Non-Alcoholic Fatty Liver Disease

A clinical and histopathological study

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Till Charlotte och Moa!

Of all knowledge, there is nothing greater than to know that You love me.

Abstract

Fatty liver has previously often been associated with excessive alcohol consumption. During the last two decades, the interest in fatty liver occurring in non-drinkers i.e. non-alcoholic fatty liver disease (NAFLD) has increased dramatically. Today, NAFLD is considered as the most common liver disease in the developed world. It is strongly associated with obesity, insulin resistance, and hypertension. Thus, NAFLD is considered as the hepatic manifestation of the metabolic syndrome.

The spectrum of NAFLD includes: simple fatty liver without necroinflammatory activity; non-alcoholic steatohepatitis (NASH), a condition characterised by hepatocellular injury, inflammation, and fibrosis; cirrhosis; and in some individuals hepatocellular carcinoma.

The degree of steatosis in liver biopsies is usually assessed by a morphological semiquantitative approach in which the pathologist uses a four-graded scale: 0–3 or none, slight, moderate and severe. In this thesis we show that there is a considerable inter- and intra-individual variation in such scoring methods and that a more standardised and quantitative approach is preferable. The area/volume of fat in liver biopsies is greatly overestimated when assessed semiquantitatively. Moreover, the point counting technique has a better reproducibility than visual evaluation and should be preferred in estimates of liver steatosis.

The long-term clinical and histopathological course of 129 consecutively enrolled NAFLD patients was studied. Mean follow-up (SD) was 13.7 (1.3) years. Survival of NASH patients was reduced compared with a matched reference population. These subjects more often died from cardiovascular and liver-related causes. Seven patients (5.4%) developed end-stage liver disease, including 3 patients with hepatocellular carcinoma. Most NAFLD patients will develop diabetes or impaired glucose tolerance in the long term. Progression of liver fibrosis is associated with more pronounced insulin resistance and significant weight gain.

During follow-up, 17 patients had been prescribed a statin. At follow-up, patients on medication with statins had significantly higher BMI. Diabetes was significantly more common among patients on medication with statins and they had significantly more pronounced insulin resistance. However, they exhibited a significant reduction of liver steatosis at follow-up as opposed to patients not taking statins. Although patients under statin treatment exhibited a high risk profile for progression of liver fibrosis, only four patients on

statin treatment progressed in fibrosis stage. It is concluded that statins can be prescribed safely in patients with elevated liver enzymes because of NAFLD.

Alcohol consumption was evaluated with a validated questionnaire combined with an oral interview. In a multivariate analysis moderate alcohol consumption, particularly when frequency of heavy episodic drinking was analysed, consistent with the diagnosis of NAFLD to be set, was independently associated with fibrosis progression in NAFLD.

The NAFLD activity score (NAS) is a newly proposed system to grade the necroinflammatory activity in liver biopsies of NAFLD patients. We evaluated the usefulness of the NAS in predicting clinical deterioration and fibrosis progression in our cohort of NAFLD patients. Although the NAS was independently associated with future risk of progressive fibrosis in NAFLD, the clinical usefulness of the score was limited due to significant overlap in clinical development between NAS-score groups.

List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Semiquantitative evaluation overestimates the degree of steatosis in liver biopsies: a comparison to stereological point counting.**
Franzén LE, Ekstedt M, Kechagias S, Bodin L
Mod Pathol 2005;18:912-916

II **Long-term follow-up of patients with NAFLD and elevated liver enzymes.**
Ekstedt M, Franzén LE, Mathiesen UL, Thorelius L, Holmqvist M, Bodemar G, Kechagias S
Hepatology 2006;44:865-873

III **Statins in non-alcoholic fatty liver disease and chronically elevated liver enzymes: A histopathological follow-up study.**
Ekstedt M, Franzén LE, Mathiesen UL, Holmqvist M, Bodemar G, Kechagias S
J Hepatol 2007;47:135-141

IV **Alcohol consumption is associated with progression of hepatic fibrosis in nonalcoholic fatty liver disease.**
Ekstedt M, Franzén LE, Holmqvist M, Bendtsen P, Mathiesen UL, Bodemar G, Kechagias S
Submitted

V **The clinical relevance of the Nonalcoholic Fatty Liver Disease Activity Score (NAS) in predicting fibrosis progression.**
Ekstedt M, Franzén LE, Mathiesen UL, Bodemar G, Kechagias S
Submitted

Contents

Abbreviations

AAT	Alfa-1-antitrypsin
AFLD	Alcoholic fatty liver disease
ALD	Alcoholic liver disease
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ApoB	Apolipoprotein B
AST	Aspartate aminotransferase
AUDIT	Alcohol use disorders identification test
AUDIT-C	The AUDIT alcohol consumption questions
BMI	Body mass index
CoA	Coenzyme A
ChREBP	Carbohydrate response element-binding protein
CI	Confidence interval
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
FFA	Free fatty acids
GGT	Gamma glutamyl transpeptidase
HbA1c	Haemoglobin A1c (Glycosylated haemoglobin)
HED	Heavy episodic drinking
^{1}H-MRS	Proton magnetic resonance spectroscopy
HOMA	Homeostasis model assessment
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
ICC	Intraclass correlation coefficient
IGT	Impaired glucose tolerance
IKK-ß	IκB kinase - ß
IL	Interleukin
INR	International normalized ratio
IR	Insulin resistance
LDL	Low density lipoprotein
NA	Not available
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Non-alcoholic steatohepatitis
NF-κB	Nuclear factor-κB
PBC	Primary biliary cirrhosis
PMN	Polymorphonuclear cell
PPAR	Peroxisome proliferator-activated receptor
PSC	Primary sclerosing cholangitis
RNA	Ribonucleic acid
SIBO	Small intestinal bacterial overgrownth
SREBP	Sterol regulatory element-binding protein
TGF-ß	Tumor growth factor - ß
TNF-α	Tumor necrosis factor - α
VLDL	Very low density lipoprotein
QUICKI	Quantitative insulin sensitivity check index

Introduction

Background

The accumulation of lipids within hepatocytes is commonly referred to as fatty liver. Fatty liver has traditionally been considered as a benign and reversible condition and to represent a non-specific response of the liver to metabolic stress of different origin. Previously, most cases of fatty liver were attributed to excessive alcohol consumption.

In 1980 Ludwig and colleagues described 20 middle-aged patients without apparent alcohol consumption with abnormal liver biochemical test results and morphological evidence of alcoholic hepatitis, i.e. moderate to severe steatosis with lobular inflammation.[1] The disease was named non-alcoholic steatohepatitis (NASH). Although the paper by Ludwig et al. is often referred to as the first report of NASH, the histopathological features seen in NASH were described earlier.[2, 3] Over the years several names have been used to describe this condition: diabetic hepatitis,[4] non-alcoholic steatonecrosis,[5] alcohol-like liver disease in the non-alcoholic,[6] non-alcoholic fatty hepatitis,[7] fatty liver hepatitis,[8] bright liver syndrome,[9] and non-alcoholic steatosis syndromes.[10] There is a strong association between the occurrence of fatty liver and insulin resistance, one of the core features of the metabolic syndrome.[11]

During the last two decades a large number of studies have challenged the benign nature of non-alcoholic fatty liver. Some patients with this condition will progress to liver cirrhosis and hepatocellular carcinoma (HCC).[12] These observations have spurred an immense interest among scientists all over the world. In 2007 more than 200 articles were published investigating different aspects of this intriguing condition.

Definition of non-alcoholic fatty liver disease

Traditionally, hepatic fat content exceeding 5% of liver weight has been considered the definition of fatty liver.[13] When the hepatic triglyceride content was measured in 345 subjects without apparent risk factors for hepatic steatosis (non-obese, non-diabetic, minimal alcohol consumption, normal liver biochemical tests, and no known liver disease) with proton magnetic resonance spectroscopy, the upper limit of normal, i. e. the 95th percentile, was 5.56%,[14] thus being in close agreement with the traditional definition.

Fatty infiltration of the liver may arise in a variety of medical conditions and can be triggered by drugs, nutrition, and infections (Table 1). However, in the majority of patients, fatty liver is, with today's scientific knowledge, attributed either to excessive alcohol consumption, i.e. alcoholic fatty liver disease (AFLD), or to overweight/obesity, i.e. non-alcoholic fatty liver disease (NAFLD).

There is no consensus on what represents "excessive" alcohol consumption with regards to scientific studies of the liver. In studies published on NAFLD the cut-off level for what is considered to be a tolerable alcohol consumption ranges from abstinence[1, 6, 15] up to 252 g/week.[16] Most commonly 140 g/week is used to differentiate between AFLD and NAFLD.

Table 1: Causes of fatty liver others than alcohol and overweight/obesity.

Nutritional	Drugs	Inborn errors of metabolism	Miscellaneous conditions
Gastrointestinal surgey for obesity	Amiodarone	Abetalipoproteinemia	Fatty liver of pregnancy
Malnutrition	Antiviral agents	Familial hepatosteatosis	Hepatitis C
Rapid weight loss	Aspirin	Galactosemia	Human immunodeficiency
Starvation	Cocaine	Glycogen storage disease	virus infection
Total parenteral nutrition	Diclorethylene	Herediteray fructose intolerance	Inflammatory bowel disease
	Ethionine	Homocystinuria	Partial lipodystrophy
	Ethyl bromide	Systemic carnitine deficiency	Severe anemia
	Glucocorticoids	Tyrosinemia	Small-bowel diverticulosis with
	Hydrazine	Weber-Christian syndrome	bacterial overgrowth
	Hypoglycin	Wilson disease	Environmental hepatotoxins
	Methotrexate		-Toxic mushrooms
	Perhexiline maleate		-Phosphorus
	Safrole		-Petrochemicals
	Synthetic estrogens		-Organic solvents
	Tamoxifen		
	Tetracycline		
	Valproic acid		

Histopathology of NAFLD

NAFLD is a spectrum of liver lesions ranging from simple hepatic steatosis to NASH with progressive fibrosis leading to cirrhosis and liver failure in some patients and eventually hepatocellular carcinoma. The different parts of this spectrum are probably best regarded as parts of a histological continuum.
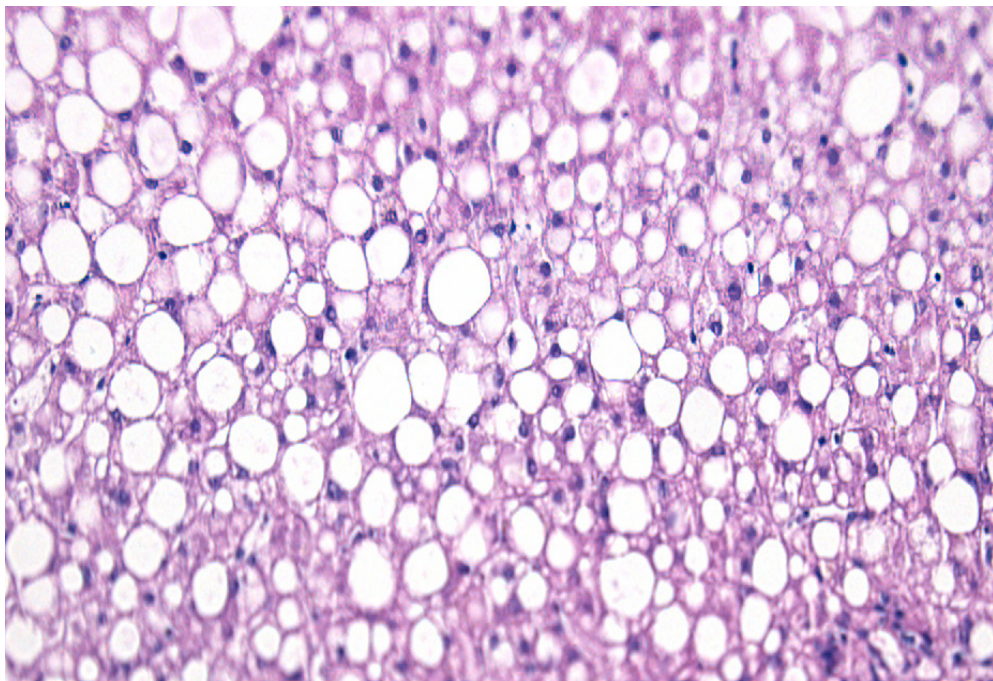
Figure 1: *Pronounced panacinar macrovesicular steatosis (Haematoxylin and eosin).*
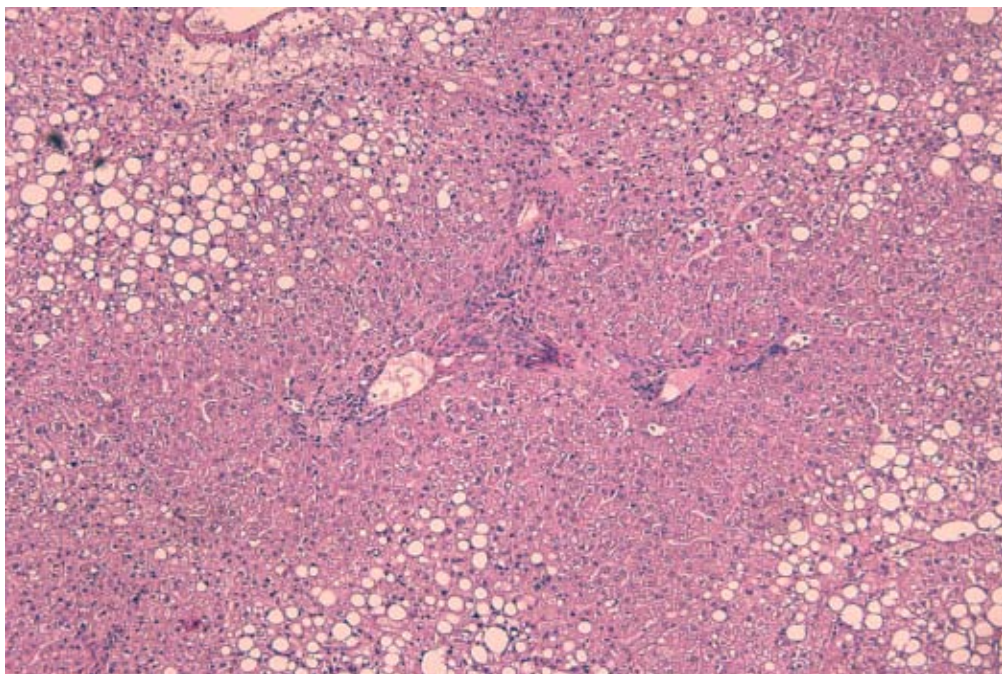
Figure 2: *Wedge biopsy obtained during bariatric surgery. A portal tract is seen in the center of the image. Note the predominantly perivenular involvement of macrovesicular steatosis (acinar zone 3) (Haematoxylin and eosin).*
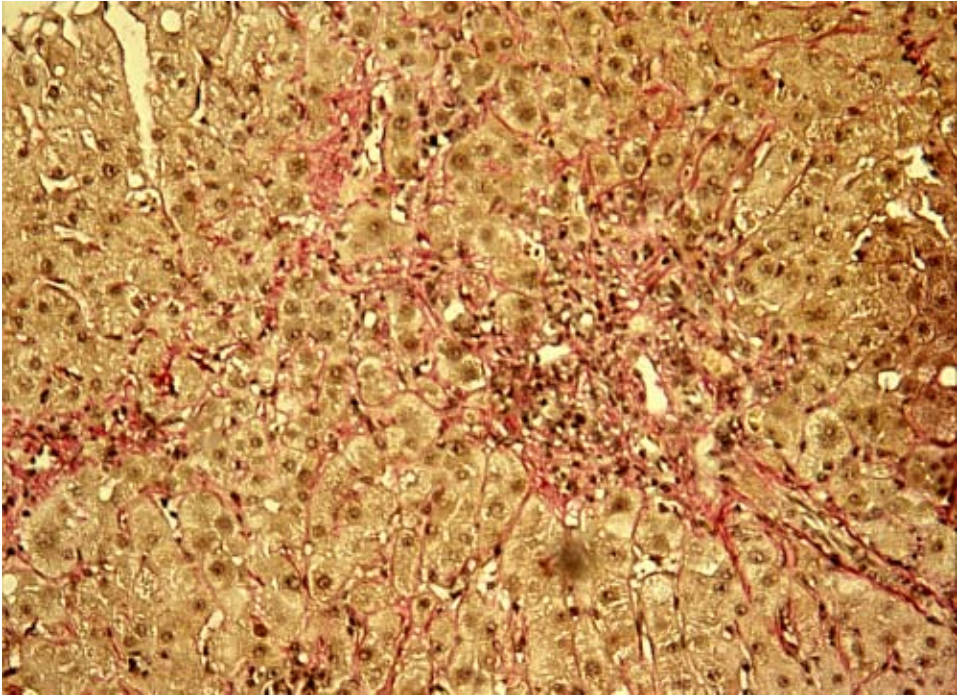
Figure 3: *Pericellular distribution of fibrosis in NAFLD. Steatosis and hepatocellular ballooning often regress as fibrosis stage progresses (van Gieson).*
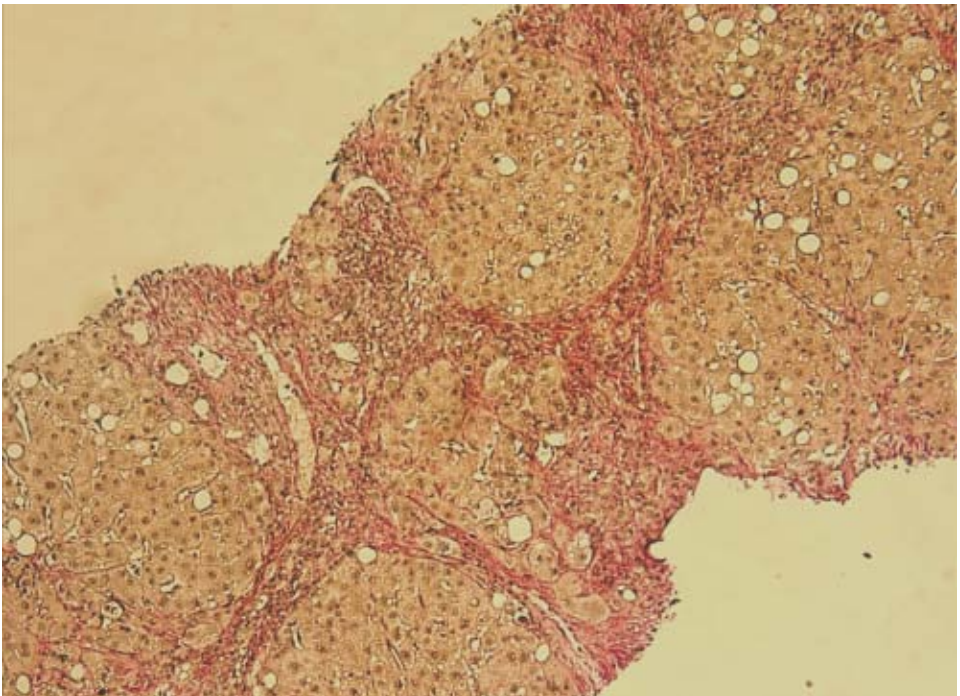
Figure 4: *Cirrhosis in NAFLD. Bridging fibrosis and regenerative nodules are present as well as mild steatosis (Van Gieson).*

