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IMPORTANCE OF IRON OVERLOAD AND STEATOSIS IN PATIENTS WITH CHRONIC LIVER DISEASE

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Importance of iron overload and steatosis in patients with chronic liver disease

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To my late father

ABSTRACT

This thesis deals with the importance of hepatic iron and fat deposition in the context of chronic liver disease, with special focus on the role of the S65C mutation in the hemochromatosis (HFE) gene, the association between non-alcoholic fatty liver disease (NAFLD) and cryptogenic cirrhosis (CC) in patients evaluated for liver transplantation, the expressions of innate and adaptive immunity in non-alcoholic steatohepatitis (NASH), and the role of the iron regulatory hormone hepcidin in dysmetabolic iron overload (DIO).

NAFLD is the most common liver disease in the Western world. Some patients with NAFLD develop NASH and are then at risk for progressive liver disease, cirrhosis and hepatocellular carcinoma. NASH involves the innate immune system, but the role of the adaptive immune system in this context is less clear. A subgroup of patients with NAFLD develops DIO, the causes of which are unknown. Mutations of the HFE gene may be associated with DIO, but results are conflicting. The role of the most recently found HFE mutation, S65C, has not been known. Finally, in end-stage NASH cirrhosis, liver fat diminishes, and patients with CC may therefore have an underlying NAFLD.

In study I the HFE S65C gene mutation was retrospectively studied in 296 patients with suspected iron overload and 250 healthy controls in order to determine the HFE S65C frequency, and evaluate whether this mutation would result in a significant hepatic iron overload or not. We found that the S65C allele was enriched in patients with high serum ferritin compared with controls, and half of the carriers of this allele had mild or moderate hepatic iron overload, but no signs of significant fibrosis.

In study II, 39 patients with CC were compared with 431 patients having cirrhosis of other etiologies, to evaluate the presence of NAFLD in patients with CC and determine survival after liver transplantation. We found that 44% of the CC patients had an underlying NAFLD. CC patients had a higher frequency of diabetes, ascites, and hyponatremia compared with those having cirrhosis of other etiologies. Weight loss was significantly higher among patients with CC, but there was no difference in patient survival between the groups.

In study III, liver biopsies from 49 patients with suspected NAFLD were classified according to the NAFLD Activity Score (NAS) and liver fat was assessed with morphometry. Biopsies were stained with various markers of T-cells, macrophages, apoptosis and cell adhesion molecules (ICAM-1). We found an increased number of regulatory T-cells (Tregs) and CD68 cells in NASH, pointing at an involvement of both the adaptive and innate immune systems. ICAM-1-positive hepatocytes were only seen in NASH livers and localized in areas with microvesicular fat, and the ICAM-1 level in serum was increased in patients with NASH.

Study IV aimed to determine the association between hepcidin and iron parameters, lipid status and inflammatory markers in NAFLD in relation to other chronic liver diseases. Serum hepcidin was analyzed in 85 patients with chronic liver disease (38 of which had NAFLD) and 38 healthy controls. Liver biopsy was performed in 67 patients and hepcidin mRNA in liver was determined with real time-qPCR in 36 patients. We found that hepcidin regulation was similar in NAFLD compared to other chronic liver diseases with various degrees of hepatic iron overload. In NAFLD hepcidin correlated to serum ferritin and liver iron, but not to BMI, CRP, NAS or steatosis. Transferrin saturation, but not hepcidin, could be used to discriminate between hyperferritinemic NAFLD patients with or without iron overload.

In conclusion, we found that the HFE S65C mutation leads to mild to moderate hepatic iron overload, but neither to clinically manifest hemochromatosis, nor extensive liver fibrosis. Re-evaluation of patient data in cryptogenic cirrhosis discovered underlying NAFLD in 44% of patients evaluated for liver transplantation. There was no difference in patient survival between cryptogenic patients and those having cirrhosis of a known etiology. In NASH, an involvement of the innate adaptive immunity is seen, and immunohistochemical markers of inflammation are localized to areas of microvesicular steatosis. Serum hepcidin levels in patients with NAFLD correlate adequately to iron parameters, but not to BMI, NAS or inflammatory markers.

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LIST OF SCIENTIFIC PAPERS

The thesis is based on the following original papers, which are referred to in the following text by their roman numerals:

I. P Holmström, J Marmur, G Eggertsen, M Gåfvels, P Stål. Mild iron overload in patients carrying the HFE S65C gene mutation: a retrospective study in patients with suspected iron overload and healthy controls. Gut 2002;51:723–730

II. J Marmur, A Bergquist, P Stål. Liver transplantation of patients with cryptogenic cirrhosis: Clinical characteristics and outcome. Scandinavian Journal of Gastroenterology 2010;45:60–69

III. C Söderberg, J Marmur, K Eckes, H Glaumann, M Sällberg, L Frelin, P Rosenberg, P Stål, R Hultcrantz. Microvesicular fat, inter cellular adhesion molecule-1 and regulatory T-lymphocytes are of importance for the inflammatory process in livers with non-alcoholic steatohepatitis. APMIS 2011;119: 412–420

IV. J Marmur, S Beshara, G Eggertsen, L Onelöv, N Albiin, O Danielsson, R Hultcrantz, P. Stål. Hepcidin levels in non-alcoholic fatty liver disease with or without hyperferritinemia. Manuscript

CONTENTS

LIST OF ABBREVIATIONS

AIH	Autoimmune hepatitis
ALD	Alcoholic liver disease
BMI	Body mass index
BMP	Bone morphogenic protein
CC	Cryptogenic cirrhosis
CLD	Chronic liver disease
DIO	Dysmetabolic iron overload
Foxp3	Forkhead box protein 3
HH	Hereditary hemochromatosis
ICAM-1	Inter cellular adhesion molecule-1
IL-6	Interleukin-6
LFA-1	Lymphocyte function associated antigen-1
MELD	Model for end-stage liver disease
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Non-alcoholic steatohepatitis
NHH	Non-hereditary hemochromatosis
OLT	Orthotopic liver transplantation
sICAM-1	Serum ICAM-1
Tf	Transferrin
TfR 2	Transferrin receptor 2
TNF-α	Tumor necrosis factor-α
TLR	Toll like receptor
Tregs	Regulatory T-cells

1 INTRODUCTION

In this thesis, we have aimed to characterize clinical features and underlying pathogenic mechanisms of iron and fat infiltration in the livers of patients with suspected iron overload and/or non-alcoholic fatty liver disease. Our patient cohorts include those with elevated serum ferritin, chronic liver disease, non-alcoholic fatty liver disease (NAFLD) with or without non-alcoholic steatohepatitis (NASH), and/or cryptogenic end-stage cirrhosis. In the Western world, NAFLD has become the most common liver disease, possibly because the prevalence of obesity and type 2 diabetes is rising worldwide. Fatty liver was considered a benign condition, but today we know that patients with NASH are at risk of progressive liver disease, and NASH-cirrhosis has become a common indication for liver transplantation. The problem of fatty liver is not limited to patients with NAFLD. Steatosis as a co-factor in chronic liver disease has been recognized in chronic hepatitis C, alcoholic liver disease, and hemochromatosis.[1, 2] Hereditary hemochromatosis is an iron overload disorder caused by mutations resulting in insufficient hepcidin secretion which in turn leads to increased uptake of dietary iron. Excess iron is stored in parenchymal organs. The liver is particularly vulnerable to the toxic effects of iron since it is the main site of iron storage. If the excess iron is not removed there is a risk of oxidative stress and fibrogenesis.[3] Iron overload has also proven to be a co-factor in other liver disease, not the least due to the link between iron stores and insulin resistance.[4] In NAFLD, iron has been proposed as a pathogenic factor for NASH development, and iron reduction therapy suggested as a treatment option. Thus, steatosis and iron overload is not only important in NAFLD and hemochromatosis, but also in chronic liver disease in general.

1.1 IRON HOMEOSTASIS

Iron homeostasis requires coordination between tissues that export iron into plasma (duodenal mucosa, macrophages) tissues that utilize iron (mainly red blood cell precursors), and tissues that store iron (such as hepatocytes, pancreatic cells and cardiac cells). The iron storage protein, ferritin, reflects iron stores in normal conditions, but not in the case of inflammation or liver damage. The amount of iron in an average adult is 3–4 g. To support erythropoiesis and other metabolic processes about 25 mg iron/ day is needed. Aged erythrocytes stand for the predominant contribution, and only 1-2 mg of dietary iron is absorbed from enterocytes in normal conditions, equaling the amount of daily loss. Iron is distributed through blood plasma, where it is bound to the iron transport protein transferrin. The body is dependent on regulation of the dietary uptake of iron, since losses are not modulated by iron excess or deficiency. The small peptide hepcidin is the master regulatory hormone of systemic iron metabolism. It is expressed in the liver and inhibits iron recycling from macrophages and enterocytes, by binding to and inducing the degradation of the cellular iron exporter ferroportin, thus lowering iron levels in serum. Consequently, deficiency of hepcidin will lead to iron overload.[5-7] Mutations in four different genes have been identified to result in hereditary hemochromatosis. These are the structural gene for hepcidin HAMP, and genes that are required for the expression of hepcidin through interaction with iron, hemojuvelin (HJV), tranferrin receptor 2 (TfR 2), and HFE. Iron regulation is also dependent on the Bone morphogenic protein (BMP) pathway, mostly BMP 6.

BMP 6 increases when liver iron concentration is high indicating that BMP6 could work as an indicator of iron storage. The BMP6 co-receptor HJV enhances the BMP receptors affinity for its ligands and boosts hepcidin transcription. Mutations in HJV decrease hepcidin to levels as low as those seen in patients with mutations in HAMP. Mutations in these genes lead to juvenile hemochromatosis, the most severe form of genetic iron overload disease. Inflammation increases levels of hepcidin since its synthesis is induced by IL-6. Decrease of hepcidin synthesis is thought to be the cause of iron overload in chronic liver disease and alcohol overconsumption.[8] In obesity, the low grade inflammation and expression of hepcidin in adipose tissue, might explain why iron deficiency is common in this condition.[9]

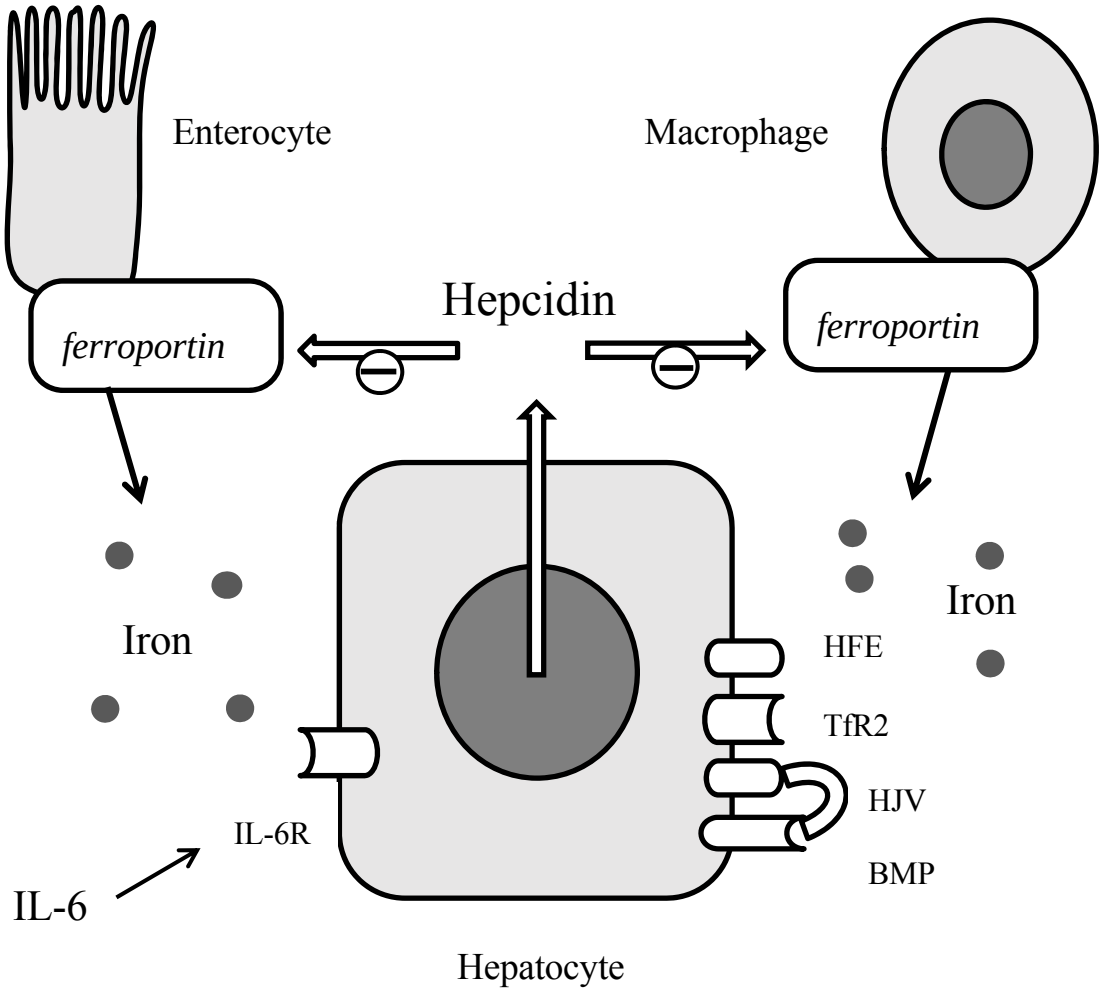

Figure 1. In normal iron homeostasis transferrin bound iron will stimulate hepcidin synthesis by interaction with HFE, TfR2 and HJV. BMP is of crucial importance to hepcidin regulation and HJV acts as a co-receptor for BMP-6. Hepcidin causes ferroportin to be internalized and thus blocks the pathway for iron transfer from enterocytes and macrophages into plasma. Hepcidin synthesis is induced by IL-6. Blood loss, anemia and hypoxia, leads to decrease in hepcidin production through erythropoetic stimuli.

