and fibrosis, is of utmost concern in NAFLD (Figure 15).

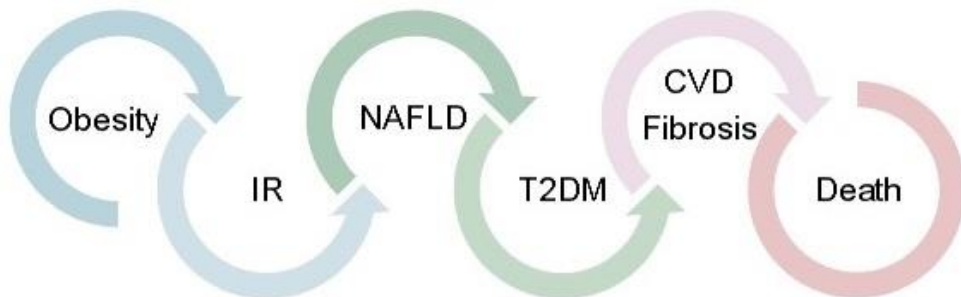


**Figure 15.** Vicious metabolic circles in NAFLD.

The results of this thesis support the need for early detection of metabolic risk factors to prevent the development of NAFLD, and the early detection of NAFLD patients with a high risk of liver-related morbidity, metabolic comorbidities, CKD and increased mortality. Non-invasive scoring systems might be a promising tool to identify these patients.

# Conclusions

- The risk of developing elevated liver functions tests and NAFLD in individuals with long-term insulin resistance is low, but not insignificant. Steatosis development in these individuals is associated with a progress of metabolic comorbidities.
- At long-term follow-up of patients with biopsy-proven NAFLD 17% had been diagnosed with cirrhosis, 6% with HCC and 14% had developed liver-related events, most commonly ascites.
- Patients with biopsy-proven NAFLD has significantly higher crude overall mortality than the general population.
- The prevalence of CKD is higher in middle-aged patients with biopsy-proven NAFLD. Long-term CKD in NAFLD is associated with a significantly higher overall mortality, compared to NAFLD patients with preserved kidney function, which is explained by metabolic comorbidities.
- Simple non-invasive fibrosis scoring systems can be used for early identification of NAFLD patients with increased risk of developing liver-related events, overall mortality, but also metabolic comorbidities and CKD.

# Future perspectives

There is an increasing interest in NAFLD, which has generated numerous publications in the past few decades. Despite a high prevalence in the general population, and with a not negligible risk of severe liver disease and a high risk of metabolic comorbidities, many questions are still unanswered. Worryingly, NAFLD is in many high risk individuals still undiagnosed, and the awareness of the disease urgently needs to increase, especially in primary care settings and in high risk clinics, for example diabetes clinics.

Comparing different NAFLD studies is a challenge, since methods of diagnosing the disease varies and the histological criteria for NASH have changed over the years. NAFLD is also a complex heterogeneous disease with multiple risk factors including environmental and genetics factors, which affects multiple molecular pathways [156]. Important clinical endpoints such as mortality, and development of cirrhosis and HCC, require large prospective studies over at least 10-15 years. Studies involving histologic improvement needs standardisation in histological scoring systems, and the biopsy specimens should be reviewed by at least two pathologists [157]. MR, instead of liver biopsy, is an option to evaluate resolution of steatosis.

Most importantly in NAFLD patients, fibrosis must be ruled out, or even better prevented. Due to logistic and economic reasons we can´t refer all individuals with a metabolic risk factor to imaging to diagnose NAFLD. Liver function tests are in many cases normal. Risk stratification is warranted [158, 159]. Firstly, NAFLD needs to be diagnosed in risk individuals, for example in patients with T2DM and obesity. Since the prevalence of T2DM in Sweden is around 4% and obesity 12% simple non-invasive scoring systems for steatosis could be a possible diagnostic methods in cases with normal liver function tests. If elevated liver function tests, US and laboratory tests to exclude other chronic liver disease should be performed. In cases with steatosis, non-invasive scoring systems for fibrosis should then be used to find the NAFLD patients with an intermediate and high risk for fibrosis, who need further evaluation with transient elastography and liver biopsy. The findings in **Paper 4** indicates that, despite fibrosis stage, non-invasive scoring systems can be used at an early stage to find risk individuals, not only regarding liver-related events and mortality, but also future metabolic comorbidities. These findings should be reproduced in larger studies. It indicates that a sub-group of NAFLD patients

