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NON-ALCOHOLIC FATTY LIVER DISEASE

AN EMERGING LIVER DISEASE

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**Karolinska
Institutet**

Stockholm 2011

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ISBN 978-91-7457-283-4

Printed by



www.reproprint.se

Gårdsvägen 4, 169 70 Solna

Till Mamma

*Success is the ability to go from one failure
to another with no loss of enthusiasm.*

Winston Churchill

ABSTRACT

The aim of the study was to evaluate what NAFLD is from a molecular perspective, what influences the disease progression and what the prognosis of the disease is.

Fatty liver has earlier often been associated with excessive alcohol intake and only in the last two decades has it been viewed as a condition in non-drinkers i.e. non-alcoholic fatty liver disease (NAFLD). Nowadays NAFLD is considered the most common cause of liver disease, showing that this is a highly modern problem that has taken epidemic forms. NAFLD is strongly associated with obesity, insulin resistance/diabetes, atherosclerosis and hypertension, thus NAFLD is considered the liver's manifestation to the metabolic syndrome. NAFLD encompasses a wide range of clinical diagnosis from simple steatosis to non-alcoholic steatohepatitis (NASH) and cirrhosis to, in some individuals, hepatocellular carcinoma (HCC).

In the first study we investigated the mortality and causes of death in a cohort of subjects with elevated serum levels of aminotransaminases. We determined the frequency of NAFLD and NASH in this population and compared the survival rate and the causes of death in NAFLD-subjects to those subjects with other liver diseases, and to the general population of Sweden. NAFLD was detected in 118 subjects of the total 256, 51 out of the 118 subjects were classified as NASH. During the follow-up period 47 (40%) of the 118 subjects diagnosed with NAFLD died. Compared with the total Swedish population, subjects with NAFLD exhibited 69% increased mortality and subjects with NASH, an increased risk with 86% and NAFLD-patients tend to in a higher extent die from liver disease.

Hyperferritinemia is quite common in NAFLD patients and in the second study we used two animal models of hepatic steatosis to investigate how iron regulatory genes are affected by steatosis alone or in combination with increased oxidative stress and inflammation. We found an increased *hamp1* expression in leptin deficient *ob/ob* mice and it seems to be caused

by up-regulation of the IL-6, STAT3, Hamp1-pathway, indicating systemic inflammation. Hepatocytes from both NAFLD mice-models were more sensitive to oxidative stress than their non fat controls.

In the third study, we evaluated biopsies from 31 NASH or borderline NASH subjects. We saw that in livers with NASH, hepatocytes with microvesicular steatosis seem to express more inflammatory markers, and in these livers an increased number of Foxp3+ T-cells (e.g. regulatory T-cells) and increased area of CD68 cells were seen. NASH patients also showed positive staining for inter cellular adhesion molecule-1 (ICAM-1) on hepatocytes and that it was localized in areas with microvesicular fat. ICAM-1 was also found to be increased in the blood circulation of NASH patients.

In the fourth study, we evaluated the role of neural cell adhesion molecule (N-CAM) in biliary type fibrosis and liver fibrosis due to parenchymatous disease. N-CAM knock out mice had attenuated liver fibrosis after bile duct ligation but not after carbon tetrachloride injections. Furthermore, hepatic stellate cells isolated from N-CAM knock-outs had impaired activation. These results suggest a role of N-CAM in biliary type liver fibrosis.

LIST OF PUBLICATIONS

- I. **Decreased Survival of Subjects with Elevated Liver Function Tests During a 28-Year Follow-Up**
Söderberg C, Stål P, Askling J, Glaumann H, Lindberg G, Marmur J, Hultcrantz R.
Hepatology. 2010 Feb;51(2):595-602.
- II. **Iron regulatory genes in two different mice models for liver steatosis.**
Söderberg C, Skogsberg J, Björkegren J, Stål P, Hultcrantz R, Sjöström M
Submitted
- III. **Microvesicular fat, ICAM-1 and regulatory T-lymphocytes are of importance for the inflammatory process in livers with NASH**
Söderberg C, Marmur J, Eckes K, Glaumann H, Sällberg M, Frelin L, Rosenberg P, Stål P, Hultcrantz R
Accepted for publication in APMIS
- IV. **Attenuated liver fibrosis after bile duct ligation and defective hepatic stellate cell activation in N-CAM knockout mice.**
Rosenberg P, Sjöström M, Söderberg C, Kinnman N, Stål P, Hultcrantz R
Accepted for publication in Liver International

LIST OF ABBREVIATIONS

ALP	Alkalinephosphate
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BDL	Bile duct ligation
BMI	Body mass index
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
CVD	Cardiovascular disease
DCT1	Divalent cation transporter 1
DMT1	Divalent metal transporter 1
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FGFR	Fibroblast growth factor
Foxp3	Forkhead box protein 3
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDNF	Glial cell line-Derived neurotrophic factor
Hamp1	The gene coding for Hepsidin
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	High fat diet
HOMA	Homeostasis model assessment
HPRT	Hypoxanthine phosphoribosyltransferase
HSC	Hepatic stellate cell
IR	Insulin resistance
ICAM-1	Inter cellular adhesion molecule 1
IGF-1	Insulin growth factor 1
IKK	I κ B Kinase
IL-6	Interleukin-6
IRE	Iron-responsive element
IRP	Iron-regulatory protein
IRS-1	Insulin receptor substrate 1
JNK1	c-Jun kinase 1
LDH	Lactate dehydrogenase
LFA-1	Lymphocyte function-associated antigen 1
MCD	Methionine- and choline deficient diet
MDA	Malone dialdehyde
Mttp	Microsomal triglyceride transfer protein
NAFLD	Non alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Non alcoholic steato hepatitis
NCAM	Neuronal cellular adhesion molecule

NIH	American national institutes of health
Nramp2	Natural resistance-associated macrophage protein 2
NTBI	Non-transferrin bound iron
PBC	Primary biliary cirrhosis
PBS	Phosphate buffer saline
PECAM	Platelet endothelial cell adhesion molecule
PSC	Primary sclerosing colangitis
SCB	Statistiska centralbyrån
SMR	Standardized mortality ratio
α SMA	α -Smooth muscle actin
T2D	Type 2 Diabetes Mellitus
T-reg	Regulatory T-lymphocytes
TNF- α	Tumor necrosis factor α
Tf	Transferrin
TfR1	Transferrin receptor 1
TfR2	Transferrin receptor 2
TLR	Toll like receptor
TGF- β	Transforming growth factor β
TBH	Tert-butyl-hydroperoxide

CONTENTS

1	Introduction.....	1
1.1	Fatty liver, NAFLD	1
1.2	Histopathology of NAFLD.....	4
1.3	Obesity (BMI) Trends in the Swedish population	5
1.4	NAFLD and Insulin Resistance (IR).....	6
1.5	Iron.....	7
1.5.1	Iron and NAFLD	8
1.5.2	Hepatic Iron Metabolism.....	9
1.6	Inflammation.....	10
1.6.1	Inflammation and cell damage in NASH	11
1.7	Liver Fibrosis.....	14
1.7.1	Mechanisms of liver fibrosis in NASH	16
1.8	Animal models of NASH	17
2	Aim	19
3	Methods, study I-IV.....	20
3.1	Patients (Study I)	20
3.2	Grading of liver biopsies for NASH (study I and III)	20
3.3	Causes of death (Study I)	21
3.4	Animals.....	21
3.4.1	<i>Mtp</i> (Study II).....	21
3.4.2	<i>ob/ob</i> (Study II).....	22
3.5	Morphometry (study III).....	22
3.5.1	Grading of Fibrosis.....	22
3.5.2	Steatosis (Study III).....	23
3.6	Immunohistochemistry (Study III, IV and V)	23
3.6.1	Paraffin embedded sections	23
3.6.2	Frozen sections	23
3.7	ELISA	24
3.8	Procedures.....	24
3.8.1	Hepatocyte isolation and cell culture (Study II).....	24
3.8.2	Incubation of hepatocytes with cytokines and induction of oxidative stress with TBH (Study II)	25
3.8.3	Cell damage assessment (Study II).....	25
3.8.4	Bile duct ligation in mice (Study IV).....	26
3.8.5	Bile flow rate (Study IV).....	26
3.8.6	Carbon tetrachloride injections in mice (Study IV)	27
3.8.7	Isolation and <i>in vitro</i> activation of hepatic stellate cells (Study IV)	27
3.8.8	Total Iron (Study II)	28
3.8.9	Quantitative Real-Time PCR (Study IV).....	28
3.8.10	Immunoblot (Study II and IV)	28
3.9	Ethical considerations.....	29
3.10	Statistics	29
3.10.1	Study I.....	29
3.10.2	Study II	30

3.10.3	Study III.....	30
3.10.4	Study IV	30
4	Results and discussion.....	31
4.1	Study I.....	31
4.1.1	Re-evaluation of the Liver Biopsies	31
4.1.2	Relative Risk of Death.....	31
4.1.3	Causes of Death.....	33
4.1.4	Summary and conclusions	33
4.2	Study II	33
4.2.1	Iron content.....	33
4.2.2	Intracellular signalling of hamp1	34
4.2.3	Iron regulatory genes	34
4.2.4	Summary and conclusions	35
4.3	Study III.....	35
4.3.1	NAS-classification and Morphometrical measurements	35
4.3.2	Apoptosis and inflammation.....	36
4.3.3	Soluble ICAM-1.....	37
4.3.4	Summary and conclusions	38
4.4	Study IV (NCAM).....	39
4.4.1	N-CAM expression	39
4.4.2	Blood chemistry tests.....	39
4.4.3	Loss of N-CAM attenuates fibrosis following bile duct ligation.	39
4.4.4	CCl ₄ induced liver fibrosis.....	40
4.4.5	Expression of myofibroblast marker α -SMA	40
4.4.6	Hepatic stellate cells isolated from N-CAM ^{-/-} mice have impaired activation after stimulation with TGF- β 1	40
4.4.7	Summary and conclusions	41
5	General discussion.....	42
6	General conclusions	49
7	Populärvetenskaplig sammanfattning.....	50
8	Acknowledgements.....	52
9	References	54

1 INTRODUCTION

1.1 FATTY LIVER, NAFLD

The liver normally contains less than 5% of fat but under certain conditions excess fat may accumulate in the liver. Fatty liver can occur for many different reasons, the most known of which is excessive alcohol consumption. Other causes are the metabolic syndrome, obesity, protein-calorie malnutrition, starvation or rapid weight loss, total parenteral nutrition, various drugs such as Amiodarone, Tamoxifen, Glucocorticoids, Tetracycline, Oestrogens, Methotrexate and Thallium. Also certain types of metabolic disorders can cause fatty liver such as Wilson disease and Glycogen storage disorders. However, today, the most common cause is non-alcoholic fatty liver disease (NAFLD) which is a disorder closely related to the metabolic syndrome and obesity. NAFLD is increasing immensely in the western world and a similar trend is seen in Asian countries. Over the last 10 years, the prevalence of obesity has doubled among the adult population and tripled in children, and according to American national institutes of health (NIH), two thirds or up to as many as 85% of the American population is now overweight or obese. It is estimated that 75 % of the overweight population or those with type 2 diabetes have NAFLD. About 20% of those with NAFLD have an inflammation in the liver so called non-alcoholic steatohepatitis (NASH). Some studies show that among patients with non-NASH (simple steatosis and steatosis with inflammation), about 40% progress to fibrosis and over half developed NASH during 4-13 years follow-up [1, 2].

The diagnosis of NAFLD is based on detection of fat in the liver by either liver histology or by imaging modalities and exclusion of any other liver disease. Imaging such as ultrasonography and computed tomography have limited sensitivity as they can only detect moderate to severe steatosis, that is when more than 30% of the liver cells (hepatocytes) are affected, and therefore does not detect mild steatosis [3]. As a result of this, it is hard to estimate the true prevalence and prognosis of NAFLD and NASH.

NAFLD is, as mentioned, highly associated with obesity but it is important to remember that this is not a condition that affects only obese individuals but can also be seen in lean persons. In a study from the US it was found in autopsies that 2.7% of the lean individuals have steatohepatitis [4].

NAFLD has been considered the hepatic manifestation to the metabolic syndrome because it's close relation with obesity, insulin resistance and many of the other factors of metabolic syndrome [5].

The metabolic syndrome is a group of risk factors. Patients who have this syndrome have been shown to be at an increased risk of developing cardiovascular disease (CVD) and/or type-2 diabetes. Metabolic syndrome is a common condition that goes by many names; syndrome X was introduced in 1988 to focus attention on cardiovascular disease risk [6] also dysmetabolic syndrome, insulin resistance syndrome, obesity syndrome, and Reaven's syndrome are common names of the same clustering.