The histopathological hallmark of NAFLD is macrovesicular steatosis, which predominantly affects the perivenular regions (acinar zone 3). In severe cases it can extend to a panacinar distribution (Figure 1-2). There is no clear cut-off for how many fat vacuoles visible in the light microscope that can be regarded as normal. It has been suggested that < 5% of hepatocytes involved should be considered normal.[17] However, this is based on assumption rather than hard evidence.

When the hepatic steatosis is accompanied by features of necroinflammation the diagnosis of NASH can be made. The most characteristic feature of necroinflammation and hepatocellular injury in NAFLD is hepatocellular ballooning, which sometimes is associated with formation of Mallory's hyaline. Mallory bodies in NAFLD are often small and poorly formed. Immunohistochemical techniques might therefore be needed to detect this histological feature.[18, 19] Lobular inflammation with a mixed infiltration of neutrophils, lymphocytes and macrophages can be detected although the severity is typically mild. Several other histopathological findings have been reported in NAFLD.[20] Fibrosis is sometimes considered as a feature of steatohepatitis and is commonly used to describe the stage of the disease.

The typical pattern of fibrosis of NAFLD is a perisinusoidal and/or pericellular distribution (Figure 3). Eventually bridging fibrosis may develop and in some patients the fibrosis progresses to cirrhosis (Figure 4). Once cirrhosis has developed, features of steatohepatitis often become less prominent.[21] Sinusoidal capillarization and portosystemic shunting has been suggested as explanation for this phenomenon.[22]

The histopathological definition of the different parts of the disease has previously not been well defined.[23] Different definitions have been used by different authors.[24, 25] The scoring system of NASH (Table 2) developed by Brunt and colleagues has been widely accepted. It unifies the lesions of steatosis and necroinflammation into a "grade" and those of fibrosis into a "stage".[26]

Table 2: Grading and staging of the histopathological lesions in NASH according to Brunt[26].

Necroinflammatory Grade

Grade 1, Mild
    Steatosis (predominantly macrovesicular) involving up to 66% of biopsy; may see occasional ballooned zone 3 hepatocytes; scattered rate intra-acinar polymorphonuclear cells ± intraacinar lymphocytes; no or mild portal chronic inflammation.
Grade 2, Moderate
    Steatosis of any degree; ballooning of hepatocytes (predominantly zone 3) obvious, intra-acinar pmn's noted, may be associated with zone 3 pericellular fibrosis; portal and intra-acinar chronic inflammation noted, mild to moderate.
Grade 3, Severe
    Panacinar steatosis; ballooning and disarray obvious, predominantly in zone 3; intra-acinar inflammation noted as scattered pmn's, pmn's associated with ballooned hepatocytes ± mild chronic inflammation; portal chronic inflammation mild or moderate, not marked.

Fibrosis Stage

Stage 1
    Zone 3 perisinusoidal/pericellular fibrosis; focally or extensively present.
Stage 2
    Zone 3 perisinusoidal/pericellular fibrosis with focal or extensive periportal fibrosis.
Stage 3
    Zone 3 perisinusoidal/pericellular fibrosis and portal fibrosis with focal or extensive bridging fibrosis.
Stage 4
    Cirrhosis.

Although this scoring system is appealing it was developed for NASH and does not encompass the entire spectrum of NAFLD. The multicenter cooperative Clinical Research Network for NASH[27] developed a histopathological scoring system in order to encompass the entire spectrum of NAFLD. The scoring protocol comprised 14 individual histopathological features. Using multiple logistic regression the NAFLD activity score (NAS) was constructed. The NAS is the unweighted sum of steatosis, lobular inflammation, and hepatocellular ballooning scores. NASH was defined as a NAS of ≥5, "borderline NASH" as a NAS of 3 or 4, and "not NASH" as a NAS of < 3.[28] This scoring system is very appealing due to its simplicity, but the authors state that "it is not intended that numeric values replace the pathologist's diagnostic determination of steatohepatitis".

Variability in assessment of histopathology in NAFLD

There are two main reasons for variability in the diagnostic information obtained by liver biopsy. First there is the variability because of sampling error and, secondly, there is both intraobserver and interobserver variation in the assessment of the histopathological findings.

The liver specimen obtained by liver biopsy represents approximately 1/50 000 of the total mass of the liver.[29] A few studies have been designed to investigate sampling variability in NAFLD. In a study by Ratziu et al. 51 patients with suspected NAFLD underwent percutaneous liver biopsy and two samples were collected from the right lobe in each patient. Substantial agreement was seen for steatosis grade (κ = 0.64), moderate agreement for

hepatocyte ballooning (κ = 0.45) and perisinusoidal fibrosis (κ = 0.43), while Mallory bodies (κ = 0.27), and lobular inflammation (κ = 0.13) displayed only slight agreement. The negative predictive value of absence of NASH (i.e. steatosis and ballooning) in the first liver biopsy was 0.78 when the second liver biopsy was used as standard. When a composite diagnosis including hepatocyte ballooning and perisinusoidal fibrosis was used, the negative predictive value was even lower (0.74). For fibrosis stage moderate agreement was seen (κ = 0.47).[30]

Intraobserver agreement is generally good for grading steatosis and moderate to substantial for assessment of fibrosis. The variability in grading necroinflammatory items is generally higher. Kappa values from studies investigating sampling variability, intraobserver as well as interobserver variability are summarized in Table 3.

Table 3: Sampling, intraobserver and interobserver variability in NAFLD

	Coefficient of concordance (κ)		
	Sampling reliability[a]	Intraobserver reliability[b]	Interobserver reliability[e]
Steatosis (grade)	0.64-0.88	0.74-0.98	0.64
Ballooning (grade)	0.20-0.45	0.62-0.64	0.50
Lobular inflammation (present)	0.13-0.32	0.37-0.58	0.21
Mallory bodies (presence)[c]	0.27	0.39	0.33
Interface hepatitis (presence)[c]	0.78	0.91	0.21
Acidophilic bodies (presence)[c]	0.07	0.34	0.17
Fibrosis	0.47-0.53	0.68-0.69	0.60
Diagnosis of NASH	0.32-0.82	0.85-0.90	NA

[a]From Ratziu et al.[30] and Merriman et al.[31], [b]From Ratziu et al.[30], [c]Not assessed in Merriman et al.[31], [d]Not assessed in Ratziu et al.[30], [e]Younossi et al.[32]

Pathophysiology of NAFLD

Throughout human history the principal threat to survival has been recurrent famine. Adipocytes enable humans to store energy for coping with cycles of undernutrition. During the 20th century, however, an unprecedented change in the pattern of caloric availability has taken place in Western societies, which together with a more sedentary lifestyle has lead to a state of chronic overnutrition in millions of people.[33] This change in nutritional state at the population level has lead to an increasing prevalence of diseases associated with overnutrition such as the metabolic syndrome and its complications.

Fat depots

Since energy intake in humans is periodic it is vital that our bodies have the capacity to store energy to be utilised when fasting. Carbohydrate is stored in the liver as glycogen and lipids

are stored in adipose tissues as triglycerides. To store energy in the form of lipids is much more efficient since the caloric value of lipid stores is about 100 times that of carbohydrate stores. The endogenous fuel stores are shown in Table 4.

Table 4: Approximate energy stores in males weighing 70 kg

Tissue	Fuel source	Grams	Kilocalories
Adipose tissue	Triglycerides	13,000	121,000
Liver	Glycogen	100	400
	Protein	300	1,200
	Triglycerides	50	450
Muscle	Protein	6,000	24,000
	Glycogen	400	1,600
	Triglycerides	250	2,250
Blood	Glucose	3	12
	Triglycerides	4	37
	Free fatty acids	0.5	5

The classical compartment for lipid storage is the subcutaneous fat tissue. In cases where the subcutaneous lipid stores are exceeded lipids may be shunted to other depots, such as intra-abdominal compartments and to insulin-sensitive tissues (i.e. muscle and the liver) that are prone to deposit lipids in specific clinical scenarios.[34] The abdominal fat depot has been associated with increased risk of insulin resistance, diabetes, and cardiovascular disease. Abdominal fat is composed of several distinct anatomic depots, which can be further subdivided into several distinct storage sites. The subcutaneous fat can be subdivided into anterior and posterior (superficial and deep) layers, and the intraabdominal fat can be divided into intraperitoneal and retroperitoneal sites. The intraperitoneal (visceral) fat depot is composed of mesenteric and omental fat. Of the body total fat content the visceral fat depots constitutes approximately 10%.[35]

Normal hepatic lipid metabolism

When eating, dietary lipids (>90% triglycerides) are digested within the gastrointestinal tract and lipolytic products cross into the enterocyte. Within the enterocytes triglycerides, cholesterol, phospholipids, and apolipoproteins are packaged into chylomicrons. Chylomicrons enter the lymph system, which drains into the venous circulation. Lipoprotein lipase in capillaries of adipose tissue, skeletal muscle, and heart hydrolyses triglycerides in the chylomicrons to fatty acids and glycerol, leaving behind "remnant" chylomicrons.

Free fatty acids enter adipocytes, muscle, and hepatocytes where they are esterified to glycerol-3-phosphate to ultimately form triglycerides for storage.[36]

Within the liver, fatty acids come from the circulation in the form of free fatty acids derived from lipolysis in adipose tissues or from the *de novo* synthesis of fatty acids from Acetyl-CoA, the regulatory building block. Dependent of energy state the fatty acids within the liver is either stored as triglycerides or they undergo β-oxidation in the mitochondria or peroxisomes of the hepatocytes, not contributing to energy storage. When the fatty acids are not used for producing energy they are converted to triglycerides for storage or to be transported into the circulation by very low density lipoprotein (VLDL). The main source of fatty acids for VLDL production comes from lipolysis within adipose tissue. *De novo* liver lipogenesis only contributes with 8%, in the feeding state (4% in the fasting state), of the fatty acids incorporated in to the VLDL particle while adipose tissue contributes with 44%, chylomicrons with 15%, and dietary acids with 10%.[37] The ability of the liver to assemble and secrete VLDL particles significantly affects the intra-hepatic lipid steady state.

Fat accumulation

The accumulation of lipids (mostly triglycerides) within the liver seen in NAFLD is the result of an imbalance in hepatic normal lipid turnover. There are several possible sites in the normal lipid metabolism where alterations can result in the appearance of hepatic steatosis: the delivery of free fatty acids to the liver; the *de novo* lipogenesis in hepatocytes; the rate of β-oxidation within the liver; and the export of triglycerides through production and secretion of VLDL (Figure 5).[38]

Influx of FFA. In plasma, there is a pool of fatty acids that circulate in nonesterified form often referred to as free fatty acids. Fatty acids from dietary intake and from lipolysis in adipose tissue are the main sources of fatty acids in this pool.[39] In subjects with insulin resistance the hormone sensitive lipase within the adipocytes is not fully suppressed by insulin. Therefore, in these individuals the result is an increased lipolysis within the adipose tissue resulting in an increased influx of free fatty acids to the liver.[40] Visceral adipose tissue releases excess free fatty acids to the portal circulation and is considered as one of the key players in the pathophysiology of hepatic insulin resistance and liver steatosis. The correlation between visceral fat and insulin resistance is well established. Unexpectedly, only
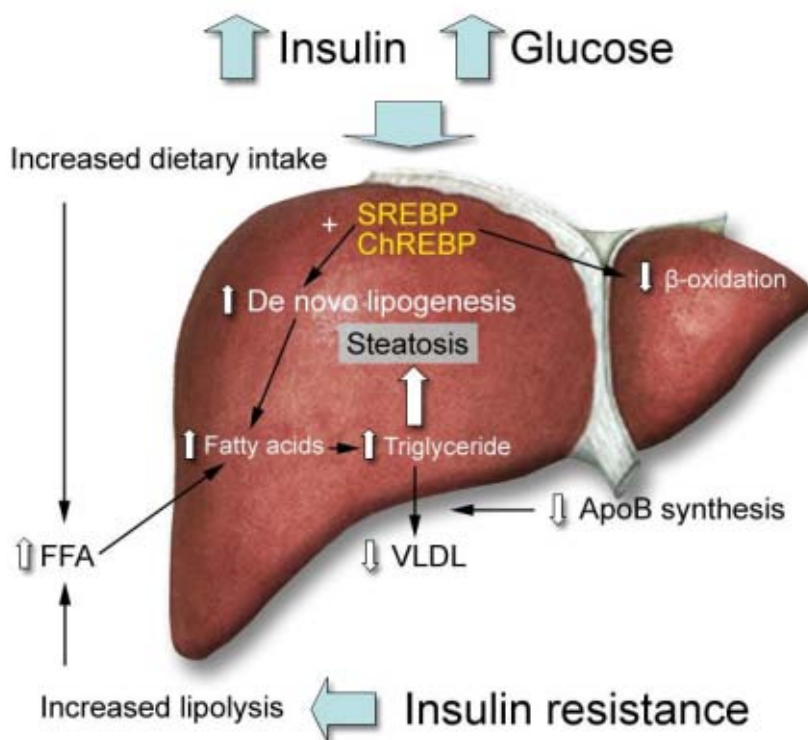
Figure 5: Hepatic steatosis is the result of an imbalance in the normal hepatic lipid turnover. As a consequence of the increased lipolysis within the adipose tissue due to insulin resistance, the liver is presented with an increased influx of free fatty acids (FFA). In insulin resistant individuals, insulin and glucose levels are increased. As a result, transcription factors such as sterol regulatory element-binding protein (SREBP) and carbohydrate response element-binding protein (ChREBP) are increased which lead to increased de novo lipogenesis as well as decreased β-oxidation. Together with an increased dietary intake in many NAFLD patients as well as impaired transportation of lipids out of the liver by very low density lipoproteins (VLDL) these factors all contribute to the excessive accumulation of lipids within the liver seen in NAFLD. (Illustration: Åsa Källstrand Thor)

approximately 5% and 20% of portal free fatty acids originated from visceral fat in lean and obese subjects, respectively. The relative amount of portal vein free fatty acids derived from visceral fat was much less than that derived from subcutaneous fat but with a higher proportion in persons with upper obesity.[41]

Hepatic lipogenesis. In patients with NAFLD, *de novo* lipogenesis is increased in the fasting state and fails to respond to changes in dietary state. Approximately one quarter of the triacylglycerol in the liver of NAFLD patients is produced by *de novo* lipogenesis compared with approximately 5% in subjects without hepatic steatosis.[39] In animal models of NAFLD,

the activity of two transcriptional factors, sterol regulatory element-binding protein (SREBP-1) and carbohydrate response element-binding protein (ChREBP), is increased.[42, 43] Both factors regulate gene expression resulting in an increased *de novo* lipogenesis. The increased lipogenesis leads to a concomitant decrease in fatty acid β-oxidation.

