GENERAL INTRODUCTION

HUMAN NASH

Prevalence and risk factors

Nonalcoholic fatty liver disease (NAFLD) is emerging as one of the most common chronic liver diseases during the last decades (1). In US, a dramatic increase in patients with NAFLD has been observed from 30% in 2008 to 46% in 2011 (2) and the prevalence of NAFLD in Asian countries is alarmingly high with 17% in China, 32% in India, 18% in Japan, 32% in Malaysia and 55% in Indonesia (3, 4). Depending on the study population and the diagnosis approach used, the prevalence of NAFLD is estimated between 20% and 40% in the general population (1, 5, 6).

NAFLD is strongly associated with metabolic comorbidities, such as obesity, diabetes mellitus, and dyslipidemia (1). Most recent studies report that NASH has a prevalence of 2-3% in the lean non-Asian population (7) and the prevalence markedly increases in obese (up to 20%) and morbidly obese subjects (up to 50%) (8, 9). However, Asian people with a low body mass index or BMI (~23.5) but higher proportion body fat already has an increased risk for NAFLD. Recent reports have shown that 15%-21% of Asian patients with NAFLD are non-obese (4), suggesting that the other risk factors or the distribution of body fat may be important for NAFLD development as well. Globally, there is a 60% to 76% prevalence of NAFLD in diabetic patients and 22% of those further develop NASH (2, 3). Histopathological evaluation showed that patients with diabetic NAFLD have more severe inflammation than non-diabetic NAFLD patients and are susceptible for rapid progression to fibrosis (8, 9). Furthermore, dyslipidemia is also associated with an increased prevalence to develop NAFLD, for instance in patients with the metabolic syndrome (10). In 2002, the National Institute of Health (NIH) reported that the presence of dyslipidemia (hypertriglyceridemia, hypercholesterolemia, or both), was associated with NAFLD in 20% to 80% of the cases (11). A South Korean study in nonobese, nondiabetic adults showed that insulin resistance and triglyceride levels are independently associated with NAFLD (12).

Diagnosis

Most persons with NAFLD are asymptomatic and the disease is usually discovered when laboratory examination shows abnormal and persistently high levels of liver enzymes, that is alanine aminotransferase (ALT) and aspartate aminotransferase (AST), during screening. However, ALT and AST are also frequently elevated in obese subjects, and it is very difficult to define the upper limits of ‘normal’ for these routine liver enzymes tests (13). Alternatively, NAFLD with bland steatosis can be diagnosed by non-invasive imaging techniques, such as ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI), however, imaging approaches are unable to detect inflammation, a required criteria for NASH diagnosis. Currently, liver biopsy is still the ‘golden standard’ for NASH diagnosis in the clinic and there is an urgent need for alternative diagnostic tools. The procedure of patient selection for biopsy-based diagnosis is illustrated in **Figure 1**. However, since liver biopsy is expensive and invasive and clearly cannot be employed for population screening, accurate noninvasive tests would be of tremendous benefit. The

apoptosis marker CK-18 has been reported recently to be a most promising noninvasive diagnostic test for NASH. In 2006, Wieckpwski et al (14) found that measurement of caspase-cleaved CK-18 levels showed a specificity of 99.9%, and a sensitivity of 85.7% for the diagnosis of NASH. Further validation studies are still needed to clarify the cut-off value for NASH detection when a commercial test for CK-18 would be employed in clinical settings.

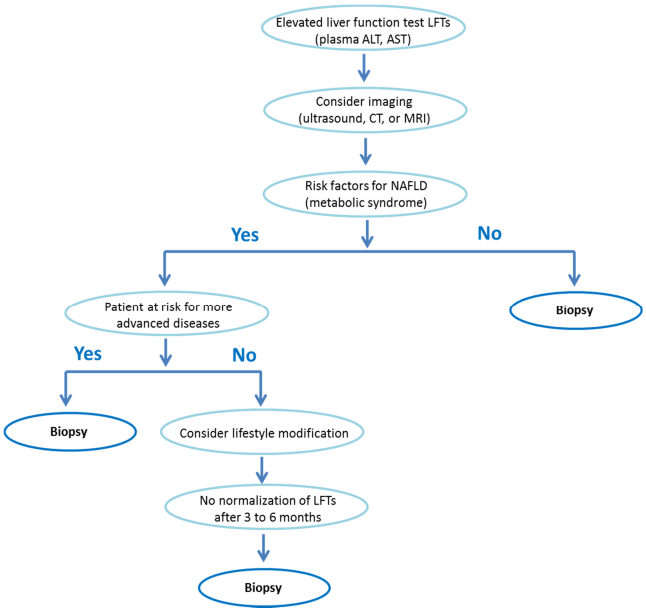

Figure 1: Selection algorithm for assigning patient to biopsy-based diagnosis. Copyright 2009, 2010 The Cleveland Clinic Foundation. All rights reserved.