1.2 IRON TOXICITY

In switching between its ferric (FeIII) and ferrous (FeII) form, iron has the ability to easily donate and accept electrons. This makes iron essential for various processes, most importantly those of oxygen transport. However, iron can also be harmful. To prevent its harmful effects, iron is bound to transferrin in the circulation and stored by ferritin. In normal conditions there are hardly any notable levels of free or labile iron. In the case of iron overload disorders, free iron catalyzes the production of highly toxic hydroxyl radicals through the Fenton and Haber-Weiss reactions.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-} \text{ (Fenton)}$$

$$Fe^{3+} + O^{2-\cdot} \rightarrow Fe^{2+} + O^2$$

Net reaction:

$$H_2O_2 + O_2^{-\cdot} \xrightarrow{Fe} OH^{\cdot} + OH^{-} + O^2 \text{ (Haber-Weiss)}$$

Figure 2. Iron catalyzes the production of hydroxyl radicals ($OH^{\cdot}$) from superoxide ($O^{2-\cdot}$) and hydrogen peroxidase (H_2O_2).

This, in turn may lead to peroxidation of organelle membrane lipids. Antioxidant defense mechanisms counterbalance this process, but as iron overload increases, they become insufficient.[10-12] In hemochromatosis massive iron overload may cause cell death and the initiation of fibrogenesis. If the excess iron is not removed there can be a progress to cirrhosis.[13]

1.3 HEREDITARY HEMOCHROMATOSIS

Hereditary hemochromatosis (HH) is the most common autosomal recessive disorder in Caucasians, affecting 1 in every 200-400 persons.[14] It may lead to enhanced iron absorption and progressive iron disposal in parenchymal organs, most notably in the liver. With time, excess iron may cause damage to parenchymal organs with an increased risk of developing diabetes mellitus, arthropathy, liver cirrhosis, and hepatocellular carcinoma.[15] HH is divided in four types. Type 1 is the HFE-related HH, also referred to as classic hereditary hemochromatosis. Type 2, also called juvenile hemochromatosis, is caused by mutations in the HJV gene (subtype A), and in the HAMP gene (subtype B). Type 3 is caused by mutations in the TfR2 gene. Type 4 is an autosomal dominant condition with heterozygous mutations in the ferroportin 1 gene.[14] These different types of HH differ greatly in phenotypic expression, but types 1-3 share inappropriate hepcidin levels as a common pathogenetic factor.[16]

Type 1 is the most common, responsible for more than 80% of cases in patients of European descent.[17] Homozygocity for the substitution of cysteine for tyrosine at amino acid position 282

(C282Y) in the HFE protein is the dominating mutation. In the H63D mutation aspartate replaces histidine at amino acid position 63 in the HFE protein.[18] The H63D mutation does not by itself cause hemochromatosis, not even in its homozygous form. In combination with the C282Y mutation, i.e. compound heterozygocity, there is a risk of iron overload. However it tends to be milder compared to that of C282Y homozygocity, and comorbid factors such as alcohol overconsumption or steatosis are probably needed for clinical disease progression.[19] C282Y heterozygocity alone is not considered to be responsible for iron overload, but there are data supportive of a protective role against iron deficiency.[20, 21] A third HFE-mutation, S65C, has been associated with mild to moderate hepatic iron overload. However, there are no studies that have associated the S65C mutation to extensive liver fibrosis.[22-25]

Treatment of hemochromatosis consists of weekly phlebotomies, removing 400-500 ml each time until S-ferritin is about 50 µg/l. When iron stores are depleted patients continue treatment with phlebotomies 2-6 times a year. The prognosis in HH is good with adequate treatment. In pre-cirrhotic, non-diabetic patients, life expectancy is normal.[26] Early detection is desirable, but screening of the general population is often argued against because of low disease penetrance. Screening of first degree relatives, especially in siblings, is usually recommended.[17, 27]

1.4 NAFLD AND NASH

Nonalcoholic fatty liver disease (NAFLD) is a common condition that defines a spectrum of alcohol-like liver disease in the absence of significant alcohol use, genetic, viral and autoimmune etiologies.[28] Within this spectrum patients with NASH (Nonalcoholic steatohepatitis) are at risk of developing progressive liver disease, cirrhosis and hepatocellular carcinoma.[29] It has also been suggested that NASH could be a leading cause of cryptogenic cirrhosis.[30, 31] Among factors that are predominantly described to be associated with NASH are insulin resistance or non-insulin dependent diabetes, obesity, and dyslipidemia.[32] Today NAFLD is generally regarded as the liver manifestation of the metabolic syndrome.[33] The term NASH was first used in 1980 to describe histopathological findings indistinguishable from those of alcoholic liver disease in obese subjects without significant alcohol use.[34] Coining of this term likely helped to put more focus on the condition in the coming years.

The common definition of NAFLD is presence of at least 5% steatosis in the liver. The histological diagnosis of NASH is dependent on multiple lesions within the liver parenchyma which has prompted the development of scoring systems. Kleiner has presented a validated histological feature scoring system addressing the lesions of NAFLD and proposed a NAFLD activity score (NAS) for use in clinical trials. This score is defined as the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3), and ballooning (0-2); thus ranging from 0-8. Fibrosis is separated from other features of activity. A NAS of 5 or more correlates with NASH, scores of 2 or less are not diagnostic of steatohepatitis. The term “borderline NASH” is used for scores of 3-4.[35] Hence, NAFLD is a clinicopathologic diagnosis, and one cannot overlook the sometimes thin line between NAFLD and alcoholic fatty liver disease. Most

studies allow for a daily intake of alcohol of less than 30 grams for men and 20 grams for women.[36] In everyday clinical practice the distinction between NASH and ASH is not always clear. Studies have shown association between moderate alcohol use and the progression of hepatic fibrosis in NAFLD. Contrary to these results, several studies have attributed light to moderate alcohol use a protective role in NAFLD.[37-39]

The prevalence of NAFLD has been estimated to 20-30% in the western world, and that of NASH to 2-3%. As obesity and diabetes in the population increases fatty liver also becomes more frequent, constituting a major health problem. However, since fatty liver is very common (in some studies up to 50% of the population) there are those who propose that the term NAFL (non-alcoholic fatty liver) should be used instead of NAFLD in many cases to avoid that such a widespread condition with a predominantly benign course will be regarded as a disease state.[40]

The pathogenesis of NASH was initially described as a two-hit process starting with the accumulation of fat in the liver. The second hit involves oxidative stress which can promote lipid peroxidation in the hepatocytes membrane causing secretion of proinflammatory cytokines (such as TNF-α and IL-6), and stellate cell activation, which results in fibrosis.[41] Insulin resistance is probably the most important factor in the development of NASH. In recent years new insights have expanded the original theory on the pathogenesis of NASH in to a multiple-hit process in which parallel events are thought to interact. This theory comprises dietary factors, gut microbiota as well as host genetics. It adds detail as well as complexity to the mechanisms underlying the progress from steatosis to steatohepatitis.[42]

1.5 INFLAMMATION AND IMMUNITY

When the body is exposed to harmful stimuli, such as pathogens, damaged cells, or irritants, it will respond with inflammation. Inflammation can be classified as either acute which occurs over seconds to days, or chronic, which occurs over longer times. Acute inflammation is the body´s initial response to harmful stimuli and is achieved by increased movement of leukocytes such as plasma cells and granulocytes from the blood into the injured tissues. This recruitment of inflammatory cells is mediated through production of chemical factors including cytokines. Prolonged, or chronic, inflammation causes a shift in the type of cells present at the site of inflammation, mainly to macrophages and lymphocytes i.e. mononuclear cells, and leads to simultaneous destruction and healing of the tissue. With intense or chronic inflammation comes the risk of scarring and organ dysfunction. Inflammation is one type of the body´s response to pathogens. It is non-specific and therefore often described as the dominating mechanism of innate immunity, as compared to adaptive immunity, which is a specific response. Innate immunity is the first line of host defense. It is rapid and has a broad impact. It includes epithelial barriers, complement protein and release of cytokines which in turn regulate the function of other cells. Innate immune responses also have the ability of recognizing molecular patterns that are shared by many microbes, for example the recognition of lipopolysaccarides by toll-like receptors. Adaptive immunity has the ability of assembling antigen-binding molecules

with specificity for individual microbial and environmental structures. It also includes immune memory. It produces long lived cells that can persist in an inactive state. Effector functions can be re-expressed rapidly when these cells encounter their antigens for a second time.[43, 44] The cells of the adaptive immune system are T and B lymphocytes. B-cells produce antibodies and can form memory cells with the ability of rapid antibody production in future encounters with the same pathogen. T-lymphocytes can be divided into subtypes according to their functions and lineage markers, such as CD4 and CD8. CD4+ cells recognize antigens through presentation of major histocompatibility complex (MHC) class II molecules and produce cytokines as helper-T-cells. CD8+ cells are activated by antigens presented by MHC class I molecules and form cytotoxic T-cells to destroy virally infected cells and tumor cells. Memory T-cells are a subset of T-cells that persist after an infection is gone, and may be either CD4+ or CD8+. Regulatory T-cells or Tregs are CD4+ cells that inhibit immune responses and prevent autoimmunity.[45]

1.6 INFLAMMATION AND CELL INJURY IN NASH

As stated above NAFLD is a clinicopathologic diagnosis, and liver biopsy is mandatory for the diagnosis of NASH. Hepatocytes are organized into plates, separated by sinusoids within the lobule. In the middle of each lobule is a central vein. Portal tracts are situated in the corners of the roughly hexagonal lobule. The inflammation in steatohepatitis is predominantly lobular, but portal inflammation may also occur. In lobular infiltrates a mix of polymorphonuclear cells and chronic inflammatory cells (including lymphocytes, monocytes, plasma cells and eosinophils) are seen. The portal infiltrates, which are not always present in adults, are composed of mononuclear cells. The inflammation in NASH may also include lipogranulomas. Hepatocellular injury may result in ballooning or acidophilic degeneration.[46]

The mechanisms of NAFLD development are not fully understood. An early model is the two-hit hypothesis presented by Day and colleagues. Accumulation of fat renders the liver susceptible to a second hit. There have been various candidates for the second hit. Since NAFLD often is described as the liver manifestation of the metabolic syndrome, insulin resistance might be the cause of both hits,[28] but as stated above, current opinion stress the probable interactions of various underlying mechanisms. There have been proposals that increased endotoxin levels could be a second hit. Obesity has been associated to gut permeability leading to increased levels of bacteria and endotoxins in portal circulation. Toll-like receptors (TLR), as part of the innate immune response, can recognize microbes as well as respond to free fatty acids and might be of importance in the pathogenesis of obesity related inflammation and insulin resistance. It has been shown that activation of TLR-4 can induce the production of pro inflammatory cytokines in macrophages and epithelial cells.[47] Much evidence supports a key role for interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) in NASH. These pro inflammatory cytokines has been found to be increased in human fat cells from patients with obesity and insulin resistance.[48, 49] Regulatory T-cells (Tregs) seem to be of importance in hepatic immune regulation. Tregs have been identified as CD4 (+), CD25 (+),

and forkhead box protein 3 positive (Foxp3 (+)). Tregs are thought to have a positive effect on tumor growth by suppressing antitumor immune cells.[50] Inadequate Treg regulation contributes to chronic hepatitis B/C virus infection and autoimmune liver disease.[51-53] Whether or not Tregs play a role in the pathogenesis of NASH had not yet been investigated at the time of our study. Tregs as well as other T-lymphocytes express lymphocyte function associated antigen-1 (LFA-1) to adhere to endothelial cells expressing inter cellular adhesion molecule-1 (ICAM-1). ICAM-1 is important for leukocyte endothelial transmigration. A positive correlation between inflammation and the levels of ICAM-1 has been shown in NAFLD.[54]

1.7 DYSMETABOLIC IRON OVERLOAD

Iron accumulation in the liver is considered to be a co-factor for progression of liver disease. Hyperferritinemia and positive liver iron stains occur frequently among patients with NAFLD. This condition was originally named "Insulin-resistance associated iron overload". Today the term dysmetabolic iron overload (DIO) is commonly used. Since oxidative stress seem to explain liver injury in relation to iron overload as well as in NASH there has been proposals stating that iron might be an important pathogenetic factor in NASH.[55] The role of the hemochromatosis mutation C282Y in relation to NASH has been investigated. George et al. found that this mutation was responsible for most of the mild iron overload found in NASH.[56] In a study by Bonkovsky et al. the prevalence of HFE-mutations (C282Y and H63D) was significantly higher in patients with NASH compared to controls. They also found that patients with C282Y mutations had more hepatic fibrosis than those without.[57] There are also contradicting studies that have failed to prove the association of HFE-mutations and NASH,[58, 59] indicating that the high prevalence of these mutations among NASH patients might be due to selection bias. Bugianesi at al. found that increased ferritin levels were a marker of severe histologic damage in NAFLD, but not of iron overload. Iron overload and HFE-mutations did not significantly contribute to hepatic fibrosis in the majority of patients with NAFLD.[60] Thus, even though the importance of HFE-mutations in the development of DIO is debated, hyperferritinemia with or without mild to moderate iron overload is still a common finding in NAFLD.[61, 62] Although it is still not known if iron reduction therapy in NAFLD-patients with iron overload can improve clinical endpoints such as fibrosis or complications of type 2 diabetes, there are studies that are supportive of such treatment due to its beneficial effects on insulin sensitivity, and observations of a tendency towards histological improvement.[63-65]

Treatment in NAFLD and NASH is aimed at improvement of metabolic control and thus the cornerstone ought to be life-style interventions (which sometimes are difficult to implement). Pharmacotherapy has not yet provided the clinician with so many options.[66] In clinical practice iron reduction therapy is often employed in patients with NAFLD and concurrent DIO.