Lipid export. Triglycerides are transported out of the liver in VLDL particles. Each VLDL particle has a diameter of 30-100 nm and contains a single molecule of apolipoprotein B (ApoB). The synthesis of ApoB is a rate-determining step in the production of VLDL within the hepatocyte. Hyperinsulinaemia, which is seen in insulin resistance, can alter the synthesis of ApoB.[44] In NASH patients the synthesis of ApoB is decreased, which might indicate that decreased ApoB synthesis is an important factor in the development of hepatic steatosis.[45]

From fat to inflammation and fibrosis

The accumulation of fat within hepatocytes has been regarded to be a benign and reversible condition. Why some individuals with fatty liver develop inflammation and/or fibrosis, i.e. NASH, which is considered as the more aggressive form of NAFLD, is not fully understood. A large number of cytokines, adipokines and altered gene expressions have been shown to play a role in progression of fatty liver to inflammation and ultimately liver fibrosis. In 1998, the so called "two hit" model was proposed by Day.[46] According to this model the "first hit" would be the development of hepatic steatosis and an assumed "second hit" would lead to inflammation and fibrogenesis. However, this theory has been challenged as knowledge of the interplay between insulin resistance, free fatty acids and adipose tissue inflammation has increased. It has been suggested that steatosis is an epiphenomenon of the injurious mechanisms rather than a true "first hit".[47]

In obesity, macrophage infiltration of the adipose tissue give rise to a pro-inflammatory milieu.[48] In this pro-inflammatory state a number of adipokines and cytokines have been found to be associated with the accumulation of fat and the presence of NASH. Since most studies are cross-sectional in design it is impossible to determine what alterations are primary or secondary. In NAFLD progression there is a complex interplay between adipose tissue, the liver, and inflammatory cells where many factors exercise control on each other. This pro-inflammatory milieu and the interplay between the different organs are schematically summarised in Figure 6.
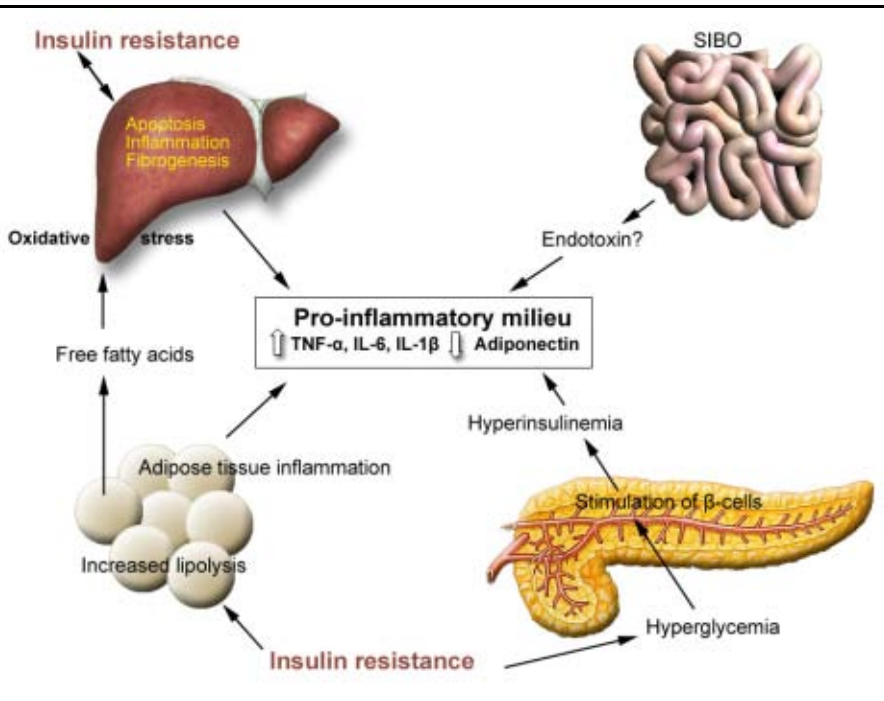
Figure 6: In the transition from NAFLD to NASH, the more aggressive part of the NAFLD spectrum, several pro-inflammatory changes have taken place. Insulin resistance affects the liver, adipose tissue, and the pancreas which give rise to a pro-inflammatory milieu. The role of small intestinal bacterial overgrowth (SIBO) is debated and might play a role. In the liver the pro-inflammatory changes give rise to increased inflammation and apoptosis which stimulate further cytokine production. Finally, activation of stellate cells leads to fibrogenesis. (Illustration: Åsa Källstrand Thor)

Some of the individual molecules and cellular processes associated with inflammation and fibrogenesis in NAFLD has gained more intense interest and are shortly summarised:

Adiponectin. Adiponectin is a cytokine exclusively produced by adipocytes. In mice, adiponectin decreases hepatic lipogenesis and increases free fatty acid oxidation.[49] In humans, several studies have shown a reverse correlation between adiponectin levels and hepatic insulin sensitivity as well as fat content in the liver.[50, 51] Adiponectin is anti-steatotic in both muscle and hepatocytes, probably by activating PPARγ and AMP-dependent kinase.[49] Adiponectin production is decreased by TNF-α as well as oxidative stress and both are considered important in the progression of NAFLD. Moreover, adiponectin suppresses the production of TNF-α making it an important anti-inflammatory agent.[52]

Leptin. Leptin is a 16-kDa peptide hormone coded by the *ob* gene secreted mainly by adipocytes of white fat tissue. The Leptin receptor, Ob-R, is a member of the class-1 cytokine

receptor family and was originally demonstrated in hypothalamic neurons.[53] In animal studies, the main role of leptin seems to be prevention of lipid accumulation in non-adipose sites, such as the myocardium, skeletal muscle, pancreas, and liver.[54] Initially leptin was characterised as a regulator of body weight and energy expenditure.[55] In NASH patients serum leptin levels are increased compared with gender and BMI matched controls. Serum leptin levels were independently associated with the amount of steatosis but not with inflammation and fibrosis.[56]

Peroxisome proliferator-activated receptors (PPARs). PPARs are members of the nuclear receptor superfamily and ligand activated PPARs induce gene expression that regulates adipogenesis, lipoprotein metabolism, glucose metabolism, and inflammation. PPARs exist in three isoforms, PPAR-α, PPAR-δ/β, and PPAR-γ. Established treatments affecting PPARs are fibrates, which are PPAR-α agonists, and the thiazolidinediones, which are PPAR-γ agonists.[57] PPAR-α is expressed in metabolically active tissues such as the liver, muscle, heart, and kidneys. Fatty acids stimulate PPAR-α to increase transcription of enzymes that induce peroxisome proliferation, lipid uptake, and increased lipid β-oxidation. PPAR-α thereby serves as a regulator against lipid accumulation in the liver. In mice, PPAR-α stimulation prevents intra-hepatic lipid accumulation and prevent the development of steatohepatitis.[58]

PPAR-γ is abundantly expressed in adipose tissue and to a lesser extent in macrophages, muscle, and liver. In adipocytes PPAR-γ modulates key glucoregulatory molecules and adipocyte differentiation promoting lipid storage in mature adipocytes, thereby preventing lipid storage in non-adipose tissues.[59] Stimulation of PPAR-γ improves insulin sensitivity and lipoprotein profile in humans.[60] Hepatic Kupffer cells also express PPAR-γ, as well as quiescent hepatic stellate cells. PPAR-γ plays a critical role in the control of inflammation and activation of stellate cell, thereby making it an interesting target for stopping the development of NASH and fibrosis development in NAFLD.[61, 62]

Tumor necrosis factor-α. Tumor necrosis factor-α (TNF-α) is considered a key player in the progression from simple fatty liver to NASH. TNF-α is produced by macrophages in the adipose tissue and is increased in obesity.[48] Free fatty acids can induce expression of TNF-α in hepatocytes through activation of NF-κB,[63] thereby linking the increased influx of free fatty acids seen in hepatic steatosis to the progression of inflammation. In adipocytes, TNF-α down regulates adiponectin production.[64] Through activation of NF-κB by IKK-β, TNF-α together

with IL-6 and IL-1β, is associated with hepatic and systemic insulin resistance, commonly associated with NASH.[65] Free fatty acids can directly activate the IKK-β/NF-κB pathway in hepatocytes, which further endorse that free fatty acids not only increase the amount of liver fat but also initiate inflammation.[63]

Apoptosis. Hepatocellular apoptosis, a highly organized and genetically controlled form of cell death, probably play an important role in liver injury and disease progression in NAFLD patients. In NASH, apoptosis of hepatocytes is significantly increased and the degree of apoptosis correlates with the severity of steatohepatitis and the stage of fibrosis.[63, 66] Apoptosis could be initiated through the so-called extrinsic (death receptor-mediated) pathway and the intrinsic (organelle-initiated) pathway. Although the relative importance of each of these pathways in human NAFLD remains to be elucidated, both these mechanisms are believed to be involved in the pathogenesis of NASH.[68] In experimental models of NASH expression of Fas, a death receptor member of the tumor necrosis factor receptor family, is increased which results in increased sensitivity to Fas-mediated apoptosis.[67] A number of apoptotic cascades in hepatocytes are induced by TNF-α.[69] The plasma level of cytokeratin-18 fragments, a product from cleavage of cytokeratin-18 by caspase-3 which is activated as one of the final steps in the apoptotic pathway, is highly associated with the diagnosis of NASH. In a study of 44 consecutive patients with NAFLD, the specificity of elevated plasma levels of cytokeratin -18 fragments for the diagnosis of NASH was 99.9% and the sensitivity 85.7%.[70]

Oxidative stress. Lipid peroxidation, i. e. the degradation of lipids whereby free reactive oxygen species "steal" electrons from lipids in cell membranes, is an important part of the different pathways resulting in cell damage in NAFLD. Lipid peroxidation products activate the transcription factor NF-κB leading to production of pro-inflammatory cytokines as well as death ligands stimulating apoptosis.[71] In NASH patients lipid peroxidation products have been demonstrated and are associated with more advanced disease.[72] Generation of reactive oxygen species come from inflammatory cells once inflammation becomes established. The large influx of free fatty acids to the hepatocytes as a result of insulin resistance leads to production of free reactive oxygen species in the mitochondria and the smooth endoplasmic reticulum. Induction of CYP2E1, an inducible enzyme of the cytochrome P450 system, has been demonstrated in and associated with liver damage in NAFLD.[73-75] CYP2E1 metabolises ethanol as well as fatty acids and both substrates also induce the expression of the enzyme.

Increased CYP2E1 activity leads to increased production of oxygen radicals when substrates are metabolised. The induction of CYP2E1 thereby contributes to lipid peroxidation within the hepatocytes. When measured non-invasively a strong correlation between CYP2E1 activity and the amount of steatosis as well as BMI was found.[76] In obesity free reactive oxygen species are also produced in adipose tissue.[73]

Endoplasmic reticulum stress. The endoplasmic reticulum (ER) plays a central role in the synthesis, folding, and trafficking of proteins. ER is sensitive to changes in homeostasis. ER stress is characterised by accumulation of unfolded proteins within the ER triggering what is referred to as the unfolded protein response. In NASH patients the unfolded protein response is altered with failure to activate downstream recovery pathways. These changes, together with free fatty acid toxicity, and mitochondrial dysfunction, lead to activation of c-jun-N-terminal kinase which results in apoptosis and inflammation.[77]

Small intestinal bacterial overgrowth. Small intestinal bacterial overgrowth has been reported in obese and diabetic patients. The endotoxin produced by bacteria in the small bowel has been suggested as a factor contributing to pro-inflammatory cytokine production in NAFLD. Data from animal models as well as limited human data seem to support that gut-derived portal endotoxin may stimulate Kupffer cell activation and production of cytokines in NAFLD patients.[78, 79]

Activation of hepatic stellate cells. Stellate cells are perisinusoidal cells located in the space of Disse (previously referred to as Ito cells, lipocytes, perisinusoidal cells, or fat-storing cells) that are vital in the development of fibrosis in chronic liver disease. Conversion of the quiescent vitamin A-storing cell into proliferative, fibrogenic, and contractile myofibroblasts is a key step.[80] The previously described inflammatory changes associated with progression of NAFLD lead to activation of hepatic stellate cells as part of normal healing processes. So far, TNF-α does not seem to activate stellate cells directly, but TNF-α probably induce fibrogenesis through activated hepatic Kupffer cells that secrete fibrogenic cytokines. One of the central cytokines produced by Kupffer cells is TGF-β, which markedly stimulates extracellular matrix synthesis in stellate cells.[81] There is evidence suggesting that even non-inflammatory pathways are involved in stellate cell activation. Profibrogenic potential has been shown for leptin, angiotensin II,[82] norepinephrine,[83] as well as hyperglycaemia and hyperinsulinaemia through up-regulation of connective tissue growth factor,[84] all associated with obesity, insulin resistance, and NAFLD.

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Iron and NAFLD

Excessive iron accumulation is harmful. In its most extreme form, iron accumulation may lead to cirrhosis, hepatocellular carcinoma, diabetes mellitus, hypogonadism, cardiomyopathy, arthritis, and skin pigmentation. Several mutations have been described to cause pathological accumulation of iron. Most known are mutations in the haemochromatosis gene (HFE).[85]

Insulin resistance is associated with hepatic iron accumulation in patients with non-homozygous HFE-gene mutations.[86] Therefore, it is not surprising that hyperferritinaemia is commonly observed in NAFLD and found to be an independent risk factor for advanced liver fibrosis.[87-89] Thus, it is hypothesised that iron play part in the pathogenesis of inflammation and liver fibrosis in NAFLD. It is believed that iron enhances oxidative stress within the liver and markers of oxidative stress have been found to be increased in NASH patients.[90] Phlebotomy improves insulin resistance in NAFLD patients.[91] In a pilot study nine NASH patients were treated with phlebotomy. A significant reduction of ferritin and ALT levels was seen.[92]

Insulin resistance

As previously described insulin resistance plays a pivotal role in the pathophysiology of both simple fatty liver and NASH. The understanding of the intracellular mechanisms associated with insulin resistance is being unravelled and there are several reviews written on this subject.[93-95]

Physiologically, insulin resistance is defined as a condition where higher than normal insulin concentrations are needed to achieve normal metabolic responses[96] or that normal insulin concentrations fail to achieve a normal metabolic response.[97] The gold standard to measure insulin resistance is the euglycaemic hyperinsulinaemic glucose clamp where the amount of glucose needed to maintain euglycaemia during infusion of insulin at a fixed rate reflects whole-body insulin sensitivity.[98] Because of the experimental complexity and the expertise required the "glucose clamp" is difficult to use in larger clinical trials. Most commonly, fasting glucose and insulin concentrations are used to assess insulin sensitivity. The homeostasis model assessment (HOMA)[99] is a measure of insulin resistance, whereas the quantitative insulin sensitivity check index (QUICKI)[100] is a measure of sensitivity, and is frequently transformed into 1/QUICKI. The formulas for calculating HOMA and QUICKI are

shown in Figure 7. Despite the simplicity of HOMA and QUICKI, a good correlation has been demonstrated between HOMA and the "glucose clamp" in normal and pathological conditions.[101]

HOMA = fasting glucose × fasting insulin / 22.5

QUICKI = 1 / [log(fasting insulin) + log(fasting glucose)]

Units: Glucose is measured in mg/dL, and insulin in pmol/L

Figure 7: The formula for calculating HOMA and QUICKI

Although lean persons can be insulin resistant, it is most commonly found in overweight or obese individuals. However, the degree of insulin resistance varies considerably amongst equally obese subjects.[102] In normal weight and moderately overweight subjects, fat accumulation within the liver was associated with several features of insulin resistance, independently of body mass index and intra abdominal and overall obesity.[103] Thus, hepatic steatosis is probably the most proximal correlate of insulin resistance, rather than the visible subcutaneous fat. In women, who has more subcutaneous fat than men[104], the same regression line was found both in men and women when the amount of hepatic fat was plotted against fasting insulin.[105]

The difference in insulin sensitivity was attributed to different patterns of lipid partitioning, where those with severe insulin resistance were characterised by increased deposition of lipids in the visceral and intramyocellular compartments.[106] The unopposed lipolysis in the adipose tissue caused by insulin resistance in the adipocytes leads to accumulation of lipids within the liver and adipose tissue insulin resistance is positively correlated with liver fat content both in type 2 diabetic patients and nondiabetic patients.[107] The hepatic steatosis is enough to induce hepatic insulin resistance by activating PKC-ε, JNK, I-κB kinase β and NF-κB.[108]

Clinical features

The clinical features of patients with NAFLD vary considerably between different cohorts of patients. Many reports come from series of NAFLD patients undergoing obesity surgery making results difficult to apply to the typical NAFLD patient of the general population. Another bias in NAFLD cohorts is that most studies have been conducted in tertiary referral centres. Few studies have explored NAFLD in the general population and none of these has included histopathological evaluation. Studies of NAFLD patients in the general population

are probably the best studies to describe the clinical features of the typical NAFLD patient. Clinical features in studies of NAFLD patient cohorts are summarised in Table 5.

