This thesis reports on novel insights in pathophysiology and non-invasive diagnostic modalities in non-alcoholic fatty liver disease (NAFLD). A specific subgroup of patients with NAFLD suffers from inflammation and/or fibrosis, also called non-alcoholic steatohepatitis (NASH). NASH may now be recognized as an important clinical entity due to its increasing incidence and the potential evolution of NASH to cirrhosis. In addition, NASH is associated diseases of the cardiovascular and metabolic systems.

Murine and human models helped in unraveling underlying pathophysiological mechanisms and specific key elements have been indicated. However, these have not yet been successfully transposed into diagnostic (bio)markers identifying patients with NAFLD at risk to develop NASH and associated metabolic disease. The non-invasive imaging techniques currently available for quantifying hepatic steatosis lack the ability to discriminate between simple fatty liver and inflammation. The individual contributions relevant to pathophysiology and non-invasive diagnostic tools described in this thesis will be summarized, discussed and put into perspective.

Global epidemic and consequences *Section 1*

Chapter 1: Overview of NAFLD

The phenomenon of excessive triglyceride accumulation in the liver in the absence of alcohol abuse has been described since the early sixties. Due to the global epidemic of obesity the number of patients with NAFLD is excessive, resulting in a significant burden on health care systems. In this first chapter an extensive overview of clinical characteristics, pathophysiology, imaging techniques and therapeutic opportunities is provided. Understanding the underlying pathophysiological mechanisms, involving genetic, environmental and biochemical factors, lays the foundation for the development of new, more potent, (non-)invasive diagnostic tools. Subsequently, targeted therapies can be explored and eventually applied.

NAFLD, diabetes and pathophysiology *Section 2*

Chapter 2: NAFLD, chronic hepatitis C and insulin resistance

Chapter 2 presents the results of an observational prospective study on patients with chronic hepatitis C (HCV). During treatment with either standard therapy comprised of pegylated interferon with ribavirin or with trial medication including amantadine and high dose interferon followed by standard therapy, 9/189 patients developed hyperglycaemia; in three patients insulin resistance (IR) suggested T2DM and in 5 positive autoantibodies were detected suggesting T1DM. Since symptoms of hyperglycaemia resemble side-effects of interferon, the first conclusion and recommendation is to assess glucose levels on a regular basis during treatment with interferon.

The second conclusion is the development of IR in the presence of HCV. Apparently, IR occurs following an inflammatory response in the liver to HCV infection. The viral core protein causes a profound impairment in insulin signaling at the level of the insulin receptor substrate tyrosine phosphorylation and phosphatidol inositol-3 kinase activation. This hypothesis is supported by a clear causal relationship between HCV and the presence of IR while after successful antiviral treatment IR improved. A negative impact of IR on the efficacy of standard interferon-containing

treatment has even been described. However, the exact underlying mechanism(s) still remain to be elucidated and might be valuable to better understand pathophysiology of IR in NAFLD. Based on these data, we developed an investigator initiated randomized controlled trial for HCV-infected patients with IR who were then randomized for a 12 week pretreatment with placebo or pioglitazone (PPAR-γ-agonist) followed by standard antiviral treatment (HEPAR-study). The rationale was to improve treatment efficacy by first ameliorating IR (through effects by pioglitazone) and then (re)treating patients for HCV with antiviral treatment. Liver biopsies and ^{1}H-MRS were planned. Unfortunately, due to upcoming new protease inhibitors and other competitive trials the enrollment process failed and this study was prematurely terminated.

Chapter 3: NAFLD is related to non-alcoholic fatty pancreas disease (NAFPD)

