The contribution of metabolic and adipose tissue inflammation to non-alcoholic fatty liver disease

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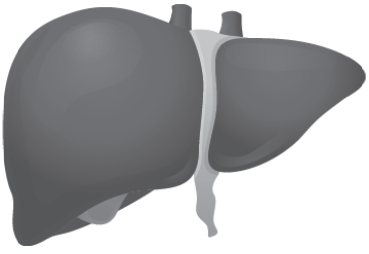
Chapter 8

Summary and general discussion



NAFLD is the most common cause of chronic liver disease worldwide. Prevalence estimates for NAFLD in general adult population are between 25% and 45%, and the disease incidence increases in parallel of obesity and diabetes [1]. Histologically, NAFLD comprises a wide spectrum of liver damage ranging from liver steatosis to non-alcoholic steatohepatitis (NASH) and fibrosis. Liver steatosis is considered benign in many cases [2], while NASH is a more severe condition that is characterized by steatosis and lobular inflammation with or without fibrosis. The progression from steatosis to NASH is associated with the presence of the metabolic syndrome (defined as central obesity accompanied by two or more of the following conditions: elevated fasting glucose concentration (reflecting insulin resistance), hypertension, raised triglyceride (TG) levels and lowered high-density lipoprotein cholesterol (HDL) levels) [1]. In particular patients with NASH have increased risk to develop other metabolic complications, such as cardiovascular disease, and have overall higher mortality [3-5]. Although the etiology of NASH remains largely enigmatic, it is generally accepted that inflammation is a central component of NASH pathogenesis. This inflammation may be triggered by metabolic surplus (excess energy or nutrients) and is also referred to as “metabolic inflammation”. White adipose tissue (WAT) is assumed to be largely involved in the development of metabolic inflammation. However, much remains unknown about the origin and underlying mechanisms controlling inflammation in NAFLD progression. The studies described in this thesis contributed to the understanding of the role of WAT in the development of NAFLD and provide insight into the molecular processes that cause metabolic inflammation.

THE ROLE OF WHITE ADIPOSE TISSUE IN NASH DEVELOPMENT

Adipose tissue has multiple roles in orchestrating adaptation to changes in nutrient availability [6]. It is not solely a reservoir for energy excess, but can act as an endocrine organ by transmitting soluble signals in the form of “adipokines” which can act locally and systemically. Moreover, the adipose tissue can interact extensively with immune cells which, under specific conditions, infiltrate in the

adipose tissue. Activation of the immune system in obese individuals in a so-called "chronic low-grade inflammatory state" is considered to be a crucial factor in the pathogenesis of metabolic diseases, such as NAFLD [7]. The basis of this view is that obese individuals have elevated plasma levels of inflammatory cytokines (e.g. TNFα, IL-6), increased chemokine levels that induce infiltration of immune cells, as well as elevated levels of general markers of inflammation (e.g. haptoglobin, SAA) [8].

Heterogeneity in the obese population

Although obesity is one of the major risk factors for NAFLD, some obese individuals appear 'metabolically healthy' despite having excessive body fat. These 'metabolically healthy' obese individuals have normal to high levels of insulin sensitivity, lower hepatic fat content, and a generally favorable cardiovascular profile [9]. This subgroup of obese individuals may maintain metabolic health as a result of their genetic profile or unclarified lifestyle features [10]. The factors that distinguish the "metabolically healthy" from the "metabolically unhealthy" obese are still poorly understood. A better understanding of the factors contributing to the metabolically healthy phenotype and the stratification of obesity phenotypes could lead to new prevention and therapeutic intervention strategies and thereby improve public health.

It has been postulated that some obese individuals are protected against metabolic complications because of a more favorable body fat distribution. In general, it is thought that increased visceral fat mass is linked to detrimental health effects. Studies have shown that increased visceral (intra-abdominal) fat is positively associated with metabolic disease [11,12], independent of overall adiposity [13]. On the contrary, subcutaneous adipose tissue is associated with more favorable levels of glucose and lipids [14].