Most NAFLD patients do not have any symptoms or signs of liver disease unless symptoms of end-stage liver disease are present. If present at all, symptoms in NAFLD patients are constitutional and non-specific. Some patients report fatigue and/or a sensation of fullness on the right side of the upper abdomen. Hepatomegaly is present in 75% of cases, but may be difficult to detect due to the high prevalence of obesity in NAFLD patients.[6] It is evident that overweight/obesity and diabetes are important risk factors for developing NAFLD. With the development of proton magnetic resonance spectroscopy the association between the amounts of fat accumulated within the liver and several risk factors, especially insulin resistance, are being elucidated.[102]

Gender

In many of the earlier NAFLD studies the majority of patients were females. In the report by Ludwig et al.[1] 65% of patients were women. However, in cohorts of NAFLD patients derived from the general population NAFLD is more prevalent in males. In the largest study by Browning et al. NAFLD was more prevalent in men than in women with a ratio of 1.1:1. This gender difference was even more obvious in white subjects. In white males 42% had increased hepatic triglyceride content compared with 24% of white women.[109] In an Israeli NAFLD cohort derived from the general population male gender was associated with the diagnosis of NAFLD even after adjusting for obesity and abdominal obesity.[110]

Obesity and diabetes

Since fat accumulation within the liver is tightly linked to insulin resistance it is not surprising that of obesity and diabetes, conditions associated with insulin resistance, are very common in NAFLD patients. Obesity is found in 39-100% of NAFLD patients, and diabetes in 5-55%.[111] The large differences seen in the prevalence of obesity and diabetes between different NAFLD cohorts are probably due to selection biases. The typical NAFLD patient is an obese middle aged individual with diabetes. However, NAFLD can be diagnosed also in lean euglycaemic patients.[25, 109, 112] Not surprisingly, NAFLD patients have suboptimal health-related fitness and lower physical activity.[113]

Laboratory abnormalities

Mildly to moderately elevated serum levels of ALT and/or AST is the most common laboratory abnormality found in patients with NAFLD. The AST/ALT ratio is usually < 1. An AST/ALT ratio of > 1 is associated with advanced fibrosis in NAFLD.[114] Serum alkaline phosphatase is usually within two times the normal range. Although ALT elevation is the most common laboratory abnormality in NAFLD patients a subset of patients present with isolated ALP elevation. NAFLD patients that present with isolated ALP elevation are more often women and are more likely to have advanced fibrosis.[115]

In the general population most NAFLD patients have normal liver function tests. In the Dionysos trial NAFLD was equally common in patients with and without suspected liver disease (elevated ALT or GGT, or positive serum markers for hepatitits B or C),[116] and 79% of NAFLD patients in the study by Browning at el had normal ALT levels.[109]

As described previously elevated levels of ferritin are commonly found in NAFLD patients. In the study of 144 patients reported by Adams et al. 11% had elevated ferritin and 11% had elevated transferrin saturation.[114]

Autoantibodies are commonly found in patients with NAFLD.[117, 118] The significance of these autoantibodies is uncertain. It has been reported that patients with autoantibodies had higher inflammatory grades and more advanced fibrosis than autoantibody negative controls.[117]

Table 5: Clinical features of NAFLD patients in different studies.

	N	Females (%)	Age (mean (range))	Obesity (%)	BMI (SD)	DM (%)	Elevated ALT (%)
Case series							
Ludwig[1] (1980)[e]	20	65	54 (38-80)	90[a]	NA	50[b]	NA
Bacon[25] (1994)[e]	33	42	47 (26-69)	39[a]	NA	21[b]	88
Matteoni[24] (1999)[e]	132	53	53 (± 13.1)	70[NA]	29.5 (± 5.8)	33[b]	NA
Angulo[114] (1999)[e]	144	67	51 (11-77)	60[c]	31.2 (20.9-57)[d]	28[b]	NA
Dixon[119] (2001)[f,g]	105	78	41 (± 11)	100[c]	47 (± 7)	19[b]	30
Bedogni[116] (2005)[f]	135	44	57 (IQR 19)	NA	30 (IQR 7.2)	NA	NA
General population							
Browning[109] (2004)	708	M1,1/1F	46 (± 10)	67	NA	18	21
Zelber-Sagi[110] (2006)	98	33	51 (± 9.5)	NA	30 (± 4.4)	21	8.2
Hamaguchi[120] (2005)	812	22	49 (± 8.2)	NA	26 (± 3.0)	NA	NA

DM, diabetes mellitus; NA, not available
[a]10% above expected (ideal) weight; [b] glucose tolerance test not perfomed; [c]BMI>30; [d]Range; [e]Retrospective; [f]Prospective; [g]Obesity surgery

Diagnosis

Abnormal liver function tests

In clinical practice patients with NAFLD are often identified by asymptomatic elevation of liver enzymes. Fatty liver is the most common cause of mildly to moderately elevated liver enzymes both in Sweden[121, 122] and elsewhere.[123, 124] Hypertransaminasaemia, if viral or other causes of liver disease have been excluded, is sometimes used as a surrogate marker for NAFLD.[125] Using elevated liver enzymes as a marker for NAFLD is simple and cheap, but has several disadvantages.

The upper limit of normal for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) is not well defined. Recently the upper limit of normal for ALT in Sweden was changed. It was raised from 47 U/L (0.8 μkat/L) to 65 U/L (1.1 μkat/L) in men and from 35 U/L (0.6 μkat/L) to 44 U/L (0.75 μkat/L) in women. These changes were based on the ALT levels in 3,000 adults living in the Nordic countries. There are reasons to believe that the increased ALT levels in the population reflect the increased prevalence of obesity and NAFLD.[126] It has been shown that ALT elevation is seen more frequently in obese.[127] In an Italian study of 3,927 subjects with normal BMI, normal serum cholesterol, triglycerides, and glucose levels, and absence of concurrent medication, the upper limit of ALT was 30 U/L (0.5 μkat/L) in men and 19 U/L (0.32 μkat/L) in women. Moreover, the full spectrum of NAFLD can be found in patients with normal ALT values.[128] When compared to ultrasonography, the sensitivity of elevated ALT for diagnosing NAFLD is 8.2% with a specificity of 98%.[110]

Ultrasonography

Ultrasonography of the liver is safe and relatively inexpensive. It has been used in a number of studies investigating the prevalence of fatty liver in a variety of settings (see "Prevalence of NAFLD"). Fatty infiltration of the liver produces an increased echogenicity when compared to the echogenicity of the kidneys. The increased echogenicity is due to the fact that fat attenuates ultrasound more than normal liver parenchyma.[129]

In patients with at least moderate steatosis the sensitivity ranges between 89-91% with specificity between 82-93%.[130-132] In a more recent study using the latest technology excellent sensitivity was reported (100%) for detecting moderate to severe steatosis. However, interobserver agreement was moderate ($\kappa = 0.43$).[133] Moreover, ultrasonography often misses to diagnose steatosis of lesser degree. In the study by Saadeh et al. sensitivity dropped from 91% to 64% when patients with mild steatosis grade were included in the analysis.[132] Therefore, when used in prevalence studies ultrasonography underestimates the prevalence of fatty liver. Moreover, ultrasonography does not have the ability to differentiate between simple fatty liver and NASH. Nor has it the capacity to detect fibrosis.[133]

Proton magnetic resonance spectroscopy

Proton magnetic resonance spectroscopy (^{1}H-MRS) is a relatively new and non-invasive technique to diagnose fatty liver. Although it is expensive and not available in all centres, especially in developing countries, it has proved itself to be a very valuable method in assessing liver fat. It has been used in surprisingly large epidemiological studies (see "Prevalence of NAFLD").

^{1}H-MRS is based on the physical phenomenon known as chemical shift. Chemical shift is caused by the slight difference in magnetic field surrounding the proton nuclei of hydrogen in water molecules (O-H bond) compared with that of protons in lipid molecules (C-H bond).[134] Therefore, this technique is especially useful in tissues with mixed water and fat content, as in hepatic steatosis.

With the hepatic triglyceride content is measured quantitatively. There is a close correlation between hepatic triglyceride content measured in vivo by ^{1}H-MRS and chemically from biopsies (R = 0.934).[135] Thus, ^{1}H-MRS must be considered the gold standard in the non-invasive.diagnosis and quantitative assessment of hepatic fatty infiltration.

As with ultrasonography, ^{1}H-MRS lack the ability to differentiate between simple fatty liver and NASH. Moreover, it gives no information on the development of fibrous tissue in the liver.

Liver biopsy

Liver biopsy is usually the most specific test to assess the nature and severity of liver disease. The diagnosis of NASH can only be made through the examination of liver tissue. In recent years liver biopsy has been challenged by non-invasive techniques to assess liver fibrosis[136], but so far liver biopsy is still considered the gold standard in staging NAFLD.

There are currently several methods available for obtaining liver tissue: percutaneous biopsy, transjugular biopsy, laparoscopic biopsy, or fine-needle aspiration guided by ultrasonography or computed tomography for diagnosis of solid lesions.

Needles for percutaneous liver biopsy are broadly categorised as suction needles (Menghini needle, Klatskin needle, Jamshidi needle), cutting needles (Vim-Silverman needles, Tru-cut needle), and spring-loaded cutting needles that have a triggering mechanism. The liver tissue obtained measures between 1.4 mm, in standard thin-bore or spring-loaded needles, up to 2 mm, obtained with Menghini or Tru-cut needles, in diameter.

Because of its invasive nature liver biopsy can cause serious complications. In a French prospective study severe complications were observed in 0.57% of patients.[137] Mortality rate among patients after percutaneous liver biopsy is approximately 1/10 000 to 1/12 000. Mortality is highest among patients who undergo biopsies of malignant lesions or in patients with cirrhosis. Other complications ranging from mild to severe are summarised in Table 6. Hospitalisation because of complications after a liver biopsy occurs in 1 to 3 % of patients. Whether the use of ultrasonography to guide the biopsy decreases the complication rates even lower, provides higher diagnostic yield, or is cost effective is still debated.[29]

Table 6: Complications of percutaneous liver biopsy

Abdominal discomfort	Intraperitoneal haemorrhage
Biliary ascites	Mild to severe pain
Bacteremia (Transient)	Peritonitis
Breakage of the biopsy needle	Pleuritis
Carcinoid crisis	Pneumothorax
Hemobilia	Pneumoperitoneum
Hemothorax	Pneumoscrotum
Hypotension due vasovagal reaction	Subcutaneous emphysema
Intrahepatic or subcapsular hematoma	Subphrenic abscess

Epidemiology

Prevalence of fatty liver

The prevalence of NAFLD varies considerably depending on the subset of patients being investigated. In obese persons fatty liver affects more than 50%[138, 139] and 100% of severely obese with diabetes.[140] Thus, the prevalence of NAFLD in the general population is linked to the frequency of obesity and diabetes.

The technique used to diagnose hepatic steatosis also influences the prevalence reported in different studies (See "Diagnosis"). Large epidemiological studies using liver biopsy in the general population cannot be performed because of the potential severe complications with this procedure. Since ^{1}H-MRS is highly sensitive in detecting fatty infiltration and has the ability to quantitatively assess the amount of fat within the liver, this method is ideal to use in epidemiological studies. Unfortunately, the use of ^{1}H-MRS in large epidemiological studies is held back by the high cost and the complicated technique. Most epidemiological studies have used ultrasonography or liver function tests to assess the prevalence of NAFLD.

Several large studies have been performed in the general population of various countries. A large study (n = 2,349) from Dallas County used ^{1}H-MRS to assess the prevalence of fatty liver. In this study, the prevalence of fatty liver was 33.6%.[14] Even in populations previously considered to have low risk of having fatty liver studies have reported high prevalence numbers. Studies investigating the prevalence of fatty liver in the general population are summarised in Table 7.

Table 7: Studies on the prevalence of fatty liver in the general population.

	Country	Number of subjects	Prevalence of fatty liver (%)
Elevated liver enzymes			
Clark et al. (2003)[141]	United States	15,676	5.4
Ruhl et al. (2003)[142]	United States	5,724	2.8
Pendino et al. (2005)[143]	Italy	1,645	3.0
Ultrasonography			
Nomura et al. (1988)[138]	Japan	2,574	14.0
Jimba et al. (2005)[144]	Japan	1,950	29.0
Fan et al. (2005)[145]	China	3,175	17.3
Bedogni et al. (2005)[116]	Italy	598	23.0
Hamaguchi et al. (2005)[120]	Japan	4,401	18.5
Zelber-Sagi et al. (2006)[110]	Israel	352	30.0
Chen et al. (2006)[146]	Taiwan	3,245	11.5
Amarapurkar et al. (2007)[147]	India	1,168	16.6
Proton magnetic resonance spectroscopy			
Szczepaniak et al. (2004)[14]	United States	2,349	33.6

Prevalence of NASH

There are no reliable data on the prevalence of NASH in the general population. A number of studies have been undertaken in obese individuals undergoing bariatric surgery. In these series the frequency of NASH varies between 14 and 56%. In hospital series of patients undergoing liver biopsy the frequency ranges from 1 to 32%.[148] These large variations on the prevalence of NASH can partly be attributed to different definitions of NASH and which histopathological findings are required for the diagnosis to be set.

In an autopsy series of 351 apparently non-alcoholic patients the frequency of NASH was 6.3%. NASH was defined as ballooning of hepatocytes with clearing of the hepatocellular cytoplasm accompanied by large-droplet steatosis. NASH was found in 18.5% of obese and in 2.7% of lean patients.[8]

Natural course

The benign nature of NAFLD has been challenged by a number of clinical studies during the last two decades. Indirect evidence comes from studies in patients with cryptogenic cirrhosis. After adjustment for age and gender, obesity and diabetes were much more prevalent than expected in patients with cryptogenic cirrhosis.[21, 149] Moreover, in patients undergoing liver transplantation because of cryptogenic cirrhosis, a significant proportion of patients develop NAFLD post-transplant.[150] These data indicate that many cases of cryptogenic cirrhosis are in fact burned out NASH. Once cirrhosis has developed, the diagnosis of NAFLD is difficult to set since the fat vacuoles within hepatocytes, the histopathological hallmark of NAFLD, have frequently disappeared.[151]

Some patients previously diagnosed with fatty liver will develop cirrhosis,[24] and the liver disease can progress to hepatocellular carcinoma in some of these patients.[152] Subacute liver failure, because of NAFLD, has been described but is probably very uncommon.[153] NAFLD patients that develop cirrhosis have a high risk of developing decompensation and/or cardiovascular disease, but the prognosis for NAFLD cirrhosis is better than for cirrhosis due to hepatitis C.[154]

Treatment

The standard of care for patients with NAFLD is lifestyle modification with weight loss as the mainstay of therapy. Several small uncontrolled trials utilising different caloric restriction regimens and combinations of carbohydrate, protein and lipid diets have been performed as well as studies on the effect of increased exercise. The benefit of lifestyle modifications in NAFLD has recently been reviewed.[38] Although there is need for controlled trials of longer duration, it seems that diets aiming at achieving about a 10% weight reduction by reducing total daily energy intake, improve both metabolic and histopathological variables in a diverse group of NAFLD patients.[155, 156] Moreover, exercise expending about 400 calories, performed 3-4 times a week, can probably improve the metabolic profile in NAFLD patients.[157, 158] Weight loss achieved through bariatric surgery improves liver histology in NAFLD patients.[159]

So far there is no established pharmacological treatment for NAFLD. Treatment strategies for NAFLD aim to improve insulin sensitivity, modify underlying metabolic risk factors, or to protect the liver from further insult by reducing oxidative stress. Multiple pharmacological interventions have been attempted with variable success. These include pentoxifylline,[160] orlistat,[161] vitamin E,[162-164] ursodeoxycholic acid,[165] and lipid-lowering agents.[166] Studies of insulin sensitizing agents such as metformin[50, 164] and thiazolidinediones,[50, 163, 167-169] have yielded promising results. In a placebo-controlled trial of pioglitazone, metabolic and histopathological improvement was seen in the 26 NASH patients receiving active treatment.[170] Similar results were reported in a French study of 63 NASH patients. Steatosis and aminotransferase levels improved significantly but there was no improvement in other parameters of liver injury.[171] Considering the safety concerns raised about the thiazolidinediones they are so far considered "promising but not ready for prime time" in NAFLD.[172]

The last few years a lot of attention has been given to the cannabinoid signalling system. Endocannabinoids regulate appetite and play a significant role in governing energy efficiency.[173] The cannabinoid receptor antagonist rimonabant improved metabolic abnormalities when tested in human obesity trials[174, 175] and in *fa/fa* rats rimonabant reduces liver fat content and nearly normalises alanine aminotransferase levels.[176] Controlled trials in humans are conducted and results are eagerly awaited.