Elevated serum ferritin seems to be a marker of histologic severity in NAFLD,[60, 67] but cannot be used for detection of patients with iron overload. Magnetic resonance imaging is a non-invasive alternative to liver biopsy in diagnosing hepatic iron overload that has proven useful,[68]

but still, the search for new markers of iron overload is important, since they would be of great use in a clinical setting.

The possible contribution of iron in disease progression in NAFLD has focused attention on the iron regulatory hormone hepcidin. Depressed hepcidin synthesis has been described in other chronic liver diseases. Alcohol may induce down-regulation of hepcidin, causing iron overload in alcoholic liver disease (ALD). Hepatitis C virus infection seems to suppress hepcidin, also causing iron retention.[69, 70] There are several studies on hepcidin in NAFLD but the results are not conclusive. Aigner et al. found increased hepatic expression of hepcidin as well as a down regulation of the iron export protein ferroportin-1 and the iron sensing molecule hemojuvelin in iron overloaded NAFLD patients.[71] Nelson et al. investigated the relationship between serum hepcidin levels, histology, including iron deposition, and HFE genotype in patients with NAFLD. They found an association between lower hepcidin levels and increased hepatocellular iron overload in patients carrying the C282Y mutation. However they also found that the HFE-genotype did not affect the physiologic up regulation of hepcidin in accordance to hepatic iron overload, thus concluding that body iron stores are the determining factor of hepcidin regulation in NAFLD.[72] There are studies, however, that come to different conclusions. Zimmermann et al. studied patients with the metabolic syndrome, with or without NASH, but no iron overload. They found higher hepcidin levels in patients compared to healthy controls. Hepcidin correlated with ferritin and lobular inflammation in all patients, and with small dense low density lipoproteins and insulin resistance index in NASH. They suggest hepcidin as a potential marker for hepatic inflammation with possible linkage to lipid and carbohydrate metabolism in NASH.[73] Barisani et al. studied iron related gene expression in DIO patients. No alternations were found as hepcidin mRNA correlated with the expression of its regulators. A significant correlation between hepcidin and indices of lipid metabolism was observed leading to speculations on interactions between hepcidin and dyslipidemia.[74] This is in line with the work by Senates et al. who studies hepcidin levels in NAFLD patients. They found hepcidin levels to be higher in patients compared to age and sex matched healthy controls. There was a significant correlation between hepcidin and total cholesterol and triglycerides, but no association to iron parameters or histology.[75] Hepcidin levels have also been studied in the case of morbidly obese patients undergoing bariatric surgery. In a study by Vuppalanchi et al. obesity was associated to hepcidin levels, but there was no correlation to NAFLD including liver histology, (However this study did not include assesement of iron stains).[76] Bekri et al found an increased expression of hepcidin mRNA in adipose tissue of obese patients. In this group the presence of diabetes or NASH did not affect hepcidin expression levels either in adipose tissue, or in the liver.[77] In summary there are different explanatory models of hepcidin regulation in DIO. Some stress the putative links to lipid metabolism and obesity which is in line with the possible involvement of pro-inflammatory cytokines in the pathogenesis in NASH, while others have found that hepcidin levels primarily reflect iron stores.

1.8 CRYPTOGENIC CIRRHOSIS

As mentioned above patients with NASH are at risk of developing progressive liver disease, cirrhosis and hepatocellular carcinoma. The term cryptogenic cirrhosis (CC) is used when no underlying etiology of the liver disease can be found, and thus is a diagnosis of exclusion. The proportion of CC, as compared to all cirrhotic patients, varies and has been estimated to between 5-30%.[78] The focus on NAFLD and NASH in recent years has evoked the question whether or not many cases of CC in fact could be "burned out NASH".[79] A more definite diagnosis can be obtained in the case of CC if a more detailed clinical evaluation is carried out prior to, or after, orthotopic liver transplantation (OLT), as has been shown in several studies.[30, 80-83] Studies have shown higher frequencies of diabetes and obesity in patients with CC compared to those with cirrhosis of known etiologies, leading to the assumption that the underlying cause is NAFLD in up to 50% in cases of CC[30, 31, 84]. However, other studies have concluded that a smaller proportion (10-20%) of CC patients have possible NAFLD as the underlying diagnosis. Instead there was a higher frequency of patients with burned out autoimmune hepatitis.[81, 83, 85] Other proposed underlying causes include unknown viral (non-A, non-B, non-C) infections, heterozygous alpha-1-antitrypsin deficiency, and alcohol abuse unapparent at the time of diagnosis. One would think that the latter could be a fairly common explanation, but studies have failed to prove alcohol abuse as a major cause in this setting.[86]

2 AIMS

The overall aims of this study were to explore the pathogenesis, histological and clinical features in patients with chronic liver disease due to steatosis and/or iron overload, with special emphasis on HFE mutations, inflammatory regulation, iron homeostasis and end-stage liver disease in the context of liver transplantation.

The specific aims of this study were

- to determine the HFE S65C frequency in a Northern European population, and to evaluate whether the S65C mutation would result in a significant hepatic iron overload or not. (Study I)
- to evaluate the presence of NAFLD in patients with cryptogenic cirrhosis evaluated for OLT, and to compare survival in OLT candidates with cryptogenic cirrhosis and those with cirrhosis of another known origin. (Study II)
- to correlate amount and type of hepatic fat to inflammation in NAFLD, and investigate if not only innate, but also adaptive immunity is involved in NASH. (Study III)
- to investigate if hepcidin levels are altered in patients with NAFLD with or without DIO compared to other patients with chronic liver disease with or without hepatic iron overload, and to see if these levels correlate to markers of inflammation, dyslipidemia and/or altered iron metabolism. (Study IV)

3 MATERIALS AND METHODS

3.1 SUBJECTS AND DATA COLLECTION

3.1.1 Study I

Patients

Patients with clinical indications of iron overload were selected from those genotyped for HFE mutations at the Division of Clinical Chemistry, Huddinge University Hospital from October 1st 1997 to September 19th 2000. All patients having: (1) serum ferritin >300 μg/l (males) or >200 μg/l (females); or (2) Tf-saturation >50% (males) or >45% (females) were included, except for those patients found by family screening or those related to another subject in the study, who were excluded. Another 17 patients were excluded who had hyperferritinemia due to acute hepatitis, acute liver failure, hepatocellular carcinoma, thyreotoxicosis, acute leukaemia, or myelodysplastic syndrome. In total, 296 patients were included in the study. Apart from HFE mutation analysis, values for serum ferritin and/or Tf-saturation, and hemoglobin count were collected retrospectively from patient files from the time of diagnosis (before any phlebotomy treatment had been initiated). In 78 cases, the exact serum ferritin value at the time of diagnosis could not be found, and in 90 patients, data on Tf-saturation were missing. Clinical data concerning iron staining of liver biopsies (if performed), and whether or not patients had undergone phlebotomy were extracted from patient files for 231 of 296 patients. Patients with hepatic iron staining of grade 1 or more or who had been treated with phlebotomies were classified as having iron overload.

In patients carrying the HFE S65C mutation, clinical data were collected from patient files. Alcohol consumption, hepatitis B and C serology, and activity levels of serum alanine aminotransferases were evaluated. In patients diagnosed as having iron overload and undergoing phlebotomies, redrawn quantities of blood were noted.

Controls

A total of 250 healthy control subjects participated in the study. None had a history of liver disease or had received multiple blood transfusions. They were recruited from hospital staff, students, and their relatives. Blood samples were collected from each subject for analysis of serum ferritin, Tf-saturation, and hemoglobin count. HFE mutation analysis was performed on all subjects.

3.1.2 Study II

A search in the computerized OLT evaluation register at the Karolinska University Hospital in Huddinge was performed in order to find adult patients evaluated for OLT between 1990 and 2004. Of the 924 evaluations found, 350 were excluded for the following reasons: (a) evaluations for re-transplantation (n = 63); (b) evaluations on non-cirrhotic patients such as

those with familial amyloidosis with polyneuropathy or acute liver failure (n= 92); (c) patients with malignant liver disease (n = 180); and (d) patients with polycystic liver disease, Caroli's syndrome or Budd–Chiari syndrome (n = 15). In patients evaluated twice or more only the initial evaluation was included. Of the remaining 574 cases, clinical data could be retrieved in 470 consecutive patients, all of whom were included in the study. Of these, 39 (8.3%) had been diagnosed as having cryptogenic cirrhosis. No patient transplanted later than December 31st 2004 was included.

In the 39 patients diagnosed with cryptogenic cirrhosis, more detailed information was obtained regarding alcohol consumption, concurrent autoimmune disease and previous response to treatment for presumed autoimmune liver disease by re-evaluation of patient files pre- and post-OLT. None of these patients reported a previous or current alcohol consumption exceeding 20 g/day in the interview protocol. Signs of autoimmunity were defined as either concurrent autoimmune disease (thyroid disease, rheumatoid arthritis, inflammatory bowel disease and vitiligo) or elevated autoantibodies and immunoglobulins. The written results from previous liver biopsies were accessible in 24 of 39 patients with cryptogenic cirrhosis.

The OLT evaluation register at the Karolinska University Hospital in Huddinge comprises prospectively registered data on all adult patients evaluated for OLT since 1989. Data has been collected from patient interviews and results of investigations performed at the time of evaluation for OLT. Data on diabetes mellitus, history of hypertension, esophageal and gastric varices, variceal bleeding episodes and hepatic encephalopathy were recorded. Child–Pugh and Model for End-Stage Liver Disease (MELD) scores had been calculated for all patients. Data also include blood and serum biochemistry (including liver tests), lipid status and viral plus immunological markers. Body mass index (BMI) at the time of OLT evaluation was corrected by a weight reduction of 5 kg if significant ascites was present. A current alcohol consumption > 20 g of ethanol per day had been recorded, and previous alcohol over-consumption (> 60 g per day) or previous healthcare for alcohol dependency had been noted. All patients had been interviewed by an experienced anesthesiologist regarding estimated weight loss in the last 12 months prior to the evaluation for OLT, and this had been documented in the protocol.

Patient survival

Survival dates were recorded until 1 January 2008. Patients not accepted for OLT were classified either as being too healthy for OLT or as having bad health and/or other diseases precluding OLT. Patients who died during the time of evaluation for OLT were recorded. For those patients who were accepted for OLT, patient survival after acceptance on the waiting list (intention-to-treat), as well as after OLT (post-transplant), was recorded.