As mentioned before, white adipose tissue (WAT) is not merely a storage site for excess energy, but can also act as a endocrine organ capable of secreting a variety of inflammatory mediators [15]. Hence, in the development of metabolic diseases such as NASH, the 'inflammatory status' of WAT may be even more important than the distribution of fat mass. In support of this, liver of obese subjects with

inflamed intra-abdominal (omental) WAT contain more fibro-inflammatory lesions than livers of equally obese subjects without WAT inflammation [16,17]. This observation suggests that inflammation in a specific WAT depot contributes to the inflammatory component in human NASH. The time-course experiment in an animal model for NASH, as described in **chapter 2**, supports this hypothesis. Herein, we explored the sequence of inflammatory events in different WAT depots and the liver in high-fat diet (HFD)-fed C57BL/6J mice that developed obesity. More specifically, we have investigated whether different intra-abdominal (i.e. epididymal and mesenteric) and subcutaneous (inguinal) WAT depots differ in their susceptibility to develop chronic inflammation. We found that the first depot that became inflamed was the intra-abdominal epididymal adipose tissue (eWAT) and this inflammation preceded NASH development. Moreover, we found that surgical excision of inflamed eWAT reduced liver inflammation, demonstrating that this WAT depot causally contributes to NASH development.

Adipose tissue expansion and inflammation

The susceptibility of eWAT to become inflamed, as shown herein, may be related to the fact that adipocytes in eWAT are more prone to hypertrophy than those in other WAT depots [18]. The deleterious effect of adipocyte hypertrophy was demonstrated in an *in vitro* experiment with isolated primary human adipocytes, [19] where only very hypertrophic cells were found to secrete MCP-1, a key mediator of immune cell recruitment into WAT. Consistent with this observation, adipocyte hypertrophy is associated with infiltration of macrophages and formation of crown-like structures (CLS), [20] a histological hallmark of inflamed WAT. Macrophage infiltration is positively correlated with the size of adipocytes both in visceral and subcutaneous fat [20,21]. However, visceral fat is more prone to macrophage infiltration compared to subcutaneous fat [22]. Moreover, it has been shown in obese individuals that visceral fat exhibit higher expression of inflammatory cytokines than subcutaneous fat [23]. These differences might explain the strong association between visceral obesity and NASH development.

Importantly, obese mice and humans with hyperplastic obesity (i.e. obesity without adipocyte hypertrophy) do not show CLS in WAT and remain insulin

sensitive [21,24]. The activation of peroxisome proliferator-activated receptor-γ (PPARγ) has an important role in adipocyte differentiation to stimulate fat storage via hyperplasia [25]. It has been shown that metabolic unhealthy obese (insulin resistant) patients have lower expression of PPARγ in visceral fat than (insulin sensitive) metabolically healthy obese individuals [26]. Subcutaneous WAT shows higher expression of PPARγ compared to visceral WAT [27], suggesting that this depot may be less susceptible to develop inflammation. Thus, pharmacological activation of PPARγ may be a suitable intervention strategy to stimulate fat storage via hyperplasia and thereby preventing adipocyte hypertrophy. Indeed, in **chapter 3** it was shown that intervention with a PPARγ-activator, rosiglitazone, stimulated hyperplasia specifically in the subcutaneous WAT and prevented adipocyte hypertrophy. Consequently, this depot did not become inflamed even though its mass was much greater than in control animals, an effect that was also observed in humans treated with rosiglitazone [28]. Thus, limited capacity for adipose tissue expansion, rather than obesity *per se* may underlie the development of inflammation [29,30] and leads to ectopic fat deposition in other organs such as liver [30].