A newly developed compound interfering with the IKK2-nuclear factor NFκB signalling pathway prevents the accumulation of lipids within the liver as well as the initiation of NASH in promising animal studies.[177]

Aims of the study

- To validate point counting as a technique to quantitatively assess the amount of hepatic steatosis in liver biopsies.

- To describe the long-term clinical and histopathological development of patients with NAFLD.

- To evaluate survival of NAFLD patients compared with the general population.

- To evaluate factors associated with future risk of fibrosis progression with special interest to alcohol, weight changes, metabolic profile and medical treatment.

- To investigate whether statin treatment could be prescribed safely in patients with NAFLD.

- To investigate which histopathological features predict future risk of developing cirrhosis and end-stage liver disease.

- To evaluate the clinical usefulness of the newly proposed NAFLD activity score in predicting fibrosis progression.

Subjects

Quantitative assessment of liver steatosis (Paper I)

Seventy-five archived liver biopsy slides stained with haematoxylin-eosin were used to evaluate point counting technique to quantitatively assess the degree of fatty infiltration. Twenty-five liver biopsies of each grade (mild, moderate, and severe) were selected.

Long-term follow-up study (Papers II, III, IV, V)

The long-term follow-up study has been performed in a cohort of 129 NAFLD patients, of whom 87 (67%) were male. Mean age at baseline was 51.0 ± 12.9 years and mean BMI was 28.3 ± 3.8 kg/m^2. Diabetes had previously been diagnosed in 11 patients (8.5%), and 14 patients (11%) had manifest cardiovascular disease at baseline.

Procedures

Selection of biopsies (Paper I)

A total of 75 liver biopsies stained with haematoxylin-eosin were selected from archived slides at the Department of Pathology at the University Hospital in Örebro. They were selected according to the original grade of steatosis diagnosed, (twenty-five of each grade, i.e. mild, moderate, and severe). No biopsies without steatosis were included in the study.

Enrolment of patients (Papers II, III, IV, V)

Baseline study (Papers II, III, IV, V)

All patients referred between 1988 and 1993 to the Department of Gastroenterology and Hepatology, University Hospital in Linköping, or to the Department of Internal Medicine, Oskarshamn County Hospital, for evaluation of persistently (>6 months) elevated liver enzymes were consecutively enrolled into a clinical study. Elevated liver enzymes were defined as elevated serum ALT and/or aspartate aminotransferase (AST) of > 41 U/L (0.70 μkat/L), and/or serum alkaline phosphatase of > 106 U/L (1.8 μkat/L). A diagnostic work-up was performed in each patient including physical examination, laboratory investigations, and liver biopsy.[121] A total of 212 patients were included in the baseline study.

Follow-up study (Papers II, III, IV, V)

All patients' records were reviewed when the follow-up study was being planed and all diagnoses were revised according to modern terminology. One hundred and forty-four patients were diagnosed with hepatic steatosis without any other concomitant liver disease or medication associated with fatty infiltration of the liver. Seven of these subjects reported at baseline, current or previous average weekly alcohol consumption of 140 g or more and were thus not considered to have NAFLD. The remaining 137 patients originally diagnosed with NAFLD constituted the NAFLD cohort of the follow-up study. Diagnoses found in the cohort of 212 patients and flow-chart of included and excluded patients at each step of follow-up are presented in Figure 3. Each subject in the study cohort was identified by linking his or her unique personal identification number to the National Registry of Population. All medical records from primary care health centres and hospitals were reviewed. Special attention was given to development of chronic diseases and signs of alcohol abuse. Subjects who had died during follow-up were identified and their causes of death were obtained by reviewing their

medical records and the information obtained from the Registry of Causes of Death. A letter was sent to each NAFLD patient's primary health care physician asking if there were any medical or other reasons not to contact the particular patient. If the primary health care physician gave consent, a letter was sent to each patient followed by a telephone call within two weeks. Those who accepted follow-up were offered clinical and biochemical investigation, ultrasonography, and a repeat biopsy of the liver.

Reference population (Paper II)

For comparison of observed survival and causes of death, a reference population of all subjects (n = 44,745) of the same age and sex living in the same county as each NAFLD patient at baseline was obtained from Statistics Sweden.

The prevalence of liver-related complications of those in the general population in the same age range (36-80 years) as the NAFLD cohort was estimated by obtaining data from the Swedish Hospital Discharge Register. Included were all individuals living in the same geographical area as the NAFLD cohort who had been hospitalized in 2004 with primary or secondary diagnoses (according to the International Classification of Diseases, Tenth Revision) of cirrhosis, chronic hepatic failure, portal hypertension, hepatorenal syndrome, ascites, oesophageal varices, or hepatocellular carcinoma.

Data collection (Papers II, III, IV, V)

All patients that accepted follow-up were seen at the Department of Gastroenterology and Hepatology, University Hospital in Linköping, or at the Department of Internal Medicine, Oskarshamn County Hospital by either Mattias Ekstedt or Stergios Kechagias.

Biochemical investigation (Papers II, III, IV, V)

Subjects had blood drawn after an overnight fast for a complete blood count and analysis of prothrombin, thyroid-stimulating hormone, transferrin, iron, transferrin saturation, ferritin, AST, ALT, alkaline phosphatase, gamma glutamyl transferase, bilirubin, total cholesterol, low-density lipoprotein, high-density lipoprotein, triglycerides, plasma glucose, serum insulin, and plasma protein electrophoresis including albumin, α-1-antitrypsin, ceruloplasmin, and immunoglobulins. In addition, blood was obtained for detection of hepatitis B surface antigen, anti-hepatitis C virus (HCV) antibodies, hepatitis B virus DNA, HCV RNA, transglutaminase antibodies, antinuclear antibodies, smooth muscle antibodies, and mitochondrial antibodies.

Moreover, genomic DNA isolated from anticoagulated venous blood was used to identify the C282Y, H63D, and S65C mutations in the *HFE* gene as well as the Z and S mutations in the *Pi* gene. Subjects were considered to have diabetes mellitus if they were receiving dietary or drug treatment for this disease. The remaining subjects had a 75-g oral glucose tolerance test after an overnight fast.

Clinical assessment (Papers II, III, IV, V)

A structured form was used to assess the clinical history, including past and present diseases, both acute and chronic. Hip and waist circumference as well as weight and length were measured in all patients and BMI was calculated (BMI = weight (kg) / length (m) / length (m)). Overweight was defined as BMI > 25 kg/m^2 but ≤ 30 kg/m^2, obesity as BMI > 30 kg/m^2, diabetes as fasting plasma glucose ≥ 126 mg/dL (6.9 mmol/L), requiring treatment, or plasma glucose >199 mg/dL (10.9 mmol/L) 2 h after oral administration of 75 g of glucose, impaired glucose tolerance as plasma glucose > 140 mg/dL (7.7 mmol/L) but ≤ 199 mg/dL (10.9 mmol/L) 2 h after oral administration of 75 g of glucose, hypertension as blood pressure ≥ 130/85 mmHg or requiring treatment, and hypertriglyceridaemia as fasting triglycerides ≥ 150 mg/dL(1.7 mmol/L). Metabolic syndrome was defined as having at least 3 of the following[178]: (1) waist circumference > 102 cm in men or > 88 cm in women; (2) fasting triglycerides > 150 mg/dL (1.7 mmol/L); (3) fasting HDL < 40 mg/dL (1.0 mmol/L in men or < 50 mg/dL(1.3 mmol/L in women; (4) blood pressure > 130/85 mmHg or a diagnosis of hypertension; (5) fasting glucose > 110 mg/dL (6.1 mmol/L) or a diagnosis of diabetes mellitus. Insulin resistance was calculated according to homeostasis model assessment.[101] Past and present medications were noted.

Assessment of alcohol consumption (Papers II, III, IV, V)

A questionnaire was constructed using the three questions of the AUDIT-C questionnaire[179]: "How often did you have a drink containing alcohol during the last three months?"(response alternatives were: daily; 4-5 times a week; 2-3 times a week; once a week; 2-3 times a month; once a month; 1-2 times the last three months; or never), "How many drinks containing alcohol did you have on a typical day during the last three months when you were drinking?" (response alternatives were: 9 drinks; 8 drinks; ... or less than one drink), and "How often did you have 5 drinks or more (men) or 4 drinks or more (women) on one occasion during the last three months?" (response alternatives were: every day; nearly every day; 3-4 times a week; 1-

2 times a week; 2-3 times a month; once a month; once in six months; or never). A drink was described with Figure 8 that was added to the questionnaire.

A drink is defined as:

| 50 cc of medium | 33 cc of strong beer | 15 cc of wine | 8 cc of strong wine | 4 cc of spirits |
| strength beer | | | | |

Figure 8: This picture was added to the alcohol questionnaire to define the different amounts of alcoholic beverages that represent "a drink".

One additional question intended to assess changes in alcohol consumption during follow-up was designed especially for this study. This question, "In what way has current (i.e. the last three months) alcohol consumption changed compared with alcohol consumption before the first liver biopsy?" concerned possible changes in overall alcohol consumption during follow-up. The response alternatives were as follows: decreased considerable; decreased some; increased some; increased considerable; or unchanged. A nurse handed out the questionnaires to the patients when they came to clinic. After completion of the questionnaire the physician (M.E; S.K.) conducted an oral interview to assert that the questionnaire was answered correctly. The patient was asked to describe his or her alcohol consumption during a typical week, changes during the year, as well as changes in alcohol consumption during follow-up. To calculate weekly alcohol consumption at the time of follow-up number of drinking occasions was multiplied with the number of drinks (i.e. 12 grams of ethanol) consumed on each occasion. Moreover, frequency of episodic drinking (more than 60 grams of ethanol in males and 48 grams of ethanol in females consumed on one occasion), and changes in alcohol consumption were registered. Evaluation of alcohol consumption was performed prior to liver biopsy.

Liver biopsy (Papers II, III, IV, V)

Liver biopsies were performed percutaneously with ultrasonography guidance using a 1.6 mm Biopince needle on an outpatient basis. All patients were monitored at the out-patient clinic for six hours after the liver biopsy had been performed.

Histopathological evaluation (Papers I, II, III, IV, V)

Paper I

The liver biopsy slides were blinded and evaluated twice regarding grade of steatosis (both macro and microvesicular) by an experienced liver pathologist (L.E.F.). The interval between the evaluations was 2 months. The degree of steatosis was graded 0-3 based on area of the section of the needle biopsy that was occupied by fat vacuoles (grade 0: no fatty infiltration; grade 1: less than 1/3 of area occupied by fat vacuoles; grade 2: 1/3-2/3 of area occupied by fat vacuoles; and grade 3: more than 2/3 of area occupied by fat vacuoles).[130]

Papers II, III, IV, and V

All biopsies at baseline and at follow-up were read by the same experienced liver pathologist (L.E.F.), who was blinded to patient details. Baseline and follow-up biopsies were read randomly during a limited period of time. Liver histology was scored according to the system developed by Brunt et al.[28] (see Table 2), except that acidophil bodies and glycogenated nuclei were not assessed and that PAS-D Kupffer cells were scored as present or absent. Steatosis grade was assessed semiquantitatively as described in the Brunt system as well as quantitatively as described in paper I.

In papers II, III, IV and V ("broad" criteria), NASH was defined as steatosis plus any stage of fibrosis or as steatosis plus lobular inflammation plus ballooning degeneration.[180] In paper V ("strict" criteria), the NAS was calculated as the unweighted sum of steatosis (0-3), lobular inflammation (0-3) and hepatocellular ballooning (0-2) scores. NASH was defined as NAS ≥ 5, "borderline NASH" as NAS <5 and ≥ 3, and "not NASH" as NAS < 3.[28]

Two different definitions of progressive fibrosis were used. In paper II and III, progressive fibrosis was defined as a higher fibrosis stage at follow-up compared with the stage at baseline, or development of symptoms of end-stage liver disease. In papers IV and V, progressive fibrosis was defined as an increase of more than one fibrosis stage, or development of symptoms of end-stage liver disease.

Quantitative assessment of steatosis (Papers I, II, III)

A Leica DMRXA 2 microscope with a Leica DC 200 digital camera was used for image capturing. In all, 10 images from each biopsy were captured and stored in a computer using the software Adobe Photoshop 6.0. The first field of view was chosen in the end of the biopsy

closest to the end of the microscopic slide. After the first image had been grabbed, the next field of view was chosen by moving along the length axis of the biopsy 1.25 fields of view in order not to get overlapping images for evaluation. This procedure was continued until 10 images had been grabbed. A point grid, consisting of 100 crosses 35 μm apart, was superimposed on each image. The final magnification on the computer screen when counting was ×400. The number of hits on fat vacuoles in hepatocytes (including both macro- and microvesicular) and normal hepatocytes was counted. Hits on damaged tissue and larger areas with connective tissue were excluded. The results are given as the percentage of biopsy area with fat deposition. Images from 20 randomly chosen specimens were recounted to assess the reproducibility of the point counting in the same images.

In all, 20 specimens were then selected randomly and a new set of 10 images was captured from each of these specimens. These new images were counted as above and the results were used to assess the reproducibility of the point counting technique when new images were resampled.

Statistics (Papers I, II, III, IV, V)

For continuous variables, differences between two groups were evaluated with the Student t test when data were normally distributed and with the Mann-Whitney U test when the assumption of normality was not met. For dichotomous variables, differences were tested using the χ^2 test corrected for continuity or Fisher's exact test. Survival curves were constructed according to the Kaplan-Meier method. One-sample log-rank tests were used for comparison with the reference population. Causes of death were compared using the z test with Bonferroni correction. In paper I, agreement for the scoring results was analysed by the kappa coefficient, both in the unweighted and the weighted form, the weights chosen as quadratic weights.[181] For quantitative steatosis assessment, agreement was analysed by the intraclass correlation coefficient (ICC). A P value < 0.05 was considered statistically significant in all papers.

Multivariate analyses (Papers III, IV, V)

In paper III, a multivariate linear regression analysis using backward elimination was performed to evaluate clinical and biochemical variables associated with change of quantitative steatosis between baseline and follow-up biopsies. At each step, the variable with

the largest probability of F value was eliminated, provided the *P* value exceeded 0.10. Sex and pharmacological treatments were coded as indicator variables.

In paper IV, clinical and biochemical variables regarded to influence fibrosis progression in NAFLD were tested in univariate and multivariate binary logistic regression analyses using stepwise forward modelling. All variables tested in the univariate analysis were included into the multivariate analysis. At each step, the predictor variable with the largest score statistic whose significance value was less than .05 was added to the model. Insulin resistance according to homeostasis model assessment (IR_{HOMA})[101] could not be calculated in 14 patients because they were under insulin treatment. Therefore, two multivariate binary logistic regression analyses were performed. In Model 1 all patients (n = 71) were included while in Model 2 only patients who were not treated with insulin (n = 57) were included. Patients were divided into two groups according to change in fibrosis stage between biopsies; either "significant progression" (progression by more than one fibrosis stage) or "insignificant change" (unchanged fibrosis stage, progression or regression by one stage). Patients that had developed end-stage liver disease were classified as having "significant-progression".

In paper V, associations between histopathological variables and significant fibrosis progression were evaluated by using univariate binary logistic regression analyses and the association between significant fibrosis progression and the NAS together with clinical variables was evaluated in a multivariate binary logistic regression analysis. A *P* value of <.05 was considered statistically significant in all papers.

Ethical Considerations

Written informed consent was obtained from all participating subjects. The study designs were approved by the local ethics committee at the University Hospital in Örebro (Paper I) and at the University Hospital in Linköping (Papers II, III, IV, V).

Results

Quantitative assessment of liver steatosis (Paper I)

After re-evaluation of the 75 liver specimens; 21 specimens obtained score 1 (mild), 20 specimens score 2 (moderate), and 34 specimens score 3 (severe). In Table 8 mean values of the point counting, range, as well as the coefficient of variation are presented according to steatosis grade.