Obesity and IR cause fatty infiltration of many organs, including pancreas (pancreatic steatosis) and liver (NAFLD). Since pancreatic tissue is difficult to obtain, a post mortem study revising liver and pancreatic histology in 80 patients was performed. The first conclusion is the clear correlation between interlobular, total pancreatic fat and the NAFLD Activity Score (NAS). The presence of intralobular pancreatic fat was related to NASH. Obesity (and the concomitant IR) seems to be an independent predictor for the presence of pancreatic fat. Thereby the suggested pathophysiological hypothesis of both conditions seems to be equal. Firstly, IR results in peripheral lipolysis, thereby increasing the portal influx of fatty acids and tissue triglyceride accumulation. Secondly, experimental and human studies have shown adipose tissue to be more active than was believed in the past. Visceral fat is now specifically known to play a key role in the metabolic dysfunctions that occur as a consequence of obesity. It produces adipokines, chemokines, and cytokines, which are collectively referred to as adipocytokines. In humans, obesity leads to an increase in leptin and a decrease in adiponectin production, which results in monocyte and macrophage infiltration and the secretion of proinflammatory cytokines (IL-6 and TNF-α). And thirdly, oxidative stress, lipid peroxidation and production of reactive oxygen species lead to a further deterioration of the inflammatory and fibrogenic state of pancreas and liver. However, this "multi-hit" hypothesis has been questioned and some authors suggest direct detrimental effects of fatty acids on metabolic activity of hepatocytes and pancreatic cells. The term non-alcoholic fatty pancreatic disease (NAFPD) can be justified. However, in which manner NAFLD and NAFPD interact and whether one condition precedes the other remains unclear.

Chapter 4: Impaired hepatic FGF19 response in NAFLD

Fibroblast growth factor 19 (FGF19) is a novel regulating protein of hepatic lipid homeostasis. It appears to influence both hepatic fatty acid oxidation (through repression of acetyl-CoA carboxylase 2) and hepatic lipid synthesis (through suppression of insulin-induced fatty acid synthesis) in isolated hepatocytes and murine models. Hypothetically, it could play a role in the development of NAFLD. This chapter describes the postprandial FGF19, triglycerides, bile salts (BS) and plasma marker for hepatic bile salt synthesis (7α-hydroxy-4-cholesten-3-one (C4)) responses in subjects with NAFLD with IR and without IR. Fasting FGF19 levels and intestinal production of FGF19 were equal between both groups. However, in those patients with IR an anticipated decline in bile salt synthesis (read: decline in C4) after a postprandial FGF19 increase was not observed. The postprandial FGF19 levels in both NAFLD subgroups were similar at all individual time points; it suggests that the hepatic response to FGF19 is impaired in IR. When taking similar FGF19 responses and different BS excursions into consideration, speculating on

impaired hepatic BS uptake is tempting. As mentioned before pro-inflammatory cytokines are released from visceral adipose tissue and have been implicated in the development of hepatic IR. Interleukin (IL) 1β and IL-6 are known to reduce the expression of the sodium-dependent receptor of BS uptake (NTCP). In this study, IL-6 may have contributed to the altered postprandial BS excursions. However, several lines of evidence rule out that diminished hepatic uptake of BS is the cause of the absent decline in BS synthesis.

Another possible explanation is that the expression of the FGF19 hepatic receptor (FGFR4) and/or the obligate signaling co-factor βKlotho is altered. FGFR4 expression was significantly decreased in fasting and in diabetic mice and reversed after insulin administration. Other suggestions are altered signal transduction at the level of the plasma membrane or intracellular relay of the FGF19 signal. There is evidence for a possible cross-talk between FGF19 and insulin; FGF19 was recently shown to reduce insulin-stimulated fatty acid synthesis and lipogenic gene expression in hepatocytes. It seems that FGF19 partly signals through the insulin-activated PI3K pathway. However, whether such interference is mutual, and whether it is maintained in the insulin-resistant state is, as yet, unknown.

Chapter 5: Hyperferritinaemia in NAFLD en T2DM

This cross-sectional study demonstrates that there are no relations between correlates of the metabolic syndrome, hyperferritinaemia and the presence of NAFLD in type 2 diabetic patients treated in a non-academic teaching hospital. Unfortunately this study has some weaknesses; for instance the absence of liver biopsies for examination of histology. Recently, the cut off values of liver enzymes (such as ALT) are under debate. Some authors have suggested adjustments to lower references to identify those patients with chronic liver disease at risk for developing fibrosis. In studies focusing on healthy, normal weight subjects the 95th percentile of normal ALT levels were lower than assessed in historical cohorts (for male < 34 IU/mL and female < 28 IU/mL). In this study, ALT levels > 28 IU/ml were accepted as increased. Despite this, a significant relation between ferritin and the presence of NAFLD could not be demonstrated. However, results from other recent studies have discussed hyperferritinaemia in NAFLD. As possible causes release from damaged hepatocytes, association with hereditary hemochromatosis and hepatic iron overload have been proposed. However, these have (also) all been rejected.