Proposed mechanisms for inflamed white adipose tissue in NASH development

The removal of inflamed WAT as demonstrated in **chapter 2**, resulted in reduced NASH development. Moreover, intervention by rosiglitazone reduced WAT inflammation and subsequent NAFLD progression as shown in **chapter 3**. Both studies, showed that histological improvement of the liver was paralleled by the reduction of circulating pro-inflammatory adipokines, including leptin and saturated fatty acids. These data support a model in which secretion of inflammatory mediators (e.g. lipids, adipokines) by inflamed WAT drive NASH development as depicted in Figure 1. Excess of nutrients leads to expansion of WAT involving adipocyte hypertrophy [29]. Once adipocytes do not further increase in size and a WAT depot has reached a maximal mass, infiltration of immune cells and CLS formation are observed. Next, the inflamed WAT produces soluble inflammatory mediators that are released into the circulation driving the development of inflammatory pathologies in other organs, including the liver. The model in Figure 1 implies that intervention

strategies that can attenuate WAT inflammation may reduce NAFLD development. Strategies aiming at reducing WAT inflammation should focus on mechanisms that allow optimal WAT expansion and prevent immune cell infiltration.

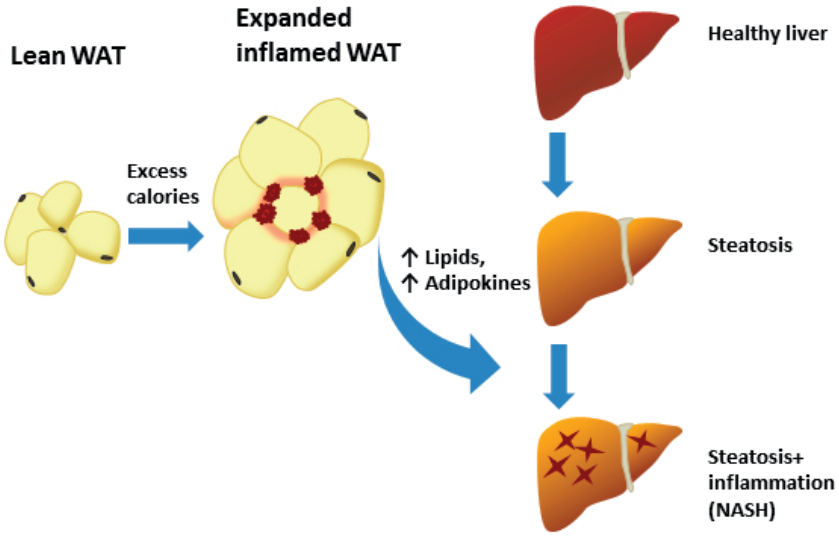


Figure 1: Limitation in expansion is critical for the development of obesity-associated inflammation and NASH. Caloric excess leads to expansion of WAT involving adipocyte hypertrophy. Infiltration of immune cells and formation of crown-like structures are observed once the adipocytes of a depot do not further increase in size and a WAT depot has reached a maximal mass. Inflamed WAT produces inflammatory mediators that can be released into the circulation driving the development of inflammatory pathologies, such as NASH. Among the inflammatory mediators are adipokines, such as TNFα, IL-6, leptin (increased) and adiponectin (decreased) as well as specific pro-inflammatory lipids like palmitic acid and stearic acid.

THE LINK BETWEEN INFLAMMATION, INSULIN RESISTANCE AND NAFLD

In 1993, Hotamisligil and colleagues were the first that described a molecular link between inflammation and obesity-associated insulin resistance [31]. They showed in rodent models of obesity and diabetes that increased expression of the pro-

inflammatory cytokine TNFα in adipose tissue correlated with insulin resistance. This study was supported by similar findings in humans, showing that elevated TNFα concentrations in both WAT and plasma were associated with decreased insulin sensitivity [32,33].