3.1.3 Study III

A computer search for in-house liver biopsies at the Department of Gastroenterology at Karolinska University Hospital in Huddinge between April 1994 and October 2004 was

performed in order to identify patients with persistent abnormal liver biochemistries for more than 6 months, and/ or clinical signs of cirrhosis of unknown cause at the time of biopsy. Four hundred and five biopsies were found and for these cases clinical data was reviewed. Exclusions were made for patients with known alcohol use in excess of 20 g/ day. Biopsies from patients with other known liver disease such as viral hepatitis, autoimmune hepatitis, drug-induced liver disease, primary biliary cirrhosis, biliary obstruction, hemochromatosis, Wilson´s disease, and α-1-antitrypsin deficiency associated liver disease, were excluded. The remaining biopsies corresponded to 110 patients who were classified as subjects with high suspicion of NAFLD based on clinical data including ultrasound and/or the presence of hepatic fat as described in the original liver biopsy protocols. For these patients laboratory data were obtained from medical and laboratory records closest to the dates of liver biopsy, such as serum alanine aminotransfrease (ALT), aspartate aminotransfrease (AST), alkaline phosphatase, gamma-glutamyl transpeptidase (GGT), ferritin, transferrin saturation, total cholesterol, total triglyceride and glucose levels. BMI was recorded for all patients. No patient had a history of ingestion of drugs known to cause hepatic steatosis, including corticosteroids, high-dose estrogens, methotrexate, calcium channel blockers or amidorone in the previous 6 months. All biopsies were re-evaluated by a pathologist blinded to patient data, and scored according to the NAFLD Activity Score (NAS) as described by Kleiner et al.[35] (See below.) From this cohort we randomly selected 31 patients with the diagnosis of NASH. In addition, we selected 18 non-NASH patients having steatosis without inflammation (n=8), inflammation and less than 5 % steatosis (n=8), and no inflammation and less than 5% steatosis (n=2), respectively.

3.1.4 Study IV

Patients with chronic liver diseases and/or hereditary hemochromatosis with or without hyperferritinemia were prospectively enrolled in this study at the outpatient clinics at the Karolinska University Hospital between January 2008 and April 2013. A total of 85 patients were included, 38 of which had NAFLD, 18 hereditary hemochromatosis (HH), ten non-hereditary hemochromatosis (NHH), and 19 patients with various other causes of chronic liver disease (CLD). Among the 18 patients with HH, 12 were HFE C282Y homozygotes and six were C282Y/H63D compound heterozygotes. NHH was defined as the clinical phenotype of hemochromatosis (elevated serum ferritin and transferrin saturation, and hepatic iron overload) but without homozygocity for the HFE C282Y mutation or compound heterozygocity for the C282Y and H63D mutations. One patient with NHH had received oral iron substitution for several years; however, none had been treated with parenteral iron substitution or blood transfusions. In the group of 19 patients with chronic liver disease, nine had alcohol overconsumption (>30 g/day), and ten patients had other causes of liver disease (primary biliary cirrhosis, hepatitis C, cryptogenic cirrhosis, methotrexate-treated psoriasis) with alcohol consumption <30 g/d. None of the patients with NAFLD, HH or NHH had reported a previous or current alcohol consumption exceeding 20 g/day.

Hyperferritinemia was defined as a serum ferritin >350 µg/L, according to the reference value of the Karolinska University Laboratory. All patients were over 18 years of age. One patient with iron deficiency (serum ferritin <30µg/L) was excluded. No patients included had been subject to treatment with iron reduction therapy before entering the study. Liver biopsy was performed in 67 out of 85 patients. MRI was used for iron assessment in 16 cases in which histology was lacking. In 21 cases there was both histology and MRI. In two cases (one HH compound heterozygote and one with CLD) both liver histology and MRI was lacking.

The NAFLD-patients were divided into three groups: (1) those with normal ferritin and without any signs of iron overload in liver biopsy or on MRI (NAFLD-N; n=15); (2) those having elevated ferritin, but no signs of iron overload in liver biopsy or on MRI (NAFLD-FERR; n=7); and (3) those with elevated ferritin and iron overload (NAFLD-DIO; n=16). The chronic liver disease patient group (CLD) was divided into: (1) those with normal iron parameters and no signs of iron overload (CLD-N; n=8); and (2), those with signs of hepatic iron overload (CLD-IO; n=11).

Controls

A total of 40 controls, recruited from hospital staff, with normal or low (<30 µg/l) ferritin levels participated in the study. None had a history of liver disease. Of these, two were excluded (elevated transaminases in one case, and compound heterozygocity and elevated ferritin in the other). A third subject had slightly elevated ferritin (413 µg/l), but was not excluded. The remaining 38 controls were divided into two groups: (1) those with normal serum ferritin (>30 µg/L; n=25) (denoted normal iron status controls) and (2) those who were iron deficient (serum ferritin <30 µg/L; n=13) (denoted iron deficiency controls).

Biochemical data was collected at the time of enrollment in the study for patients and controls. Blood samples were collected before 10 A.M. in the morning. Subjects were not fasting but had had a light breakfast. Routine blood chemistry analyses at the Karolinska University Hospital were used.

Body mass index was calculated and HFE-mutation analysis was performed on all subjects.

3.2 ASSESSEMENT OF LIVER BIOPSIES

3.2.1 Assessment of liver biopsies in study I

Liver biopsy had been performed in seven of the 14 patients carrying the HFE S65C mutation. These biopsies were re-evaluated in order to refine the data from the written protocols in those cases where precise indications concerning iron score and fibrosis stage were lacking. Iron deposition in hepatocytes was described using the “hepatocyte iron score” (HIS) as described by Deugnier et al.[13], with the following modifications: grade 0= no stainable iron, grade 1= faint bluish color with small non-coalescent iron granules in zone 1 hepatocytes, grade 2= iron granules in the majority of zone1 hepatocytes, occasionally coalescent, grade 3= marked iron deposition with coalescent granules, and grade 4= massive

iron deposition in hepatocytes of the entire lobule. Sinusoidal cell iron deposits were described by the "sinusoidal iron score" (SIS) and scored as present =1 or absent =0. Fibrosis was staged as follows: stage 0= absent, stage 1= non-extensive portal fibrosis, stage 2= extensive portal fibrosis, stage 3= bridging fibrosis, and stage 4= cirrhosis.

3.2.2 Assessment of liver biopsies in study II

The written results from previous liver biopsies were accessible in 24 of 39 patients with cryptogenic cirrhosis. Biopsies displaying steatosis or steatohepatitis in the written protocol were re-evaluated and classified by an experienced pathologist according to Brunt et al.[87]

3.2.3 Grading of liver biopsies for NAFLD and NASH (study III and IV)

All liver biopsies were re-evaluated by an experienced pathologist blinded to clinical data. Liver histology was scored in accordance with the system developed by Kleiner and Brunt et al.[35, 88] Thus the classification was based on the basis of macro- and microvesicular steatosis, lobular inflammation, and ballooning degeneration. The stage of fibrosis was also recorded.

Degree of steatosis was graded 0-3 based on the area of the biopsy occupied by fat: (grade 0: < 5%, grade 1: 5-33%, grade 2: 34-66%, and grade 3: >67% of the area occupied by fat). Lobular inflammation was graded 0-3 based on the number foci/ 200 magnification (grade 0: none, grade 1: <2 foci, grade 2: 2-4 foci, and grade 3: >4 foci). Ballooning was graded 0-2. (0: where no ballooned cells were seen, 1: ballooned cells few or inapparent, and 2: many ballooned cells or easily noted.)

NAS was calculated as the unweighted sum of steatosis (0-3), lobular inflammation (0-3), and hepatocellular ballooning (0-2).

Patients diagnosed with NASH had a NAS-score of ≥5. In study III the term borderline NASH was used to define (eight) patients with the score of 4.

3.2.4 Determination of siderosis in study IV

Siderosis was determined for all patients semi-quantitatively on histopathologic examination of Perls' stained liver biopsy samples adapted from Deugnier et al. [89] to match available levels of magnification:

A score from 0 to 4 for iron in hepatocytes was determined: (0) granules absent or barely discernible at a magnification of 400X; (1) barely discernible granules at a magnification of 200X but easily confirmed at a magnification of 400X; (2) discrete granules at 100X magnification; (3) discrete granules easily confirmed at magnification of 40X, but barely discernible at a magnification of 20X; (4) granules obvious at a magnification of 20X, and barely visible for the naked eye. RES-iron was also determined and scored as (0) none, (1) mild, (2) or more than mild, as described by Nelson et al. [90]. In this study, these two scores were transformed into a histologic iron score (HIS) ranging from 0 to 5, comprising the score for iron in hepatocytes (0-4), plus one point for RES iron in those cases where it had been

determined as more than mild, or a half point where it has been determined as mild. Iron overload was defined as a histologic iron score of ≥1.

3.3 BIOCHEMICAL ANALYSES, MORPHOMETRY AND MAGNETIC RESONANCE IMAGING

3.3.1 Biochemical data

Unless otherwise indicated, standard laboratory routine methods at the Karolinska University Hospital, have been used.

3.3.2 Mutation analysis in study I

Human genomic DNA was extracted from peripheral blood leucocytes using Qiagen Blood and Cell Culture DNA Midi Kit (Qiagen GmbH, Hilden, Germany). In the control material, identification of mutations in the HFE gene causing the amino acid exchanges C282Y, H63D, and S65C was carried out by restriction fragment length polymorphism (RFLP), essentially as described previously.[22, 91, 92] Electrophoresis was performed on precast polyacrylamide gels (GeneGel Excel 12.5/24 Kit) using the GenePhor DNA Separation System (Pharmacia Biotech AB,Uppsala,Sweden). Bands were visualized by silver staining (PlusOne DNA Silver Staining Kit; Pharmacia Biotech AB, Uppsala, Sweden). All substitutions detected by RFLP were confirmed, either by repeating RFLP testing (C282Y) or by automatic sequence analysis (H63D, S65C). In the patient material automatic DNA sequence determination was used, corresponding to the first half of exon 2 and the whole of exon 4, using the ABI Prism Big Dye Primer Cycle Sequencing Kit on an ABI Prism 377 DNA Sequencer (PE Applied Biosystems, Norwalk, Connecticut, USA). Screening for the Y250X mutation in the TfR2 gene was performed in 44 patients by restriction enzyme digestion, according to Camaschella and colleagues.[93]

3.3.3 Serum levels of ICAM-1 in study III

Serum levels of ICAM-1 were measured with ELISA (Human sICAM-1/CD54 Immunoassay; RnD Systems, MN, USA).

3.3.4 Morphometric study of fat content in study III

Fat volume density was determined using a point counting method with a 11 x 11 grid in x 200 magnification in Nikon Eclips E800 (Nikon, Solna, Sweden) according to Weibel et al.[94] The area of fibrosis was determined with a computer software program, Image J (public domain, NIH, MD, USA). The size of the hepatocytes depends on how much fat they contain, which thereby influences the number of cells per defined area. In order to compare the number of inflammatory cells between patients with different degree of steatosis, a method was developed to correct the number of cells positive for inflammatory markers to the whole 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 50% fat the number of positive cells seen are in fact half of what we should see because of the fat

occupying 50% of the area. The formula: estimated true number of positive cells = number of positive cells counted/ (1-percentage of fat) was used in an attempt to approximate the number of cells.

3.3.5 Immunohistochemistry in study III

Paraffin-embedded liver sections from the 49 patients were stained with specific antibodies. Sections were deparaffinized with xylene and then ethanol. After rehydration, sections were blocked in 0.3–3% H_2O_2, put in unmasking solution Vector, H-3300 (Vector laboratories, Buringame, CA, USA), pH 6 and heat activated by press cooker for 10–30 min, treated with IMPRESS serum block and incubated with primary antibody overnight at 4°C. For secondary antibody IMPRESS was used. The bound antibody was revealed by addition of DAB and then counterstained with haematoxylin.

For immunostaining the following was used: IMPRESS (Vector Laboratories, Buringame, CA, USA) system, for Cleaved Caspase-3 (Asp175, rabbit a-human 1:200; Cell Signalling, Danvers, MA, USA), CD3 (DAKO, 0452, rabbit a-human, 1:1000; DAKO, Stockholm, Sweden), ICAM1 (CD54, Cell signaling 4915, rabbit a-human, 1:30), CD68 (DAKO M0814, mouse a-human, 1:1000; DAKO), and TLR4 (eBioscience 14-9917-82, mouse a-human, 1:20; eBioscience, San Diego, CA, USA).

The ICAM1-stainings were considered positive for hepatocytes when the staining covered the entire outer cell membrane of hepatocytes. The Foxp3: Standard IHC-protocol for paraffin-embedded tissue with 3% H_2O_2-blocking in methanol, unmasking solution Vector, H-3300, pH 6, heat activated by press cooker, blocking with serum, avidin and biotin, primary antibody (Foxp3 mouse a-human, Abcam ab 20034; Abcam, Cambridge, UK), concentration 10 µg /mL and incubated overnight in 4°C. For secondary antibody biotinylated horse a-mouse (BA-2001, 1:200; Vector) was used. Apoptosis was evaluated immunohistochemically using ApopTag (ApopTag Peroxidase In Situ Apoptosis Detection Kit, S7100; Chemicon International, Billerica, MA, USA). ApopTag was performed according to the manufacturer's instructions and stained with DAB, then counterstained with Hx and was calculated in three different areas; fat area /tissue (defined as none of the other), inflammatory lobular area and portal zones (PZ) (hepatocytes one or two cell rows form portal inflammation tracts).The results of immunohistochemical staining with specific antibodies were calculated in the entire section and then divided with the number of fields viewed in the microscope at magnification 40 x (1.0 mm^2).