Histopathology

Histopathologic features

The hallmarks of NASH are steatosis and inflammation (**Figure 2**). Steatosis is always a component of NAFLD and is characterized as an accumulation of intrahepatic lipids in the form of triglycerides and cholesterylesters within hepatocytes (15). Using hematoxylin and eosin (HE) staining or specific stainings for lipids identification such as Oil Red O, intrahepatic fat accumulation can be observed in different forms, namely: 1) macrovesicular steatosis: large fat droplets fill the cytoplasm of hepatocytes, displacing the remaining contents of the cell and the nucleus to the periphery, or 2) microvesicular steatosis: small fat droplets accumulate around the nucleus of the hepatocytes (16). Lobular inflammation is a defining characteristic of NASH livers and foci of inflammatory cell infiltrates, which consist of Kupffer cells, monocytes, and polymorphonuclear leukocytes, are typically detected. Hepatocellular injury is another important feature of NASH and is usually identified as cellular ballooning. Hepatocellular ballooning refers to enlarged hepatocytes, with rarefied and reticulated-like cytoplasm. The ballooned hepatocytes are predominantly located in the neighborhood of steatotic hepatocytes.

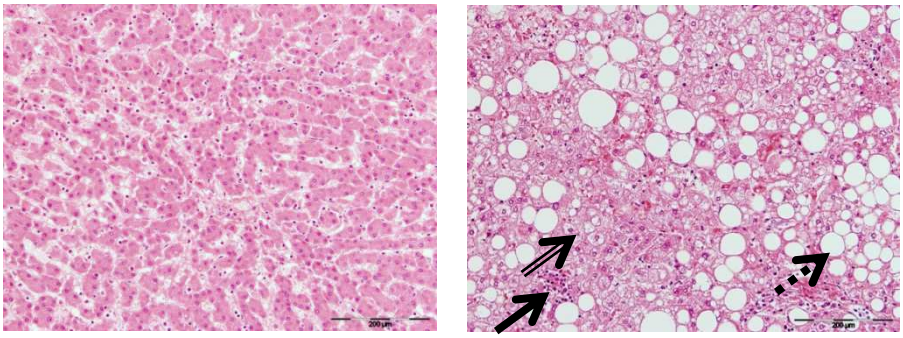

Figure 2: characterization of steatosis and inflammation in human NASH. Macrovesicular steatosis (⇢), microvesicular steatosis (⟹), foci of inflammatory cell infiltrates indicates inflammation (→)

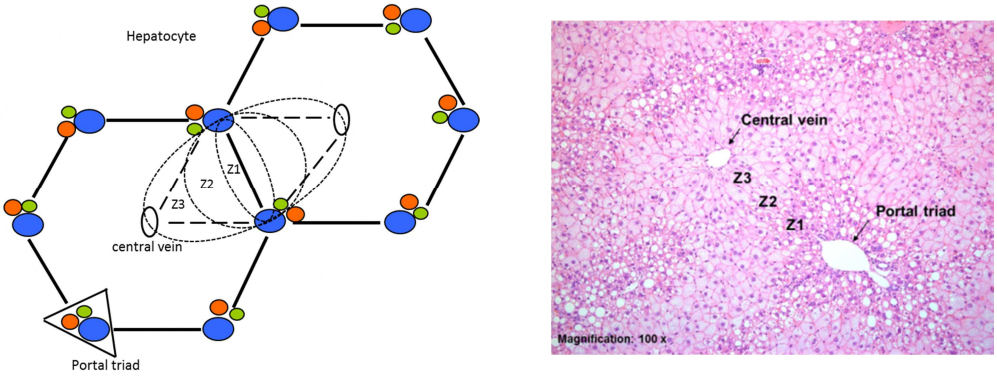

Figure 3: zonation of the liver: schematic diagram (left) and histological (HE) cross- section (right).

The liver parenchyma can be functionally divided into three zones, Z1, Z2 and Z3 (**Figure 3**), which differ with respect to oxygen supply. Z1 is the periportal area, where oxygen- and nutrient enriched blood enters the tissue via the hepatic artery and the portal vein. Z2 is the midzonal area, and Z3 is the pericentral area. The incoming blood flows through sinuses to the central vein. Adult and pediatric patients (2-19 years old) show a different zonal steatosis pattern as shown in **Table 1**.

Table 1: Different steatosis pattern in adult and pediatric patients (2-19 years old).