would need regular follow-up to prevent, find or treat future metabolic comorbidities and liver related complications. Which scoring systems to use for risk-stratification needs to be clarified in larger studies. Simple scores, as in **Paper 4**, are easily available and can be used in a primary care setting. Combining different scores, perhaps in a stepwise fashion might be an option [160]. Future availability of more complex scores are also a possibility. Adding genetics to the risk stratification, where PNPLA3 for example is associated with progression to cirrhosis and HCC, might be one possibility [99]. Avoiding liver biopsy for the diagnosis of NASH requires more sensitive and specific biomarkers.

In many cases alcohol consumption is a contributing factor to liver damage in NAFLD patients, and many patients with alcohol overconsumption have metabolic risk factors, why it is difficult to separate the two entities NAFLD and AFLD. Future studies need better assessment of alcohol consumption, for example repeated analyses of PEth [145]. NAFLD might in the future be regarded as a metabolic liver disease, with more or less alcohol as a contributing factor. The name BAFLD, Both Alcoholic and Non-Alcoholic Fatty Liver Disease, has been proposed for these patients (Figure 16) [161].

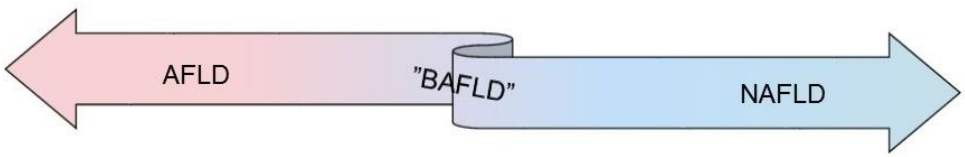


**Figure 16.** NAFLD, AFLD or BAFLD (Both Alcoholic and Non-Alcoholic Fatty Liver disease).

So what is the need of finding fibrosis if we have no specific treatment for advanced disease, apart from life style intervention as in all cases of NAFLD? Currently there are several phase 3 clinical trials in NAFLD and hopefully we will have a therapy in advanced NAFLD in the near future. Also, in cases of cirrhosis HCC surveillance and gastroscopy for detecting oesophageal varices should be considered [159].

Developing CVD, a significant future risk in NAFLD, is potentially life-threatening and prevention is of utmost concern. Whether NAFLD is an independent risk factor for CVD is under debate. If so, a diagnosis of NAFLD could in the future be added to scores for CVD risk, such as Framingham Coronary Heart Risk Score or the European Heart SCORE, which calculates the gender-specific 10 year risk of CVD [119, 162]. Also, if NAFLD is independently associated with an increased risk of CVD does this mean that the threshold for treating for example hyperlipidaemia should be lower in NAFLD patients? The results in **Paper 1** indicates that if you do not progress in metabolic diseases, in this case insulin resistance to T2DM, the risk of elevated liver function tests and moderate-severe NAFLD is low. Prevention of

metabolic comorbidities is therefore vital. Termination of the viscous hepatic and metabolic circles is a future challenge.

# Populärvetenskaplig sammanfattning på svenska

Non-alcoholic fatty liver disease (NAFLD), eller icke-alkoholorsakad fettleversjukdom, är den vanligaste leversjukdomen i världen. Den är starkt kopplad till insulinresistens och det metabola syndromet. NAFLD definieras som leverförfettning som överstiger 5% av leverns vikt. För diagnos krävs att man utesluter överkonsumtion av alkohol och andra sekundära orsaker till leverförfettning, som till exempel läkemedel. De flesta med NAFLD har inga symtom, blodprover för leversjukdom är i många fall helt normala, och oftast upptäcks fettinlagring med t.ex. ultraljudsundersökning. Inte bara insulinresistens, utan även bland annat kosthållning, stillasittande livsstil, genetiska faktorer är bakomliggande faktorer för uppkomst av NAFLD.

Förekomsten av NAFLD bedöms i Europa att ligga på ca 20% av befolkningen, en siffra som troligtvis kommer att öka i takt med att förekomsten av metabola sjukdomar som fetma och diabetes ökar i samhället. Ju fler metabola sjukdomar en patient har desto större är risken att utveckla NAFLD.