Origin of insulin resistance

Given the obvious connection between obesity and adiposity, studies have mainly focused on obesity-driven inflammation in WAT during the development of insulin resistance. However, obesity is also associated with the development of inflammation in other metabolic tissues, such as the liver. While inflammatory processes in both the WAT and liver are associated with the development of insulin resistance [34], it remains unclear to what extent both organs contribute to obesity-induced (systemic) insulin resistance. Since it is difficult to untangle this question in human subjects, we studied the inflammatory processes in both WAT and liver in a HFD-induced animal model for NAFLD (**Chapter 4**). In this time-course study, we observed that the development of tissue-specific insulin resistance was paralleled by increased infiltration of inflammatory cells in both, WAT and liver. However, adipose-specific insulin resistance was already observed after 6 weeks of HFD feeding, while hepatic insulin resistance occurred much later in time (after 24 weeks of HFD feeding). These findings support the view that hepatic inflammation contributes less to whole body insulin resistance compared to WAT inflammation, at least in early stages of diet-induced obesity, as previously hypothesized by others [15,35]. Moreover, it has been shown the degree of adipose tissue insulin resistance is associated with progressive NASH in patients [36], supporting the view that insulin resistance in WAT is an early disease symptom that may contribute to NASH development [37].

Inflammatory mechanisms underlying insulin resistance

As mentioned earlier, insulin resistance is associated with inflammation in WAT. More specifically, WAT inflammation is characterized by infiltration of macrophages that form CLS. The number of adipose tissue macrophages (ATM) is positively associated with the progression of insulin resistance [38].

The chemokine monocyte chemoattractant protein (MCP)-1 and its receptor C-C chemokine receptor-2 (CCR2) play a pivotal role in the recruitment of macrophages. In support of this, MCP-1 and CCR2 knockout mice exhibit reduced ATM content and insulin resistance [39-42]. Furthermore, prophylactic treatment with a CCR2 antagonist reduced macrophage content in WAT and hyperinsulinemia [43]. Other studies have demonstrated that CCR2 antagonists can also improve NASH [44], however, this has not been tested in a therapeutical setting so far. Therefore, we have examined in **chapter 4** whether CCR2 inhibitor, propagermanium, would attenuate NASH development in mice with manifest disease symptoms (i.e. WAT inflammation and insulin resistance). We observed that propagermanium intervention reduced insulin resistance and WAT inflammation. Moreover, propagermanium reduced macrovesicular steatosis and lobular inflammation, indicating an attenuation of NASH development. However, the beneficial effects were much more pronounced in the early intervention group compared to the late intervention group (started after 6 weeks vs. 12 weeks of HFD feeding). Hence, CCR2 inhibitors may be beneficial to treat insulin resistance and NASH, but only when administered early in the disease development. Based on existing literature [45-47], it is likely that disease pathways other than MCP1/CCR2 become upregulated at later stages of disease process (e.g. RANTES/CCR5) and that interventions merely targeting MCP1/CCR2 become less efficient. Therefore, NASH patients may benefit more from a treatment that is directed at both, the CCR2 and CCR5 pathways. The use of such a dual-CCR2/CCR5 antagonist is currently being examined in a large randomized phase 2b trial in NASH [47].

It should be noted that not only the number of ATM, but also the inflammatory phenotype of this immune cell population differs during obesity, which are typically referred to as M1 and M2 macrophages. M1 macrophages are considered pro-inflammatory as they secrete pro-inflammatory cytokines (e.g. TNFα, IL-1β), whereas M2 macrophages secrete anti-inflammatory cytokines (e.g. IL-10) [48]. In particular the accumulation of M1 macrophages, which express the CD11c surface marker, have been implicated in the development of insulin resistance [34,38]. In support of this notion, CD11c depletion in obese mice results in a rapid normalization of glucose and insulin tolerance and decreased inflammatory markers in WAT [49].

Various studies have described a shift in ATM subsets from M2 in lean mice to M1 in obese mice [48,50]. In obese humans, however, the ATM phenotype is less polarized, as both M1 and M2 markers can be detected in human ATMs [51-53]. Despite a 'mixed' ATM phenotype, these macrophages are thought to contribute to the chronic inflammatory process in obesity as they can produce extensive amounts of pro-inflammatory cytokines [53]. It has been assumed that macrophages exhibit phenotypic plasticity in response to their surrounding milieu [54]. For instance, it was shown that progressive lipid accumulation in macrophages favors M1 polarization [55]. The transcriptional factor PPARγ appears to be a key player in this macrophage polarization [56]. Indeed, we observed that administration of PPARγ activator rosiglitazone leads to decreased expression of M1 and increased expression of M2 markers in WAT as shown in **chapter 3**. This suggests that rosiglitazone partly exerts its insulin sensitizing effect by preventing M1 polarization in WAT.