Patient	Steatosis		
	Predominant pattern	**Zonal location**	**Severity**
Adult	mixed macro- and microvesicular steatosis	pericentral, Z3	+
Pediatric	macrovesicular steatosis	periportal, Z1	++

Scoring system for pathologic evaluation

In 2005, the NASH Clinical Research Network (NASH-CRN) has developed and validated a system of histological evaluation for the full spectrum of NAFLD that could be useful in clinical trials (17). This scoring system of Kleiner et al. (17) separates the grading (activity) from staging (fibrosis) and is also called NAFLD activity score (NAS). The grading is feature-based and encompasses steatosis, inflammation and hepatocellular ballooning (18). Notably, this scoring system of NASH not only relies on the presence of the different features mentioned above, but also on the zonal pattern that develops within the liver. The NAFLD activity score is however not applicable for diagnosis of NASH, but developed for monitoring the severity of the disease in order to assess putative changes in disease progression during the duration of a clinical trial.

Regular intervention and treatment

Lifestyle modification

In clinical practice, the approach for NAFLD and NASH treatment begins with advices on lifestyle modification to induce weight loss. The goal of this primary treatment is to improve steatosis (which is often a reversible condition) and prevent the development of fibrosis, which can lead to cirrhosis and its complications. A Randomized controlled trial (RCT) with 31 obese NASH patients receiving intensive lifestyle changes for 48 weeks showed that subjects with ≥7% weight loss had significant improvement in steatosis, lobular inflammation and ballooning (19). It has been suggested that body weight loss between 5~10% is beneficial for improvement of hepatic steatosis and liver histology (19, 20). Weight loss is commonly achievable by calorie restriction, especially the reduction of calories from dietary carbohydrates, combined with regular exercise. In 2012, the American Association for the Study of Liver Disease (AASLD) proposed a dietary guideline for patients with NAFLD, which recommends an approach of calorie restriction with a low-carbohydrate (40%-45%) or low-fat (<30%) daily calorie intake, to reduce hepatic triglyceride content and to improve insulin resistance (21, 22). A mild and significant reduction in intrahepatic triglyceride content was reported in a recent randomized clinical trial (RTC) after 16 weeks of 150-300 min (per week) of moderate intensity exercise (23). Another RCT showed similar reduction in hepatic fat content, as well as an improvement of BMI and insulin resistance after following aerobic exercise and anaerobic resistance training for 4 months (24). In practice, the initiation of an exercise program is recommended based on the current activity level of the patient, however, it is hard to reach a consensus on the type, intensity and duration of exercise which are optimal for patients with NAFLD or NASH.

Vitamin E

Although the most safe treatment for NAFLD or NASH is lifestyle modification, weight loss is still difficult to maintain in the long term (25). Therefore, additional supplementary therapy is often necessary. Vitamin E (α-tocopherol) is an antioxidant and vitamin E-800 international units (IU)/day is currently considered as a first-line treatment for non-diabetic, non-cirrhotic NASH patients (21). The largest clinical trial to date, PIVENS, which

tested 800 IU/day vitamin E in 84 subjects with NASH for 96 weeks, showed significant reductions in ALT (P<0.001), AST (P<0.001), hepatic steatosis (P<0.005), and lobular inflammation (P<0.02), but no significant improvement in fibrosis (P<0.24) (26). After 2 years, the follow-up analysis showed that beneficial changes in ALT were more frequent among vitamin E treated subjects (48%) than placebo (16%) recipients (P<0.001). Among vitamin E recipients, ALT responses were associated with decreases in NAS (P<0.001), but not with fibrosis scores (P=0.34) (27). Although vitamin E has consistently shown to have an effect in improving serum ALT and AST as well as liver histology in patients with NASH, one concern is whether it increases all-cause mortality. Since there are conflicting data from different clinical trials, additional RCTs are needed to determine long-term efficacy and safety of vitamin E (28).

Thiazolidinediones

When vitamin E is not effective, administration of pharmacological agents (e.g. pioglitazone rosiglitazone) as adjunctive therapy is widely applied in the clinic. Pioglitazone and rosiglitazone belong to the class of thiazolidinediones (TZDs). TZDs can improve insulin sensitivity by activating the nuclear transcription factor peroxisome proliferator activated-receptor-gamma (PPAR-γ), thereby upregulating gene transcription for several proteins involved in glucose and lipid metabolism (29). A pilot study in 18 non-diabetic NASH patients showed that 30 mg/day pioglitazone for 48 weeks led to a significant reduction in transaminases (ALT, AST), and a significant improvement in NAS (P<0.05) (30). A recent meta-analysis that included 5 RCTs showed that pioglitazone significantly improved steatosis (OR 4.05, 95% CI 2.58-6.35) and inflammation (OR 3.53, 95 % CI 2.21-5.64), but not fibrosis (OR 1.40, 95% CI 0.87-2.24) (3). The long-term safety of TZDs has been debated regarding the risk of cardiovascular disease (31), congestive heart failure (32) and bladder cancer (33). In a recent meta-analysis (32) of 19 trials enrolling a total of 16.390 patients with T2DM, pioglitazone treatment was associated with a significant reduction (18%) in the primary outcome of death, myocardial infarction or stroke (P<0.005), however, there was also a higher rate of congestive heart failure with pioglitazone (2.3% vs.1.8% in the control group, P<0.002). Therefore, in September 2010, the European Medicines Agency (EMA) recommended that the drug should be suspended from the European market because the benefits of rosiglitazone no longer outweighed the risks. In US, rosiglitazone only could be sold with a prescription from a certified doctor and patients were required to be informed of the risks associated with its use through specified pharmacies. Until November 2013, the FDA lifted its earlier restrictions on rosiglitazone after reviewing the results of several other clinical trials, which did not provide evidence for an increased risk of heart infarct associated with the use of the drug.