The National Heart Lung and Blood Institute (NHLBI) estimated in 2004 that in the U.S. over 47 million adults (25%) have the metabolic syndrome. It can affect anyone at any age, but the risk increases with age and is most frequently seen in those with significant overweight, with most of their excess fat in the abdominal area.

There is debate regarding whether obesity or insulin resistance is the cause of the metabolic syndrome or if they are consequences of a more far reaching metabolic derangement. A number of markers of systemic inflammation, including C-reactive protein, are often increased, as are fibrinogen, interleukin 6 (IL-6), and others. IL-6 are most likely increased because of obesity, about one third of the total circulating IL-6 originates from adipose tissue [7] and an increase in IL-6 has been associated with

obesity and insulin resistance [8]. There is also a debate concerning whether the sum of metabolic syndrome actually is greater than its parts. The general agreement is that the metabolic syndrome precedes both type 2 diabetes and cardiovascular diseases, thus providing a tool to predict both cardiovascular disease and type 2 diabetes [9, 10] but it is not clear that the metabolic syndrome actually performs better than its individual compartments [11].

Criteria for Clinical Diagnosis of Metabolic Syndrome

Clinical Measure	WHO [12]	ATP III [13]	AHA/NHLBI [14]
Waist Circumference		≥102 cm in men, ≥88 cm in women	Same as ATP III
BMI	BMI >30 kg/m ²		
Triglycerides	≥150 mg/dL	Same as WHO	Same as WHO
HDL-C	<35 mg/dL in men, <39 mg/dL in women	<40mg/dL in men, <50 mg/dL in women	Same as ATP III
Blood Pressure	≥140/90 mm Hg	≥130/85 mm Hg	Same as ATP III
Glucose	IGT, IFG, or T2D	Fasting >110 mg/dL (IFG)	Fasting ≥100 mg/dL (IFG)
Insulin Resistance	YES	NO	NO
Microalbuminuria	YES	NO	NO
<p>Notes: WHO requires insulin resistance plus two additional risk factors for diagnosis; ATP III requires three of five risk factors for diagnosis. AHA/NHLBI recommends that triglycerides, HDL-C, and blood pressure should be considered abnormal when drug treatment is prescribed.</p> <p>Abbreviations: BMI=Body mass index; IGT=Impaired Glucose Tolerance; IFG=Impaired Fasting Glucose; T2D=Type 2 Diabetes</p>			

1.2 HISTOPATHOLOGY OF NAFLD

NAFLD is a spectrum of conditions from simple hepatic steatosis to NASH with extensive fibrosis leading to cirrhosis and eventually in some cases hepatocellular carcinoma (HCC). Liver biopsy remains the gold standard for the diagnosis, grading and staging of NAFLD and NASH. However, given that liver biopsy is associated with a small risk of complications, as well as sampling variability, the urgent need for non-invasive diagnostic biomarkers is being pursued. In many studies, a staining system developed by Kleiner et al. [15] is utilized to evaluate liver biopsies from NAFLD patients. The scoring system evaluates the percentage of fat content, inflammatory foci and swollen hepatocytes, i.e. ballooning. The scoring system also evaluates the disease activity by grading of fibrosis.

The hallmark of NAFLD is the histopathologic finding of macrovesicular steatosis. In severe cases this can be found through out the whole biopsy (fig 1.). There is no clear cut off for the number of fat-containing hepatocytes allowed in a normal liver but a number of <5% ha been suggested and is for now accepted as some kind of gold standard. However this is just a speculation and not based on hard evidence.

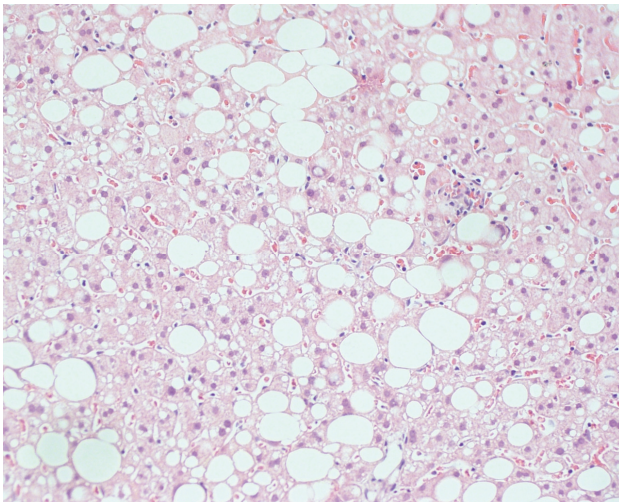


Figure1. *Pronounced macrovesicular steatosis in NAFLD*

(Haematoxylin/Eosin-staining)

When steatosis is found together with inflammatory infiltrates and other signs of necroinflammation the diagnosis of NASH can be made. Lobular inflammation with a mixture of neutrophils, lymphocytes and macrophages are often found but the severity is often mild (fig 2.). A characteristic feature of NASH is swelling of hepatocytes, ballooning. Since it is difficult to distinguish between a hepatocyte swollen due to excessive fat accumulation and a hepatocyte suffering from necroinflammation, an immunohistochemical method to be able to accurately determine the cause of the morphological change is highly desirable. Sometimes fibrosis is considered as a feature of steatohepatitis and it is commonly used to describe the stage of the disease.

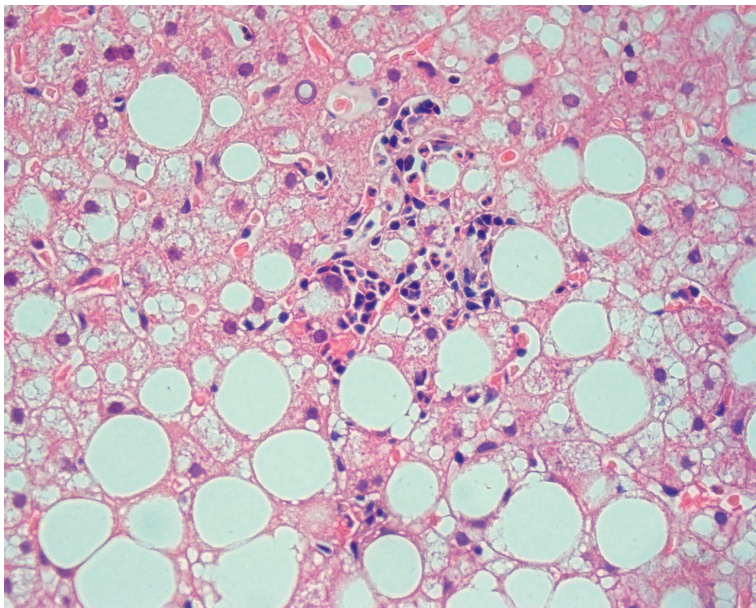


Figure 2. *Lobular inflammation in NASH (Haematoxylin/Eosin-staining)*

1.3 OBESITY (BMI) TRENDS IN THE SWEDISH POPULATION

Since 1980 Statistics Sweden (Statistiska Centralbyrån, SCB) has regularly collected data about weight and height in a cohort of Swedes. From that it has been possible to follow the progression of body mass index (BMI) and in that way follow overweight and obesity in the Swedish population. Numbers from SCB shows that more than every second man

and every third woman in Sweden today are overweight (BMI 25-30), which is an increase with about 50% since 1980. The number of obese (BMI >30) Swedes has during the same period increased with 100%, from 5% to 10%, among both men and women. One should have in mind that these numbers are based on self-reporting from telephone interviews and it is known that people tend to underestimate these kinds of numbers. One study that is not based on subjective measures is a study from Gothenburg where 50 year old men have been weighted and measured every 10th year from 1963 and onwards [16]. During the 40 years the study has been going on, the 50 year old men has become on average 9 kg heavier but also 4 cm taller. The number of overweight and obese individuals increased from 44% to 60% and the number of obese more than doubled from 6% to 14%.

The Swedish board of agriculture (Jordbruksverket) has published statistics on sales from food consumptions from 1960-2006. These data demonstrate increased consumptions of sweets such as sodas from 20L to 90L soda/person and year, chocolates and candy from 6kg to over 15kg per person and year during the last 40 years [17]. Statistics from National food administration (Livsmedelsverket) show that 25% of children's daily energy intake comes from candy, sodas, snacks, ice cream or deserts [18]. Unless drastic changes will take place, food habits combined with statistics saying that physical activity is decreasing will lead to an increasing prevalence of overweight and obesity.

1.4 NAFLD AND INSULIN RESISTANCE (IR)

The first definition came already in the 1960s, when measurement of insulin concentrations became available. Insulin resistance is not a disease as such, but rather a state or condition in which a person's body tissues have a lowered level of response to insulin. As a result, the body produces larger quantities of insulin to maintain normal levels of glucose in the blood. There are a few ways to measure IR and the gold standard is to perform a euglycaemic hyperinsulinaemic glucose clamp. The subject is

given an infusion of insulin and the blood is then buffered with glucose to maintain normal blood glucose level, the amount glucose needed then reflects whole-body insulin sensitivity [19]. This is however a rather difficult measurement and very time-consuming so most commonly fasting insulin and glucose concentrations are used to approximate the insulin resistance. Homeostasis model assessment, HOMA [20], is a measurement of insulin resistance which is calculated by the formula $HOMA = (\text{fasting glucose} \times \text{fasting insulin}) / 22.5$ and has a good correlation with the glucose clamp [21]. Obesity and diabetes has initially been held responsible for NAFLD but IR is perhaps now considered the most important mechanism throughout the progression and prevalence of NAFLD [22]. Some patients develop hepatic steatosis without evidence of obesity or IR [23], but the proportion of lean, non-IR NAFLD patients is small. The underlying mechanisms that contribute to obesity-induced IR are not entirely clear. Suggestions have been made that obesity may lead to hepatic IR through activation of pro-inflammatory macrophages in adipose tissue that secrete pro-inflammatory cytokines such as IL-6 and TNF- α [24]. IL-6 blocks the insulin signalling pathway [25] and inflammatory stress can up-regulate the production of IL-6 in adipocytes via activation of c-Jun kinase (JNK1) [26]. Patients with NAFLD often have decreased plasma levels of adiponectin which is negatively related to hepatic IR and hepatic inflammation [27].

1.5 IRON

Iron is essential for many vital functions and there is no regulated way by which excess iron can be disposed of in humans. Normally, the iron level is regulated by decreasing the body's uptake of iron from the gut. Therefore, iron toxicity may arise when the body's needs and storage capacity are filled. There are many causes of iron overload in humans, both genetic and acquired, e.g. hemochromatosis and post-transfusion iron overload respectively, both of which have traditionally been associated with severe hepatic deposits of iron. NAFLD and NASH among others are

conditions in which iron deposits are mild to moderately increased which however may have clinical relevance [28]. In chronic liver diseases, iron deposits are found either in hepatocytes, Kupffer cells or in both. Together with oxygen, iron can form free oxygen radicals, causing oxidative stress, which in turn leads to serious cell and tissue damage. Therefore, each organism (and every cell) needs to keep the iron concentration at a moderate level. The dual challenge of avoiding iron deficiency and iron overload has resulted in a tightly controlled and complex regulation of iron homeostasis. Alternation of iron pools is the result of a complex network of events, acting at the transcriptional and translational level to change the expression of proteins involved in transport, uptake, utilization, and storage of iron.

1.5.1 Iron and NAFLD

Hepatic iron in excess is associated with insulin resistance [29] and therefore also commonly observed in patients with NAFLD. Hyperferritinemia, associated with mild hepatic iron accumulation, is frequently observed in about one-third of NAFLD cases examined [29-31]. Hepatic iron accumulation is an independent risk factor for advanced liver fibrosis in NAFLD [31, 32]. Iron is suspected to enhance hepatic damage as associated with non-alcoholic fatty liver disease [33] and iron reduction therapy has been shown to have positive effects on both NAFLD disease activity and insulin sensitivity [30, 34].

Moreover, a condition known as insulin resistance-associated hepatic iron overload (IR-HIO) has been described [29]. It has been previously shown that insulin stimulates ferritin synthesis, and facilitates iron uptake, whereas iron, in turn, influences insulin signalling, reduces the hepatic extraction and the metabolism of insulin [35]. In addition, hepatic iron overload in experimental NAFLD models is associated with lipid peroxidation products and increased necro-inflammation [36].