Enbart fettinlagring i levern är troligtvis godartat, men NAFLD kan progrediera till Non-Alcoholic Steatohepatitis (NASH), med inte bara fett utan även inflammation och cellskada i levern. Vidare kan fibros (ärrbildning) och till och med skrumplever uppkomma, och fibrosutveckling är klart kopplat till symptomgivande allvarlig leversjukdom med ökad dödlighet som följd, i första hand i hjärt-kärlsjukdom. Det finns idag ingen annan rekommenderad behandling än att justera metabola riskfaktorer, som till exempel att gå ner i vikt. Vilka patienter med enbart fettinlagring som riskerar att utveckla fibros, och hur vi tidigt ska identifiera dessa är inte helt klart.

Den översiktliga målsättningen med avhandlingen var att beskriva långtidsutvecklingen av NAFLD avseende lever-relaterade komplikationer, metabola sjukdomar och dödlighet.

I **delarbete 1** undersökte vi förekomsten av förhöjda leverprover, och om dessa förhöjda prover kunde förklaras av NAFLD, hos individer med mångårig insulinresistens. Insulinresistens förekommer hos majoriteten med NAFLD, medan det omvända förhållandet är oklart. Vi identifierade individer som deltagit i den

populationsbaserade kohorten Malmö Kost Cancer (MKC) 1991-1996, där en subgrupp utreddes med blodprover för bedömning av insulinresistens (formeln HOMA-IR, d.v.s. faste-insulin x faste-blodsocker/22.5) och genomgick förnyade undersökningar. Den fjärdedel med högst värden av HOMA-IR och utan diabetes (305 stycken) bedömdes som insulinresistenta och samtliga bjöds in för att kontrollera leverprover till vår studie, i medel 17 år efter inklusion i Malmö Kost Cancer. Enbart 25 individer hade förhöjda leverprover och när dessa undersöktes vidare med utvidgad blodprovstagning, ultraljud eller skiktröntgen av levern, samt läkarundersökning, hade enbart 5 individer NAFLD. De som diagnosticerades med NAFLD hade högre förekomst av det metabola syndromet, var mer insulinresistenta eller hade utvecklat diabetes.

I **delarbete 2** var målsättningen att beskriva naturalförloppet vid NAFLD, inklusive utveckling av lever-relaterade komplikationer, metabola sjukdomar och dödlighet. Samtliga patienter som genomgått leverbiopsi och fått diagnosen NAFLD 1978-2006, och som deltagit i den populationsbaserade kohorten Malmö Förebyggande Medicin (MFM) 1974-1992 identifierades. Vid inklusion i MFM ingick blodprovstagning, ett extensivt frågeformulär om bland annat sjukhistoria och livsstil, samt en klinisk undersökning. Totalt undersöktes 33 346 individer. Sammanlagt 36 individer med biopsi-verifierad NAFLD identifierades. Journalgranskning gjordes på samtliga, och resterande MFM användes som kontroll-grupp. Medeluppföljningstiden var 27 år. Sammanlagt 25% av NAFLD-patienter utvecklade skrumplever och 14% primär levercancer. Dödligheten var signifikant ökad i NAFLD-gruppen jämfört med en kontrollgrupp, och den vanligaste dödsorsaken var hjärt-kärlsjukdom följt av lever-relaterade komplikationer.

Även **delarbete 3** utgick från leverbiopserade patienter med NAFLD, men nu inkluderades samtliga som fått diagnosen 1978-2006 från ett lokalt biopsiregister. Målsättningen var att studera kopplingen till kronisk njurfunktionsnedsättning vid NAFLD, vilken verkar ha en ökad förekomst jämfört med normalpopulation, och om det påverkar dödligheten. Sammanlagt 120 patienter inkluderades och en extensiv journalgranskning gjordes. Blodprover från biopsitillfället och senaste uppföljningen fram till 2016-12-31 eller död registrerades. Som kontroll-grupp användes en subgrupp från MFM, där bedömning av njurfunktion fanns vid inklusion 1991-1996 och vid uppföljning 2002-2006. Medel-uppföljningstiden för NAFLD-patienter var 19.5 år. Förekomsten av kronisk njurfunktionsnedsättning var enbart signifikant högre i den högsta åldersgruppen (>55 år) vid inklusion jämfört med kontroller (25% jämfört med 9.4%), men ingen signifikant skillnad sågs vid uppföljning. NAFLD-patienter med långvarig kronisk njurfunktionsnedsättning hade däremot en signifikant högre dödlighet jämfört med de NAFLD-patienter som hade bevarad njurfunktion. Statistiska analyser visade dock att den högre dödligheten kunde förklaras av en ökad förekomst att metabola sjukdomar som t.ex.

diabetes samt fibros-utveckling hos dessa patienter, inte av njurfunktionsnedsättningen i sig.