Agents in clinical trials

Ezetimibe

Ezetimibe, as a potent inhibitor of cholesterol absorption, inhibits Niemann–Pick C1-like 1 (NPC1L1)-dependent cholesterol transport in liver (34). The beneficial effects of ezetimibe on improvement of liver steatosis and insulin resistance have been observed in mice fed a high fat diet (35) and Zucker obese fatty rats (36). An open-label study enrolled 45

patients with liver biopsy-proven NAFLD for treatment with ezetimibe (10 mg/day) for 24 months. It was found that ezetimibe therapy significantly lowered serum ALT and CRP levels, whereas no significant changes were observed in adiponectin, leptin, and resistin levels. Histological features of steatosis score (P=0.0003), necro-inflammatory score (P=0.0456), ballooning score (P=0.0253) and NAS (P=0.0007) were significantly improved relative to baseline (the trial had no placebo control group). However, the fibrosis stage was not significantly (P=0.6547) changed (37). A recent RCT trail showed that ezetimibe (10 mg/day) treatment for 6 months significantly lowered the level of serum total cholesterol and also improved hepatic ballooning score and fibrosis stage, but significantly increased hepatic long-chain fatty acids and HbA_{1c} compared to the control group (38). Collectively, the effect of ezetimibe on NASH still needs further examination in future studies.

Metreleptin

In February 2014, the US Food and Drug Administration (FDA) approved orphan drug MYALEPT™ (metreleptin for injection), which is indicated as an adjunct to diet as replacement therapy for the treatment of complications of leptin deficiency in patients with lipodystrophy. Metreleptin is recombinant human leptin. In a recent human study, NASH patients received subcutaneous administration of metreleptin daily for 2 years (39). Besides a significant improvement in metabolic profile, ALT and AST, patients treated with metreleptin also showed significant improvements in steatosis (reduction of mean score from 1.8 to 0.9) and ballooning injury scores (from 1.2 to 0.4), as well as a 44.2% reduction in NAS ($p<0.0001$) while fibrosis remained stable (39). Another ongoing RCT has been performed by the University of Michigan and is expected to complete in September 2015. The aim of this trail is to determine if the one-year treatment of metreleptin can improve NAFLD or NASH in patients with concurrent lipodystrophy, By taking pre-treatment and post-treatment liver biopsies and measuring various metabolic markers, this trial will evaluate the safety and effectiveness of metreleptin for the treatment of NAFLD/NASH.

L-carnitine

In mammals, L-carnitine is a conditionally essential nutrient that can be synthesized endogenously from lysine and methionine or obtained from the diet, primarily from red meat (40). L-carnitine is indispensable for energy metabolism and has been proposed as a supplement to treat a variety of health conditions including heart attack and heart failure (41, 42). *In vitro* and *in vivo* studies found that L-carnitine enhances both lipolysis and fatty acid oxidation (43) and that it reduces serum fatty acid levels by increasing beta-oxidation in hepatocytes (44, 45). Recently, dietary supplementation of L-carnitine (1000 mg/day) was investigated in 74 NASH patients, over a period of 6 months (46). Laboratory parameters in patients treated with L-carnitine showed significant improvements in plasma risk factors, among which a decrease of serum ALT/AST, TNF and CRP levels, and an improvement of insulin resistance. All patients (36) showed a significant reduction in steatosis, parenchymal inflammation, and hepatocellular injury, and 32 patients (86%) had improvement in fibrosis. Another recent RCT trail has been performed by Tehran University of Medical Sciences with the estimated completion year of 2015. After 2 years

treatment of L-carnitine (1000 mg/day), the level of ALT, AST and liver elasticity in NASH patients will be measured to evaluate the effect of L-carnitine on NASH (28).