1.5.2 Hepatic Iron Metabolism

Approximately 40% of the storage iron, or about 400 mg for a normal adult man, is located in the liver [37, 38]. Under normal circumstances, the majority of the iron (98%) is found in hepatocytes [39]. The remaining 2% is mostly found in Kupffer cells, and very small amounts in stellate cells, endothelial cells and bile duct cells. During iron overload the amount of iron stored in Kupffer cells can increase considerably, but hepatocytes remain the major storing place. The liver can take up iron in several ways but under normal conditions the majority of iron in the circulation is bound to Transferrin (Tf), so the main source is diferric Tf [40]. The liver can also take up iron from circulating heme, hemoglobin and ferritin. Studies on hepatocytes have showed that there are both high-affinity saturable and low-affinity unsaturable components [41-43]. The high affinity uptake of Tf is mediated by Tf receptor 1 (TfR1) [44]. After Tf has bound to TfR1 on the cell surface the complex is endocytosed. Tf is then reduced after a proton pumping by ATPase [45]. The iron released from Tf is then carried across the membrane of the vesicle to the cytoplasm by the ferrous iron transporter divalent metal transporter 1 (DMT1). There are some regulation of TfR1 gene at transcriptional level but most of the regulation is carried out on mRNA level by the iron regulatory protein (IRP)/iron responsive element (IRE) system [46]. The IRE system acts by that the TfR1 mRNA 3' region contains a series of loops, the IRE, to which the IRP1 and IRP2 binds. When intracellular iron levels are low the IRPs bind to the IREs on TfR1 3' and protects the mRNA from endonuclease degradation and more TfR1 can be synthesized. When cells have an excess of iron the opposite is seen and TfR1 expression is reduced, protecting the cell from accumulating more iron. With heavy iron loading, such as the case in hemochromatosis, TfR1 can be almost undetectable in liver tissue [47-49]. TfR2 is a homologue to TfR1 and it is suggested that TfR2 is responsible for the low affinity process [50-52]. TfR2 is like TfR1 a membrane bound protein and binds to Tf, but the affinity to diferric Tf is 25-fold lower than that of TfR1. TfR2 also has a more restricted tissue distribution; high expression is found on hepatic

parenchymal cells [53, 54]. TfR2 does not, unlike TfR1 contain any IREs in its mRNA and is therefore not regulated by cellular iron content [53].

Most cells take up iron via serum Tf by receptor-mediated endocytosis. However hepatocytes and hepatoma cells, among others can also take up non-transferrin bound iron (NTBI) from serum although how this is mediated is poorly understood. The role of NTBI in the normal individual is limited because of most serum iron is normally bound to Tf. Under certain conditions when Tf is fully saturated with iron, a substantial amount of NTBI may be present. NTBI is rapidly cleared from the circulation by the liver, mainly hepatocytes [55, 56]. Findings showed that when divalent cations such as; Cu^{2+} , Zn^{2+} and Co^{2+} were added in high concentrations, uptake of ferrous iron was inhibited. This indicated that NTBI shared the same pathway with other divalent cations [57]. More recent studies have showed that divalent metal transporter 1 (DMT1) is responsible for iron transport from Tf-recycling endosomes to the cytoplasm [58]. DMT1, also called divalent cation transporter 1 (DCT1) or natural resistance-associated macrophage protein 2 (Nramp2), transports cations such as Fe^{2+} , Zn^{2+} , Mn^{2+} , Co^{2+} , Cu^{2+} and Ni^{2+} , is the major duodenal ferrous iron transporter [59, 60].

1.6 INFLAMMATION

The classical definition of inflammation is the body's response to diverse injuries. Inflammation can be classified as either acute or chronic. Acute inflammation is typical in the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes from the blood into the injured tissues. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells which are present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process. The process of acute inflammation is initiated

by cells already present in all tissues, mainly resident macrophages, dendritic cells, Kupffer cells and mast cells. The mediator molecules also alter the blood vessels to permit the migration of leukocytes, mainly neutrophils, outside of the blood vessels (extravasation) into the tissue. The acute inflammatory response requires constant stimulation to be sustained. Inflammatory mediators have short half-lives and are quickly degraded in the tissue. Hence, inflammation ceases once the stimulus has been removed.

The main cells involved in chronic inflammation are macrophages and lymphocytes i.e. mononuclear cells. With the aid of chemical mediators such as lymphokines, macrophages engulf, neutralize or kill foreign antigens. Lymphocytes are the predominant cell in chronic inflammation. Activation of low-grade chronic inflammation appears to be a common factor for obesity, type 2 diabetes and cardiovascular disease.

1.6.1 Inflammation and cell damage in NASH

In NAFLD/NASH two types of inflammation are seen; either lobular or portal or a mixture of these. Lobular infiltrates consists of a mixture of acute (polymorphonuclear leukocytes) and chronic (mononuclear cells, including lymphocytes, monocytes and plasma cells and eosinophils) cell types. Portal inflammation can range from absent to marked in NASH [61]. NASH patients often have an increase in Kupffer cell aggregates and the dominating place is zone 3 [62].

ICAM-1 (Intercellular Adhesion Molecule-1, CD54) is known for its importance in stabilizing cell-cell interactions and facilitating leukocyte endothelial transmigration. The primary receptors for ICAM-1 are integrins which mediate cell-cell interactions and allow for signal transduction. Inflammatory responses will up-regulate the expression of ICAM-1 thereby increasing the adhesive nature of leukocytes and endothelial cells. While the selectins instigate a rolling behavior over the endothelial layer, the ICAM-1 interaction with leukocyte Lymphocyte

function-associated antigen 1 (LFA-1) or Mac-1 actually stabilizes the leukocyte for extravasation. The arrested leukocytes then begin diapedesis, the process of crossing the endothelial layer, which is mediated by PECAM (platelet endothelial cell adhesion molecule, CD31), a protein expressed both on leukocytes and the intercellular junctions of endothelial cells [63]. Expression of ICAM-1 in livers with NASH has been shown to be significantly higher in either the cytoplasm or in the lobular inflammation. The level of ICAM-1 has also been shown to have a positive correlation with both lobular inflammation and severity of necroinflammatory activity. Patients with NAFLD had significantly higher hepatic immunoreactivity of ICAM-1 than those with a normal liver. Considering that ICAM-1 antigen is important in mediating immune and inflammatory responses, it has been speculated that an altered hepatic immune response may participate in the hepatic inflammation observed in NASH [64].

Forkhead box protein P3 (FOXP3) is an acetylated and phosphorylated protein (transcription factor) active in human regulatory T cells (Tregs) [65]. In contrast to CD8⁺ cytotoxic T-cell lymphocyte, which generally exert a suppressive influence on tumor growth, Tregs are thought to have a positive effect on tumor growth through suppression of antitumor immune cells. CD4⁺CD25⁺ Tregs constitute a minor but functionally unique population of T-cells that maintain autoimmunity. Tregs can inhibit immune response mediated by CD4⁺CD25⁻ and CD8⁺ T-cell in vitro by contact-dependent and cytokine-independent mechanism [66-68]. FOXP3 is critical for the development and function of Tregs in mice and humans [69] and is still the only marker for evaluating real Tregs that have a suppressive function. The number of Tregs increases during the progression of established cancers as well as that of their precursor lesions. Furthermore, the prevalence of Tregs is significantly correlated to poor patient survival, independent of other prognostic factors [70].

CD3 is a surface glycoprotein and part of a big complex on T-cells This complex plays an important role in coupling antigen recognition to several

intracellular signal-transduction pathways, and could therefore be used to examine the infiltration of T-cells in a tissue.

Caspase 3 is a member of a family of evolutionarily conserved cysteine protease proteins known as caspases. Many of these enzymes are part of a proteolytic cascade that plays a central role in cell death by apoptosis. Caspase-3, -6 and -7 have been classified as executioners because of their capacity to cleave crucial substrates, thus killing the cell [71]. One of the essential substrates cleaved by executioner caspases is Poly (ADP-Ribose) Polymerase (PARP), an abundant chromatin-associated protein involved in maintaining DNA stability and repair [72]. To be active, caspase 3 requires proteolytic cleavage. In normal cells, caspase 3 should exist as a procaspase in which the potential cleavage site is intact.

The presence of a “leaky gut” has been shown in patients with obesity which lead to increased bacterial and endotoxin levels in the portal circulation. Lipopolysaccharide has been shown to be involved in NASH [73]. Once the microbes have breached physical barriers they are recognized by toll like receptors (TLRs) that activates immune cell responses. Mammalian TLRs include intracellular and transmembrane receptors that recognize microbial proteins, nucleic acids, carbohydrates, and lipids to activate host defence mechanisms. Activation of TLR-4 triggers multiple intracellular signalling pathways and is important for the amplification of and maintenance of inflammatory signals and fibrosis [74, 75]. It has become increasingly apparent that the toll-like receptors (TLRs), and in particular TLR4, may be involved in the initiation and activation of kinases including IKK (IkB Kinase) and JNK (c-Jun N terminal Kinase), leading to the secretion of inflammatory cytokines and inhibitory phosphorylation of IRS-1 (Insulin Receptor Substrate 1), which promotes insulin resistance [24, 76].

1.7 LIVER FIBROSIS

Liver fibrosis is the accumulation of tough, fibrous scar tissue in the liver. The formation of scar tissue is a normal response to injury, but in fibrosis this healing process goes awry. When liver cells, hepatocytes, are injured due to any type of injury such as viral infection, alcohol over-consumption, diabetes, obesity, toxins, trauma, or other factors, the immune system is activated to repair the injury. Also hereditary diseases such as hemochromatosis, α 1-anti-trypsin deficiency and cystic fibrosis cause liver fibrosis as well as autoimmune disorders such as primary biliary cirrhosis, primary sclerosing cholangitis and autoimmune hepatitis [77]. The injury or death of hepatocytes stimulates inflammatory immune cells to release cytokines, growth factors and other substances. The chemical messengers induce activation of hepatic stellate cells (HSC) and the production of collagen, glycoproteins (such as fibronectin), proteoglycans and other substances that are deposited in the liver. This builds up the extracellular matrix (non-functional connective tissue). At the same time, the process of breaking down or degrading collagen is impaired. In a healthy liver, the synthesis (fibrogenesis) and breakdown (fibrolysis) of matrix tissue are in balance. Fibrosis occurs when excessive scar tissue builds up faster than it can be broken down and removed from the liver.

In early stages of fibrosis few people experiences any symptoms at all and the liver function is still preserved. As the liver injury and inflammation continue, more scar tissue builds up and connects with existing scar tissue. This can eventually interfere with the metabolic functions of the liver. And if the disease progresses, it will lead to cirrhosis, an end-stage condition when the liver is severely scarred, this restricts the blood flow, causes portal hypertension and the ability for the liver to function is impaired. (fig3.)

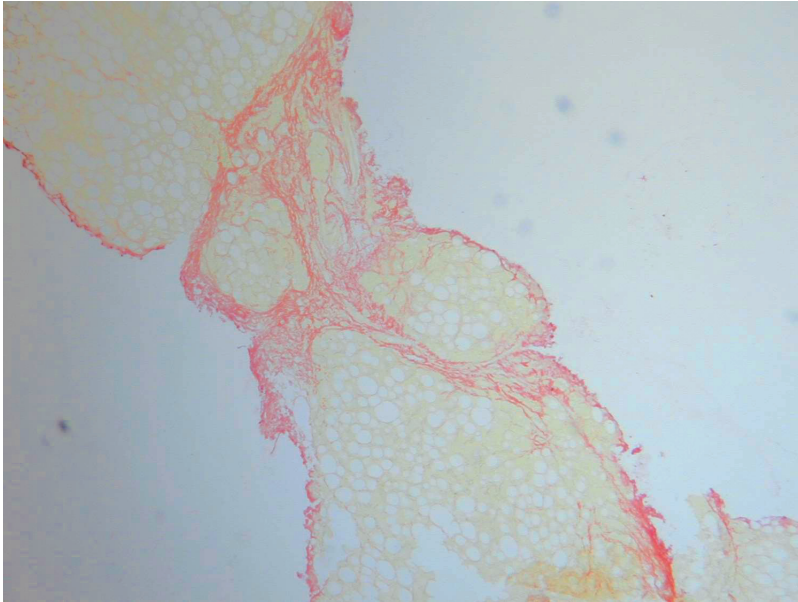


Figure 3. *Cirrhosis in NAFLD. Regenerative nodules and severe steatosis (Sirius staining)*

To the general public cirrhosis is mostly associated with excessive alcohol consumption but it marks the end-point of a number of liver diseases [77, 78]. Morbidity and death due to complications from cirrhosis represents a major global health issue [79]. The prevalence of cirrhosis follows the prevalence of the diseases causing it. For example there are about 360 million people with hepatitis B infection and about 123 million with hepatitis C in the world. Deaths related to these infections have been estimated to over 900 000 per year [80-82].