3.3.6 Quantitative assay of hepcidin in serum samples in study IV

Freshly drawn serum samples from the 85 patients and 38 healthy controls were stored at -70°C until analysis. Samples were analyzed for hepcidin by a competitive ELISA kit (Bachem, Peninsula Laboratories, LLC, CA, United States). Reference ranges established in 83 normal subjects showed hepcidin levels that ranged 8-76 and 2-50 µg/L for men and

women, respectively (2,5-97,5 percentiles). The results were significantly different between genders.

3.3.7 Analysis of cytokines in study IV

IL-6 and TNFα were measured using Bio-plex Pro Human Cytokine Group 1 kit (Bio-rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Briefly, plasma/serum was diluted 1:4 using Bio-plex sample diluents. To obtain the nine point (including blank) standard curve, the kit standard was reconstituted and diluted fourfold. The 10x IL-6 and TNFα coupled beads was diluted in kit assay buffer and added to all standard and sample wells. The plate was incubated on shaker 30 min. After washing IL-6 and TNFα biotinylated detection antibodies were added and the plate was incubated as above. In the final step PE-conjugated Streptavidin was added and the plate was run on a Magpix instrument (Luminex Corporation, Austin TX, USA) and analyzed with xPonent software (Luminex).

3.3.8 Analysis of hepcidin mRNA in liver biopsies in study IV

Thirty-nine liver biopsies were collected from selected patients, immediately immersed in RNA-later and stored at -70°C until processed. Total RNA was retrieved from 36 of the 39 utilized liver biopsies with a dry weight of 0.3-5.9 mg using the RNAqueous -4PCR kit (Ambion PN AM1914). Recovered quantities of RNA ranged from 13-200 ng/μL. The quality and quantity of the extracted RNA was verified with the Bio-Rad Experion 700-7000 electrophoresis system, and only samples with an RQI > 8 were included in the study. cDNA synthesis was carried out with the High Capacity Reverse Transcriptase Kit (Applied Biosystems), using 65-930 ng of total RNA per sample.

Quantative analysis of liver mRNA: Determination of specific mRNA levels was performed on the 7500 Fast Real Time PCR System (Life Technologies), utilizing three endogenous controls (GAPDH, Cyclophilin and HPRT) and a reference sample (liver RNA from a patient not eligible for this study). Two different primer pairs were used for the hepcidin analyses (denoted Hepcidin Harvard and Hepcidin Saku). All cDNA samples were diluted 1:5, and utilizing a sample volume of 2 μL, each sample was run in triplicates with power SYBR Green PCR master mix (Life Technologies PN 4367659) and 0.1 μM of each primer. Relative expression levels were calculated by the ΔΔCt method, utilizing the 7500 Software v.2.0.6. The threshold was set to 0.2, utilizing a defined baseline between 3 and 13 cycles for all PCR primer sets. The efficiency was set to 90 % for Cyclophilin and GAPDH, 110 % for HPRT and 100 % for both the hepcidin primer pairs. Replicates with a S.D. >0.5 were omitted from the analysis. The efficiency of the PCR reactions were evaluated by using a 6-fold dilution series of the reference sample, and was found to vary between 90-110%.

3.3.9 Magnetic resonance imaging in study IV

Magnetic resonance (MRI) imaging was used for detection and quantification of liver iron overload in 35 patients and correlated to histology in 17 of these. Liver iron was assessed

semi-quantitatively as has been described by Gandon et al.[95] (Their calculation algorithm is available at http://www.radio.univ-rennes1.fr/Sources/EN/HemoCalc15.html).

In the correlation analyses of serum hepcidin to liver iron content, MRI iron was approximated to histologic liver iron (HIS) score as follows: <60 µmol/g iron = HIS 0; 60-100 µmol/g = HIS 1; 101-150 µmol/g = HIS 2; 151-200 µmol/g = HIS 3; 201-250 µmol/g = HIS 4; >250 µmol/g iron = HIS 5.

3.4 STATISTICAL ANALYSIS

Student's t-test for unpaired data was used for comparing two groups assuming a normal distribution. The Mann-Whitney test was used when comparing non-parametric data between two groups. Numerical values of laboratory parameters were analyzed using one-way ANOVA and validated for equal variance and normal distribution. Kruskal-Wallis ANOVA was used when the assumptions of normal distribution did not hold. Results were presented as mean ± SEM or mean and range. All p-values were presented as two-tailed.

The relationship between two categorical variables was examined with Chi2-test or Fisher's exact test (when applicable). Pearson´s correlation or simple linear regression was used for correlations.

In study II, patient and graft survival after acceptance for OLT (intention-to-treat survival) and after liver transplantation (post-transplant survival) were assessed by Cox regression analysis with a score test to calculate the hazard ratios, and Kaplan–Meier survival plots.

In study IV, the correlation between two numerical variables was analyzed with simple linear regression validated for linearity, variance between observations and for normal distribution. In the cases where the assumptions did not hold the Spearman's rank order correlation was used instead. Multiple linear regression was used for variables that were significantly correlated in the simple linear regression in study IV.

A p-value < 0.05 was considered statistically significant.

3.5 ETHICAL APPROVAL

Study I was approved by the ethics committee at Huddinge University Hospital. Studies II-IV, were approved at the ethics committee at Karolinska University Hospital.

4 RESULTS

4.1 STUDY I

The HFE S65C mutation was found in 14 patients and eight controls. In controls, the S65C allele frequency was 1.6%. The S65C allele frequency was enriched in non-C282Y non-H63D chromosomes from patients (4.9%) compared with controls (1.9%) ($p<0.05$).

Table 1. Number of patients and controls with the C282Y and H63D mutations, and allele frequencies of the S65C mutation in alleles without the amino acid substitution C282Y or H63D.

C282Y	H63D	No of patients (n=296)	No of controls (n=250)	S65C alleles (patients)	S65C alleles (controls)
+/+	-/-	84	1	-	-
-/-	+/+	7	7	-	-
+/-	+/-	21	2	-	-
+/-	-/-	30	27	2/30 (0.067)	1/27 (0.037)
-/-	+/-	52	41	3/52 (0.058)	1/41 (0.024)
-/-	-/-	102	172	9/204 (0.044)	6/344 (0.017)
				14/286 (0.049)**	8/412 (0.019)**

**p=0.0449 (Fisher´s exact test)

Serum ferritin was significantly increased in controls carrying the S65C mutation compared with those without HFE mutations. Fifty per cent of controls and relatives having the S65C mutation had elevated serum ferritin levels or transferrin saturation. The number of iron overloaded patients was significantly higher among those having HFE S65C compared with those without any HFE mutation. Half of patients carrying the S65C mutation (7/14) had evidence of mild or moderate hepatic iron overload but no signs of extensive fibrosis in liver biopsies.

Table 2. Genotype, biochemical iron parameters, and clinical data in unrelated patients carrying the S65C variant.

Pat No.	Sex	Genotype	Age (y)	Ferritin (µg/l)	TS (%)	Diagnosis
1	M	C282Y/S65C	24	238	77	Mild iron overload
2	F	C282Y/S65C	60	324	48	Healthy, surveillance
3	F	H63D/S65C	72	265	55	Healthy, surveillance
4	M	H63D/S65C	49	536	31	Mild iron overload, AAT def.
5	M	H63D/S65C	68	1463	53	Moderate iron overload, diabetes mellitus
6	F	S65C/N	55	251	41	Mild iron overload
7	M	S65C/N	64	566	44	Mild iron overload
8	F	S65C/N	63	205	26	Mild iron overload
9	F	S65C/N	72	364	41	Steatosis
10	M	S65C/N	58	621	28	NASH, mild iron overload
11	F	S65C/N	71	211	56	Ferritin normalized
12	M	S65C/N	64	537	26	Diabetes, hypertension, angina pectoris
13	M	S65C/N	69	690	48	Emphysema. Alcohol consumption 40-60 g/d
14	F	S65C/N	47	50	50	Healthy

TS, transferring saturation; NASH, non-alcoholic steatohepatitis

Screening of relatives revealed one S65C homozygote that had no signs of iron overload. Compound heterozygosity with S65C and C282Y or H63D did not significantly increase the risk of iron overload compared with S65C heterozygosity alone.

4.2 STUDY II

Seventeen (44%) of the cryptogenic patients had NAFLD in a prior liver biopsy and/or clinical features of the metabolic syndrome. Two patients had occult alcohol over consumption and one patient had burnt-out AIH.

Cryptogenic patients had significantly higher frequencies of diabetes, ascites, and hyponatremia. There was no difference in BMI, however weight loss the last 12 months was significantly higher among patients with cryptogenic cirrhosis.

Table 3. Clinical data of patients with cryptogenic cirrhosis and of those having cirrhosis of other etiologies evaluated for liver transplantation. (data are expressed as mean ± SD or as number of patients with percentages in parentheses)

	Cryptogenic cirrhosis (n=39)	Cirrhosis of other etiologies (n=431)	*p*
Females	21/39 (54)	171/431 (40)	NS
Age at time of evaluation (y)	49 ± 9	49 ± 11	NS
BMI (kg/m^2)	24.5 ± 4.8	24.6 ± 4.1	NS
Self-reported weight loss in last year (kg)	10.1 ± 13.8 (11.9% of BW)	4.3 ± 6.5 (5.5% of BW)	< 0.01
Diabetes	10/39 (26)	50/431 (11.6)	< 0.05
Ascites	33/37 (89)	279/411 (68)	< 0.01
Sodium (135-145 µmol/l)	134 ± 6	136 ± 6	< 0.05

BW, bodyweight; NS, not significant

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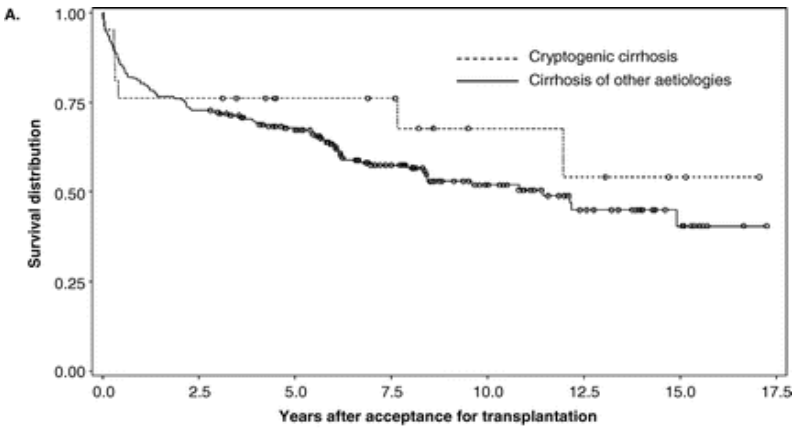
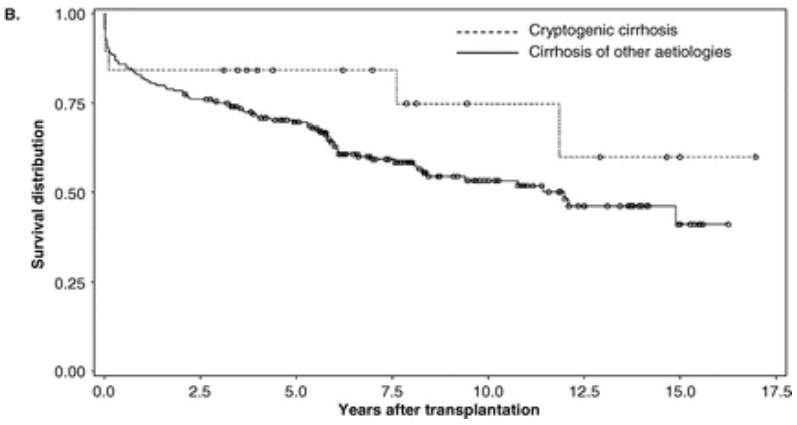
Patient survival was similar between cryptogenic patients and cirrhotics with a known etiology.



(A) Kaplan–Meier survival plot demonstrating survival after acceptance for OLT in patients with cryptogenic cirrhosis (n = 21) and those with cirrhosis of other aetiologies (n = 223) (Cox regression analysis). There is no statistically significant difference between groups (hazard ratio 0.71, p = 0.37).



(B) Survival after OLT in cryptogenic cirrhosis (n = 19) versus cirrhosis of other aetiologies (n = 202) (hazard ratio 0.55, p = 0.18).

Figure 3. Patient survival

4.3 STUDY III

Scoring of the amount of fat both by estimation according to NAS-classification and by using a morphometric method showed discrepancies that could be attributed to the presence of microvesicular fat. Microvesicular fat was increased in high NAS patients and also correlated with the total volume of fat. ICAM-1 positive hepatocytes were seen in NASH and were absent in non-NASH patients. In addition, ICAM-1 positive hepatocytes were localized to areas with microvesicular fat. The sICAM-1 was significantly higher in NASH-patients than in non-NASH patients.