Polyphenols

Polyphenols are often concentrated in leaf tissue and consist of 1-25% of the dry green leaf mass (47). A high amount of polyphenols has been found in green tea (about 35% of its total dry weight) (48). Studies from Park HJ et al. indicate that green tea extract attenuated hepatic steatosis by decreasing adipose lipogenesis and enhancing hepatic anti-oxidant defenses in ob/ob mice (49), as well as that it suppressed hepatic NFκB activation and inflammatory responses in diet-induced obese rats (50). Although several human studies have been conducted on the anti-obesity effects of dietary polyphenols (51, 52), the effects of dietary polyphenols on NAFLD are missing. In a recent RCT study, 44 participants (age 18–25 y, BMI ≥23.1 kg/m^2) were given 250 mL of either bayberry juice or placebo twice daily for 4 weeks. Compared with placebo, the consumption of bayberry juice significantly decreased the plasma levels of TNF-α ($P<0.001$) and IL-8 ($P=0.022$), although no significant changes in plasma TGs, TC, LDL-C, fasting glucose level, insulin concentration, or HOMA-IR were found (53). Due to variation among subjects (age, gender, ethnicity), chemical forms of the dietary polyphenols used and confounding factors such as other weight-reducing agents, future RCT are warranted to evaluate the efficacy of dietary polyphenols on NAFLD/NASH.

EXPERIMENTAL NASH

Taken together, the major current challenges for the study of human NASH are: 1) lack of specific and efficient non-invasive methods that detect inflammation and fibrosis in the liver and 2) lack of effective and reliable therapeutic approaches that are based on understanding the pathogenesis of NASH. Furthermore, studies of NASH in humans have limitations or ethical issues regarding the collection of liver biopsies from patients and the administration of drugs to patients because of safety concerns. Animal models of NASH that mimic human disease can thus provide crucial mechanistic information, not only for elucidating the pathogenesis of NASH but also for examining therapeutic effects of various agents.

Available diet-inducible animal NASH models

Ideally, animal models should mimic both histopathology and pathophysiology of human NASH. Recently, several review articles on animal models of NAFLD/NASH have been published (54-56). An overview of the metabolic and hepatic characteristics of current diet-inducible NASH animal models is provided in **Table 2**. C57BL6 mice fed a HFD acquire a human NASH-like metabolic and histological phenotype, but a very long feeding period (>50 weeks) is required. MCD diet-induced NASH shows similar pathohistological features as human NASH, but does not develop the metabolic syndrome characteristics of human risk groups and therefore do not exhibit the metabolic context which is seen in most human NASH patients. Fructose with/without HFD or ALIOS diet feeding leads to elevated plasma insulin, resistin, and leptin levels, as well as increased plasma ALT levels, liver TNF-

α and procollagen mRNA, indicating an inflammatory and profibrogenic response to injury. These diets also lead to hepatic steatosis and inflammation with a similar histological featuresas observed in patients with NASH. LDLR$^{-/-}$ mice fed a HFD or HFC also develop a human-like NASH with respect to the metabolic context and histological phenotype, and this strain of mice is prone to develop fibrosis. Although the available animal models do not reflect the full spectrum of human NAFLD pathology, they are useful for verifying hypotheses on the pathogenesis of NASH and for testing pharmaceutical compounds in intervention studies.

Table 2: Metabolic and pathological characteristics of animal NAFLD/NASH models. Important phenotypic and histological hallmarks are compared and the severity of a particular parameter is indicated as "+", "++", "+++", absence of a particular feature is indicated as"-".

	Phenotypic hallmarks			Histological hallmarks				
Model	Obesity	IR	Hypertriglyceridemia	Steatosis		Inflammation	Fibrosis	Ref.
				Predominate pattern	Severity			
C57BL6, HFD*	+	+	+	microvesicular	++	+	+	(57)
C57BL6, HFD+fructose**	+++	++	+++	macrovesicular	+++	+	not described	(58)
C57BL6, ALIOS diet	+++	++	++	macrovesicular	++	+	not described	(59)
C57BL6, MCD diet	-	-	-	macrovesicular	+++	+++, lymphocytes & neutrophils	++	(60-62)
Ob/ob, HFD	+++	++	++	not described	++	-	-	(63, 64)
ApoE2 knock-in, HFC	+	-	++	not described	++	++, macrophages	upregulation of collagen synthesis genes	(65, 66)
ApoE$^{-/-}$, HFC	+	+	++	macrovesicular	++	++, macrophages	not described	(67, 68)
LDLR$^{-/-}$, HFD	++	++	++	not described	++	++	+	(69)
LDLR$^{-/-}$, HFC	+++	++	+++	macrovesicular	+++	+++, macrophages	++	(66, 70)
hCRP, HFD	++	++	++	macrovesicular	++	++	+	(71)

IR: insulin resistance; HFD: high fat diet; HFC: high fat high cholesterol diet; MCD: methionine choline deficient diet

**C57BL6 wild type mice hardly develop NASH, the features shown in the table were present only when they were fed for up to 50 weeks.*

***Alios diet: 45% kcal from fat (30% from partially hydrogenated vegetable oil (28% saturated fatty acids, 57% monounsaturated fatty acids, 13% polyunsaturated fatty acids) + high fructose corn syrup equivalent (55% fructose, 45% glucose by weight) (42 g/l as gel-water)*

Note: Since the severity of NASH largely depends on how long a study is performed, studies with 8-15 weeks feeding period are included in the table (except for C57BL6 on MCD diet, which were fed the MCD diet for 50 weeks).