Inflammation can be triggered by cytokines (e.g. TNFα) that instigate inflammatory signaling through classical activation of their cell surface receptors [34]. Alternatively, the inflammatory process can be initiated by 'danger signals', such as saturated fatty acids, that activate the NLRP3 inflammasome complex [57]. Upon inflammasome activation, caspase-1 initiates the maturation of the cytokines IL-1β and IL-18. Genetic ablation of components of the inflammasome (e.g. caspase-1) has been shown to ameliorate HFD-induced obesity and insulin resistance [58], suggesting that the inflammasome is an important mediator in the development of metabolic disease. In **chapter 5**, we examined the therapeutical value of a caspase-1 inhibitor in obesity-associated NAFLD development in LDLr-/-.Leiden mice. Treatment with this inhibitor did not affect obesity or fat mass, but did reduce inflammation in WAT, which was paralleled by improvement of whole-body insulin resistance. Moreover, intervention with caspase-1 inhibitor attenuated steatosis, inflammation and fibrosis in the liver. The inflammasome is present and of relevance in multiple tissues (e.g. WAT, liver) and cell types (e.g. hepatocytes, macrophages). Future studies should therefore address whether the observed improvement of NAFLD in LDLr-/-.Leiden mice is orchestrated by direct effects of caspase-1 inhibition on the liver or via indirect effects on WAT.

Relationship between insulin resistance and NAFLD

Obesity-associated insulin resistance is thought to play a causal role in the pathogenesis of NASH, since it is strongly associated with NAFLD severity [59,60]. However, the relationship between insulin resistance and NASH is still poorly understood. Ectopic lipid accumulation in the liver has been considered to cause insulin resistance [61]. On the other hand, insulin resistance is thought to cause NAFLD development [37]. Notably, whole body insulin resistance determined by HOMA-IR or increased plasma insulin levels may merely reflect insulin resistance in adipose tissue, rather than hepatic insulin resistance. Moreover, liver steatosis is rare in metabolic healthy obese with normal insulin-sensitive adipose tissue [36], highlighting the importance of adipose tissue in NAFLD.

Reducing inflammation in adipose tissue, via macrophage polarization or inhibiting inflammatory components (e.g. inflammasome) have been shown to improve systemic insulin resistance [62,63] and, as shown herein, is frequently accompanied by improvement in NASH. As the degree of insulin resistance in adipose tissue is associated with NASH severity [36], it is thus likely that NASH development can be attenuated by improving adipose tissue function (i.e. inflammation, insulin resistance).

PRECLINICAL NASH MODELS TO EXAMINE DIFFERENT ASPECTS OF DISEASE

NAFLD is considered a complex, multifactorial disease and its progression is difficult to study in patients. Clinical research into disease mechanisms are constrained by ethical considerations, particularly with respect to obtaining tissue biopsies (e.g. liver, WAT other than subcutaneous) and by limited ability to study interactive disease pathways over time. Although animal work contributed greatly to our understanding of the mechanisms underlying NAFLD progression, to date no optimal animal model exists that reflects all the disease aspects observed in humans. Ideally, experimental NASH models should mimic both human pathophysiology and histopathology. As multifactorial origins and processes are thought to contribute to

NASH development, animal models investigating the etiology of NAFLD have been restricted to studying specific aspects of the disease.