As mentioned earlier, many cases develop liver fibrosis without any symptoms for the patient, but when the progression of the disease has progressed to severe fibrosis or cirrhosis complications appear. Clinical symptoms of decompensated liver cirrhosis are ascites, jaundice, bleeding from, esophageal varices and itching. The prognosis for decompensated cirrhosis is poor and one year survival rate is only 60%. Liver cirrhosis also increases the annual risk of developing hepatocellular carcinoma, (HCC), to 1.4-3.3% [83].

A special type of fibrosis is the biliary type of fibrosis. It develops in response to bile duct injury causing cholestasis. This could be the result of stone impaction or diseases affecting the smaller bile ducts such as primary sclerosing cholangitis (PSC), cystic fibrosis and primary biliary cirrhosis (PBC). This shows in histological stainings as proliferation of small bile ductules, inflammation and fibrosis in conjunction to the small bile ducts with an increased number of myofibroblastic cells [84].

Neural cell adhesion molecule (NCAM) is as ICAM a member of the superfamily of immunoglobulin-like adhesion molecules. NCAM was the first neural adhesion molecule to be discovered and it is also the most studied one. NCAM has many different functions such as regulation of cell sorting, migration and proliferation. There are more than 20 isoforms of NCAM and all isoforms have five immunoglobulin domains in the extracellular region. NCAM adhere to molecules such as FGFR, L1, GDNF, N-cadherin and extracellular matrix components such as agrin and proteoglycans [85]. T-lymphocytes express NCAM and it is then denoted as CD56. CD56 positive lymphocytes are suggested to contribute to hepatocellular damage in chronic hepatitis C infection [86]. NCAM is expressed in many tissues in connection with injury and is associated with fibrosis in liver, heart and kidney [87-89]. Mice deficient in NCAM show brain defects such as increased anxiety and cognitive dysfunction [90]. NCAM deficient mice appear, despite the defect, relatively normal and are fully viable and fertile. In normal adult human liver NCAM is expressed on a few nerve fibers. However when the liver gets injured, cholangiocytes, activated HSC and periportal fibroblasts as well as intermediate cells of the ductual reaction express NCAM [87, 91-95]. The function of NCAM in liver injury or fibrogenesis has yet not been studied.

1.7.1 Mechanisms of liver fibrosis in NASH

Insulin resistance in patients with NAFLD may affect HSCs as these cells express receptors both for insulin and insulin-like growth factor-1 (IGF-1), two proteins promoting cell division in HSCs. IGF-1 also increases

collagen type 1 gene expression [96]. Also connective tissue growth factor is up-regulated by insulin and glucose [97]. Connective tissue growth factor does not only stimulate HSCs but also extracellular matrix production and profibrogenic activity by transforming growth factor- β [98]. Inflammation is part of the wound-healing process during fibrogenesis. When activated, Kupffer cells release pro-inflammatory and fibrogenic factors such as chemokines and cytokines [99]. One such cytokine is the transforming growth factor- β (TGF- β) which then again stimulates the pro-fibrogenic activity in HSCs. Pro-inflammatory cytokines are controlled by pro-inflammatory pathways including c-Jun N-terminal kinase (JNK), a pathway which has been linked to apoptosis, inflammation and insulin resistance [100, 101]. In experimental NASH jnk knock-out mice showed less hepatic inflammation and fibrosis which could be interpreted as that JNK1 in Kupffer cells contributes to liver fibrosis by inducing chronic liver inflammation [102]. More detailed knowledge of the molecular mechanisms regarding fibrosis in NASH is needed in order to develop therapeutic approaches.

1.8 ANIMAL MODELS OF NASH

Animal models are widely used in pre-clinical research. They can either be used to induce a disease that mimic the disease to study or be manipulated to study the effects of a specific gene. Shutting down a gene can be done in different ways; some times this happens spontaneously, the gene can be technically erased and the animal can also be technically prepared so that a gene can be turned off after the animal is born. This is usually used when the effects of one specific gene is to be studied.

NAFLD and NASH is a clinical spectrum that develops over years and even decades and results from accumulation of risk factors. A good animal model should mimic both the histological pattern with fatty liver, inflammation and fibrosis, and the animal model should also mimic the metabolic disturbances related to NAFLD such as insulin resistance and systemic inflammation. Presently there is no animal model that reflects

all these aspects but there are a few that can mimic some of the aspects. There are two major types of animal models available to day; dietary and genetic models. The most commonly used dietary model is the methionine- and choline-deficient (MCD)-diet which results in intrahepatic steatosis, liver inflammation, oxidative stress and changes in cytokines and adipokines [103-106]. Fibrosis in this model appears after at least 4 weeks of feeding [107]. A major disadvantage of this model is that it lacks the characteristics of obesity and insulin resistance; in fact the animals loose about 50% of their body weight over 4 weeks [103]. Another dietary model is the high-fat diet (HFD) in which the majority of calories comes from fat (45-75%). These animals become obese, insulin resistant and develop get hepatic steatosis but the liver damage is not as severe as in the MCD-model [108, 109]. Among the genetic models some of the most common models are those interfering with leptin-signalling pathways. There are three types of leptin deficient mice, two with a mutation in the receptor (*db/db* and *fa/fa*) and one with a mutation in the ligand (*ob/ob*). They display similar but not identical features [110, 111]. *ob/ob*-mice develop extensive obesity and fatty liver whereas obese humans rather have hyperleptinemia[112]. Since leptin has profibrogenic effects, fibrosis in *ob/ob*-mice is delayed significantly [113]. Leptin was also showed to regulate the innate immune system and *ob/ob* mice are at risk to develop liver injury [114].

2 AIM

The general aim of this thesis is to improve our knowledge about NAFLD and NASH, with regard to long-time prognosis, immunological and histological features in NASH livers, and the association to iron metabolism, adhesion molecules and fibrosis.

Specific aims:

- To determine the frequency of NAFLD and NASH in a Swedish population with elevated liver function tests. To study the mortality and causes of death among those subjects with NAFLD and compare the survival and causes of death to those subjects with other liver diseases, and compared to the general population.
- To evaluate the effects of inflammation and oxidative stress on iron-regulatory gene-expression and signalling in two animal NAFLD models.
- To evaluate if the amount and type of storage of fat in hepatocytes is of importance for hepatocyte injury and if not only the innate immunity but also the adaptive immunity is involved in NASH.
- Since NCAM is expressed in injured liver and is associated with fibrosis, we wanted to examine if loss of NCAM would affect fibrosis development after injury of the liver parenchyma and/or fibrosis development in biliary type liver fibrosis.

3 METHODS, STUDY I-IV

3.1 PATIENTS (STUDY I)

Between 1980 and 1984, 256 subjects with unexplained elevated serum levels of ALT, and therefore referred to our unit, have been characterized in a retrospective and a prospective study by Hultcrantz and coworkers [115, 116]. Inclusion criteria were persistently elevated levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) for longer than 6 months. Subjects with symptoms or clinical signs of liver disease were excluded, as were those with serum levels of alkaline phosphate (ALP) (greater than twice the upper normal limit, 4.2 kat/L) and those exhibiting clinical or laboratory signs of kidney disease. Unless otherwise stated in the medical chart, the patient was assumed not to over consume alcohol. A great deal of effort was put into uncovering any such over consumption at the time.

3.2 GRADING OF LIVER BIOPSIES FOR NASH (STUDY I AND III)

All liver biopsies had been performed on all of the subjects percutaneously with a 1.6-mm Menghini-type needle. All of these biopsies were re-evaluated employing a modern classification by two of the authors (C.S. and R.H.), as well as by a third reference person (H.G.), all of whom were blinded to the patient details. Liver histology was scored in accordance with the NAS-system developed by Kleiner and Brunt et al. [15] The classification of NASH was made on the basis of steatosis (both macro and microvesicular), lobular inflammation, and ballooning degeneration, and fibrosis was scored to determine the stage (progression) of this condition.

The degree of steatosis was graded 0-3 based on the area of the biopsy that was occupied by fat (grade 0: fatty infiltration <5% of the area, grade 1: more than 5% but less than 1/3 of the area, grade 2: 1/3-2/3 of the area and grade 3: more than 2/3 of the area occupied by fat vacuoles). Lobular inflammation is graded 0-3 based on the number of foci/200 magnification (grade 0: No foci/200x, grade 1: <2 foci/200x, grade 2: <2-4 foci/200x and

grade 3: >4 foci/200x). Ballooning was graded 0-2 where 0 stands for no balloon cells, 1: few balloon cells and 2: many balloon cells/prominent ballooning is seen. NAS is calculated as the unweighted sum of steatosis (0-3), lobular inflammation (0-3) and hepatocellular ballooning (0-2).

For grading disease activity in connection with chronic hepatitis, we used the scoring system developed by Batts and Ludwig [117]. Iron content was evaluated according to Scheuer [118] as: no staining, weak, moderate, or intense staining, and localization predominantly in Kupffer cells or in hepatocytes.

3.3 CAUSES OF DEATH (STUDY I)

Since all Swedish residents are assigned a unique 10- digit national registration number, and these identification numbers are recorded in the nationwide and virtually complete Cause of Death Registry [119] it is possible to find out which patients are still alive and who has deceased and what the cause of death was. Through this registry, information concerning all deaths during the study period (1980 to July 9, 2008), including dates and causes of death (coded according to the International Classifications of Diseases versions 8, 9, and 10 [120-122]) could be obtained.

3.4 AMIMALS

All mice were males and in the age of 8-12 weeks at sacrifice and they were all housed in a pathogen free barrier facility (12 h light/12 h dark cycle), and were fed rodent chow containing 4% fat. Mice were sacrificed or perfused at 10-12 weeks of age and the liver was collected for analysis.

3.4.1 *Mttp* (Study II)

In the *Mttp*^{fllox/fllox}*Mx1-Cre*^{+/-} mice that were used in this study, the gene for microsomal triglyceride transfer protein is floxed (*Mttp*^{fllox/fllox}) and can

be recombined in the liver upon induction of Cre-recombinase (Mx1-*Cre*^{+/+}) with polyinosinic-polycytidylic ribonucleic acid (pI-pC; 1 µg/µl; Sigma). This recombination results in termination of hepatic VLDL synthesis, leading to a reduction in plasma triglycerides levels and an increase in hepatic lipid stores with a 3-fold increase in liver triglycerides, as described [123, 124]. Two weeks before sacrifice, *Mttp* was recombined (*Mttp*^{Δ/Δ}) with pI-pC, and littermate wild-type mice (*Mttp*^{fl_{ox}/fl_{ox}}) were injected with phosphate-buffered saline. The study mice had been backcrossed at least six times (>95% C57BL/6, <5% 129/SvJae). All mice used were male and the mice were fasted overnight before blood sampling. Total plasma cholesterol and triglyceride concentrations were determined with colorimetric assays (INFINITYtriglyceride/ cholesterol kits, Sigma) 2 weeks after the final pI-pC injection.

3.4.2 *ob/ob* (Study II)

Homozygous leptin-free *ob/ob* (on a C57BL/6 background) and C57BL/6J male mice were obtained from Taconic (Denmark) at 5-8 weeks of age and were housed within the animal facility with free access to food and water. The *ob/ob*-mice are from approximately age 4 weeks insulin resistant which later around week 16 progresses to diabetes. The *ob/ob* obese mice were age-matched with wild-type C57BL/6J mice for each experiment.

3.5 MORPHOMETRY (STUDY III)

3.5.1 Grading of Fibrosis

The area of fibrosis was determined with a computer software program, Image J (public domain, NIH). Twenty consecutive images from each biopsy (in cases where the biopsy was smaller than 20 images the whole biopsy was used) were stacked together and converted in to grey scale. The images were corrected for background by setting a threshold where black is converted in to red, the area of fraction (the red colour) was measured for all 20 pictures and a mean value was calculated.

3.5.2 Steatosis (Study III)

The size of the hepatocytes depends on how much fat they contain which thereby influences the number of cells per defined area. We wanted to compare the number of inflammatory cells between patients with different degree of steatosis. In order to do this we developed a method to correct the number of positive cells to the amount of cells by using the known area of fat. That is, if a biopsy contained 0% fat this would be the “true value” since no area was occupied by fat, but if a biopsy contains of 50% fat the number of positive cells seen are in fact half of what we should see because of the occupying 50% of the area. We used the formula: “estimated true number of positive cells” = number of positive cells counted / (1 - % of fat) in an attempt to approximate the number of cells.

3.6 IMMUNOHISTOCHEMISTRY (STUDY III, IV AND V)

Immunohistochemistry has been performed both on frozen sections and paraffin embedded tissue from both animals and human liver biopsies.