Pathogenesis of NASH

Intrahepatic effects of lipid imbalance on NASH

Triglycerides (TGs) are utilized as metabolic fuel via fatty acids oxidation. In liver, TGs can either be stored in hepatocytes or can be exported as very low density lipoprotein (VLDL). An increase of TGs in hepatocytes, which ultimately leads to hepatic steatosis, can be due to 1) increased dietary intake of free fatty acids, 2) increased *de novo* lipogenesis, 3) increased recirculation of non-esterified fatty acids to the liver from the peripheral tissues (e.g. adipose tissue or skeletal muscle), or 4) failure of hepatic clearance of fatty acids due to either impaired esterification to TGs and export as VLDL, or impaired hepatic mitochondrial β-oxidation (16). In the next paragraphs, the dietary intake and the contribution of different dietary components (HFD, dietary cholesterol and dietary carbohydrate) on NASH development will be discussed.

HFD

Studies in humans (15) and animals (72) have shown that different types of fatty acids can lead to different outcomes with respect to liver injury. Nutritional surveys showed that the dietary intake of saturated fats (SF) was significantly higher in NAFLD and NASH patients than in healthy controls (73, 74). Consistent with this, a high fat diet (HFD), that consists of >24% of energy from fat, is commonly used to induce hepatic steatosis in animal NASH models. In a high fat diet rat model, Buettner et al. (72) compared different high fat diets: coconut oil (primarily SF), olive oil (primarily monounsaturated fatty acids, MUFA), lard (mixture of SF and MUFA), and fish oil (primarily polyunsaturated fatty acids, PUFA). After 12 weeks, the first three diets significantly worsened hepatic steatosis as well as elevated plasma TG, whereas the fish oil (PUFA)-based diet did not.

However, growing evidence suggests that HFD alone is not sufficient to induce NASH (75, 76). A refined time course analysis (77) of HFD feeding in rodents showed that the inflammatory response of the liver is transient and that the liver can adapt its metabolic program to the HFD overload. Therefore experimental steatosis hardly progresses into the extensively inflamed conditions of NASH as they are observed in patients. This suggests that additional inflammatory triggers are important to experimentally induce an inflamed fatty liver.

Dietary cholesterol

The role of cholesterol on the development of NASH is recently getting more attention. In a large, nationally epidemiological study in the United States, dietary cholesterol consumption was independently associated with the development of cirrhosis (78). Another recent, pilot trial of ezetimibe, as an intestinal cholesterol absorption inhibitor, found improvements in hepatic steatosis and inflammation in humans with NASH (37, 79). Animal data are also emerging to support a role for dietary cholesterol in the progression

towards NASH. Hofker MH and colleagues (66) suggested that dietary cholesterol induced hepatic inflammation independently of hepatic steatosis in a hyperlipidemic mouse model with NASH. A study by Ioannou at al. (80) recently compared different diets (high fat, high cholesterol and high fat+high cholesterol) to examine their effects on NASH development. After 30 weeks of diet feeding using C57BL/6J mice, only high fat+high cholesterol diet led to significant levels of hepatic steatosis, hepatic inflammation, and perisinusoidal fibrosis with a similar profile of risk factors as seen in human NASH. These effects were associated with adipose tissue inflammation and a reduction in plasma adiponectin levels. Interestingly, an investigation of dietary records by Yasutake et al. (81) revealed that cholesterol intake was significantly greater in both obese and non-obese NAFLD patients than in healthy controls. Surprisingly, non-obese NAFLD patients even had higher cholesterol consumption than obese NAFLD patients, suggesting that cholesterol intake may cause NAFLD independent of obesity. Based on available human and animal data, it has been hypothesized that cholesterol-induced liver injury could be an important cause of NASH independent of obesity, insulin resistance or even steatosis as proposed recently (82). However, the exact molecular mechanism of the effects of dietary cholesterol on NASH pathogenesis is still unknown.