Histopathology of NASH

Histopathologically, fat accumulation observed in human NAFLD can manifest in two forms: macrovesicular- or microvesicular steatosis. Macrovesicular steatosis is characterized by the presence of a large lipid droplets that displaces nucleus to the periphery of liver cells, while microvesicular steatosis consists of large numbers of smaller droplets surrounding a central nucleus. In human NAFLD, the most frequent type of steatosis is macrovesicular steatosis, but a mixed pattern (macrovesicular and microvesicular) steatosis has been reported as well [64]. However, it is unclear whether a distinct type of fat storage i.e. macrovesicular or microvesicular steatosis, contributes to NAFLD progression. In **chapter 7**, we studied whether a potential relationship exists between the type of steatosis and the onset of hepatic inflammation in different experimental models of NASH. We found that macrovesicular, but not microvesicular, steatosis was positively correlated with the number of inflammatory aggregates across different disease models (i.e. ApoE3.Leiden.CETP, C57BL/6J, LDLr-/-.Leiden mice). Currently, it is unknown what factors or mechanisms that involve macrovesicular steatosis could drive disease progression. Future research should focus on how lipid droplets are formed, e.g. whether small lipid droplets characterizing microvesicular steatosis reflect newly synthesized fat droplets or if the aggregation of smaller lipid vacuoles become larger over time. Moreover, it is unknown which lipids are stored in macrovesicular steatosis and whether they differ from the lipids that accumulate in microvesicles. The latter may be of importance, because emerging data indicate that accumulation of specific types of lipids in liver cells causes lipotoxic hepatocellular injury and inflammation [65]. Therefore, it is possible that the large lipid droplets in macrovesicular steatosis contain certain toxic lipids or lipid metabolites that are absent or less abundant in liver regions with microvesicular steatosis.

Animal models of NASH

The methionine choline deficient (MCD) diet-induced NASH model is a frequently used model to study liver disease in rodents. Although this model develops pronounced (macrovesicular) steatosis, inflammation and fibrosis reflecting human histopathology, it lacks metabolic features associated with human NASH. The MCD model is associated with features that are atypical for NASH patients, i.e. weight loss, increased insulin sensitivity and low serum triglyceride (TG) levels [66]. Thus, to mimic human risk groups for NASH development, we herein used different mouse strains and diets that reflect a similar disease phenotype as observed in humans.

Inbred C57BL/6J mice fed a high-fat diet (HFD) are a frequently used model to study diet-induced obesity and associated co-morbidities, such as insulin resistance and NAFLD [67]. The development of metabolic inflammation and organ dysfunction in this model is relatively slow. Therefore, it is well-suited for longitudinal studies investigating different stages of disease development (as shown in **chapter 2, 4 and 7**). However, these mice do not develop dyslipidemia as seen in humans and they exhibit relatively low plasma TG and cholesterol levels with low levels of the atherogenic VLDL and LDL. In fact, the majority of cholesterol is confined to HDL particles. Moreover, the development of NASH is quite mild in HFD-fed C57BL/6J mice. It is likely that changes in lipid metabolism in the liver related to dyslipidemia are necessary to aggravate NASH. As dyslipidemia is considered a risk factor in NAFLD, we have used other animal models to capture this aspect of disease.

In contrast to wild-type mice, experimental models of atherosclerosis show resemblance with the human plasma lipoprotein profile. Examples are the transgenic LDL receptor deficient (LDLr-/-) mice and ApoE*Leiden (E3L) mice. LDLr-/- mice lack the LDL receptor, which is required for clearance of chylomicrons, VLDL and LDL particles. Deficiency of this receptor, results in elevated TG and cholesterol levels upon a Western-type diet. Originally, LDLr-/- mice were frequently used for atherosclerosis research. Notably, when fed a HFD, LDLr-/- mice develop obesity, hypertriglyceridemia, insulin resistance and show gradual and progressive development of NASH with fibrosis (**Chapter 5 and 7**). This makes these mice an ideal model to investigate NASH in the context of metabolic disturbances (i.e.

obesity, insulin resistance, dyslipidemia) that characterizes many NASH patients. Moreover, these mice respond to insulin sensitizing drugs, such as rosiglitazone, similarly to humans [68], as shown in **Chapter 3**.