3.6.1 Paraffin embedded sections

Immunostaining using the IMPRESS system were used for Cleaved Caspase-3, CD3, ICAM1, CD68 and TLR4. In brief, sections were deparaffinized with xylene and then ethanol in decreasing concentrations. After rehydration, sections were blocked in 0,3-3% H₂O₂, put in unmasking solution Vector, H-3300, pH 6 and heat activated by press cooker for 10-30 min, treated with IMPRESS serum block and incubated with primary Ab over night at 4°C. For secondary Ab we used IMPRESS. The bound antibody was revealed by addition of DAB and then counterstained with hematoxylin.

3.6.2 Frozen sections

Mouse liver was immediately after retrieval put in ice-cols Histocon (HistoLab Products), snap frozen in liquid nitrogen, embedded in tissue-

tec and cut in sections 4-6 μ m. Fixated in 2% paraformaldehyde in phosphate buffered saline containing 0.1% Triton X100 (Sigma Aldrich) for 10 minutes at room temperature. The sections were immunostained with primary antibody over night at 4°C. The slides were then triple washed and incubated with secondary antibody for 60 minutes at room temperature. Stainings with biotinylated secondary antibody was after washing visualized by incubation with streptavidin.

3.7 ELISA

Serum levels of sICAM-1 were measured with ELISA (RnD Systems, Human sICAM-1/CD54 Immunoassay). The assay was performed according to the manufactures instructions.

3.8 PROCEDURES

3.8.1 Hepatocyte isolation and cell culture (Study II)

Primary mouse hepatocytes were isolated using an in situ collagenase perfusion technique. Mice were deeply anesthetized with Isofluran and perfusion solutions were administrated via the right atrium of the heart and let to flow out through the caval vein that was cut below the liver. Briefly, the liver was first perfused, at 4 ml/min, with 50 ml Krebs-Ringer buffer (Sigma K-4002) including 100 U/ml penicillin, 100 μ g/ml streptomycin (Gibco, Invitrogen), 0.01 M HEPES and 0.01 % EDTA equilibrated at 96% O₂. Subsequently, using the same rate, the liver was perfused with 0.025% w/v collagenase (Sigma) solution in DMEM-F12 buffer, at 96% O₂, containing 100 U/ml penicillin, 100 μ g/ml streptomycin and 0,01 M HEPES. The resulting cell suspension was filtered and centrifuged at 50g for 4 min, 4°C. The pellet was then washed in cold Williams E medium including 100U/ml penicillin and 100 μ g/ml streptomycin, three times at 50g. The final pellet was re-dissolved in Medium 199 (Gibco, Invitrogen) containing 5% fetal bovine serum, 1 μ M hydrocortisone and 4 units/l of insulin. Cell viability was estimated using Trypan-blue exclusion with results > 95% viability for all experiments

described here. 2,5 million cells were plated on collagen (collagen type I, 0,01% in 0,1 M acetic acid) coated 35 mm dishes in Medium 199 including 5% fetal bovine serum, 1 μ M hydrocortisone and 4 units/l of insulin for 4 h. Cells were rinsed three times and the medium replaced with a serum-free version including 0.1% BSA for at least 16h, (37°C, 5% CO₂) before stimulation.

3.8.2 Incubation of hepatocytes with cytokines and induction of oxidative stress with TBH (Study II)

Hepatocytes were subjected to cytokine stimulation with TNF- α (10ng/ml) or IL-6 (20ng/ml) diluted in conditioned media containing medium 199 including 0.1% BSA, 1 μ M hydrocortisone and 4 units/l of insulin. Stimulation was continued for 24h (37°C, 5% CO₂). Following incubation, cells were rinsed several times with ice-cold PBS and used immediately for analysis. Tert-butyl-hydroperoxide (TBH) was diluted to concentration between 0.05 and 0.5 mM in Medium 199 including 0.1% BSA, 1 μ M hydrocortisone and 4 units/l of insulin. The mixture was then used to replace the growth-medium of the isolated hepatocytes for 2h (37°C, 5% CO₂). After incubation, cells were rinsed several time with ice-cold PBS and used at once for analysis.

3.8.3 Cell damage assessment (Study II)

Lactate dehydrogenase (LDH) levels in cell culture medium were measured using the LDH optimized assay kit (Sigma) according to the manufacturer's instructions. Briefly, an aliquot of growth medium was removed from each plate and incubated with LDH assay mix. The results were analyzed on a 96-well plate spectrophotometer by measuring the absorption at 490- and 650 nm. Results were expressed as a percentage compared to the controls from each group containing total LDH from lyzed cells.

Free malondialdehyde (MDA) concentrations were measured to estimate the level of lipid peroxidation in cell culture medium. Medium from cell culture dishes was ultra-filtrated, snap frozen in liquid nitrogen and stored at -70°C pending analysis. A free MDA standard was prepared to corresponding MDA concentrations of 0.1- 4.0mM, using 1,1,3,3-tetraethoxypropane (Sigma). The concentrations of MDA in samples were then calculated from the standard curve obtained in this interval. The HPLC system consisted of a Waters 600 delivery system (Waters Chromatography Division, Millipore Corporation, Milford, MA). A Waters 486 UV detector was used at 267 nm. The column consisted of a Waters carbohydrate analyzer column (125 Å, 10 µm) with a mobile phase consisting of Acetonitrile/0.03 M Tris -HCl (pH 7.4), 4:1 at a flow rate of 2.4 ml/min. The data were recorded in a Waters Millennium computerized chromatography system.

3.8.4 Bile duct ligation in mice (Study IV)

Under deep anaesthesia with Isofluran, the abdomen was opened by a mid-line incision. The hepatoduodenal ligament was identified and the common bile duct carefully mobilized by blunt dissection. The common bile duct was then ligated with non-resorbable thread and cut between ligatures. Controls were sham operated with laparotomy and identification, but no ligation, of the common bile duct [125]. Mice were given buprenorphine subcutaneously before and after surgery for analgesia. At two weeks after surgery, mice were sacrificed by exsanguinations under deep anaesthesia with Isoflurane followed by cervical dislocation.

3.8.5 Bile flow rate (Study IV)

Bile flow was measured as follows: after a midline incision as described above, the common bile duct was ligated and the gallbladder cannulated with a polyethylene tube (PE-10). Bile flow rate was then measured by collecting bile for 20 minutes and thereafter adjusting for body weight.

3.8.6 Carbon tetrachloride injections in mice (Study IV)

Carbon tetrachloride (2mg/kg body weight) was mixed 2:5 with sterile mineral oil and given intraperitoneally twice weekly for three weeks. Controls received injections with only mineral oil. Mice were given buprenorphine subcutaneously before and after injection for analgesia. Mice were sacrificed after three weeks.

3.8.7 Isolation and *in vitro* activation of hepatic stellate cells (Study IV)

Isolation of HCS was performed as described earlier [126]. C57BL/6J and N-CAM^{-/-} mice were deeply anesthetized with Isofluran. Perfusion solutions were administrated via the right atrium of the heart and let to flow out through the caval vein that was cut below the liver. Firstly Krebs-Ringer buffer supplemented with 0.01MHepes and 0.01% EGTA was supplemented to remove blood. Secondly the liver was perfused with pronase (2.5mg/ml) and 35ml collagenase (0.074mg/ml). The liver was then removed, minced and transferred to a solution with pronase (0.6 mg/ml) and DNase (0.16mg/ml). The mixture was then incubated at 39°C with shaking for 30 min. After a low speed centrifugation the hepatocytes was removed. HSC were isolated further by ultracentrifugation through a Larcoll (Sigma Aldrich) gradient. HSC were then plated on Petri dishes in DMEM-F12 supplemented with 10% FBS, penicillin and streptomycin (100U/ml and 100µg/ml respectively). The HSC were cultured for 20 days at 37°C and 5% CO₂ where after they were analyzed or used in experiments. At the first passage, 95-99% purity of the HSC were confirmed by morphological appearance, vitamin A specific fluorescence in polarized light and immunofluorescence for α-smooth-muscle actin. After gentle wash with PBS, the medium was replaced with new medium containing 10ng/ml of recombinant TGF-β1 (R&D Systems). The HSC were stimulated for 0h (control), 4h or 72h before analysis.

3.8.8 Total Iron (StudyII)

Total iron content in the liver tissue of *ob/ob* mice and controls was measured by flame absorption spectrometry (Varian SpectrAA 220 atomic absorption spectrometer) using background correction. Samples were dried in 50°C for 3 days and then hydrolyzed in concentrated HNO₃ overnight before analysis [127].

3.8.9 Quantitative Real.Time PCR (Study IV)

First strand cDNA synthesis from isolated RNA was performed using 1,5 µg RNA, 500 µM of dNTPs, 3,5 µM of random primers, 200 units of SuperScript™ II reverse transcriptase in 1X first strand buffer including 10mM DTT and 40 units of RNase out™. The reaction mix was incubated at 42°C for 50 min. Gene expression was analyzed using TaqMan® Universal PCR Master Mix and exon-to-exon spanning probes. Probes were obtained commercially from Applied biosystems.

The amplification was performed at 95-60°C for 50 cycles in an ABI Prism 7000 sequence detection system. Samples were run in duplicate or triplicate and results were normalized to the house-keeping genes HPRT or GAPDH which had been chosen due to their lack of response to the stimuli used.

3.8.10 Immunoblot (Study II and IV)

Immunoblots were performed with standard techniques and are describes in detail in Study III and V. Briefly the tissue was first minced and subsequently homogenized, in homogenisation buffer. Equal amount of protein extracts was separated on SDS-PAGE gel and subsequently blotted over to nitrocellulose membrane (Pierce). After blocking in 5% fat-free dry milk, the membrane was incubated in appropriate primary antibody solution overnight, 4°C with gentle shaking. After washing the membranes extensively and incubated in appropriate secondary antibody conjugated to HRP. Results were visualized using the SuperSignal West

Pico ECL system (Pierce). Ponceau staining (Sigma Aldrich) and actin antibody (Sigma) was used to evaluate good transfer and equal loading of protein.

3.9 ETHICAL CONSIDERATIONS

For patient studies ethical approval was given from regional ethics committee at the Karolinska Institutet in Stockholm.

Experiments performed on animals were performed after a priori approval by the local ethics committee for humane use of research animals in Northern Stockholm.

Carbon tetrachloride (CCl₄) was used with specific permission from the Swedish Environmental Protection Agency.

3.10 STATISTICS

3.10.1 Study I

To assess the relative risk of death, we employed standardized mortality ratios (SMR), that is, the ratio between the observed numbers of deaths in the cohort compared with the number expected on the basis of mortality rates for the general population. The expected numbers of deaths were calculated by adding all person-years accumulated in the cohort into strata (sex, age [in 5-year groups]) and calendar year of follow-up [in 5-year intervals]) and then multiplying the stratum-specific person-years by the corresponding incidence rates for the entire Swedish population. Ninety five percent confidence intervals (CI) were calculated assuming that the observed events followed a Poisson distribution. Follow-up began at the date of the initial liver biopsy and ended on July 9, 2008, or, if earlier, the date of death. Kaplan-Meier curves are used to depict the mortality in the cohort graphically. All analyses were conducted stratum-specific using SAS statistical software.

3.10.2 Study II

All statistical analysis was done using SPSS (Ver 16. SPSS Inc.), and results were obtained using students t-test, paired or non-paired depending on the experiment. Results are presented as mean and SD.

3.10.3 Study III

All statistics were performed with GraphPad Prism 4. When two groups are analysed, we used student's t-test for unpaired parametric data. For correlation we used Pearson's correlation. Results are presented as mean \pm SEM or mean and range. All p-values are presented as 2-tailed and on 95% confidence interval.

3.10.4 Study IV

Statistical tests were performed with GraphPad version 4.01 (Prism Software). Two tailed Students t-test was used for single comparisons.

4 RESULTS AND DISCUSSION

4.1 STUDY I

4.1.1 Re-evaluation of the Liver Biopsies

Fatty liver was detected in 143 of the 256 subjects, including 25 (10%) with alcoholic fatty liver disease and 118 (46%) exhibiting NAFLD. Of those, 51 (20%) were classified as NASH and 67 (26%) as non-alcoholic bland steatosis. Cirrhosis was present in 9% at inclusion.

4.1.2 Relative Risk of Death

During this follow-up, 113 (44%) of the 256 subjects died, which corresponded to an 80% increased risk of death in comparison with the general population. Of the 118 subjects with NAFLD, 47 died. Compared with the total Swedish population, adjusted for sex, age, and calendar period, subjects with NAFLD exhibited a 69% increased mortality (standardized mortality ratio [SMR] 1.69; 95% confidence interval [CI], 1.24-2.25); subjects with bland steatosis, a 55% increase (SMR, 1.55; 95% CI, 0.98-2.32; $P=0.062$); and subjects with NASH, 86% (SMR, 1.86; 95% CI, 1.19-2.76; $P=0.007$). In the case of the AFLD/ASH subjects, a 294% increased risk of death was observed (SMR, 3.9; 95% CI, 2.41-6.09). Chronic infection with hepatitis C or B was associated with an increased risk of death (Table 3; Fig. 2C). By contrast, subjects with autoimmune hepatitis or another diagnosis did not exhibit reduced survival, although certain subsets contained too few subjects to allow valid comparison.