Table 8: Basic characteristics of the point counting technique, stratified for the three grades of the scoring reults.

	Grade 1 (n = 21)	Grade 2 (n = 20)	Grade 3 (n = 34)
% Area fat globules Mean (Range)	2.2 (0.2-4.7)	9.2 (5.4-14.5)	23.1 (9.0-45.3)
Coefficient of variation (%)	134	69	47

An uneven distribution of fat vacuoles between images was found. This was especially seen with low-grade steatosis. A substantial overlap (n = 18) was found between score groups 2 and 3 regarding the area of fat globules measured by point counting. No morphological characteristics were found that made them scored to either group.

The intraobserver agreement for the semiquantitative assessment, performed with 2 months apart, was 81% (95% CI 72-90%) and the unweighted kappa was 0.71 (95% CI 0.58-0.85). Weighted kappa with quadratic weights was 0.87 (95% CI 0.98-1.00). When the images of 20 randomly chosen specimens were reassessed by point counting and compared to the initial counting, the ICC value was 0.99 (95% CI 0.98–1.00). In all, 10 images from each of 20 randomly chosen specimens were captured a second time and the concordance calculated. The ICC value was found to be 0.95 (95% CI 0.87–1.00).

Long-term follow-up study (Papers II, III, IV, V)

Study population (Papers II, III, IV, V)

At baseline, 137 patients were diagnosed with NAFLD. Eight subjects were reclassified as having alcoholic liver disease at follow-up based on information in their medical records or self-reported alcohol consumption ≥ 140 g/week. Therefore, the final cohort constituted of 129 NAFLD patients. During follow-up, 25 patients had died. Of the 104 patients alive and

Subjects evaluated during 1988-1993 because of chronically elevated serum aminotransferases and/or alkaline phosphatase	212 subjects	Hepatitis C (n = 26) PBC (n = 14) PSC (n = 8) Autoimmune hepatitis (n = 5) AAT-deficiency (n = 2) Normal liver biopsy (n = 8) Pathologic liver biopsy without steatosis (n = 5) Alcohol consumption > 140 g/w at baseline (n = 7) Reclassified as alcoholic liver disease during follow-up (n = 8)
Subjects with NAFLD at baseline	129 subjects	Died during follow-up (n = 25) Alive but refused follow-up (n = 16)
Subjects with NAFLD at baseline that accepted follow-up	88 subjects	No liver biopsy at follow-up (n = 20) Refused (n = 15) Decompensated cirrhosis (n = 2)[a] Cirrhosis at baseline (n = 3)[b]
Subjects with NAFLD at baseline that underwent liver biopsy at follow-up	68 subjects	

[a]Both patients were diagnosed with hepatocellular carcinoma at follow-up. One patient died shortly after diagnostic work-up at follow-up. [b]One patient developed hepatocellular carcinoma and underwent orthoptic liver transplantation during follow-up. PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; AAT, α_1-antitrypsin.

Figure 9: Number of patients at each step of the follow-up study. Reasons for exclusion are presented at each level.

eligible for follow-up, 88 agreed to participate in the follow-up study, and 68 underwent repeat liver biopsy.

Of the twenty patients that did not undergo liver biopsy at follow-up, fourteen patients refused, one patient was treated with warfarin, three patients had cirrhosis at baseline and therefore repeat liver biopsy was considered unnecessary. One of these three patients had developed hepatocellular carcinoma and had undergone successful orthotopic liver transplantation. Finally, two patients did not undergo repeat liver biopsy because they had developed end-stage liver disease during follow-up (ascites and hepatocellular carcinoma in both patients) (Figure 9). One patient that underwent liver biopsy at follow-up had developed ascites prior to follow-up and underwent liver biopsy at follow-up in order to confirm that ascites was of hepatic origin and one patient developed ascites shortly after the liver biopsy was performed. In total, five patients that accepted follow-up had developed end-stage liver disease. Follow-up started March 10, 2003 and was completed September 30, 2005. Mean follow-up time ± SD was 13.7 ± 1.3 years from time of diagnosis of NAFLD, with a total of 1,202 person-years.

Clinical characteristics (Papers II, III, IV, V)

Generally, NAFLD patients were predominantly middle-aged men with a high prevalence of overweight or obesity. Clinical and biochemical characteristics of patients, at baseline and at follow-up, are shown in Table 9. There were no significant differences in baseline clinical, biochemical, and histopathological variables in the 16 patients that were alive but did not come to follow-up compared with the participants in the follow-up study, nor were there any significant differences between those who refused a repeat liver biopsy compared with those who accepted.

Table 9: Clinical and biochemical characteristics of cohort at baseline and at follow-up [Mean ± SD or n (%)]

	At baseline (n = 129)	At follow-up (n = 88)
Age (years)	51.0 ± 12.9	61.0 ± 11.0
Sex (male)	87 (67 %)	62 (70 %)
BMI (kg/m^2)	28.3 ± 3.8	29.1 ± 4.7
Overweight	72 (56 %)	49 (56 %)
Obese	37 (29 %)	29 (33 %)
Previously diagnosed diabetes	11 (8.5 %)	37 (42 %)
Diabetes diagnosed at consultation visit	**NA**	14 (16 %)
IGT diagnosed at consultation visit	**NA**	18 (20 %)
Hypertensive	93 (72 %)	83 (94 %)
Metabolic syndrome	NA	52 (59 %)
ALT (U/L)	76 ± 43	60 ± 35
Elevated ALT	114 (88 %)	61 (69 %)
AST (U/L)	45 ± 23	35 ± 15
Elevated AST	56 (43 %)	20 (23 %)
AST/ALT ratio	0.6 ± 0.2	0.7 ± 0.3
AST/ALT ratio above one	7 (5 %)	11 (13 %)
ALP (U/L)	61 ± 33	65 ± 37
Elevated ALP	12 (9 %)	4 (4.5 %)
Bilirubin (mg/dL)	0.64 ± 0.30	0.78 ± 0.33
Elevated bilirubin	1 (0.8 %)	3 (3.4 %)
Albumin (g/dL)	4.1 ± 3.4	4.2 ± 4.1
Low albumin	6 (4,7 %)	3 (3.4 %)
Platelet count (x10^9/L)	188 ± 98	235 ± 67
Low platelet count	27 (21 %)	2 (2.2 %)
Prothrombin (INR)	1.0 ± 0.1	1.0 ± 0.2
Elevated prothrombin	5 (3.9 %)	2 (2.2 %)
Ferritin (µg/L)	232 ± 317	192 ± 159
Elevated ferritin	42 (33 %)	28 (32 %)

Abbreviations: NA, not available; BMI, body mass index; IGT, impaired glucose tolerance; HDL, high density lipoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; INR, international normalized ratio; Elevated ALT defined as > 41 U/L; elevated AST as > 41 U/L; elevated ALP as > 106 U/L; elevated bilirubin as > 1.5 mg/dL; low albumin as < 3.6 g/dL, low platelet count as < 140 x10^9/L, elevated prothrombin time as > 1.2, and elevated ferritin as > 275 µg/L in men or > 130 µg/L in women.

Histopathology at baseline and at follow-up (Papers II, III, IV, V)

At baseline, 71 patients (55%) fulfilled the "broad" criteria for NASH. Four of these patients had cirrhosis. Twelve patients (9%) had steatosis with unspecific inflammation, and 46 (36%) had simple steatosis. Patients with NASH were significantly older than patients with steatosis, with or without unspecific inflammation (54.5 ± 12.4 vs. 46.7 ± 12.3 years, respectively, *P* = 0.001). There were no other significant differences between the two histopathological groups. Mean NAS was 2.45 (± 1.00), and NASH (NAS ≥5) was diagnosed in 2 (1.6%), "borderline NASH" (NAS 3-4) in 69 (53%), and "not NASH" was diagnosed in 58 (45%) patients.

At follow-up, liver biopsies were obtained from 68 patients after 13.8 ± 1.2 years (range 10.3-16.3 years). NASH ("broad" criteria) was diagnosed in 44 patients (65%). Mean NAS was 2.1 ± 1.2, and NASH was diagnosed in 1 (1.5%), "borderline NASH" in 22 (32%), and "not NASH" in 45 (66%) patients. The decrease in NAS during follow-up was not statistically significant. Overall, quantitative steatosis was significantly lower at follow-up (12.5% ± 9.7% vs. 8.8% ± 7.4%, *P* = 0.004).

In the 68 patients that underwent liver biopsy at follow-up, 27 patients (41 %) increased in fibrosis stage, 30 (43 %) did not change, and 11 (16 %) regressed. Individual fibrosis scores, at baseline and at follow-up, are presented in Table 10, together with the baseline fibrosis stage in the three patients that did not undergo liver biopsy at follow-up because of end-stage liver disease.

Table 10: Changes in fibrosis stage between first and second biopsies in patients that underwent repeat liver biopsy or developed end-stage liver disease (n = 71).

	Fibrosis stage at Follow-Up				
Fibrosis stage at Baseline	**F0 (n = 24[b])**	**F1 (n = 22[b])**	**F2 (n = 11[b])**	**F3 (n = 7[b])**	**F4 (n = 7[b])**
F0 (n = 36)	19 (53%)	8 (22%)	6 (17%)	3 (8%)	0
F1 (n = 19)	5 (26%)	9 (47%)	3 (16%)	1 (5%)	1 (5%)
F2 (n = 11)	0	5 (45%)	1 (9%)	2 (18%)	3 (27%)[d]
F3 (n = 4)	0	0	1 (25%)	1 (25%)	2 (50%)[d]
F4 (n = 1)[c]					1 (100%)[d]

[b]Number of NAFLD patients in each fibrosis stage at follow-up, including three patients that did not undergo repeat liver biopsy, but developed end-stage liver disease.
[c]Repeat liver biopsy was not performed in patients with cirrhosis at baseline.
[d]One patient in each group did not undergo repeat liver biopsy because of end-stage liver disease.

Survival (Paper II)

A total of 26 subjects with NAFLD died during the follow-up period (including one patient who died shortly after diagnostic workup at follow-up). Of these, 19 had NASH ("broad criteria") at baseline, including one patient with cirrhosis, and seven had steatosis with or without unspecific inflammation. At the end of the follow-up period, survival of NAFLD patients was significantly lower than that of the reference population (78% vs. 84%, respectively; $P < 0.006$; Fig. 9A). Subgroup analysis showed that survival among NASH ("broad criteria") patients was significantly lower than the corresponding reference population (70% vs. 80%, respectively, $P < 0.01$; Fig. 9B), whereas survival did not differ significantly between patients with steatosis with or without unspecific inflammation and the corresponding reference population (Fig. 9C).

Causes of death and liver-related morbidity and mortality among NAFLD patients (Paper II)

Of the 129 patients with NAFLD, two patients (1.6 %) died from liver-related causes (metastatic hepatocellular carcinoma that had developed in a cirrhotic liver, and variceal hemorrhage, respectively). Liver-related death in the reference population was 0.2 %. This difference did not attain statistical significance ($P = 0.06$). The remaining 24 subjects died of causes unrelated to liver disease (cardiovascular diseases n = 16, extrahepatic malignancies n = 5, respiratory diseases n = 1, neurological diseases n = 1, renal diseases n = 1). Comparing the causes of death of NAFLD patients with those of the reference population, death from
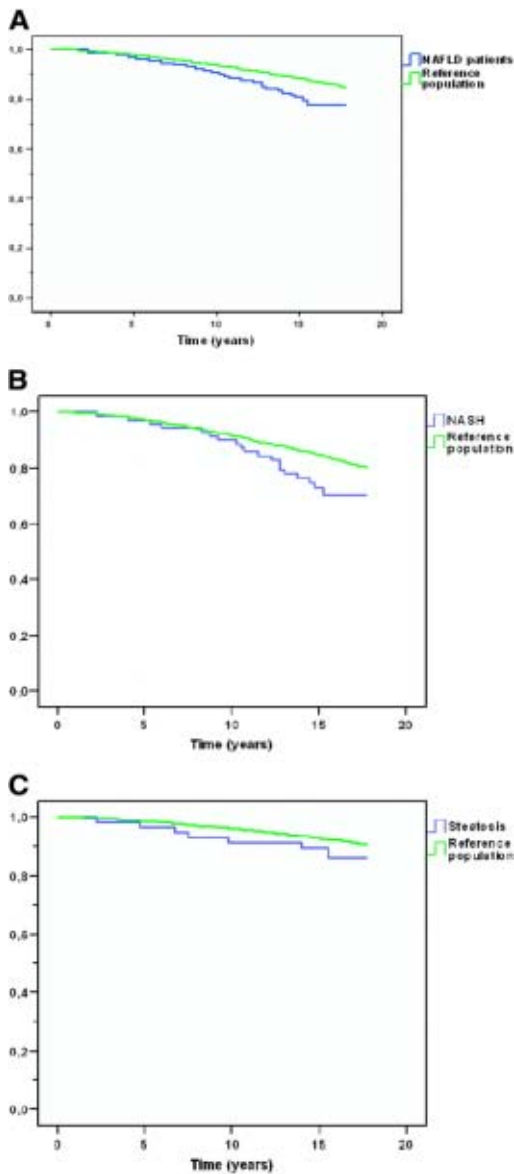
Figure 10: *(A) Overall survival of all NAFLD patients. (B) Survival of NASH patients. (C) Survival of patients with simple steatosis with and without unspecific inflammation. Survival was compared with that of a matched reference population.*

cardiovascular diseases was significantly more common among NAFLD patients (12.4 % vs. 6.7 %, respectively, $P = 0.04$).

When causes of death were analysed separately for NASH patients ("broad criteria"), both liver related death (2.8% vs. 0.2%, respectively, $P = 0.04$), and death from cardiovascular disease (15.5% vs. 7.5%, respectively, $P = 0.04$) were more common compared with the corresponding reference population.

Of the NAFLD patients who died during follow-up, two had developed cirrhosis-related complications prior to death. One female patient subsequently died from variceal haemorrhage, and one male patient who died from acute myocardial infarction had previously developed ascites, and cirrhosis was diagnosed with post-mortem biopsy. Among the 88 NAFLD patients who participated in the follow-up study, 5 had developed cirrhosis-related complications (ascites in two patients, ascites and hepatocellular carcinoma in two patients, of whom one died shortly after diagnostic workup at follow-up, and hepatocellular carcinoma in one patient who had undergone successful orthotopic liver transplantation during follow-up). Thus, of 129 NAFLD patients, 7 (5.4%) developed cirrhosis-related complications during follow-up. There were no

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significant differences in baseline clinical, biochemical, and histopathological parameters between the seven patients who developed end-stage liver disease during follow-up and the 15 patients who died from cardiovascular disease without previous development of end-stage liver disease.

Metabolic and cardiovascular characteristics of study cohort (Papers II, III, IV, V)

At baseline, most patients were overweight or obese. Fasting plasma glucose was not measured at baseline, and thus the prevalence of diabetes at onset of the study cannot be reported. At follow-up, 69 patients (78%) had diabetes or impaired glucose tolerance, and sixteen patients (18%) had manifest cardiovascular disease (Table 9).

Histopathology at baseline versus clinical outcome (Papers II, III, IV, V)

Of the 71 patients with NASH ("broad criteria") at baseline, 7 (10%) developed end-stage liver disease during follow-up, whereas none of the 58 patients with steatosis with or without unspecific inflammation developed complications related to chronic liver disease during follow-up. Mean baseline NAS was higher in those seven patients that developed end-stage liver disease although this did not reach statistical significance (3.1 ± 0.9 vs. 2.4 ± 1.0; $P = 0.062$). None of the two patients with NASH ("strict criteria") according to NAS at baseline developed end-stage liver disease. Subgroup analysis showed that no patient who had NAFLD without fibrosis (n = 60) or stage 1 fibrosis (n = 31) at baseline had developed complications related to chronic liver disease during follow-up. One of the 4 patients (25%) with cirrhosis at baseline, 3 of the 22 patients (14%) with stage 2 fibrosis at baseline, and 3 of the 12 patients (25%) with stage 3 fibrosis at baseline developed end-stage liver disease during follow-up. Forty-two patients with NASH ("broad criteria") at baseline returned for follow-up. Of these, 30 patients (71%) had diabetes. Of the patients with steatosis with or without unspecific inflammation, 21 (46%) had diabetes. This difference was statistically significant ($P <0.01$). Moreover, manifest cardiovascular disease at follow-up was significantly more common among patients with NASH ("broad criteria") at baseline than among those without NASH (29% vs. 9%, respectively, $P <0.02$).

Fibrosis progression (Papers II, III, IV, V)

The presence of necroinflammatory changes at baseline was not associated with progression in fibrosis stage at follow-up. A separate analysis of the 36 patients without fibrosis at

baseline who underwent liver biopsy at follow-up showed that only a small number exhibited histological features associated with hepatic necroinflammation at baseline. Despite this, 17 patients (47%) had developed fibrosis at follow-up.