Transgenic ApoE*Leiden (E3L) mice exhibit a humanized lipid metabolism and lipoprotein profile upon a Western-type diet that is supplemented with cholesterol. The expression of a mutated form of human ApoE results in an impaired clearance of ApoE-containing lipoprotein particles. In contrast to other experimental models for atherosclerosis (e.g. LDLr-/- and ApoE-/- mice), these mice respond to lipid-modulating drugs in a more human-like manner [69]. Moreover, prolonged treatment with a high-cholesterol diet results in the development of NASH with fibrosis in context of dyslipidemia [70]. However, metabolic disease development in E3L mice occurs in absence of obesity, WAT inflammation and insulin resistance. Even though obesity is a major risk factor in NASH development, NASH can also occur in lean individuals [71]. In these patients, the most important metabolic risk factor is considered dyslipidemia [72]. Moreover, epidemiological studies link dietary cholesterol intake to the risk and severity of NAFLD [73,74], and in particular higher cholesterol intakes are observed in lean NASH patients [73]. This potentially makes the E3L mouse model a suitable model to study cholesterol-driven NASH development independently of obesity.

The role of diet in animal models of NASH

It is generally accepted that diet has a crucial role in inflammation [75] and the development of metabolic diseases [76]. More specifically, the intake of saturated fatty acids (SFA) is associated with a greater risk of NAFLD [77], while consumption of polyunsaturated fatty acids (PUFAs) is associated with reduced disease risk [78]. As such, we investigated in **chapter 6** whether (isocaloric) replacement of dietary saturated fat with pumpkin seed oil (rich in unsaturated fat) would attenuate NAFLD and atherosclerosis development. In addition, we examined whether phytochemicals present in unrefined (virgin) pumpkin seed oil exerts additional health effects over the refined oil. We showed that pumpkin seed oil reduces dyslipidemia and attenuates NAFLD and atherosclerosis development in E3L mice. The reduced hepatic fat content by pumpkin seed oil may be the result

of increasing fatty acid β-oxidation, as well as inhibiting *de novo* hepatic fatty acid synthesis. Notably, mice receiving virgin pumpkin seed oil showed additional effects on systemic inflammation markers and hepatic inflammation. This suggests that phytochemicals present in virgin oil may have putative anti-inflammatory properties leading to more pronounced effects on disease endpoints. Numerous studies in humans have reported that various phytochemicals can indeed reduce systemic inflammatory markers (reviewed in e.g. [10,79]), but the effects of phytochemicals on disease endpoints often remain unknown. Moreover, further research is needed to determine how phytochemicals can exert beneficial effects on metabolic inflammation. An important question is whether specific phytochemicals account for the reported health effects of phytochemical-rich foods or whether the natural combination of multiple phytochemicals is important to achieve health effects.

It should be noted that the metabolically-triggered inflammatory response underlying NASH development may differ depending on the type of dietary nutrient consumed. For instance, the intake of dietary fat leads to adipocyte hypertrophy and WAT inflammation, and ultimately can lead to the development of NASH (**Chapter 2**). By contrast, increased intake of cholesterol *per se* does not necessarily lead to WAT dysfunction (unpublished results of **chapter 6**), but it can induce NASH. As demonstrated herein (**chapter 6 and 7**) and by others [35,80], dietary cholesterol is a strong inducer of inflammatory and pro-fibrotic genes in the liver. Consistent with the pro-fibrotic effect of dietary cholesterol observed in E3L mice [70], high-fat/high-cholesterol diet (HFC)-fed E3L.CETP mice show onset of fibrosis after already 12 weeks of diet feeding. More specifically, HFC feeding resulted in the activation of pro-fibrotic signaling pathways in liver controlled by TNFα, PDGF and TGFβ as shown by transcriptome analysis in **chapter 7**. The same pathways were activated in mouse liver with high (1% w/w) but not low (0.25% w/w) dietary cholesterol [81], suggesting that the pro-fibrotic effect was attributable to dietary cholesterol itself and not to the fat content of the diet. It is likely that NASH-inducing diets containing high concentrations of cholesterol accentuate specific inflammatory pathways, while other inflammatory pathways that have been associated with human NASH development (e.g. IL-6 and leptin signaling [82]) have a relative lower contribution to disease progression in this model of cholesterol-induced NASH.