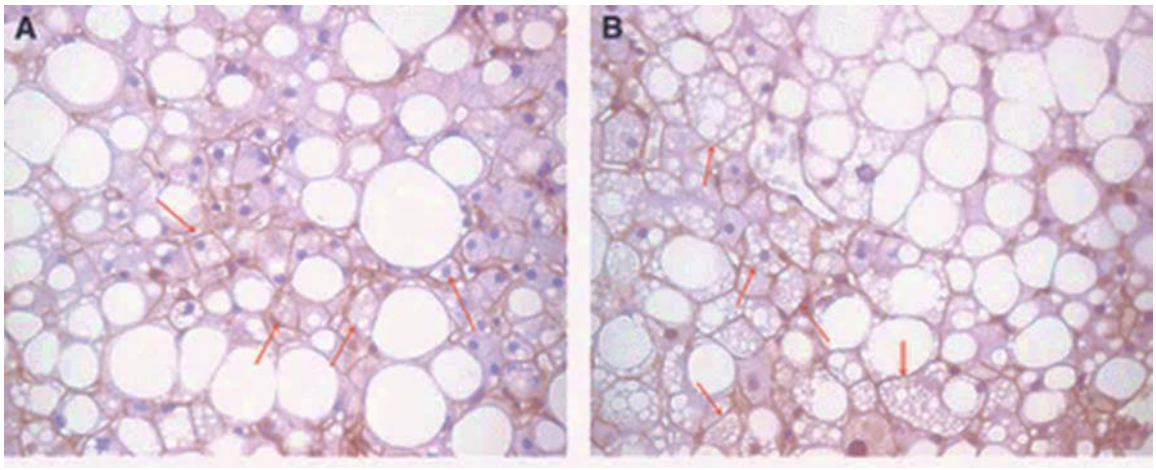
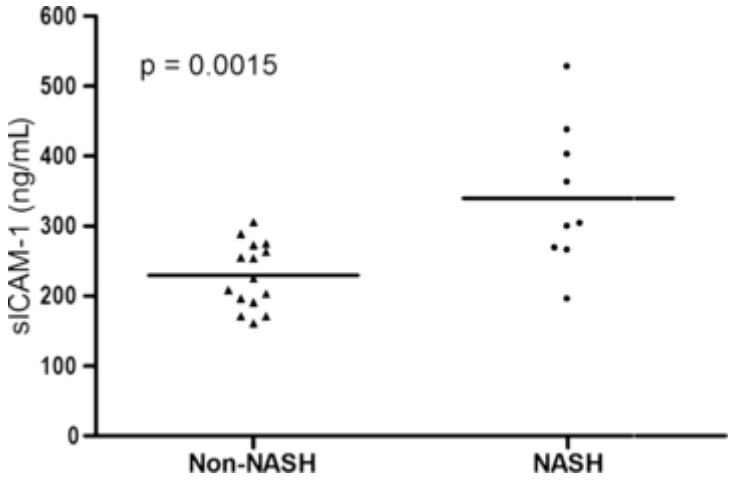


Figure 4. Immunohistochemical stainings of ICAM-1 (A and B). Positive staining was found around hepatocytes in areas of microvesicular fat in biopsies from NASH-patients.

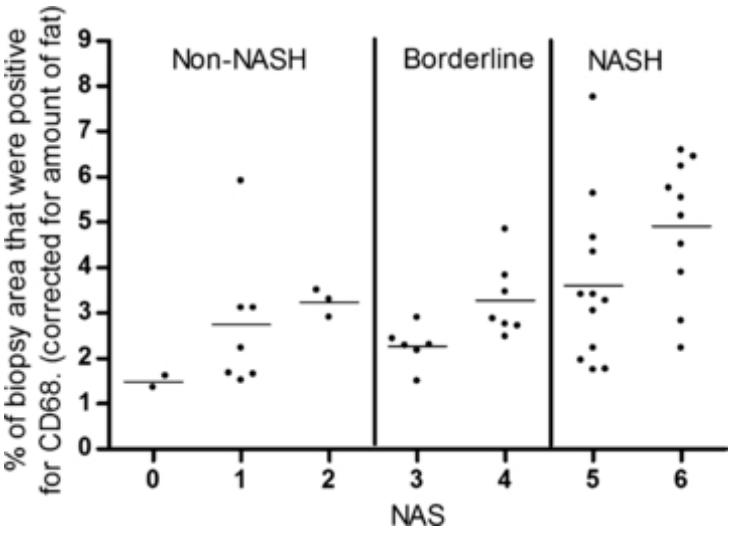


Serum levels of sICAM-1 in patients with NASH and non-NASH (controls). Patients with NASH had significantly higher serum levels of sICAM-1 than non-NASH subjects, p = 0.0015.

Figure 5.

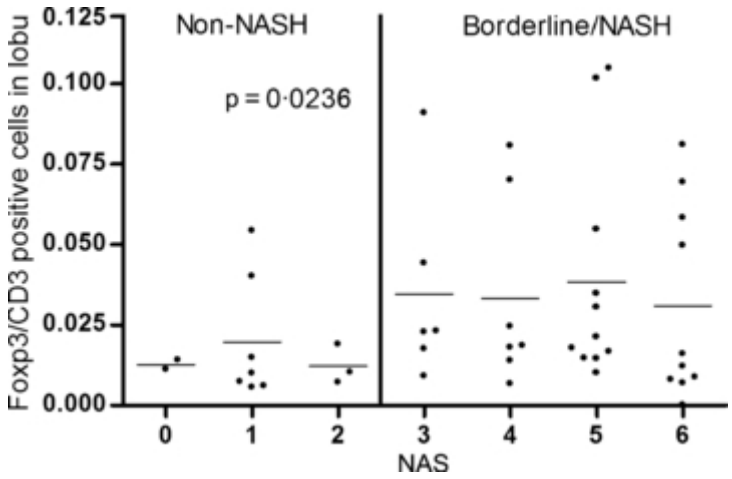
Patients with NASH had larger areas of CD68 positive cells when corrected for the amount of fat. NASH-patients also displayed a higher amount of Foxp3 positive cells, than non-NASH

patients. The quota of Foxp3/CD3 positive cells differed significantly between NASH/ borderline-NASH-patients and non-NASH patients (p=0.0236).



The area of CD68 positive cells in biopsies corrected for the amount of fat in the tissue. Results are grouped according to NAS results. NASH-patients did have significantly larger area of CD68 positive cells than non-NASH and Borderline NASH, p = 0.0011.

Figure 6.



The ratio of Foxp3/CD positive cells in the lobule from IHC, biopsies grouped according to NAS results. There was a significant difference in the quota between non-NASH and borderline/NASH-patients, p = 0.0236.

Figure 7.

4.4 STUDY IV

Serum hepcidin values for the different patient groups and controls are shown in Figure 8.

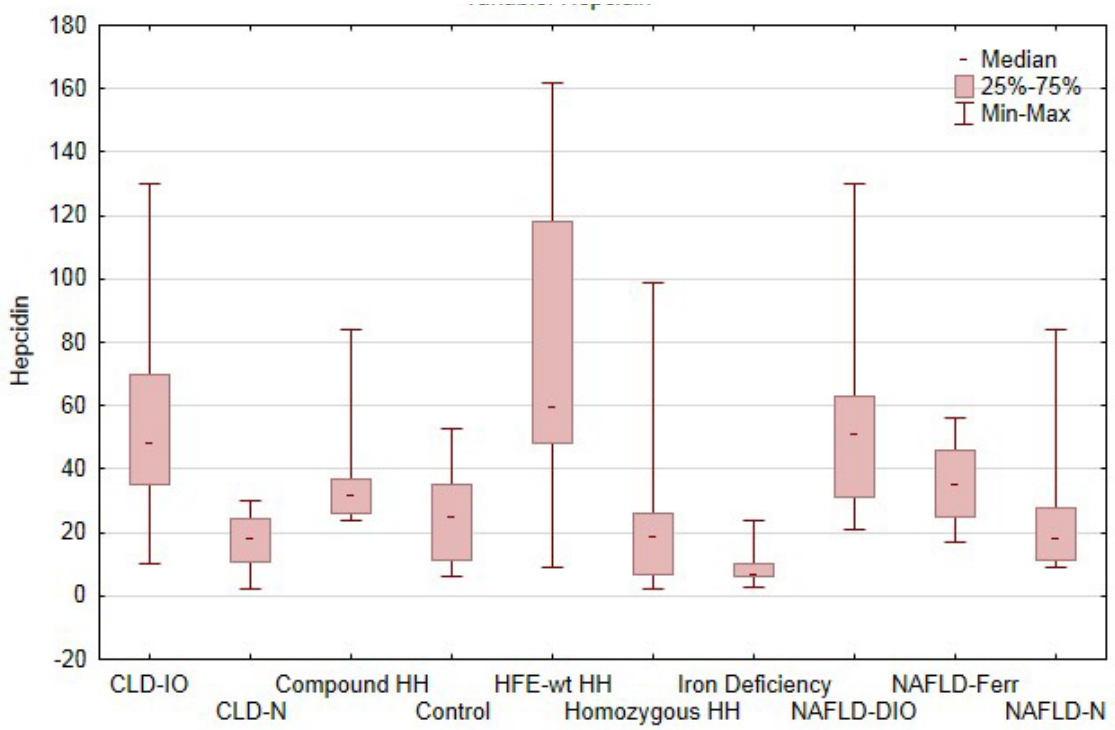


Figure 8. Serum hepcidin levels in the different patient groups. The box plots show the median, the interquartile range and the min-max values. Hepcidin levels were significantly increased in non-hereditary HH (in the graph denoted as HFE-wt HH), and for NAFLD-DIO, compared with iron deficiency controls, NAFLD-N, homozygous HH and chronic liver disease with normal iron stores (CLD-N) (Kruskal-Wallis ANOVA, $p<0.05$).

Simple linear regression showed a good correlation between histologic iron score and hepatic iron content determined by MR ($r^2=0.77$, $p<0.01$). There was also a good correlation between serum hepcidin and hepcidin mRNA ($r^2=0.39$, $p< 0.01$).

Hepcidin levels were increased in patients with iron overload (except for patients with HH), including NAFLD-DIO, and correlated to liver iron stores. Ratios between hepcidin and iron score is shown in Figure 9. NAFLD-FERR had significantly higher ratios compared to other groups.

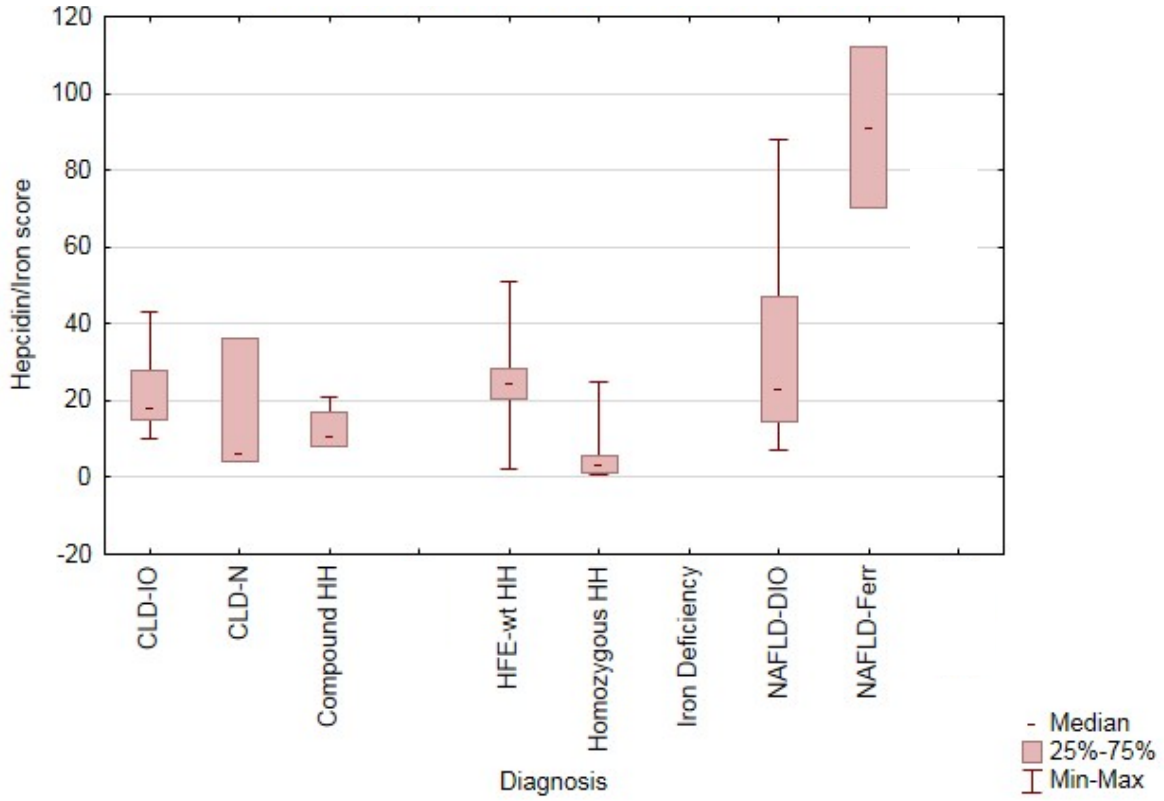


Figure 9. The ratios between serum hepcidin and hepatic iron contents ("iron score"). The calculation of iron scores are described in Methods. Patients with a hepatic iron score of 0 (NAFLD-N) are excluded from the ratio calculation. Patients with homozygous HH had significantly lower ratios, and NAFLD-FERR significantly increased ratios, compared with the other groups.(Kruskal-Wallis ANOVA, $p<0.05$). (NHH is denoted as HFE-wt HH in the graph).