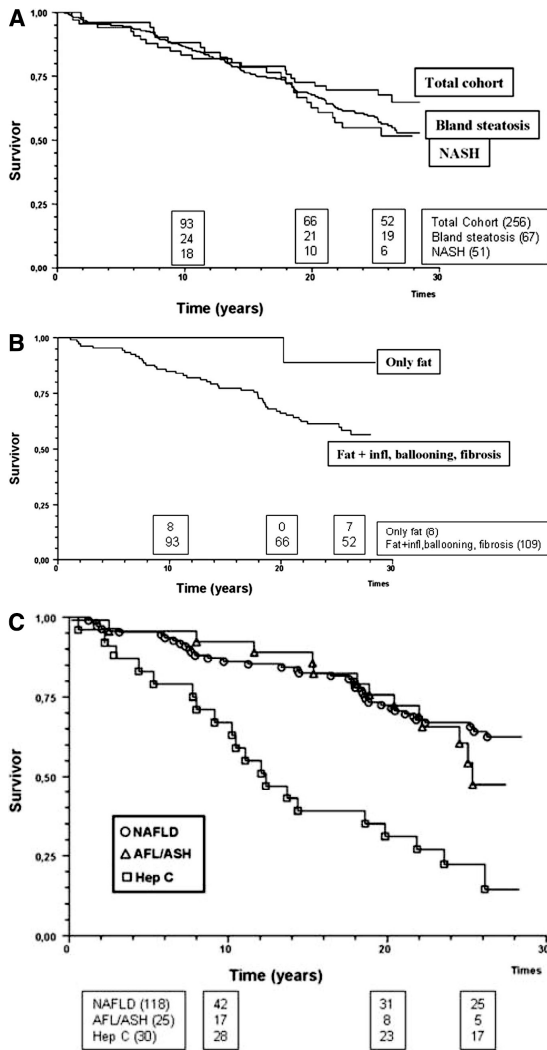


Figure 4. Overall survival of subjects in the study with NASH or bland steatosis and the entire cohort. (A) Overall survival of subjects with NASH or bland steatosis and the entire cohort of 256 subjects. Of the 118 subjects with NAFLD, 47 died. Twenty-three patients of the 67 with bland steatosis died (SMR, 1.6; 95% CI, 1.19-2.76; $P=0.007$). (B) Overall survival of subjects with NAFLD, divided into those with only fatty liver or subjects with fat and any type of inflammation, ballooning or fibrosis. (C) Overall survival among subjects with NAFLD compared with those with alcoholic fatty liver and hepatitis C.

4.1.3 Causes of Death

47 NAFLD patients died during follow up and 14 (30%) of them died of cardiovascular disease, 13 (28%) of extra hepatic malignancies, and nine (19%) of liver disease. Liver diseases are in our material in third major cause of death for NAFLD patients compared to normally on 11th place in Swedish population. On the whole, patients with NAFLD die of liver-related causes to a greater extent than the general population, but we still see cardiovascular disease and extrahepatic malignancies to be the primary and secondary causes of deaths among these patients. The frequency of HCC is almost 1000-fold higher in this group than what has been reported for Sweden earlier.

4.1.4 Summary and conclusions

We conclude that in our cohort of subjects with elevated serum levels of liver enzymes who underwent consecutive liver biopsies 28 years ago, 46% could be diagnosed as suffering from NAFLD. Overall survival was reduced in subjects with NAFLD and NASH, whereas bland steatosis with or without severe fibrosis was not associated with any increase in mortality risk in comparison with the general population. Patients with NASH had a lower risk of death than those with alcoholic liver disease or chronic viral hepatitis but a higher risk than those suffering from autoimmune and metabolic liver diseases.

Patients with NASH are at increased risk of death compared with the general population. Liver disease is the third most common cause of death among patients with NAFLD.

4.2 STUDY II

4.2.1 Iron content

Since patients with NAFLD and NASH quite commonly have hyperferritinemia we expected increased iron contents in the livers from animals in our mouse models with fatty liver. However, on the opposite,

our mice with fatty livers actually had less amount of iron in their livers than the controls. We cannot rule out however, that the flame absorption spectrometry technique underestimates iron content when there is an increased fat content in the same specimen

4.2.2 Intracellular signalling of hamp1

We found that the STAT3 protein expression which is a member of the IL-6/STAT3/Hamp signalling pathway was significantly up-regulated in *ob/ob*-animals with fatty liver compared to controls. However, no significant changes could be detected in Smad4 expression. Neither could any significant difference be found when comparing BMP6 or Smad 1/5/8 expression between *ob/ob* animals and their controls. IL-6 has earlier been reported to be increased in blood circulation in *ob/ob*-mice and an increase may trigger an up-regulation of the IL-6/STAT3/Hamp signalling-pathway which results in increased hepcidin levels and lowering uptake of iron from the intestines.

4.2.3 Iron regulatory genes

We wanted to describe the effect that oxidative stress and pro-inflammatory cytokines has on iron regulatory genes. There were no significant differences in gene expression following TBH incubation (oxidative stress). After incubation with IL-6, there was an increased expression of hamp1 in *ob/ob* mice hepatocytes but not of similar magnitude compared to normal mice. In hepatocytes from *Mtpp^{ΔΔ}* and *Mtpp^{flox/flox}* mice both cell types reacted with increased expression of hamp1 following incubation with IL-6. Both animal types also decreased their expression of the same gene following incubation with TNF- α . No other gene expressions were affected by either IL-6 or TNF- α . When we looked at whole liver samples both Tfr2 and ferritin gene expression were decreased in *ob/ob*-mice compared to control animals ($p=0,008$ and $0,045$ respectively). For the other genes analyzed, the differences in expression

were not significant. For the liver tissue samples in *Mttp* mice, no significant differences were seen between the *Mttp^{ΔΔ}* mice and the *Mttp^{flox/flox}* in any of the analyzed genes. It seems as if fat loaded cells are resistant to further uptake of iron into the cell. Also, it seems to be a tendency towards binding the existing internal iron to ferritin. This may explain why obese mice (*ob/ob*) contain less iron in the liver. Possibly, this could be viewed as a defence mechanism against oxidative stress which is in line with the reports on iron reduction therapy being beneficial in respect to NAFLD disease development

4.2.4 Summary and conclusions

We saw increased *hamp1* expression in *ob/ob* mice, and it appears to be caused by up-regulation of IL-6, STAT3, *hamp1*-pathway, indicating systemic inflammation. *ob/ob* and *Mttp^{ΔΔ}* mice hepatocytes were more sensitive to oxidative stress, seen with MDA and LDA, but no specific alterations were seen in gene expression from incubation with pro-inflammatory cytokines.

4.3 STUDY III

4.3.1 NAS-classification and Morphometrical measurements

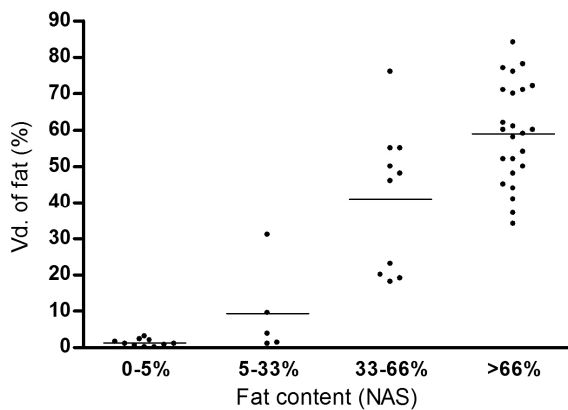


Figure 5. The amount of fat scored with NAS-classification and by point counting for fat amount.

Results from point counting are given in volume density (*Vd*,%).

The amount of fat was scored both according to NAS-classification and also more precisely by morphometry using point-counting (fig 5.). The mean volume density of fat was $58.8\% \pm 13.0\%$ in the NASH patients. The two values differed in some patients and this was mainly because of presence of microvesicular fat, which is more difficult to estimate in the NAS score.

Fibrosis correlated well between the two scoring systems; area measurement by software program and NAS.

Patients with the highest grade of lobular inflammation according to the NAS-method were not those with the highest numbers of inflammatory cells in the tissue. The number of lobular inflammatory foci was not correlated to the number of CD3 positive cells, nor did it correlate to the number of PMNs

4.3.2 Apoptosis and inflammation

The amount of cells expressing cleaved Caspase 3 was low and did not differ between the different zones in the liver. Apoptotic bodies as seen with cells positive for the Apoptag antibody were mainly located in the hepatocytes with big fat droplets rather in those with micro-vesicular fat. Apoptosis has earlier been suggested to drive the inflammation but we could not find any evidence for that in our material. There was no statistical significant correlation between the NAS-score and the number of apoptag positive cells, $p=0.54$.

ICAM-1 staining around hepatocytes was seen in almost all NASH patients and it was localized mainly in areas with hepatocytes with microvesicular fat. The areas with positive hepatocytes differed from patient to patient. Non-NASH biopsies did not show any ICAM-1 positive hepatocytes. This suggests that ICAM-1 is some what involved in the inflammatory process in the livers from NASH patients.

There were an increased number of cells positive for Foxp3 in the NASH patients and also those with fat or inflammation. Higher foxp3/CD3 quota

correlated to higher NAS-score. Cells positive for Foxp3 were distributed in both the lobule and in the portal tracts. There was no preferred localisation in the lobules and these cells were sometime more frequent in central areas of lobules and sometimes in the periportal areas. Presence of regulatory T-cells has been shown in previous work in liver diseases and found to be increased in inflammatory liver diseases such as autoimmune hepatitis and HCV. The role of Tregs is to control the immune system which is well in line with our findings demonstrating higher amounts of Tregs in the NASH group than those with only fat. NASH patients also had lower numbers of CD3 positive cells.

We found no differences in the number of macrophages but when we looked at the area of Kupffer cells it was twice the area seen in non-NASH livers. We did not see any difference in the number of TLR 4 expressed in the different patient groups and nor was it correlated to the amount of fat or inflammation. This speaks against that NASH is induced by bacterial influences from the gut or from other areas in the body, at least in our material, something that has been suggested in previous publications.

ICAM-1 positive hepatocytes were seen in NASH patients and were localized in areas with microvesicular fat. Non-NASH biopsies were negative for ICAM-1 positive hepatocytes.

4.3.3 Soluble ICAM-1

We found that sICAM-1 were significantly higher in NASH-patients (339.8 ± 34.07) than in non-NASH (229.5 ± 12.14), $p=0.0015$ (fig 6). No correlation between the BMI of the patients and the level of sICAM1 was seen.

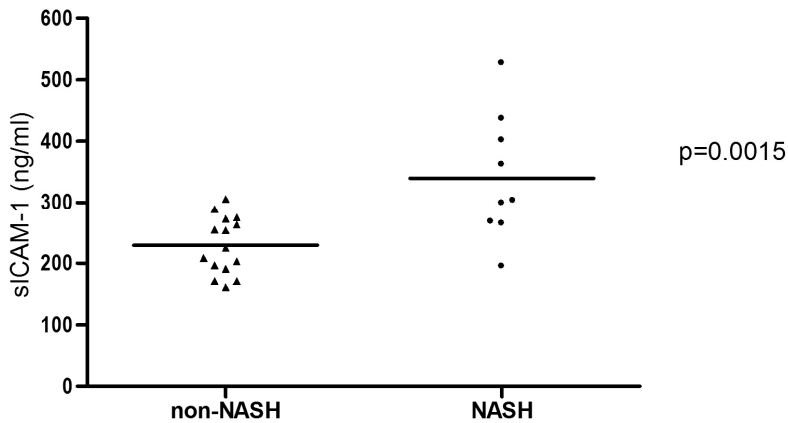


Figure 6. Serum levels of sICAM-1 in patients with NASH and non-NASH (controls) Patients with NASH had significantly higher serum levels of sICAM-1 than non-NASH subjects, $P=0,0015$

4.3.4 Summary and conclusions

We found that liver content of fat is difficult to estimate and that the presence of fat seems more important than the actual amount of it. In liver tissue with NASH, hepatocytes with microvesicular steatosis seem to be expressing more inflammatory markers. As in many other types of liver diseases NASH patients and borderline NASH patients have more regulatory T-cells. Inflammation seems to be important and affecting especially hepatocytes with microvesicular fat, which is seen by expression of ICAM-1 which also could be seen in serum samples. ICAM is known for facilitating leukocyte endothelial transmigration. Inflammatory responses will up-regulate the expression of ICAM-1 and thereby increase the adhesive nature of leukocytes. We thereby speculate that, since we see an increased expression of ICAM-1 among hepatocytes with microvesicular fat that these areas perhaps also has an increased amount of released inflammatory cytokines.