Dietary carbohydrate

Several human and animal studies suggest that carbohydrates, such as sucrose and fructose, are possibly causative to NAFLD development (83). Nutrition reports have shown that the mean daily consumption and mean frequency of soft drinks is at least two fold higher in NAFLD patients than in healthy subjects (84, 85). High amount of fructose consumption is thought to be one of the most important dietary contributors to the pathogenesis of NAFLD. In a large clinical study performed by the NASH Clinical Research Network, dietary fructose consumption was found to be associated with induction of histological features of NASH, including hepatic inflammation and hepatocyte ballooning and fibrosis stage (86). Animal studies have shown that excess intake of fructose is closely associated with obesity and steatosis, through the activation of sterol regulatory element-binding protein-1c (SREBP-1c), a transcription factor that enhances the expression of enzymes associated with fatty acid synthesis (87, 88), and with increased visceral adiposity (89) and insulin resistance (58). Some animal studies have shown that high-fructose feeding is associated with upregulation of markers of inflammation, macrophage activation and oxidative stress. A recent study form Bhattacharjee demonstrated that fructose-induced NAFLD is associated with recruitment of T cells and NK cells in mice (90).

Intrahepatic effects of inflammation leading to NASH

Pro-inflammatory cytokines

Pro-inflammatory cytokines are important contributors to the development of NASH (91). TNF-α secreted by adipocytes and hepatocytes, was the first cytokine known to be elevated in NAFLD patients (92). Human data has shown that TNF-α is elevated in NAFLD patients compared with both obese and non-obese controls (93). Crespo et al. (94) identified increased mRNA expression of TNF-α and TNF-α receptor, p55, and showed that circulating levels of these molecules are associated with fibrosis in NAFLD patients. In

leptin-deficient (ob/ob) mice fed a high-fat diet, dietary manipulations that favor pathways which quench TNF-α improved insulin sensitivity, hepatic steatosis, and inflammatory cells infiltration in the liver (95).

Data from human studies have shown that increased systemic IL-6 levels are associated with increased inflammation and fibrosis in NAFLD patients (96). Wieckowska et al. found that IL-6 expression was increased in both hepatocytes and Kupffer cells and these levels positively correlated with both the inflammatory activity and the stage of fibrosis in NAFLD patients (97). Evidence from human and animal studies indicate that pro-inflammatory (TNF-α, IL-6)-induced insulin resistance and lipid overloading of liver cells both involve activation of stress-related protein kinases such as IKKβ, an activator of the proinflammatory transcription factor NFκB) and Jun N-terminal kinase (JNK), an activator of AP-1. These pathways subsequently increase inflammatory mediators like CRP, TNF-α, IL-6, and IL-1β, which further can contribute to liver injury (16, 98). IKKβ activation leads to NFκB translocation to the nucleus, resulting in a feed–forward loop that promotes increased expression of pro-inflammatory cytokines and other mediators of inflammation that augment hepatic inflammation and insulin resistance (99). Activation of NFκB has been observed in human NASH (100) and NASH mouse models (101). Animal studies have shown that pro-inflammatory cytokines (e.g. TNF-α, IL-1β) released from NFκB-activated Kupffer cells could also activate NFκB in adjacent hepatocytes (102). The involvement of hepatic NFκB in the progression of bland steatosis to NASH has recently been proposed in transgenic mice selectively expressing constitutively active IKKβ in hepatocytes (98). The JNKs (1 and 2) can be stimulated via activation of TNF superfamily death-signaling receptors (e.g. Fas, TNF-R1, TNF-related apoptosis-inducing ligand death receptors) and TLRs, or they can directly activated by lipotoxic molecules and oxidative stress (103). Videla et al. (100) showed that, in liver, NFκB and AP-1 DNA binding is significantly increased in NASH patients. In animal studies, removal of JNK1 or IKKβ suppressed pro-inflammatory gene expression and cytokine release (104, 105). Farrell et al. (106). showed that both JNK1 and JNK2 are activated in a NASH mouse model and, conversely, italso been shown that abolishing JNK activation can attenuate macrophage accumulation, hepatocyte apoptosis and liver injury in the context of NAFLD (102). However, the exact nature of most of the inflammatory mediators that cause the transition from bland steatosis to NASH is still largely unknown.

Oxidative stress

Oxidative stress, refers to an imbalance in the production of reactive oxygen species (ROS) and protective antioxidants, and has been considered to be an important contributor to the development of NASH (16). Oxidative damage is significantly correlated with inflammation and increased in the livers of patients with NAFLD when compared with controls (107). Sanyal et al. (108) demonstrated that immunohistochemical staining for 3-nitrotyrosine, a marker for oxidative stress, was elevated in NAFLD patients as compared to controls and was markedly higher in NASH patients than in patients with bland steatosis. Mitochondrial dysfunction leading to uncoupling of oxidative phosphorylation and resulting in ROS generation has been reported in NASH subjects (108, 109). Furthermore, in NASH patients increased activity of the mitochondrial cytochrome P450 2E1 (CYP2E1) has been observed (110). Studies in rats using the methionine choline

deficient diet to induce NASH found increased CYP2E1 activity (111). Using CYP2E1 knock-out mice, Abdelmegeed et al. recently showed that CYP2E1 plays a role in NASH development (112) and the authors suggest that the CYP2E1 enzyme may have a capacity of directly generating ROS. Moreover, upregulaton of cytokine receptors, especially TNF-α and antigens derived from gut flora via activation of NADPH oxidase system, also may play a role in the progression of NASH (16, 113).