Från samma lokala leverbiopsiregister inkluderades även patienter till **delarbete 4**. Målsättningen var att ta reda på om enkla icke-invasiva score-system för bedömning av fibros i levern kan användas för att tidigt identifiera NAFLD-patienter med framtida risk för metabola sjukdomar, lever-relaterade komplikationer och dödlighet. Totalt 144 patienter inkluderades, och medeluppföljningstiden var 19 år. Redan vid biopsitillfället hade 18.1% avancerad ärrbildning i levern (fibros-stadie 3-4). Vid uppföljning, baserad på extensiv journalgranskning fram till 2016-12-31 eller död, hade 17% diagnosticerats med skrumplever, 6% med primär levercancer och 14% hade lever-relaterade komplikationer, framför allt vätska i bukhålan. Fyra väl validerade enkla score-system beräknades med blodprovssvar från biopsitillfället (NAFLD fibrosis score (NFS), FIB-4 index, APRI och BARD). NFS predikterade signifikant alla inkluderade framtida utfall (diabetes mellitus, kardiovaskulär sjukdom, kronisk njurfunktionsnedsättning, lever-relaterade komplikationer och total dödlighet), och även FIB-4 index med undantag av diabetes. APRI och BARD predikterade färre utfall. När resultaten av respektive score-system delades upp i tre grupper (låg, medelhög och hög risk för avancerad fibros, enligt tidigare studier) sågs även här klart ökad risk för de inkluderade utfallen vid stigande score för NFS och FIB-4 index, till viss del för APRI, men inte för BARD. Sammanfattningsvis kan icke-invasiva score-system användas till att tidigt identifiera risk-individer med NAFLD, men det är inte klart vilket score som ska användas, om score ska kombineras eller om utveckling av befintliga score är nödvändigt.

Sammantaget i avhandlingen sågs en klart ökad risk att utveckla skrumplever vid NAFLD, vilket hos biopserade patienter sågs hos cirka 17%. Det fanns en klart ökad risk att utveckla primär levercancer i denna population, vilket 6% gjorde. Symptomgivande allvarlig leversjukdom, med t.ex. förvirring på grund av leversjukdom eller vätska i bukhåla sågs hos 14%.

Vid långtidsuppföljning var den vanligaste metabola sjukdomen högt blodtryck, vilket 66% hade diagnosticerats med. Förekomst av diabetes sågs hos 54%, och NAFLD-patienter med avancerad ärrbildning i levern hade särskilt ökad risk för diabetes. Kardiovaskulär sjukdom förekom hos 54 % (här definierat som kärlkramp, hjärtinfarkt och blodpropp till hjärnan). Kronisk njurfunktionsnedsättning bedömdes föreligga hos 37.5% vid uppföljning.

Dödligheten var signifikant ökad vid NAFLD jämfört med en kontrollgrupp från MFM, och signifikant ökad vid NAFLD med långvarig kronisk njurfunktionsnedsättning jämfört med NAFLD med bevarad njurfunktion, vilket i det senare fallet berodde på en ökad förekomst av metabola sjukdomar. Icke-

invasiva score-system, framför allt NFS och FIB-4 index, kan användas för att tidigt identifiera risk-patienter avseende ökas mortalitet.

Resultaten i denna avhandling indikerar behovet av att tidigt upptäcka metabola risk-faktorer för att förhindra progress till metabola sjukdomar vilket ökar risken för NAFLD. Det indikerar också ett behov av att tidigt identifiera NAFLD-patienter med progress av metabola sjukdomar, med en ökad risk för lever-relaterade komplikationer och en ökad dödlighet, där användning av icke-invasiva score-system kan vara ett sätt.

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