Other research has pointed out the changes in up-and downregulation of genes involved in fatty acid and iron metabolism during disease progression. The more severe the liver disease the more iron metabolism related genes were up-regulated and serum ferritin increased. Others studied the sole iron exporter located in enterocytes, ferroportin-1 (FP-1). Negative correlations between TNF-α-concentrations, BMI and FP-1 expression could be demonstrated. A clinical study from the United States' NASH Clinical Research Network proved increased serum ferritin to be associated with a diagnosis of NASH, and worsened histological activity and is an independent predictor of advanced hepatic fibrosis among patients with NAFLD.

Novel markers indicating presence of inflammation, fibrosis, oxidative stress or hepatocyte apoptosis alone or combined in panels are under research. Potential markers are ferritin (inflammation), fibroblast growth factor 15/19 (regulating protein in lipid homeostasis), hyaluronic acid (fibrosis), lipid peroxidation products (such as gluthatione peroxidase (oxidative stress)) and cytokeratine 18 (hepatocyte apoptosis). An ideal biomarker or a combination of these

in diagnostic panels (e.g. European Liver Fibrosis) should be helpful in monitoring progression of NAFLD over time, its response in therapeutic trials and in establishing the prognosis. However, more research is needed since none of the commercially available markers are fulfilling these criteria.

Chapter 6: Hepatic unsaturated fatty acids in NAFLD assessed by 3.0 T ^{1}H-MRS

[1]H-Magnetic resonance spectroscopy (MRS) is a promising imaging modality capable of assessing hepatic steatosis by measuring the nuclear chemical shifts generated by hydrogen atoms of fatty acid chains in a magnetic field. This chapter describes the feasibility of using 3.0 Tesla MRS in patients with well-defined NAFLD. Using amplified magnetic fields higher spectral resolution is achieved resulting in more detailed information on different fatty acid components. Two ratios are calculated; ratio 1 representing hepatic unsaturated fatty acids (UFA) (at 5.4 ppm) and ratio 2 representing total hepatic fat (hepatic triglyceride content (HTGC)). The total hepatic UFA was well correlated with features consistent with the presence of NAFLD (e.g. HOMA-IR (indicator for severity of insulin resistance) and HTGC). Even, in T2DM patients a significant higher total hepatic UFA was measured. Assessing (poly)unsaturated fatty acids (PUFA) is feasible using 3.0 Tesla MRS in patients with NAFLD. The increased hepatic UFA in T2DM patients can be explained by a decreased ω-3/ω-6 ratio with a substantial hepatic accumulation of ω-6 PUFA. These finding are in agreement with previously obtained biochemical data from gas chromatography. However, a major limitation of this study is the absence of liver histology.

Ω-6 PUFA is notorious for pro-inflammatory effects and might be therefore a potential factor in developing NASH. In animal models imitating NASH, ω-6 PUFA is increased and positively correlated with the amount of lobular inflammation. Intervention trials studying the effect of administrating ω-3 fish oil to patients with NASH are ongoing.

Chapter 7: Morbidly obese patients undergoing gastric bypass surgery: assessment with open-system ^{1}H-MRS

Given the obesity epidemic increasingly more patients are not suitable for fitting in a standard MR scanner due to the bore's limited aperture of 55-60 centimeters. This study focused on the use of open bore MRS in morbidly obese patients (BMI > 35 kg/m^2) undergoing gastric bypass surgery. In 38 patients MRS revealed an accuracy of 89%, correlated well with hepatic fat quantification by liver histology (r=0.85, p<0.001) and offered adequate discrimination between all grades of hepatic steatosis. Unfortunately, liver biopsies after surgery could not be performed. Another limitation is the possible discrepancy between the location of liver biopsy versus the MRS voxel position. However, this technique remains useful in quantifying and grading hepatic steatosis in more obese patients. Whether increased magnetic fields will result in more specific discrimination of various fatty acids is unclear.