In 1998, Day and James proposed the 'two-hit theory' (114) and described a possible pathogenic mechanism for the development of NASH: extensive lipid accumulation as a 'first hit' is followed by inflammation (as 'second hit') causes the progression of the disease towards NASH. On the other hand, clinical researchers occasionally found patients with NASH without obvious steatosis. Furthermore, steatosis could be improved in ob/ob mice when treated with anti-tumor necrosis (TNF)-α antibody (95). After a series of debates on the proposed sequence of events, either sequentially or in parallel, the concept of 'multiple parallel hits' has recently been considered (115). More recently, Jou et al. (116) proposed hepatocyte death and lack of repair as a possible 'third hit'. This theory proposes that combined oxidative/metabolic stress and in imbalanced cytokine production, may lead to an increase of hepatocytes death. These multiple hits can occur simultaneously and are intertwined; this may eventually lead to NASH (16, 117).

OUTLINE OF THE THESIS

Metabolic stress caused by excess dietary intake is considered to be an important contributor to NASH in humans, but the exact molecular mechanism of metabolic overload-induced NASH is largely unknown. Therefore, the aim of this thesis was to better understand the role of metabolic overload and metabolic inflammation in the development of NASH.

The NAFLD activity score (NAS) used in clinical studies has not been validated for animal models of NASH. In ***Chapter 2***, we developed a generic scoring system for the assessment of NAFLD in mice. This new scoring system allows to grade the histological features present in the various NASH mouse models currently used in preclinic research. Our scoring system is based on the human NAS system, and the inter- and intra-observer reproducibility was analyzed and found to be reliable.

After this methodological optimization and in order to get insight into the nature of the chronic inflammatory component that drives the development of NASH, we examined in ***Chapter 3*** the effect of non-metabolic triggers (LPS, IL-1β administered by slow-release minipumps) and metabolic dietary triggers (carbohydrate, cholesterol) of inflammation on the progression of bland steatosis to NASH. We showed that HFD feeding followed by metabolic triggers induced extensive steatosis and specific inflammatory components (neutrophils, AP-1) which results in a human-like NASH phenotype, while long-term LPS and IL-1β stimulation on top of HFD feeding did not. We next analyzed the distinct types of steatosis, microvesicular and macrovesicular steatosis, during NAFLD development over time, and investigated how steatosis is related to the onset of hepatic inflammation. More specifically, we performed in ***Chapter 4*** a time-course study using a metabolic inflammatory trigger (HFD+cholesterol). Histological hallmarks of NASH, such as macro-

vesicular/micro-vesicular steatosis, inflammatory cell infiltrates, NFκB activation and fibrosis were evaluated, and the correlations between the specific forms of steatosis, hepatic inflammation and activation of inflammatory transcription factors were established.

Next, we examined specific nutritional and pharmacological interventions that may attenuate diet-induced experimental NASH. In ***Chapter 5***, the effect of Mirtoselect, a standardized anthocyanin-rich bilberry extract was evaluated on the progression of NASH. We showed that Mirtoselect has profound effects on intrahepatic inflammatory processes resulting in a significant attenuation of NASH and associated fibrosis. In ***Chapter 6***, we studied the effect of an anti-inflammatory drug, salsalate, on diet-induced NASH in a short-term and a long-term study. Salsalate exhibited strong NASH-reducing properties and almost fully attenuated hepatic steatosis and inflammation.

Besides in liver, chronic metabolic overload may also develop in other organs which potentially contribute to NAFLD development. In ***Chapter 7***, we analyzed the development of metabolic stress in white adipose tissue and liver over time and found that white adipose inflammation preceded liver inflammation and NAFLD. The nature of the metabolic adaptations in WAT and liver were remarkably similar. The same transcriptional regulators were found to be activated to orchestrate the complex responses to metabolic overload. Finally, we investigated how long-term HFD-induced metabolic overload (t=42 weeks) would affect metabolic and inflammatory processes in the kidney in ***Chapter 8***. Drugs with established anti-inflammatory properties (rosuvastatin and rosiglitazone) were tested regarding their potency to reduce metabolically-induced organ inflammation and to improve kidney function.

Finally, the findings from the above studies and their implications are discussed in ***Chapter 9***.

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