In NAFLD, hepcidin correlated to serum ferritin ($r^2=0.20$, $p< 0.01$) and liver iron ($r^2=0.27$, $p< 0.05$) but not to BMI, CRP, NAS or steatosis. Patients with NAFLD-DIO had significantly higher transferrin saturation (0.40±0.08) than NAFLD-FERR (0.25±0.10), $p< 0.05$. The hepcidin-to-liver iron ratio was highest in NAFLD-FERR, and there was a trend towards increased inflammatory markers in NAFLD-FERR. Serum hepcidin correlated inversely with serum leptin in NAFLD patients ($r^2=0.14$, $p< 0.05$). There was a trend towards increased portal inflammation in NAFLD-FERR, but without statistical significance. Steatosis, lobular inflammation, ballooning, fibrosis and NAS-score did not differ between groups. Clinical and laboratory findings in patients with NAFLD are demonstrated in Table 4.

Table 4. Clinical and laboratory findings in patients with NAFLD and dysmetabolic iron overload (DIO), elevated serum ferritin but normal iron stores (NAFLD-FERR), and normal serum ferritin (NAFLD-N), respectively (mean ± S.D.)

	NAFLD-DIO (n=16)	**NAFLD-FERR** (n=7)	**NAFLD-N** (n=15)
BMI (kg/m^2)	28.1±2.4	29.4±2.7	31.4±5.0
Serum ferritin (µg/L)	816±285*	621±170	156±78
Serum hepcidin (µg/L)	53±28*	37±13	24±19
Ratio hepcidin/ferritin	0.07±0.04*	0.06±0.04*	0.19±0.19
Transferrin saturation (%)	0.39±0.09*#	0.25±0.10	0.27±0.07
Liver iron score	2.13±0.92*#	0.14±0.24	0.03±0.13
	n=13:	**n=6:**	**n=11:**
Triglycerides (mmol/L)	1.95±0.90	1.83±1.09	2.89±1.09
Cholesterol (mmol/L)	5.25±0.71	5.25±0.84	5.18±0.96
	n=13:	**n=6:**	**n=9:**
Leptin (µg/L)	15.8±8.65	21.2±17.0	16.2±9.09

*=$p<0.05$(vs. NAFLD-N)

#=$p<0.05$(vs. NAFLD-FERR)

5 GENERAL DISCUSSION

The rapid global increase of obesity and type 2 diabetes mellitus is paralleled by an increase in NAFLD, thus representing a challenge to general practitioners as well as hepatologists. The state of knowledge has improved substantially over the years, and what was considered a completely benign condition a couple of decades ago, is now recognized as a major health problem. Why some patients with NAFLD progress to NASH with the risk of developing end stage cirrhosis and hepatocellular carcinoma, while others do not is still not fully understood. Dysmetabolic iron overload is common in NAFLD, and iron overload as a potential pathogenic factor has attracted much interest in this diagnosis as well as in chronic liver disease in general.

The HFE gene was identified in 1996, and homozygocity for the HFE C282Y mutation accounts for approximately 90% of hereditary hemochromatosis (HH) in Sweden.[96, 97] Compound heterozygocity for C282Y/H63D is much less common and is also associated to less severe iron overload compared to C282Y homozygocity.[98, 99] Also, a third mutation in the HFE gene, S65C was found a few years later. At the time of our study (Study I), it´s clinical importance was still controversial. We found that the S65C allele was enriched in non-C282Y and non-H63D chromosomes from patients with clinical signs of iron overload compared to healthy controls. These findings were in line with results reported in a French study by Mura et al.[22] In control subjects we could also confirm the HFE mutation frequencies from other studies in subjects of Northern European ancestry.[20, 22, 100] When we studied patients carrying the S65C mutation in detail we found that half of them had signs of mild to moderate hepatic iron overload, but no signs of extensive fibrosis. When investigating the relatives of one patient we found a S65C homozygous subject (the patient's mother) who had signs of iron deficiency, probably due to menstrual blood loss. The patient's brother who carried the same genotype: C282Y/S65C, had normal ferritin and only slightly elevated transferrin saturation. We concluded that S65C is likely to constitute a negligible risk for iron associated liver cirrhosis. And as stated above, there are no studies that have associated the S65C mutation to extensive liver fibrosis. In retrospect, 4 out of 14 patients in our study had either NAFLD or diabetes. A potential role for the H63D mutation in NAFLD pathogenesis has been suggested by Nelson et al.[72], if such a link could be attributed to the S65C mutation is this far entirely hypothetical. However, the question sheds light on the complexity of chronic liver disease where multiple factors probably are likely to interact in the disease process.

The finding of the iron regulatory hormone hepcidin helped clarify the context of iron overload in several conditions, for instance, the hepcidin deficiency in hereditary hemochromatosis, and the impaired synthesis in chronic liver disease such as alcoholic liver disease. The role of hepcidin in the case of NAFLD and DIO seems more complicated. As described above, there are studies that conclude that the hepcidin regulation in NAFLD is normal, while others stress the putative link to fat and inflammation. This discrepancy is not completely surprising when recapitulating the topic of hepcidin regulation. There are four

pathways controlling the hepcidin production in hepatic cells: First the plasma iron regulation pathway involves circulating transferrin-bound iron that will compete with HFE in binding to transferrin receptor 1 (TfR 1), which promotes hepcidin production via the formation of the TfR 2/HFE complex. Secondly, in the erythropoetic pathway, blood loss, anemia and hypoxia will lead to erythropoetic stimuli and a subsequent decrease in hepcidin production, thus making more iron available. Thirdly, the inflammatory regulation pathway is mainly induced by IL-6 and leads to hepcidin excess, causing anemia. Finally, the control of HJV on the BMP/SMAD signaling pathway has been described as mandatory in hepcidin regulation.[101, 102]

In our study (Study IV) we studied hepcidin levels, inflammatory markers and lipid profiles in patients with various liver diseases and ferritin levels, focusing on NAFLD. The advantage of this approach is that we can compare several carefully defined subgroups. A disadvantage is the low number of subjects in some of the groups (i.e. NAFLD-FERR). We investigated serum hepcidin levels in 85 patients with NAFLD, hemochromatosis and other chronic liver diseases, and in 36 of these we correlated serum hepcidin to mRNA in liver tissues. We found a good correlation between hepatic hepcidin mRNA and serum hepcidin measured by ELISA. Among the 23 NAFLD patients having elevated ferritin in our study, the majority (16 patients) had DIO, whereas seven patients were found to have elevated ferritin but normal iron stores. In all NAFLD patients, hepcidin levels correlated strongly to iron indices such as serum ferritin and transferrin iron saturation, as well as to hepatic iron contents. The hepcidin-to-hepatic iron score ratio was significantly increased in patients with elevated ferritin and normal iron stores (NAFLD-FERR). One may speculate that the increased hepcidin levels in these patients are a result of a low-grade chronic inflammation, simultaneously increasing serum ferritin levels, and there was a trend towards increased TNFα and IL-6 levels among these patients. Thus, a subgroup of NAFLD patients may have a low-grade inflammation contributing to increased serum ferritin and hepcidin levels, which would then not lead to hepatic iron accumulation and DIO. This is paralleled by the observation of a higher fraction of portal inflammation in biopsies from NAFLD-FERR patients. Unfortunately, the number of patients in this “inflammatory-NAFLD” group is too small in the present study to draw firm conclusions regarding the contribution of low-grade inflammation to hyperferritinemia and elevated hepcidin in NAFLD. Our study was not designed to discriminate between NAFLD-DIO and “inflammatory-NAFLD”, but rather to study hepcidin levels in NAFLD patients compared with patients having other liver diseases, with or without iron overload. Thus, further studies are needed on a larger NAFLD cohort to explore the hypothesis of the existence of two NAFLD subgroups: “NAFLD-DIO” and “inflammatory NAFLD with hyperferritinemia”. When calculating the hepcidin levels in relation to the hepatic iron content, i.e. the ratio between hepcidin and liver iron score, patients with DIO had a similar ratio as patients with other chronic liver diseases with iron overload, that is patients with other chronic liver disease and iron overload and non-hereditary hemochromatosis, indicating that hepcidin synthesis in DIO is regulated similarly as in other chronic liver diseases.

We found that transferrin saturation was significantly higher in NAFLD patients with DIO compared to those with increased ferritin and normal iron stores. This result indicates that transferrin saturation might be used as a marker to differentiate between these two patient groups. Such a readily available marker could be of good use in clinical practice, since dysmetabolic iron overload may need treatment with phlebotomies, which is not the case in "inflammatory NAFLD with hyperferritinemia".

In our study, we found no correlation of serum hepcidin with BMI, serum cholesterol, serum triglycerides, hepatic steatosis, or NASH activity score (NAS). As mentioned previously there are studies that have reached different results. The studies by Barisani et al, and Senates et al. found correlations between hepcidin and cholesterol and triglycerides suggesting interactions with lipid metabolism.[74, 75] In studies on morbidly obese patients, obesity was associated to hepcidin levels, but these studies have not found correlations to NAFLD including histology or diabetes.[76, 77] The NAFLD patients in our study had only slightly increased BMI as compared to patients with other liver diseases, and the situation in morbidly obese subjects with markedly increased body fat may prove different. The majority of our NAFLD patients had either iron overload or a possible "inflammatory NAFLD with hyperferritinemia", which are conditions that strongly induce hepcidin synthesis, which could mask a weaker association between hepcidin and cholesterol or triglycerides. There is a tight relationship between iron deficiency and obesity. Proinflammatory cytokines such as IL-6 are secreted by the adipose tissue and can induce hepcidin expression. Furthermore there is a production of hepcidin in adipose tissue, although it is not clear if this could represent a significant proportion.[9, 103] Interestingly, we found an inverse correlation between serum hepcidin and leptin, which is in conflict to other studies performed on obese subjects. Again, the situation in our patient cohort differs from those findings, since our patients have only slightly elevated BMI but all had significant hepatic steatosis and the majority elevated serum ferritin. Our finding has to be confirmed by others in the same context; i.e. NAFLD with liver disease but only moderate overweight.

HFE mutations have been described as being more common in patients with NASH. In the present study, we could not find an increased frequency of C282Y or H63D mutations in NAFLD patients with dysmetabolic iron overload as compared to patients with other liver diseases, or healthy controls. However, the H63D mutation was enriched in NAFLD patients with normal iron stores. This finding indicated that this mutation may play a role in hepatic steatosis, as has been mentioned above.

The "two hit" hypothesis has for long been used as the explanatory model in the pathogenesis of NASH. Insulin resistance plays a central role in the "first hit" leading to hepatic steatosis. The "second hit" involves oxidative stress, which in turn leads to the development of steatohepatitis and fibrosis. The close connection to the metabolic syndrome has led to the proposal that insulin resistance could be the cause of both hits. The simplicity of this model is obviously appealing, but it has been expanded into the "multiple parallel hits" hypothesis in order to take a number of different processes that might contribute to liver inflammation

into consideration. Among these are inflammatory mediators derived from adipose tissue and the gut, as well as immune system activation. A more detailed model may also encompass such observations as inflammation preceding steatosis in certain cases.[47, 104]

The NASH activity score (NAS) was introduced in 2005 by Kleiner et al. to be used in studies on NAFLD and NASH. The score is based on the unweighted sum of three parameters: hepatic fat content, lobular inflammation and ballooning. A score of ≥5 correlates with the diagnosis of NASH[35]. NAS is widely used, but there are those who argue that NAS omits crucial information. For example it takes no account of fibrosis, which has been addressed in studies of NAS in the context of disease progression and mortality in NAFLD.[105, 106] In addition, Younossi et al. performed a study in which the original pathologic criteria for NAFLD subtypes (as had been described by himself and colleagues in 1998[107]) demonstrated the best predictability for liver related mortality in this patient group.[108] Moreover the term NAFLD covers a broad spectrum of liver disease. The majority are patients with simple steatosis. Some will develop steatohepatitis, and these are in turn at risk for progression with fibrosis. In this perspective NAS might seem blunt, since a patient with pronounced steatosis and very mild inflammation can receive the same score as a patient with only mild steatosis and inflammation and/ or signs of necroinflammatory activity i.e. ballooning.