4.4 STUDY IV (NCAM)

4.4.1 N-CAM expression

After a single CCl₄ injection in wild type mice, highest level of N-CAM was found after 72 h. N-CAM was expressed by all bile ducts and also by mesenchymal cells around blood vessels and bile ducts. In wild-type mice exposed to CCl₄ injections twice weekly for three weeks, N-CAM was detected mainly in myofibroblastic cells, both in portal areas and around necrotic areas.

At 72 hours after bile duct ligation (BDL), wild-type mice showed positive staining for N-CAM in cholangiocytes and mesenchymal cells of the portal areas. 2 weeks after BDL, proliferating bile ductules and periportal fibroblasts were N-CAM positive but the larger bile ducts were N-CAM negative. In mice that were sham-operated or injected with mineral oil alone, occasional N-CAM positive cells were seen. All N-CAM^{-/-} mice were negative for immunoblotting and immunofluorescence with anti-NCAM.

4.4.2 Blood chemistry tests

Serum bilirubin was significantly higher in 2-week BDL N-CAM^{-/-} and showed a trend towards higher values in sham-operated, 3-week CCl₄ or mineral oil-injected N-CAM^{-/-} mice. ALT was significantly higher in 3-week CCl₄-injected mice.

4.4.3 Loss of N-CAM attenuates fibrosis following bile duct ligation.

Two weeks after BDL wild-type mice had developed a pronounced liver fibrosis with expansion of the portal tracts and formation of fibrous septa between the portal areas. N-CAM deficient BDL mice had milder fibrosis with less bridging and less expansion of the portal areas. Wild-type mice had more formation of bridging fibrous septae and thickness of fibrosis surrounding the bile ducts. In N-CAM^{-/-} mice a significantly larger area of bile infarcts was seen as compared with wild-type, 2,2% vs. 0,78%, p=0.02. Bile flow rate was equal between genotypes.

The finding of an increased amount of bile infarcts and increased serum-bilirubin in the two week BDL N-CAM knock-outs indicate that loss of N-CAM is associated with an increased vulnerability to cholestasis.

4.4.4 CCl₄ induced liver fibrosis

No evident difference in the amount of liver fibrosis was seen between N-CAM knockouts and wild-type mice that were subjected to CCl₄ injections. CCl₄ treated mice of both genotypes had moderate partially bridging fibrosis. Control mice injected with mineral oil alone had normal liver histology without fibrosis.

4.4.5 Expression of myofibroblast marker α -SMA

N-CAM deficient BDL mice had milder fibrosis with less bridging and less expansion of the portal areas.

In CCl₄ treated mice, α -SMA positive cells were distributed around the interlobular margins as well as in regeneration areas. No significant difference were seen in the distribution or amount of SMA positive cells between N-CAM^{-/-} and wild type in neither BDL nor CCl₄ injected mice.

4.4.6 Hepatic stellate cells isolated from N-CAM^{-/-} mice have impaired activation after stimulation with TGF- β 1

The 140kD isoform of N-CAM, which is expressed by activated HSC, has been reported to be important for cell migration [128] and hence it is possible that loss of N-CAM has a negative effect on recruitment of activated HSC to the portal areas.

The 140kD isoform of N-CAM was present at start of stimulation with TGF- β 1 followed by a slight increase after 72h in HSC isolated from wild-type mice. No N-CAM protein was found in HSC from N-CAM^{-/-} mice.

All isolated HSC showed normal viability throughout the experiments and no signs of increased apoptosis were seen.

Following stimulation with TGF- β 1, α -SMA levels decreased at both 4 and 72 hours in N-CAM^{-/-} HSC. In wild-type derived HSC, a moderate increase of α -SMA was seen at the same time points.

Desmin had in N-CAM^{-/-} HSC, after 4 hours of TGF- β 1 stimulation, decreased significantly and at 72h and was almost undetectable. In contrast to this, a marked temporal increase of desmin was found in HSC from wild-type mice.

The clear impairment of activation of N-CAM deficient HSC as measured by SMA and desmin supports the hypothesis that N-CAM is important in differentiation of HSC and possibly other types of cells to fibrogenic cells in the injured liver.

4.4.7 Summary and conclusions

The results indicates a role of N-CAM in cholestatic liver disease since the loss of N-CAM resulted in decreased hepatic collagen and fibronectin deposition in mice subjected to BDL. Animals exposed to repeated CCl₄ injections and therefore a hepatocellular injury did not show the same alterations. It also indicates a roll of N-CAM in HSC activation since N-CAM null mice show impaired activation in vitro.

5 GENERAL DISCUSSION

Fatty liver was in the beginning thought upon as a benign disease but more and more reports are indicating that this is perhaps not the fact. In the society today major topics for discussions are how obesity, diets physical activity are affecting not only our quality of life but also relates to diseases such as diabetes and coronary heart diseases. However little is mentioned in the debate how this also has a strong relation to liver diseases. In the first paper of this thesis we performed the longest follow-up of patients who originally demonstrated elevated serum levels of hepatic enzymes and were subsequently shown by biopsy to be suffering from NAFLD. Four major strengths were pointed out in this work: First, all of the subjects were enrolled consecutively during a defined period. Second, all underwent liver biopsy at the time of referral, so that the diagnoses of NAFLD are based on histological criteria. Third, re-evaluation of the initial biopsy findings was performed in all cases. And finally, all deceased subjects could be followed-up through the Cause of Death Registry, so that there were no losses during follow-up. The study showed that of all patients enrolled because of elevated liver enzymes, 46% was due to NAFLD. Patients with NAFLD had decreased survival compared with the general Swedish population. Our data confirmed previous studies by Adams [129] and Ekstedt [1]. In both these studies as well in our own, liver related death including HCC was the third most common cause of death. HCC is a major health problem worldwide, with more than 500,000 cases diagnosed annually [130]. Whereas the incidence of HCC has been increasing during the last 5 to 8 years, the survival of those affected has not changed significantly during the past two decades. With obesity and NAFLD becoming more and more common, with enhanced prevalence among younger people, the associated rise in relative risk of mortality and terminal liver disease will be of considerable significance to public health in the future. In the current investigation, four subjects with NAFLD/NASH died of complications of cirrhosis, and five died of HCC. If NASH then is caused by obesity, insulin resistance

and sedentary lifestyle, it thus seems reasonable to recommend changes in lifestyle for all subjects with NAFLD. Of the nine subjects diagnosed with NAFLD and concomitant cirrhosis at the time of inclusion in the current study, only one remained alive.

It seems as the prognosis for patients with NAFLD is highly dependent on the fibrosis stage in the biopsy. We found that when patients with cirrhosis or severe fibrosis are excluded from the group with bland steatosis, deaths attributable to either cirrhosis or liver cancer are much less common. It has in shorter studies been shown that patients with no fibrosis at baseline only a few will develop end-stage liver disease [131, 132], but one has to remember that fibrosis and NAFLD has a slow progression and it has been shown in a study with repeated liver biopsies [1] that out of patients with no fibrosis at baseline about 40% later at follow up about 10-14 years later have developed fibrosis. Perhaps younger individuals who get the diagnosis fatty liver without fibrosis, it even though the risk for end stage liver disease at the moment looks minimal, should be carefully followed up to reassure that they are not developing fibrosis later on.

The scoring system developed by Kleiner et al. [15] combines the three parameters hepatic fat content, lobular inflammation, and ballooning, all of which contribute equally to the NASH score. Thus, a patient with pronounced steatosis and relatively moderate inflammation could receive the same NASH score as one with mild steatosis but intense inflammation and ballooning. One disadvantage to this scoring system is that it does not take fibrosis into consideration. Another problem with the scoring system is that as no one yet knows what is driving the disease progression and the scoring systems equally weigh steatosis and inflammation, it is hard to say if the NAS score actually is of any value for the physician. Is it as discussed before steatosis and the fibrosis grade that are important to investigate for the disease progression and mortality or are inflammation of importance as well? Data available on prognosis based on NAS score is still limited [15, 133]. We found an increased amount of Kupffer cells in

the tissue of NASH patients and when activated, Kupffer cells release pro-inflammatory and fibrogenic factors such as chemokines and cytokines [99]. One such cytokine is the transforming growth factor- β which then could stimulate the profibrogenic activity in HSCs. As mentioned in the introduction pro-inflammatory cytokines are controlled by pro-inflammatory pathways such as c-Jun N-terminal kinase (JNK), which pathway has been linked to apoptosis and inflammation [100, 101]. In experimental NASH jnk knockout mice showed less hepatic inflammation and fibrosis which could be interpreted as that JNK1, in Kupffer cells, contributes to liver fibrosis by inducing chronic liver inflammation [102]. These experimental data thus links the development of fibrosis to inflammation elicited from Kupffer cell cytokine release. Proinflammatory cytokines have been shown to be capable to induce ICAM1 expression on cell lines *in vitro* [134]. In non-NASH livers, we found that ICAM1 expression is largely confined to sinusoidal lining cells with only faint staining on part of hepatocyte membranes. But in NASH or borderline-NASH patients, ICAM-1 shows positive hepatocyte staining for the whole membrane.

Another interesting finding in our study was that ICAM-positive cells largely were located in the areas of microvesicular fat deposits. Thus our data indicate that locally released cytokines might be more common in areas of microvesicular fat.

Patients with NASH also had higher levels of sICAM-1 than patients with borderline NASH or non-NASH. This has been shown before [135] and it is also known that obese patients may have higher sICAM-1 than subjects with normal BMI [136, 137]. Interestingly, earlier studies also showed that sICAM-1 levels are elevated in alcoholic liver disease [138, 139] and it was possible to correlate to the severity of liver damage [140]. As noted earlier, whether the circulating levels of sICAM-1 originate from the liver is hard to say but we and others [135] have shown that NASH livers express elevated levels of ICAM-1. Whether or not sICAM-1 could be used as a new non-invasive tool to diagnose NASH needs to be studied further.

Previous work have demonstrated the presence of regulatory T-cells in liver diseases and it was found to be increased in inflammatory liver diseases such as autoimmune hepatitis and chronic hepatitis C [141]. Our findings are in line with those seen in other inflammatory liver diseases, since our patients with NASH had higher amounts of Tregs as compared to those having only fat without inflammation. However in a study where mice were fed high fat diet to induce liver steatosis, steatosis was associated with the depletion of hepatic Tregs and led to up-regulation of the inflammatory TNF- α signalling pathway [142]. Our finding with increased amount of Tregs is interpreted as a attempt to regulate the inflammation and may suggest an immunological disturbance, still leading to an increased inflammation in these livers. Tregs had in the experiment with high fat diet, an increased susceptibility to oxidative stress-induced apoptosis. After treatment with an antioxidant, Treg apoptosis was reduced; the number of hepatic Tregs increased and the liver inflammation decreased [142].

As mentioned in the introduction, hepatic iron excess is associated with insulin resistance [29] and also commonly observed in patients with NAFLD. There is contradictory evidence that hepatic iron may play a role in the pathogenesis of NAFLD/NASH. Some studies have shown abnormal serum ferritin and/or transferrin saturation and elevated hepatic iron concentration in NASH [143]. George *et al.* have proposed the hypothesis of iron related liver injury in NASH [32]. They showed that 41% of patients in their study had increased hepatic iron and above 20% had hepatic iron concentrations in the upper limit of normal. They also showed that the increased hepatic iron content was associated to the severity of fibrosis. Others have reported high frequencies of hyperferritinemia and increased hepatic iron in NASH patients [144]. Serum levels of an indicator of oxidative stress (TRX) were increased and in proportion with the hepatic iron load. These findings suggest that iron could be a cofactor for the oxidative stress in NASH by enhancing the lipid peroxidation and fibrogenesis in NASH. In contrast to this, many others [145-148] have shown that only a minority of NASH patients have significant iron

accumulation. Nor did they find a connection between iron overload and aggressive histological, clinical outcome or degree of fibrosis. From these results the authors draw the conclusion that increased serum ferritin in NASH is a non-specific effect of hepatic necroinflammation. Ferritin is known to increase in serum when it is released from damaged hepatocytes. It is also possible that increased serum levels of ferritin come from iron-independent oxidative stress such as free fatty acid lipid peroxidation and cytokine release.