Those patients who progressed in fibrosis stage at follow-up had significantly higher ALT (P =0.005), significantly higher AST (P =0.003), and significantly lower platelet count (P =0.003) at follow-up. Moreover, at follow-up subjects with progressive fibrosis significantly more often had a weight gain exceeding 5 kg (P =0.02), they were significantly more insulin resistant according to homeostasis model assessment (P <0.04), and they had significantly more pronounced hepatic fatty infiltration (P =0.03). We were not able to find any association between baseline clinical and biochemical parameters with future progression of fibrosis.

Statin treatment and fibrosis progression (Paper III)

Of the 68 patients that underwent repeat liver biopsy, seventeen patients were on medication with statins (statin cohort), compared with 51 patients that were untreated (no statin cohort). Of the 25 patients that died prior to follow-up, four patients had been prescribed a statin. None of these four patients had developed end-stage liver disease. None of the patients that developed end-stage liver disease was treated with statins.

At baseline, cholesterol levels (264 ± 86 vs. 230 ± 46, respectively, P = 0.04) and BMI (30.2 ± 3.5 vs. 27.2 ± 3.8, respectively, P = 0.006) were significantly higher in the statin cohort. Other clinical and liver-related biochemical parameters did not differ significantly between the two cohorts. At follow-up, the statin cohort continued to have higher BMI, diabetes and manifest cardiovascular disease were more common, and the statin cohort had more pronounced insulin resistance. As expected, patients on statins had significantly lower cholesterol, and lower LDL. Clinical and biochemical variables of the two cohorts at follow-up are presented in Table 11.

Pair wise comparisons between baseline and follow-up showed no significant changes in BMI and biochemical parameters in the cohort consisting of patients that had not been exposed to statins. In the statin cohort cholesterol was significantly lower at follow-up (176 ± 39 vs. 264 ± 86 mg/dL, respectively, P = 0.001) but other parameters analysed at baseline were not significantly different at follow-up.

At baseline, the statin cohort had significantly more pronounced hepatic fatty infiltration (20.4 ± 7.5% vs. 10.3 ± 9.0%, respectively, *P* = 0.001). There was a significant reduction of quantitative steatosis in the statin cohort between baseline and follow-up (20.4 ± 7.5% at baseline vs. 11.1 ± 8.9% at follow-up, *P* = 0.001). As a result of this reduction fatty infiltration in the statin cohort was not significantly different from the no statin cohort at follow-up. Patients that had not been prescribed a statin did not change significantly in hepatic quantitative steatosis over time.

In the statin cohort, four patients (24%) progressed in fibrosis stage while among patients that had not been prescribed a statin 23 (45%) progressed in fibrosis stage. Those four patients in the statin cohort that progressed in fibrosis stage had no significant reduction of fatty infiltration (18 ± 6.1% at baseline vs. 16 ± 9.0% at follow-up) as opposed to the 13 patients that remained stable or regressed in fibrosis stage (21 ± 8.2% at baseline vs. 9.3 ± 7.9% at follow-up, *P* = 0.001). During follow-up the no statin cohort, as a group, exhibited significant progression of fibrosis stage while the statin cohort remained, as a group, stable.

Table 11: Clinical and biochemical characteristics of cohort at baseline and at follow-up according to statin treatment [Mean ± SD or n (%)]

	No statin (n = 51)	Statin (n = 17)	*P*
Age (years)	60.1 ± 12.0	62.5 ± 8.5	NS
Time period between first and second liver biopsy (years)	13.8 ± 1.2	13.8 ± 1.2	NS
Duration of statin exposure before second liver biopsy (years)		6.1 ± 4.8	
Statin prescribed: simvastatin/atorvastatin/pravastatin		11/5/1	
BMI (kg/m^2)	28.3 ± 4.7	30.8 ± 4.0	0.044
IGT/Diabetes	14 (27%)/22 (43 %)	0 (0%)/15 (88 %)	0,004
Metabolic syndrome	26 (51 %)	13 (76 %)	NS
Hypertensive	48 (94 %)	16 (94 %)	NS
Cholesterol (mg/dL)	213 ± 41	176 ± 39	0.002
LDL (mg/dL)	134 ± 33	101 ± 35	0.001
HDL (mg/dL)	54 ± 23	45 ± 9.5	0.03
Triglyceride (mg/dL)	157 ± 98	149 ± 60	NS
ALT (U/L)	61 ± 35	63 ± 40	NS
AST (U/L)	35 ± 17	36 ± 13	NS
AST/ALT ratio	0.7 ± 0.3	0.7 ± 0.3	NS
ALP (U/L)	66 ± 45	55 ± 27	NS
Glucose (mg/dL)	117 ± 33	150 ± 47	0.002
QUICKI	0.34 ± 0.03	0.32 ± 0.03	0.04
HbA1c (%)	4.9 ± 1.0	5.7 ± 1.3	0.014
Quantitative steatosis original biopsy (%)	10 ± 9.0	19 ± 7.5	0.0001
Quantitative steatosis (%)	8.2 ± 7.0	11 ± 8.9	NS

Abbreviations: NA, not available; BMI, body mass index; IGT, impaired glucose tolerance; HDL, high density lipoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; INR, international normalized ratio; Elevated ALT defined as > 41 U/L; elevated AST as > 41 U/L; elevated ALP as > 106 U/L; elevated bilirubin as > 1.5 mg/dL, low albumin as < 3.6 g/dL, low platelet count as < 140 x10^9/L, elevated prothrombin time as > 1.2, and elevated ferritin as > 275 μg/L in men or > 130 μg/L in women.

Alcohol consumption and fibrosis progression (Paper IV)

Alcohol consumption and heavy episodic drinking (HED) in NAFLD patients according to change in fibrosis stage are shown in Table 12. Patients that developed end-stage liver disease are presented separately (Table 12). A trend towards higher weekly alcohol consumption at follow-up was seen when the 17 patients (12 patients, who progressed by more than one stage during follow-up and 5 patients that had developed end-stage liver disease), who were classified as having significant progression in fibrosis stage, were compared with the 54 patients with insignificant change in fibrosis stage (38 (0-134) vs. 17 (0-138) g/week, $P = 0.061$). The proportion of patients reporting HED at least once a month was significantly higher among those with significant progression in fibrosis stage (8 (47%) vs. 6 (11%) patients, respectively, $P = 0.003$). One (6%) patient with significant progression in fibrosis stage reported increased alcohol consumption during follow-up compared with 6 patients (11%) with insignificant change in fibrosis stage. The corresponding figures for decreased alcohol consumption during follow-up were 5 (29%) vs. 20 (37%) patients ($P = 0.62$). All patients reporting decreased alcohol consumption at follow-up denied previous alcohol consumption exceeding 140 g per week. Six patients (9%) were total abstainers. Of these, one progressed in fibrosis stage, three were stable, and two regressed (Table 12).

Table 12: Alcohol consumption, frequency of HED and change in alcohol consumption according to change in fibrosis stage during follow-up [Median (Range) or n (%)].

	Change in fibrosis stage during follow-up					
	-1 (n = 11)	**0** (n = 30)	**1** (n = 13)	**2** (n = 8)	**3** (n = 4)	**End-stage*** (n = 5)
Alcohol consumption (g/week)	2.0 (0-63)	17.5 (0-138)	18.0 (2-121)	24.0 (6-113)	100.5 (10-123)	38 (0-134)
HED once a month or more often (n)	0 (0%)	4 (13%)	2 (15%)	4 (50%)	3 (75%)	1 (20%)
Abstainers (n)	2 (18%)	3 (10%)	0 (0%)	0 (0%)	0 (0%)	1 (20%)
Increased alcohol consumption (n)	1 (9%)	3 (9%)	2 (15%)	0 (0%)	0 (0%)	1 (20%)
Decreased alcohol consumption (n)	7 (64%)	11 (37%)	2 (15%)	2 (25%)	1 (25%)	2 (40%)

HED was defined as more than 60 grams of ethanol in males and 48 grams of ethanol in females consumed on one occasion. *Five patients, of whom three patients did not undergo repeat liver biopsy, had developed end-stage liver disease during follow-up.

In the univariate binary logistic regression analysis HED, ($P = < 0.001$), and IR_{HOMA}[17] ($P = 0.030$) were found to be significantly associated with significant progression in fibrosis stage. Weekly alcohol consumption almost attained statistical significance ($P = 0.055$). In Model 1 (n = 71) of the multivariate binary logistic regression analysis, HED ($P < 0.001$) was independently associated with significant progression in fibrosis stage. In Model 2 (n = 57),

HED ($P < 0.001$), and IR_{HOMA} ($P < 0.01$) were independently associated with significant progression in fibrosis stage (Table 13).

Table 13: Univariate and multivariate binary logistic analyses evaluating factors associated with significant fibrosis progression. Comparisons are made between patients with significant progression in fibrosis stage (n = 17) and patients with insignificant change in fibrosis stage (n = 54).

Univariate logistic regression			
	Regression coefficient (±SE)	Odds ratio (95% CI)	*P*
Weekly alcohol consumption	0.012 (± 0.006)	1.012 (1.000-1.025)	0.055
Sex	0.314 (± 0.645)	1.368 (0.387-4.842)	0.627
BMI	0.073 (± 0.059)	1.076 (0.958-1.208)	0.216
ED at least once a month	1.962 (± 0.651)	7.111 (1.986-25.465)	0.003
Diabetes	0.181 (± 0.558)	1.198 (0.401-3.579)	0.746
Weight gain	0.006 (± 0.032)	1.006 (0.945-1.070)	0.850
IR_{HOMA}(n = 56)	0.290 (± 0.134)	1.336 (1.029-1.737)	0.030

Multivariate logistic regression			
Model 1* (n = 71)	Regression coefficient (±SE)	Odds ratio (95% CI)	*P*
ED at least once a month	1.962 (± 0.651)	7.111 (1.986-25.465)	0.003
Model 2* (n = 57)	Regression coefficient (±SE)	Odds ratio (95% CI)	
ED at least once a month	2.913 (± 0.843)	18.404 (3.526-96.042)	<0.001
IR_{HOMA}	0.354 (± 0.144)	1.424 (1.075-1.888)	<0.01

*IR_{HOMA} could not be calculated in 14 patients because they were under insulin treatment. Therefore, two multivariate logistic regression analyses were performed. In Model 1 all patients (n = 71) were included while in Model 2 only patients who were not treated with insulin (n = 57) were included.

NAS and fibrosis (Paper V)

There was a positive correlation between the NAS and fibrosis stage both at baseline (Correlation coefficient 0.233, $P = 0.008$) and at follow-up (Correlation coefficient 0.437, $P < 0.0001$).

During follow-up, 17 NAFLD patients progressed more than one fibrosis stage or developed end-stage liver disease and were considered having progressive fibrosis. NAS was significantly higher in patients with progressive fibrosis compared with patients with stable fibrosis (2.9 ± 0.9 vs. 2.2 ± 0.9; $P = 0.017$). Of the 71 patients that completed follow-up, 34 patients had "borderline NASH" at baseline, and 37 patients had "not NASH" at baseline. Progressive fibrosis was more frequent in the "borderline NASH" group than in the "not

NASH" group although this did not attain statistical significance (30% vs. 18%, P = 0.28). The risk of fibrosis progression in the two groups was not influenced by presence or absence of liver fibrosis at baseline (data not shown). The association between progressive fibrosis and all histopathological variables was tested with univariate binary logistic regression analyses. Only NAS was significantly associated with progressive fibrosis (P = 0.023), and lobular inflammation (which is part of the NAS) almost attained statistical significance (P = 0.074). Regression coefficients and odds ratios are presented for each individual variable in Table 14.

In a multivariate binary logistic regression analysis, including the NAS, age, sex, and diabetes, the NAS was independently associated with fibrosis progression (Table 14).

Table 14: Predictors of fibrosis progression by univariate and multivariate binary logistic regression analyses. Comparisons are made between patients with significant progression in fibrosis stage (n = 17) and patients with insignificant change in fibrosis stage (n = 54).

	Regression coefficient (±SE)	Odds ratio (95% CI)	P
Univariate binary logistic regression			
Steatosis grade	0.565 (± 0.364)	1.759 (0.862-3.587)	0.120
Quantitative steatosis	0.028 (± 0.030)	1.029 (0.970-1.091)	0.342
Lobular inflammation	1.178 (± 0.961)	5.571 (0.847-36.659)	0.074
Hepatocellular ballooning	1.293 (± 0.870)	3.643 (0.661-20.062)	0.138
Mallory bodies	1.955 (± 1.259)	7.067 (0.599-83.374)	0.120
Iron	-0.423 (± 0.593)	0.655 (0.205-2.091)	0.475
Fibrosis stage	0.323 (± 0.268)	1.382 (0.817-2.336)	0.227
NAS-score	0.757 (± 0.333)	2.132 (1.109-4.098)	0.023
Multivariate binary logistic regression			
NAS-score	0.930 (± 0.396)	2.536 (1.167-5.510)	0.019
Age (years)	0.049 (± 0.033)	1.050 (0.984-1.121)	0.143
Sex	0.815 (± 0.755)	2.259 (0.515-9.914)	0.280
Diabetes	1.254 (± 0.748)	3.505 (0.809-15.187)	0.094

General discussion

In person-years, this is the largest reported study of a follow-up series of biopsy-proven NAFLD patients originally referred because of elevated liver enzymes. Although we cannot rule out unknown biases in referral we believe that our study has several methodological strengths. First, all patients referred because of elevated liver enzymes were consecutively enrolled. Second, all patients underwent liver biopsy at baseline, and thus the diagnosis of NAFLD was based on histopathological criteria. Third, clinical and histopathological follow-up exceeded 10 years for all patients, and time to follow-up did not vary considerably between patients.

One of the main findings of the follow-up study is that the cohort of NAFLD patients had decreased survival compared with an age, sex, and geographically matched cohort from the general population. Our data confirm the results of a previous study by Adams et al. who reported that survival in a cohort of NAFLD patients in Olmstead County, Minnesota, was lower than the expected survival in the general population.[182] In their study the NAFLD diagnosis was confirmed by imaging studies in most patients rather than histopathologically, and follow-up time was highly variable. In both cohorts of patients liver-related death was the third most common cause of death. We were able to show that in NASH (broad criteria) patients, liver-related death was a significantly more common cause of death than in the general population. When survival was analysed separately for NASH (broad criteria) patients and patients with steatosis with or without unspecific inflammation, survival was not significantly decreased in the steatosis group. A benign clinical course in patients with pure fatty liver was reported in a Danish study as well (Table 15).[16]

Table 15: Studies on survival of NAFLD patients compared with the general population

	Diagnosis	N	Average follow-up (Range)	Age (SD or Range)	BMI (SD or Range)	No. of deaths (%)	Decreased survival
Dam-Larsen (2004)[16]	Bland steatosis	109	16.7 (0.2-21.9)	39 (19-80)	42 (19-72)	27 (24.8)	No
Adams (2005)[182]	NAFLD	420	7.6 (0.1-23.5)	48 ($\pm$ 15)	33.4 $\pm$ 6.1	53 (12.6)	Yes
Ekstedt (2006)[183]	NAFLD	129	13.7 (2.1-16.3)	51 ($\pm$ 13)	28.3 $\pm$ 3.8	26 (20.2)	Yes

Histological progression was seen in 40% of patients that underwent repeat liver biopsy in our cohort. Three other studies have evaluated the histological progression in NAFLD patients (Table 16).[151, 184, 185] It is evident that a number of NAFLD patients do have a progressive liver disease, and it seems as if fibrosis in some patients could regress over time. Because of

the significant sampling variability described in NAFLD[30, 31] these changes must be interpreted with caution.

Table 16: Studies on fibrosis development in NAFLD patients with sequential liver biopsies

	N	Mean time between biopsies years (range)	Progressed n (%)	Stable n (%)	Improved n (%)
Harrison (2003)[184]	22	5.7 (1.4-15.7)	7 (32)	11 (50)	4 (18)
Fassio (2004)[185]	22	4.3 (3-14.3)	7 (32)	11 (50)	4 (18)
Adams (2005)[151]	103	3.2 (0.7-21.3)	38 (37)	35 (34)	30 (29)
Ekstedt (2006)[183]	68	13.8 (10.3-16.3)	27 (40)	30 (44)	11 (16)

Compelling evidence that NAFLD is a disease that could seriously harm the liver was that more than 5% of patients did develop end-stage liver disease during follow-up. The numbers of patients that develop end-stage liver disease in different studies depend highly on the inclusion criteria used when selecting the NAFLD cohort (Table 17). Interestingly, 3 of the 7 patients who developed end-stage liver disease during follow-up were diagnosed with hepatocellular carcinoma. All 3 had previously been diagnosed with diabetes. These findings are in accordance with those of a previous report that identified diabetes as an independent risk factor for hepatocellular carcinoma.[186] Our data primarily indicate that the association between diabetes and hepatocellular carcinoma is a result of the high prevalence of NAFLD in patients with diabetes.