In order to furhter characterize the importance of fat and inflammatory cell distribution in the liver parenchyma we performed a study on 49 patients with the clinical diagnosis of NAFLD (Study III). Our goal was to investigate if the type and amount of fat is of importance to the inflammatory process in NASH, and we also wanted to investigate if both the innate and adaptive immunity is involved in NASH. The amount of fat was scored both by estimation according to NAS-classification and calculated with a morphometric method as described in the methods section. We found that these two values differed in some patients and concluded that this discrepancy could be attributed to the presence of microvesicular fat. In NAFLD macrovesicular fat is more predominant, but microvesicular fat has been correlated to higher NAS as well as more advanced fibrosis.[109] This is interesting, since our study showed that ICAM-1 positive hepatocytes were located in areas of microvesicular fat deposits. Therefore one could speculate that the presence of microvesicular fat may represent a more severe form of NASH. Moreover NASH-patients had higher levels of ICAM-1 in serum compared to patients with borderline NASH and non-NASH patients. Thus sICAM-1 might be interesting in the quest for non-invasive diagnostic tools in the case of NASH. We found an increase in the number of Foxp3 positive cells in NASH patients, and higher Foxp3/ CD3 ratio correlated to higher NAS. Since Foxp3 is the most specific marker of regulatory T cells (Tregs), this finding supports the involvement of adaptive immunity in NASH. Tregs are involved in the negative control of various immune responses, such as viral hepatitis and hepatocellular carcinoma. The finding of more Tregs and less CD3 cells in NASH-patients could indicate that CD3 cells are diminished by Tregs in order to decrease inflammation. (CD3 is used as a general T-cell marker). CD68 is a useful marker of cells of the macrophage lineage. When the area of CD68 positive cells in biopsies was corrected for the amount of fat we found

higher values in NASH-patients in comparison to non-NASH and borderline-NASH patients, indicating involvement of innate immunity. However, in our study, we did not find any difference in TLR-4 positive cells between groups, which would contradict the idea of gut microbiota as an important pathogenic factor in NASH.

Cleaved Caspase-3 and ApopTag was used for detection of apoptosis, and did not differ between groups. Thus, in our study, apoptosis could not be found to be driving inflammation.

With chronic hepatic inflammation comes the risk of progressive fibrosis and cirrhosis. In some cases the cause of the inflammatory process is unclear. Among patients with liver cirrhosis, the percentage of those having cryptogenic cirrhosis (CC), i.e., cirrhosis of unknown etiology, varies, but has been estimated to be 5-30%. However, CC is diagnosed in only 5-7% of patients undergoing orthotopic liver transplantation (OLT) due to cirrhosis.[81] This discrepancy could be explained by a more thorough work-up at the evaluation of patients for OLT, or it could indicate that the lack of a more specific diagnosis is a disadvantage in end stage liver disease. It is hardly surprising that the focus on NASH over the two last decades has prompted the question if this condition could be the underlying cause in many cases of CC. Histopathological findings are crucial for the diagnosis of NASH. Consequently, there might be an underestimation of cases, since features of NASH may disappear during the development of cirrhosis.[110] Studies on NAFLD as the likely underlying cause in CC have shown varying results, and there is also a difference between American and European studies, in which the latter have shown lower frequencies of suspected NAFLD.[30, 31, 81, 83-85]

In our study (Study II) we addressed these questions by comparing 39 CC patients to 431 patients with cirrhosis of other (known) etiologies who had been evaluated for OLT between 1990 and 2004. We wanted to estimate the frequency of possible NAFLD in a Swedish material, not the least due to the geographical variation in previous studies. We also wanted to compare the severity of liver disease and patient survival in OLT candidates in order to find out if the diagnosis of CC could be a disadvantage in this setting.

In our material re-evaluation of clinical data led us to reach an underlying diagnosis in 51% of CC patients. Seventeen (44%) of these patients were considered to have possible underlying NAFLD. Conditions associated to NAFLD such as type 2 diabetes or a history of obesity was encountered in an additional seven patients. Only one patient was found with probable autoimmune hepatitis, and two patients with occult alcohol abuse. Consequently, our findings are in line with the results from American studies.[30, 31, 80, 81, 84-86, 111, 112] The reason for the discrepancies between studies are not known, but possible explanations may be differences in patient populations, diagnostic work-ups, and whether or not patients were investigated in an OLT setting.

When comparing CC patients to patients with cirrhosis of known etiologies we found increased frequencies of ascites, hyponatremia and reported weight loss in the CC group. These findings suggest that CC patients have a more advanced liver disease at the time of

referral for OLT. However, we lack stronger evidence in this case, since there was no difference in Child-Pugh or MELD-scores between the groups.

Malnutrition is an independent risk factor for poor survival after OLT.[113] The patients with CC in our study were not malnourished and their BMI was similar to other patients with liver cirrhosis of known etiology. However, weight loss during the year before evaluation for OLT was significantly higher in the CC group. This is possibly a sign of deterioration of the liver disease in these patients and may signal a need for OLT evaluation.

When comparing the acceptance rate for transplantation there were no differences between CC patients and patients with cirrhosis of known etiology. We could observe a tendency towards a higher degree of rejections for OLT due to poor health and/ or concurrent disease in the CC group, but the difference was not statistically significant. Nor did the comparison of survival after being accepted for OLT or after OLT show any difference between the groups. When looking on at the subgroup of CC patients with possible NAFLD, the survival results were similar. Our results are in line with many other authors that have concluded that CC, as well as NASH-cirrhosis are favorable indications for OLT.[82, 85, 114, 115]

6 CONCLUSIONS

In Study I, the frequency of the HFE S65C mutation in a Northern European population was 1.6%, and it was found to have the potential of causing mild to moderate hepatic iron overload, but not extensive liver fibrosis.

Re-evaluation of data in patients with cryptogenic cirrhosis (CC) from the time of evaluation for OLT, in study II, resulted in a probable underlying etiology in more than half of patients, NAFLD being the most common diagnosis (44%). In spite of a tendency toward more severe liver disease at the time of evaluation for OLT, the survival after OLT for patients with CC did not differ from that of patients with liver cirrhosis of known etiology.

In study III, the amount of microvesicular fat increases with NAS. In liver tissue from NASH-patients, hepatocytes with microvesicular steatosis express ICAM-1. The increased number of CD68 cells and regulatory T-cells seen in liver tissue from NASH-patient indicate that there is an involvement of both innate and adaptive immunity.

In study IV, hepcidin correlates to iron indices and iron stores, but not to BMI, steatosis, or NAS, in NAFLD patients, with or without mild to moderate iron overload. Hepcidin regulation in NAFLD did not differ from that seen in other chronic liver diseases apart from hereditary hemochromatosis.

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8 POPULÄRVETENSKAPLIG SAMMANFATTNING

I denna doktorsavhandling undersöks olika aspekter av fett- och järnöverskott i levern hos patienter med kronisk leversjukdom.

Det första arbetet handlar om betydelsen av en då relativt nyupptäckt mutation, dvs. förändring i ett arvsanlag. I detta fall handlar det om en förändring i den så kallade HFE-genen. Mutationer i HFE-genen är den vanligaste förklaringen till den ärftliga formen av järnupplagringssjukdomen hemokromatos, på våra breddgrader. Sedan tidigare vet vi att de flesta fall av ärftlig hemokromatos beror på dubbel uppsättning av C282Y mutationen i HFE-genen. En variant med C282Y mutationen och H63D mutationen i kombination, kan också förekomma. I denna avhandling har vi tittat närmare på en tredje mutation i HFE-genen, nämligen S65C. Studien utfördes på patienter med tecken till järnöverskott. Friska kontroller fanns med som jämförelsematerial. Vi kom fram till att S65C mutationen var ungefär lika vanlig i befolkningen i Stockholmsregionen som i andra jämförbara delar av världen (såsom USA och Västeuropa). Vi kunde också se att de patienter som var bärare av S65C-mutationen kunde ha ett lätt till måttligt järnöverskott i levern. Däremot fann vi inte någon betydande bindvävsomvandling i leverbiopsier (vävnadsprov) från dessa patienter. Studien talar således för att bärarskap av S65C mutationen kan vara en förklaring till lätt till måttligt järnöverskott i levern, men att risken för mer avancerad leversjukdom pga. sådant bärarskap torde vara försumbar.

Den andra studien fokuserar på patienter med skrumplever av oklar anledning, så kallad kryptogen levercirros. Tanken bakom denna studie var bland annat att ta reda på om icke-alkoholorsakad fettleversjukdom kan vara den bakomliggande orsaken i fall av kryptogen levercirros. Studien omfattar noggrann genomgång av de protokoll som används i samband med att patienter med skrumplever med olika bakomliggande orsaker utreds för eventuell levertransplantation. Upprinnelsen till denna frågeställning kommer av att icke-alkoholorsakad fettleversjukdom är ett tillstånd som rönt mycket stort intresse på senare år. Även i Sverige används vanligen den engelska terminologin i detta fall. Man talar om NAFLD (Non Alcoholic Fatty Liver Disease) och NASH (Non Alcoholic SteatoHepatitis). NASH är en underdiagnos till NAFLD. Att fettlever är vanligt har man vetat sedan lång tid tillbaka, emellertid betraktades icke alkoholorsakad fettleversjukdom som ett i stort sett ofarligt tillstånd så sent som på 1980-talet. Senare forskning har visat att patienter med NASH löper risk att utveckla levercirros och levercancer. När vi gick igenom journaler och protokoll från patienter med kryptogen levercirros kom vi fram till att det fanns en sannolik bakomliggande orsak i hälften av fallen. Den orsak, eller diagnos, som var allra vanligast var just NAFLD: 17 av totalt 39 patienter. Två andra orsaker var så kallad autoimmun hepatit (1 patient) och alkoholmissbruk som var okänt vid tiden för levertransplantationsutredningen (2 patienter). Vidare ville vi ta reda på om det kan vara en nackdel att sakna en specifik diagnos, dvs. att läkarna inte vet varför en patient har levercirros. Vi jämförde de 39 patienterna som vid levertransplantationsutredningen hade diagnosen kryptogen levercirros med alla andra levercirrospatienter med kända diagnoser (exempelvis virala hepatiter, alkoholleversjukdom,

autoimmuna leversjukdomar). Vi noterade att patienter med kryptogen leversjukdom var lite sämre i sin leversjukdom vid tiden för utredning jämfört med de andra, men trots detta blev de inte levertransplanterade i lägre omfattning. Överlevnaden efter levertransplantationen visade sig vara lika god för patienter med kryptogen levercirros som för patienter med känd orsak till levercirros. När vi tittade närmare på den andel av patienter med kryptogen levercirros som sannolikt hade NAFLD som bakomliggande orsak fick vi samma resultat. En skillnad mellan patienter med kryptogen levercirros och de andra var hur mycket patienterna hade gått ned i vikt under det senaste året. Patienter med kryptogen levercirros hade gått ned mer i vikt. Detta skulle kunna vara ett tecken på en försämring i leversjukdomen och kan vara en anledning för läkare att överväga en levertransplantationsutredning.

I den tredje studien tittade vi närmare på mängden och typen av fett i leverbiopsier från patienter med NAFLD för att ta reda på om detta har betydelse för inflammation och levercellskada hos dessa patienter. Vi ville också ta reda på om den så kallade adaptiva immuniteten och inte bara den ospecifika immuniteten är involverad vid NASH. Med adaptiv immunitet avses den del av immunförsvaret som har förmågan att känna igen och även minnas det som är främmande på en detaljerad nivå. Vi jämförde patienter med NASH-diagnos, patienter som låg på gränsen till att ha NASH och andra patienter som hade fett och inflammation av olika grad. Vi kunde se att NASH-patienterna hade mer mikrovesikulärt, dvs. findroppigt fett jämfört med de andra patienterna. Den inflammatoriska markören ICAM-1 var ökad hos NASH-patienterna och lokaliserad till områden med mikrovesikulärt fett. Genom att bland annat mäta markörer för så kallade regulatoriska T-celler kom vi fram till att både adaptiv och ospecifik immunitet torde vara av betydelse vid NASH.

Den fjärde studien handlar om det järnreglerande hormonet hepcidin och dess betydelse vid NAFLD. Vid så kallad HFE-relaterad hemokromatos, som beskrivits ovan, produceras inte tillräckligt med hepcidin. Hepcidin motverkar upptag av järn från tarmen. Således riskerar patienter med hemokromatos att med tiden drabbas av ett potentiellt skadligt järnöverskott. Vi vet också att hepcidin påverkas av inflammation och att hepcidin inte bara produceras i levern, utan även i fettväven. Järnöverskott i levern är vanligt förekommande hos patienter med NAFLD. Även om järnöverskottet i detta fall oftast är mildare än hos hemokromatospatienter skulle det kunna ha betydelse för sjukdomsutvecklingen hos vissa NAFLD-patienter. Vi ville därför undersöka hur hepcidin-nivåer i blod och levervävnad hos NAFLD-patienter avspeglar järnöverskott i levern, inflammation, samt blodfetter och övervikt. Vi jämförde NAFLD-patienter med och utan järnöverkott i levern med andra leversjuka patienter och hemokromatospatienter. Vår studie visade att hepcidinregleringen hos NAFLD-patienter inte skilde sig från regleringen hos andra patienter, (förutom de med HFE-relaterad hemokromatos, vilket var förväntat). Vi kunde inte se att hepcidinnivåerna hos NAFLD patienterna påverkades av övervikt eller blodfetter, vilket vissa andra studier kommit fram till.

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