Future perspectives

All in all, the ongoing obesity epidemic necessitates the development of non-invasive tools in order to stratify those patients at risk for NASH and related medical conditions. Since the "golden

standard", liver biopsy, is liable to sampling error and complications (bleeding, bile leakage and discomfort) and is impractical for application in large cohort screening studies, new reliable non-invasive tools are needed.

Recent reports, including research reported in this thesis, have pointed out the feasibility of MRS for adequate quantification of hepatic steatosis. To date however, these techniques lack the ability to discriminate patients at risk for developing cirrhosis.
Future studies will need to explore the natural history of NAFLD, the interplay between hepatic steatosis and fibrosis. Whether MR techniques can be used as non-invasive tools to study disease progression by proton-density fat fractioning (e.g. ω-3 versus ω-6 UFA) and consecutive changes in fat distribution, needs to be investigated.
Positive emission tomography (PET) scanning is a nuclear medicine imaging technique used in many areas of medicine (oncology, neuroimaging and cardiology). After administration of a positron-emitting radionuclide, the system detects the emitted γ-rays and reconstructs a three-dimensional image or picture of functional processes in the body. Depending on the radionuclide used different aspects can be depicted. The most often used nuclide is fluorodeoxyglucose (FDG), a glucose-analogue. Only few studies using FDG-PET could demonstrate a minor difference in hepatic uptake between patients with NAFLD and controls. Therefore it seems that with FDG-PET one cannot select patients with steatohepatitis. One recent study using FEDAC, a TSPO radioligand (a mitochondrial transmembrane protein modulating mitochondrial function), has clearly shown an increased TSPO expression in NAFLD and a this closely correlates with the extent of disease severity.
Whether newer techniques in scanning or the use of other radionuclides (e.g. FEDAC) will give better results needs further research.

Recently, more genetic, transcriptomic, proteomic, metabolomic and lipidomic studies have been performed in order to understand the pathogenesis of NAFLD and NASH. A genome-wide association study (GWAS) in large patient populations by Romeo and coworkers hase resulted in a step forward. This study demonstrated a very strong association between hepatic fat content, inflammation, and a variant (rs738409) of the PNPLA3 gene. The specific effect of this gene variant remains vague and more studies are currently underway aiming to understand the role of this gene in lipid metabolism. However, it has been associated with a wide spectrum of phenotypes seen in NAFLD and NASH.
Other analyses using highly advanced transcriptomic, proteomic, metabolomic and lipidomic technologies also made great progress in identifying pathways involved in the pathogenesis of the disease as well as potential new diagnostic markers. Examples are the over-expression of keratin sulfate proteoglycan in patients with progressive features of NASH and the reduced expression of fatty acid binding protein-1 in NASH compared with simple steatosis. Another study identified five protein markers that correlate with NAFLD associated fibrosis and HCC. When taking lipidomic studies into consideration, peroxisomal polyunsaturated fatty acid (PUFA) metabolism and free radical mediated linoleic acid oxidation have been associated with NAFLD and its progression. An association between bile salts and glutathione metabolites has been demonstrated in plasma metabolomic studies.
The data collected from all these different studies suggest involvement of multiple metabolic and pathogenesis pathways resulting in the development of NASH.

For the next years, key elements for the direction of future research may be the identification of the linkage between all these significant genes, transcripts, lipids, metabolites and proteins and to finally determine the key pathophysiological pathways involved in the development of hepatic

steatosis, inflammation and fibrosis, transition to NASH, and progression to liver cirrhosis and HCC.

Eventually, this field of study combined with genetic information and results of (new) non-invasive imaging tools (such as ^{1}H-MRS) can be used to personalize drug administration and response monitoring as well as nutritional strategies for early prevention. In order to accomplish these goals worldwide initiatives on collaboration between basic scientists and clinical physicians (hepatologists and endocrinologists) have been funded (NASH Clinical Research Network (NASH-CRN) in the United States and Fatty Liver: Inhibition of Progression (FLIP) in Europe). Results will be announced in the next decades on different international forums and will give opportunities to constrain this threatening epidemic.