Progression of NAFLD is slow and the prognosis of patients depends highly on the initial fibrosis stage. Among patients with no fibrosis at baseline few will progress to end-stage liver disease.[16, 24] This has led to the common opinion that patients with simple steatosis have a benign prognosis. The results of the present study suggest that this view should be altered. None of the 91 patients that had no, or stage 1 fibrosis, in their initial liver biopsy developed end-stage liver disease. Considering that follow-up exceeded ten years in all patients this is reassuring. On the other hand, of the 36 patients without fibrosis at baseline 17 (47%) patients had developed fibrosis at follow-up. Three (8%) of these patients had developed bridging (F3) fibrosis.

Table 17: Studies on survival of NAFLD patients compared with the general population

	Diagnosis	N	Average follow-up	Cirrhosis at baseline	End-stage liver disease	Liver related death
Teli (1995)[187]	Bland steatosis	40*	11	0 (0)	0 (0)	0 (0)
Dam-Larsen (2004)[16]	Bland steatosis	109	16.7	0 (0)	1 (0.9)	1 (0.9)
Lee (1989)[15]	NASH	39	3.8	NA	1 (2.6)	1 (2.6)
Powell (1990)[188]	NASH	42	4.5	1 (2.4)	1 (2.4)	1 (2.4)
Cortez-Pinto (2003)[189]	NASH	32	5.9	3 (8)	NA	3 (9.4)
Matteoni (1999)[24]	NAFLD	98	8.3	20 (15)	NA	9 (9.2)
Adams (2005)[151]	NAFLD	420	7.6	NA	13 (3.0)	7 (1.7)
Ekstedt (2006)[183]	NAFLD	129	13.7	4 (3.1)	7 (5.4)	2 (1.6)
Hashimoto (2005)[190]	NASH (F3-F4)	89	3.7	43 (48)	10 (11)	8 (9.0)
Sanyal (2006)[154]	NASH cirrhosis	152	10	152 (100)	69 (45)	29 (19)

Abbreviations: NA, not available
*Six patients had fatty liver due to cachexia.

Traditionally the histopathological variables in NAFLD are assessed through a visual and semiquantitative estimation. In our studies steatosis grade has been assessed quantitatively through point counting. In a separate study, we scrutinized the discrepancies between semiquantitative grading of steatosis and point counting, as well as the reproducibility of the methods. Few earlier studies have considered and compared the semiquantitative and the quantitative approaches to assess the degree of steatosis in liver biopsies. Auger et al.[191] evaluated an automated analysis based on thresholding of unstained areas, automatic omission of sinusoidal empty spaces with a 'form factor' and manual exclusion of vessels with red blood cells. The semiquantitative grading was performed by two pathologists estimating the percentage of fatty hepatocytes using a 10-graded scale. They found that the pathologists' scoring varied between 0 and 80% whereas the automatic calculated densities were much lower and varied between 0 and 15%. Kumar et al.[192] studied biopsies from patients with hepatitis C and their highest semiquantitative score was 2. They found that the maximum value of score 2 was approximately 11% area of the biopsy occupied by fat. In our study of 75 liver biopsies with steatois, we found a mean fat density value of 23.1% and a maximum value of 45.3% in the score 3 group. These studies show that the amount of steatosis is greatly overestimated when assessed with semiquantitative estimation.

In a recent study, patients with severe steatosis were more likely to have steatohepatitis.[193] The authors suggest that steatosis severity could be used as a surrogate endpoint in drug development. If this will be true, a quantitative assessment of steatosis severity, at least in clinical trials, should be preferred. In our study, the steatosis grade at baseline was not significantly higher in patients with significant fibrosis progression.

Another main finding of our study, consistent with the increased cardiovascular mortality among NAFLD patients, is that most NAFLD patients (78%) were diagnosed with diabetes or

impaired glucose tolerance (IGT) at follow-up. Although we do not know with certainty how many NAFLD patients had diabetes or IGT at baseline, these findings indicate that liver enzyme elevation due to NAFLD is strongly associated with future onset of type 2 diabetes or IGT. These data confirm previously published data from another Swedish cohort of patients. After a mean follow-up time of 2.8 years, 23% of a total of 80 patients with NAFLD had developed diabetes or impaired glucose tolerance.[194] In this study, only two patients had diabetes at the baseline investigation. Together, these results clearly show that fatty infiltration of the liver precedes the onset of diabetes. Therefore, surveillance of metabolic complications in these patients is recommended.

The clinical development of NAFLD patients is highly variable. Many patients have a stable disease and will never experience any liver related complications whereas a minority will die because of cirrhosis. To find variables that predict disease progression in NAFLD is of utmost importance. Of the 7 patients that developed end-stage liver disease, none had fibrosis stage <2 at baseline. The highest risk of developing end-stage liver disease is found in the subset of patients with advanced fibrosis. Non-invasive techniques to detect advanced fibrosis in NAFLD patients have been developed and evaluated in fairly large cohorts of patients. Among different panels of clinical and/or biochemical variables[195-197] or imaging techniques, the most promising today seems to be transient elastography (Fibroscan).[198, 199]

In two of our studies we have used significant fibrosis progression as end-point. Significant fibrosis progression was defined as histological progression of more than one fibrosis stage or development of end-stage liver disease. There are two reasons for this definition. When cirrhosis is diagnosed in the index biopsy (stage 4) no progression in fibrosis stage is possible per definition even though cirrhosis is a broad clinical spectrum in itself.[80] Therefore, it is important to include the development of symptoms of end-stage liver disease in the definition of disease progression. Secondly, fibrosis stage in NAFLD has been shown to be influenced by sampling variability.[30, 31] However, a difference in fibrosis of more than one stage was only noted in 6-12 % of paired biopsies. Thus, we believe that most, if not all, NAFLD patients that were classified as having significant progression of fibrosis stage had a "true" histological and, in those cases who developed end-stage liver disease, also clinical deterioration.

In the three previous histological follow-up studies published, no histopathological variable was associated with fibrosis progression, except that low initial fibrosis score was predictive

of progression in the Adams study.[151, 184, 185] We were able to show that the NAS (unweighted sum of steatosis, ballooning, and lobular inflammation scores) was independently associated with significant fibrosis progression. This is a promising observation that warrants further studies. The use of NAS in the clinical setting is likely to be limited by the fact that there was a significant overlap in clinical course between patients in the different histological groups. Although only two out of 129 patients were classified as having NASH at baseline when the NAS was used as diagnostic criterion, seven patients (5%) developed end-stage liver disease during follow-up. Moreover, 17 patients (24%) that underwent repeat liver biopsy had progressed by more than one fibrosis stage during follow-up. A higher proportion of patients with "borderline NASH" at baseline developed significant progression of fibrosis stage during follow-up. However, the proportion was not statistically significant from those who had "not NASH" at baseline. It was evident that some patients classified as having "not NASH" at baseline progressed significantly in fibrosis stage during follow-up, including two patients that developed end-stage liver disease. These data strongly challenge the view that NAFLD patients not fulfilling the criteria for NASH have a benign course.

The greatest challenge in conducting NAFLD studies is to obtain true assessment of each patient's alcohol consumption. Hayashi et al. reported that some NAFLD patients actually had ALD when total lifetime alcohol intake was assessed.[200] This finding is supported by the fact that eight out of 137 patients originally diagnosed with NAFLD in our study, were reclassified as having ALD at follow-up. The AUDIT-C is a well-established and validated instrument in assessing alcohol consumption.[201] However, the proportion of high consumers and reported mean consumption have been reported to be higher when using a period specific normal week approach compared with the AUDIT-C alone.[202] In the present study we used the AUDIT-C in combination with an interview using the period specific normal week approach. We believe that this approach gives one of the most reliable assessments of alcohol consumption possible with reasonable time consumption.

Patients with significant fibrosis progression in our study more frequently reported heavy episodic drinking at least once a month. The association was even stronger when other variables known to be associated with progression of NAFLD were tested in a multivariate analysis. Our data show that a more thorough evaluation of alcohol consumption, including not only overall consumption but also drinking pattern, is necessary in NAFLD patients. It may be argued that "admitting" to occasional consumption of more than 60 or 48 grams of ethanol at least once a month indicates that overall weekly alcohol consumption exceeds 140

grams, and thus that these patients cannot be classified as having NAFLD. However, we believe that we used the most reliable methods available to assess alcohol consumption and that all patients fulfilled current NAFLD criteria used by most authors.

The major limitation of this study is that detailed assessment of alcohol consumption was performed on a single occasion. Preferably, alcohol consumption should have been assessed with the same technique at baseline and at follow-up as well as at regular intervals during the follow-up period. However, this type of study is very difficult to perform considering the slow progression of fibrosis in NAFLD and the long follow-up time needed. Because of the retrospective nature of alcohol consumption assessment in this study, conclusions must be drawn with caution. However, a prospective study of the long-term influence of alcohol on fibrosis progression in NAFLD is, due to its demanding nature, not likely to be carried out in the near future.

Currently a diagnosis of NAFLD is set when fatty infiltration of the liver is found and other known causes are excluded. Alcohol consumption below a specific threshold is needed for the diagnosis of NAFLD to be set. In our study, even alcohol consumption below this threshold is associated with fibrosis progression. The association between fibrosis progression and alcohol consumption is even stronger when drinking pattern is analysed. These data together with the significant overlap in clinical long-term prognosis between the histopathological subgroups of the disease show that a revision of the definition of NAFLD and NASH is urgently needed. Instead of using a "negative" definition, where the disease is diagnosed by excluding specific criteria, a "positive" definition should be adopted. Presence of insulin resistance is probably the most important feature that should be considered in this definition. In such a revision a new name for the disease is preferred, as previously suggested.[203]

Due to the high prevalence of diabetes, hypertension, and hyperlipidaemia, the risk of developing cardiovascular disease is significant among NAFLD patients. Protection from this potentially life threatening complication must be of the utmost concern in the care of these patients. Because of the undisputable role for statin treatment in primary and secondary prevention of cardiovascular disease[204], many NAFLD patients, with diabetes or manifest cardiovascular disease, are being treated with statins. Theoretical possibilities have been raised that statins given for long-term may cause silently progressing liver disease.[205] We found that statins given for long-term do not aggravate hepatic histology in NAFLD. Diabetes mellitus and insulin resistance have been associated with fibrosis progression in NAFLD.[206]

In the present study, NAFLD patients that had been prescribed a statin had significantly more often diabetes, they were more insulin resistant, had higher BMI, and were more often treated with insulin compared to those patients who were not treated with statins. Despite exhibiting a higher risk profile for progression of liver fibrosis, only 24% of NAFLD patients on statin treatment progressed in fibrosis stage.

With the increasing prevalence of NAFLD, particularly at younger ages, the modestly increased relative risk of mortality and the low absolute risk of end-stage liver disease presented in this study may be of considerable public health significance in the near future. Given the strong association between insulin resistance and NAFLD, it is reasonable to recommend lifestyle modifications to all patients with NAFLD. Not only do such modifications reduce the risk of developing type 2 diabetes[207, 208] but an intense dietary intervention, in particular, may also improve liver histology in those with NAFLD.[209] In Figure 10, a suggested management of NAFLD patients is shown.

- Screen for components of the metabolic syndrome

- Treat hyperglycemia, hypertension and dyslipidemia

- Encourage lifestyle modification

- Consider treatment with statin

- Consider liver biopsy if suspicion of severe fibrosis

- Consider HCC-surveilance if severe fibrosis is present

- Include in clinical trials

Figure 10: Suggested management of NAFLD patients.

Conclusions

- NAFLD patients have a lower survival compared with the general population. Higher mortality is primarily a result of cardiovascular disease.

- NAFLD may progress to end-stage liver disease. The risk is low but clinically significant.

- The majority of NAFLD patients will develop diabetes or impaired glucose intolerance. All NAFLD patients should be screened for metabolic complications.

- Statin treatment does not aggravate NAFLD histology.

- Alcohol consumption, especially when frequency of episodic drinking is assessed, is associated with fibrosis progression in NAFLD.

- Point counting is a simple and reliable method to quantitatively assess the severity of steatosis in NAFLD.

- The non-alcoholic fatty liver disease activity score (NAS) is independently associated with fibrosis progression. However, there is considerable overlap in clinical progression between NAFLD subgroups.

Sammanfattning på svenska

Fettlever ansågs tidigare oftast bero på överkonsumtion av alkohol. Under de senaste två decennierna har intresset för fettlever hos patienter som inte dricker för mycket alkohol, ickealkoholisk fettleversjukdom [NAFLD (non-alcoholic fatty liver disease)], ökat dramatiskt. Idag anses NAFLD vara den vanligaste leversjukdomen i den utvecklade delen av världen. Det finns ett starkt samband mellan fettlever, övervikt, insulinresistens och högt blodtryck. Fettlever anses därför utgöra en del av det så kallade metabola syndromet. NAFLD är ett spectrum av tillstånd som innefattar ren fettlever, utan inflammatorisk aktivitet, NASH (non-alcoholic fatty liver disease) som karaktäriseras av hepatocellulär skada, inflammation samt fibros (bindvävsinlagring/ärrvävnad) som kan leda till cirros (ofta kallad skrumplever), leversvikt och hepatocellulär cancer.

Graden av fettinlagring i levern bedöms oftast morfologiskt med en semikvantitativ metod där patologen använder en fyrgradig skala: 0-3 eller ingen, mild, måttlig och uttalad. I denna avhandling visar vi att det föreligger en betydande inter- och intra-individuell variation i denna metod och att en mer standardiserad kvantitativ metod är att föredra. Våra resultat visar att ytan/volymen av fett överskattas när man bedömer den semikvantitativt. Punkträkning har en högre grad av mer reproducerbarhet jämfört med en visuell uppskattning och är därför att föredra.

Den andra delen av avhandlingen beskriver den kliniska och histologiska långtidsutvecklingen hos 129 konsekutivt insamlade patienter med NAFLD. Medeluppföljningen var 13,7 år. Överlevnaden hos patienter med NASH var sämre jämfört med en matchad bakgrundspopulation. Patienterna dog oftare av hjärtkärlsjukdomar och leversjukdomar. Sju patienter (5,4 %) utvecklade klinisk leversjukdom, varav 3 patienter fick hepatocellulär cancer. Majoriteten av NAFLD patienter utvecklade diabetes eller patologisk glukostolerans under uppföljningen. Ökad bindvävsinlagring sågs hos patienter med ökad insulinresistance och mer än 5 kg viktuppgång.

Under uppföljningstiden hade 17 patienter blivit insatta på blodfettssänkande medicinering (statinbehandling). Vid uppföljningen hade patienter som behandlades med statiner signifikant högre BMI, mer ofta diabetes och mer uttalad insulinresistance. Trots att statinbehandlade patienter hade en mer uttalad riskprofil för ökad bindvävsinlagring var det endast fyra patienter som under uppföljningstiden hade ökat sin bindvävsinlagring. Vi anser

därför att det är säkert att förskriva statiner till patienter med förhöjda leverblodprover orsakade av NAFLD.

Alkoholkonsumtionen hos patienterna utvärderades med ett validerat frågeformulär kombinerat med en intervju. I en statistisk multivariat analys var alkoholkonsumtion, särskilt när man analyserade frekvensen av så kallad "heavy episodic drinking", oberoende associerat med ökad bindvävsinlagring.

Nyligen presenterades ett aktivitetsscore, "NAFLD activity score" (NAS), för att gradera de necroinflammatoriska komponenterna i leverbiopsier hos NAFLD-patienter. Vi utvärderade om NAS kan prediktera klinisk och histologisk försämring i vår cohort av NAFLD-patienter. Även om NAS var oberoende associerat med framtida försämring av leverhistologin så begränsas den kliniska användningen av ett betydande överlapp i kliniskt förlopp mellan NAS-score grupper.

Våra data visar att NAFLD kan progrediera till klinisk leversjukdom. Risken är låg men kliniskt relevant. Mer än tre fjärdedelar av patienterna kommer att utveckla diabetes eller patologisk glukostolerans. Alla NAFLD-patienter bör screenas för metabola komplikationer. Statinbehandling förvärrar inte leverhistologin. Alkoholkonsumtion, särskilt när man analyserar frekvensen av "heavy episodic drinking", ökar risken för ökad bindvävsinlagring. Punkträkning är en enkel och pålitlig metod för att kvantitativt värdera mängden fett i leverbiopsier. NAS är oberoende associerat med förvärrad bindvävsinlagring men är inte kliniskt användbart.

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