In our second paper on iron regulatory genes we aimed to establish if iron metabolism is altered in mice with liver steatosis. Instead of finding increased hepatic iron in our mice with fatty liver we actually found less. We conclude that iron probably does not play a significant role in the development of NAFLD and NASH in our models. We interpreted our results from gene expression assays to be an effect of systemic inflammation from the adipose tissue. *Ob/Ob* mice have excessive amount of fat tissue and increased IL-6 levels in the blood circulation [149], which could explain the increased hamp1 expression in their livers. IL-6 increase may trigger an up-regulation of the IL-6/STAT3/HAMP signalling-pathway which results in increased hepcidin levels and a decreased uptake of free iron, in line with a recent publication by Fatih et al [150]. Animals with less fat tissue did not show the same alterations. Thus, in conclusion we suggest that the increased ferritin levels seen in this animal model of fatty liver do not reflect a true iron overload but rather a chronic inflammation, since ferritin also acts as an acute-phase reactant. This chronic inflammation thus displays high levels of IL-6 and hepcidin. In humans the clinical picture is more complex. Indeed, many NAFLD patients with hyperferritinemia have signs of chronic inflammation and a normal iron content of the liver, whereas others have an increased liver iron content, the so-called insulin-resistance hepatic iron overload. The serum levels of hepcidin in these two subgroups of hyperferritinemic NAFLD patients are presently unknown.

NCAM only had an effect on BDL induced fibrosis. Fibrosis plays a central role when predicting the prognosis of NAFLD patients. NCAM is as earlier mentioned, associated with fibrosis in liver, heart and kidney [87-89]. NCAM is expressed by activated hepatic stellate cells (HSC), portal fibroblasts, cholangiocytes and hepatic progenitor cells during liver injury and has a functional role in liver disease and fibrogenesis. By performing repeated injections of CCl₄ we aimed to induce a similar type of damage that can be seen in NAFLD, although necrosis becomes more severe in the animal model chosen compared with that seen in human NASH. NCAM appears to play a role in liver fibrosis induced by cholestasis since mice lacking NCAM experienced less collagen and fibrinogen deposition. Periportal fibroblasts are major contributors of fibrosis induced by cholestasis and HSC, even though they are activated, to a less extent, contribute with matrix deposition [125, 151, 152]. The loss of NCAM could be associated with an increased vulnerability to cholestasis since mice lacking NCAM, after bile duct ligation, showed increased amount of bile infarcts and increased serum bilirubin. HSC migrate in biliary fibrosis towards the portal areas where they contribute to fibrosis [151]. The impairment of activation of NCAM deficient HSC supports the hypothesis that NCAM is important for the differentiation of HSC and possibly other cell types to fibrogenic cells in the injured liver. How NCAM modulates fibrogenesis is not clear. One possible mechanism is that NCAM takes place in integrin signalling and function [128, 153]. The integrin, α V β 6 is a fibronectin receptor specifically expressed by epithelia, and it is induced in bile ducts concomitant with N-CAM after acute biliary obstruction. In mice mutant for β 6, BDL-induced fibrosis was decreased and accompanied by impaired TGF- β signalling. In the livers of CCl₄-treated mice, no expression of α V β 6 on difference in fibrosis was seen. This raises the possibility that N-CAM contributes to fibrosis through interaction with integrin functions. The similar effects caused by loss of NCAM and abolished integrin function or signalling in *in vitro* studies of liver fibrosis and activation of HSC suggests a connection between integrins and NCAM in liver fibrogenesis.

6 GENERAL CONCLUSIONS

(Study I) Of subjects with elevated liver function tests 46%, was due to NAFLD. Patients with NAFLD were at increased relative risk of death compared with the general Swedish population. We saw that patients with fatty liver, to a higher extent than the normal population died from liver related causes. Liver disease was the third most common cause of death among patients with NAFLD.

(Study II) Exposure to pro-inflammatory cytokines *in vitro* did not show great effect on the iron regulatory genes in either of the mouse model. However, there was an increased hamp1 expression in obese mice (*ob/ob*-mice) that appear to be caused by up-regulation of IL-6, STAT3, Hamp1-pathway, indicating systemic inflammation. Hepatocytes from mice with liver steatosis were more sensitive to cell damage in response to oxidative stress than control animals.

(Study III) Hepatocytes with microvesicular steatosis seemed to be more affected by inflammation than hepatocytes with macrovesicular fat as shown by expression of more ICAM-1 on the cell surface. In liver tissue from NASH-patients, we found an increased number of regulatory T-cells (e.g. Foxp3+ cells) and an increased area of CD68 cells compared to non-NASH controls. This indicates an involvement of both the innate and the adoptive immune systems

(Study IV) Loss of N-CAM does not result in decreased hepatic fibrosis in animals exposed to hepatocellular damage whereas it did in mice subjected to BDL. HSC isolated from N-CAM null mice showed impaired activation *in vitro*. This indicates a role of N-CAM in liver fibrosis caused by bile duct damage.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Syftet med avhandlingen var att utreda varför fett i levern orsakar svår leverskada hos vissa personer. Fett i levern har tidigare ansetts vara relativt ofarligt under förutsättning att det inte varit orsakat av överkonsumtion av alkohol. Idag är fettlever den vanligaste orsaken till leversjukdom i världen. Fettlever är en bred term och inkluderar allt från enkel fettinlagring till leverinflammation och bindvävsbildning.

I första arbetet fann vi att personer med fettlever hade en ökad risk att dö under uppföljningstiden jämfört med normalbefolkningen. Om personerna dessutom hade en leverinflammation ökade risken ytterligare. I Sverige ligger leverrelaterad dödlighet på 11:e plats som dödsorsak men hos patienter med fettlever är leverrelaterad dödlighet tredje vanligaste dödsorsak.

Ökad järninlagring i levern hos personer med fettlever är relativt vanligt. I arbete två studerade vi hur järnomsättningen förändras då isolerade leverceller från feta möss utsattes för inflammatoriska stimuli eller fria radikaler. Hos feta möss fann vi ökat uttryck av den gen i levern som minskar järnupptag från tarmen. Den ökade mängden järn i levern vid fettlever beror troligen således inte på ett ökat upptag av järn från tarmen, utan på en ökad cellskada med lokal ansamling av järn.

I vår tredje studie har vi undersökt inflammatoriska molekyler i levern vid fettlever. Vi visar att leverar med inflammation och mikro fett i levercellerna uttryckte fler inflammatoriska markörer. Patienter med leverinflammation hade också ökad mängd av inflammationsmolekylen ICAM-1 i serumprover.

Det fjärde arbetet handlar om bildandet av ärrvävnad i levern. Adhensionsmolekylen NCAM uppträder vid leverskada hos celler som medverkar i läkningsprocessen och bildandet av bindväv. Med hjälp av möss som saknar molekylen N-CAM kunde vi se att den typen av skada som uppkommer vid fettlever, nämligen hepatocytiska, inte är beroende

av molekylen N-CAM. N-CAM verkar snarare ha en påverkan på gallgångsceller och den typen av skada ses normalt inte hos fettleverpatienter.

8 ACKNOWLEDGEMENTS

This research program was carried out at the Karolinska University Hospital and Karolinska Institutet, Stockholm, Sweden. During 2006-2011. I am truly grateful to all who have contributed and helped me in different ways on this journey. I would particularly like to thank the following persons:

Professor **Rolf Hultcrantz**, min huvudhandledare. Stort tack för att du har trott på mig från första början. Den här resan har gått både upp och ner men du har alltid stöttad och väglett mig. Stort tack också för att du har introducerat mig i ett för mig helt nytt, spännande forskningsområde. Med min bakgrund proteinkemist visste jag knappt vad en levercell var och än mindre om patologi. Mycket har varit helt nytt för mig och det har varit en spännande resa. Du har haft en bra balans av guidning och låtit mig testa på eget bevåg. Jag kommer till och med nästan sakna våra sena kvällar och pratstunder vid mikroskopet.

Bihandledare docent **Per Stål**. Stort tack för alla de timmar du har lagt ner på att läsa arbeten och abstract till kongresser, samt för många intressanta samtal.

Kristina Eckes, vår ovärderliga laboratorieassistent, det här hade aldrig varit möjligt utan dels ditt otroliga tålmod och dina enorma labbkunskaper. Jag är otroligt tacksam för att ha fått jobba så nära och mycket med dig. Utan dig hade heller inte labbet varit en sån varm arbetsplats som det är idag.

Övriga medlemmar i levergruppen **Mattias Sjöström** och **Peter Rosenberg**, Tack grabbar för all hjälp med arbetena och trevliga diskussioner kring stort och smått.

Josefin Skogsberg, vilken härlig forskaranda! Smart, trevlig och dessutom alltid tid att hjälpa till. Du har varit ovärderlig under den här tiden, dessutom så har du varit ett utmärkt öl och pokersällskap.

Övriga nuvarande och tidigare kollegor: Professor **Per Hellström**, en tillgång på labbet med dina visioner och spännande idéer. **Mia**, vad skulle den här tiden ha varit utan dig? Jag har fått en vän för livet som jag haft väldigt mycket roligt med och som jag hoppas jag kommer få dela många, fler stunder och år med. **Tobbe**, din galning, tack för alla skratt du bidragit med under åren, hoppas vi kan fortsätta att ses och kanske åka lite längd ihop i framtiden. **Linda** och **Wiveca** tack för alla diskussioner om stort och smått och alla timmar av korsordslösande, det finns visst en väldig massa ord att lära sig fortfarande. **Sofie Berg** och **Therese Ek** tidigare kollegor som jag verkligen uppskattar, det var ni som drillade in mig i miljön på Karolinska, Sofie vilken härlig envishet och bestämdhet och Therese underbart att se sådan livsglädje och hur mycket man kan lyckas att kombinera samtidigt om man bara vill. **Pelle** och **Dick** även ni fantastiska öl och pokersällskap.

Läkare och systrar nere på Gastrocentrum, ett speciellt tack till forskningssköterskorna, tack tjejer för ett ständigt glatt humör, alltid kul att komma till er!

Stort tack till **Kickan** på djuravdelningen för alla timmar av slit med avel och hjälp till virriga forskare. Du har en unik hand med djuren och det är härligt att se.

Annika Bergkvist tack för att du räddade mig på min första kongress, jag var ensam, vilsen och rädd i den stora staden Helsingfors. Tack för att du snällt lät mig bli ett plåster ☺

Urologgruppen: **Petra, Mirjana, Nasrin, Lotta, Lotta och Katarina** tack för all hjälp och alla trevliga diskussioner i lunchrummet. Utan er hade det varit riktigt tomt. Speciellt tack till Petra som kommit med en massa bra tips (och en rackarns massa godsaker) och blivit lite av en mentor.

För att lyckas med det här så krävs också ett härligt liv utanför jobbet och jag skulle vilja tacka mina pluggkompisar från Uppsala. **Hanna** och **Fredrik**, vilka härliga spel och drinkkvällar vi åstadkommit. **Åsa, Åsa, Lotta, Lotta, Aida, Emmi, Cicci** och **Nicklas** utan er hade jag nog inte klarat mig igenom studierna. Härliga kvällar på nationer och en massa partaj hemma i korridorerna. **Anders**, du började som en handledare och har blivit en vän för livet. Jag kommer dock alltid att vara lite avis på dig för dina år på Harvard. Hoppas att jag kommer få låna din hjärna många gånger till.

Älta-brudarna, **Anneli, Hellman, Stenvall** och **Anna-Karin**, kul att vi fortfarande lyckats hänga ihop trots att vi börjar bli lite äldre, vi ses inte så ofta längre tyvärr, men när vi ses så känns det precis som tiden stått still, det är härligt!

Stort tack till **Johan Hindemo**, utan Johan hade den här avhandlingen inte haft en framsida. Stort tack för att du kunde hjälpa mig så snabbt!

Min familj: Ni alla undrat hur länge man kan läsa på Universitet, vad jag egentligen kommer att bli, och när tusan jag egentligen blir färdig. Tack för allt stöd och nyfikna frågor! **Mamma** och **Pappa**, tack för allt stöd, ni betyder allt. Mamma, trots allt pushande så blev jag tyvärr inte civilingenjör, hoppas du är stolt ändå. Du betyder mycket för mig! En person som alltid ställer upp och sätter sig själv i andra hand, tack för all hjälp! **ommy** och **Chris**, min bonusfamilj, underbart med nya tidens familjekonstellationer! Tack för alla avkopplande stunder! Min lillasyster **Ellinor**, trots att du många gånger tyckt att du har en ganska konstig storasyster. Jag saknar ibland den där speedade lilla tjejen, men vi har ju så mycket mer utbyte av varandra nu för tiden! Jag tycker om dig så mycket.

Calle, allt är så mycket roligare och enklare när man är två! Även om det ibland har varit många mil emellan oss har jag alltid känt ditt stöd! Jag älskar dig!

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