Although dietary composition obviously represents one of the most important causes of NASH, the translational aspect of diets used in experimental NASH models can be debated. For example, the recommended dietary cholesterol intake for humans is no more than 300 mg/day according to the dietary guidelines for Americans [83]. At a meal size of one kilogram, this amounts to 0.03%, while diets supplemented with cholesterol ranging from 0.15% up to 2% are often used [35,84]. **Chapter 5 and 7** demonstrate that NASH and liver fibrosis can also be induced in LDLr-/- mice with a high-fat diet containing 45 energy percent from fat with only trace amounts of cholesterol (0.03%), which is translational to human diets [85]. Moreover, as shown in **chapter 7** these animals develop pronounced liver fibrosis (>20% perihepatocellular fibrosis) when the HFD treatment is prolonged (34 weeks). Importantly, **chapter 3** shows that high-fat feeding in LDLr-/- mice results in expression of several inflammatory genes that are associated with severity of human NAFLD [86]. These findings implicate that this models reflects certain processes that are also relevant in human disease development.

Taken together, many animal models have been used to study the pathogenesis of NASH and may reflect different aspects of disease, but no model completely recapitulates the characteristics of NASH in humans. Notably, the composition of the diet plays a critical role in how NASH develops, as liver inflammation may be triggered directly (e.g. via dietary cholesterol) or indirectly (e.g. via WAT). Hence, depending on the strain and diet, animal models may represent different etiologies of disease development. Although there is a clear medical need to develop novel therapies for NASH, the evaluation of therapeutic strategies is hampered by lack mechanistic insight in human disease development. Therefore, identifying causative factors in disease progression is of critical importance to further improve preclinical models of human NASH.

CONCLUDING REMARKS AND FUTURE RECOMMENDATIONS

The incidence of NAFLD is increasing dramatically along with the pandemic of obesity worldwide. Therefore, therapeutic strategies in NAFLD that target obesity can be of importance to avoid or reverse the observed deleterious health effects.

Lifestyle changes related to weight reduction are at the basis of any treatment strategy for metabolically-oriented diseases. However, many patients fail to implement lifestyle changes and pharmaceutical or nutritional interventions may be the alternative to decrease the disease burden. In this thesis, we show that a 'simple' switch in the type of fatty acid consumed can reduce metabolic overload and inflammation in the liver. Furthermore, we identified several new targets (e.g. CCR2 and caspase-1) that could be considered for pharmacological intervention.

NAFLD is a complex disease, in which mechanisms underlying disease progression are poorly understood. The complexity of this disease is highlighted by the fact that not all obese patients show disease progression, even though obesity is a major risk factor of NAFLD. The inflammatory tone of white adipose tissue, rather than its quantity, may underlie the development of obesity-associated diseases. The inflammatory state of intra-abdominal white adipose tissue shows to be a predictive marker for development of (systemic) insulin resistance and is associated with NAFLD progression. This is of great clinical significance, since it suggests that collection of intra-abdominal WAT, rather than subcutaneous fat, may have a great potential for the diagnosis of NASH. However, it should be noted that WAT inflammation in itself may not be the cause of NASH development, but rather the release of (pro-inflammatory) adipokines and fatty acids by inflamed WAT.

The lack of non-invasive biomarkers for the diagnosis of NASH is problematic in current medical practice. Liver biopsies are currently the 'golden-standard' for the diagnosis of NASH. This diagnosis is not only invasive, but also expensive and unsuitable as a tool for population screening. Hence, the most urgent need in the field of NAFLD is the discovery of biomarkers that would help a) to diagnose the stage of the disease, and b) to monitor disease progression. We potentially identified specific saturated fatty acids in the circulation associated with NASH

development that could serve as non-invasive biomarker. Future studies should validate whether these markers are suitable for the use in humans.

In conclusion, this thesis highlighted the importance of WAT in the development of NASH. Additionally, this thesis provides evidence for the contribution of specific molecular mechanisms in the development of 'metabolic inflammation' in NASH and highlights CCR2 and caspase-1 as potential targets for therapeutical intervention.

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