Universiteit
Leiden
The Netherlands

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

Mulder, P.C.A.

Citation
Mulder, P. C. A. (2017, February 16). *The contribution of metabolic and adipose tissue inflammation to non-alcoholic fatty liver disease*. Retrieved from https://hdl.handle.net/1887/46137

Version:	Not Applicable (or Unknown)
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/46137

Note: To cite this publication please use the final published version (if applicable).

Cover Page

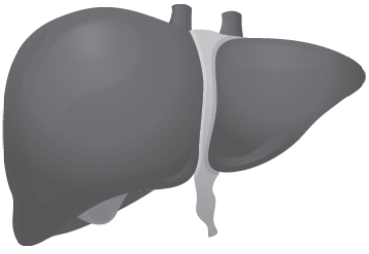
Universiteit Leiden

The handle http://hdl.handle.net/1887/46137 holds various files of this Leiden University dissertation

Author: Mulder, P.C.A.
Title: The contribution of metabolic and adipose tissue inflammation to non-alcoholic fatty liver disease
Issue Date: 2017-02-16

Chapter 1

General introduction



Current lifestyle trends in modern society, characterized by excess energy consumption and reduced physical activity have propelled the incidence of obesity to epidemic proportions [1]. Obesity is associated with increased risk for type 2 diabetes (T2D) and comorbidities, including non-alcoholic fatty liver disease (NAFLD) [2].

NAFLD is a metabolic disorder characterized by fat accumulation in the liver in the absence of chronic alcohol consumption [3]. Clinically, NAFLD encompasses a broad spectrum of liver conditions ranging from fat accumulation (steatosis) to steatosis with inflammation (non-alcoholic steatohepatitis, NASH), which can further progress to fibrosis, cirrhosis and ultimately to hepatocellular carcinoma [4]. Patients with NAFLD are at risk to develop other metabolic complications such as cardiovascular disease (CVD) [5-7] and have a higher overall mortality [7,8].

1. PREVALENCE AND DIAGNOSIS OF NAFLD

Recent estimates suggest that NAFLD is present in 30% of the general population [2,9,10]. NAFLD is tightly linked to obesity as recent estimates suggest that up to 90% of obese patients have NAFLD [11]. Furthermore, the presence of the metabolic syndrome (defined as central obesity accompanied by two or more of the following metabolic risk factors: elevated fasting glucose concentration (reflecting insulin resistance), hypertension, raised triglyceride (TG) levels and lowered high-density lipoprotein cholesterol (HDL) levels), is associated with more progressive disease [12].

NAFLD is often an asymptomatic disease and the majority of patients with NAFLD are identified by increased liver enzymes (alanine aminotransferase, ALT; aspartate aminotransferase, AST) during a routine blood test [13]. Although liver enzymes (i.e. ALT) levels have shown to be a good predictor of hepatic steatosis [14], ALT levels can be found normal in patients with severe liver pathology [15]. While steatosis can be diagnosed by non-invasive imaging, such as ultrasound and magnetic resonance imaging (MRI), none of these techniques can detect inflammation. Consequently, invasive liver biopsy is currently the gold-standard for

diagnosing the advanced stages of NAFLD (NASH and fibrosis) as well as monitoring disease progression [16]. Differentiating NASH from simple steatosis is important, because longitudinal studies have shown that patients with steatosis have similar life expectancy to that of the general population of same age and sex [17], while NASH patients have significantly higher total mortality rates [8]. Moreover, the degree of fibrosis in NASH patients is associated with a higher risk of liver-related morbidity and mortality [7,18].

1.1 Pathophysiology of NAFLD

1.1.1 Histopathology

A distinct hallmark of NAFLD is steatosis, a histological manifestation of fat deposition in the form of triglycerides within hepatocytes [4]. Morphologically, steatosis can manifest in two forms of lipid accumulation, i.e. macrovesicular or microvesicular steatosis. In macrovesicular steatosis, hepatocytes contain a large, single vacuole of fat which fills the cytoplasm and displaces the nucleus to the periphery (see [19] and references therein) (figure 1). By contrast, hepatocytes with microvesicular steatosis contain many small lipid droplets in the cytoplasm [19] (figure 1).

The histological features of NASH include steatosis, hepatocellular injury (usually characterized by hepatocellular ballooning), and lobular inflammation [20] (figure 1). Hepatocellular ballooning refers to swelling of hepatocytes with rarefied cytoplasm and is associated with cytoskeletal injury [21]. The ballooning hepatocytes are mainly located near steatotic hepatocytes and are often, but not always, associated with perisinusoidal fibrosis. Lobular inflammation in NASH is characterized by the presence of inflammatory aggregates, which are typically composed of a mixture of innate and adaptive immune cells, such as neutrophils, lymphocytes and macrophages [22].

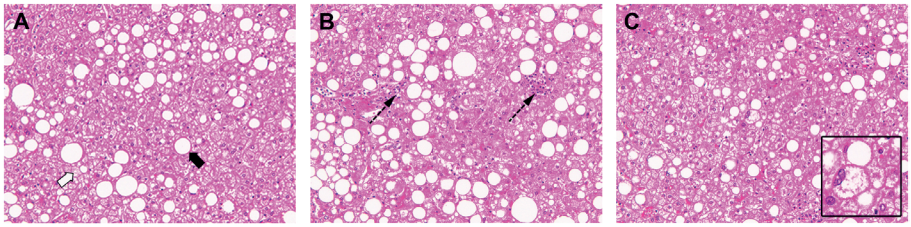


Figure 1. Histological subtypes of human NAFLD. Histological cross-sections of human liver stained with hematoxylin-eosin showing (A) Simple steatosis, predominantly in the form of macrovesicular steatosis (closed arrow) and some microvesicular steatosis (open arrow). (B) Non-alcoholic steatohepatitis (NASH) characterized by steatosis with lobular inflammation (dashed line arrow). (C) NASH characterized by the additional presence of hepatocellular ballooning (insert, magnification x400). (Unless specified otherwise, microphotographs: magnification x200).

1.1.2 Grading and staging of disease

Besides the diagnostic purpose, a liver biopsy is frequently used to assess the severity of disease. In 2005, the NASH Clinical Research Network developed and validated a semiquantitative scoring system for the evaluation of serial biopsies from NAFLD patients in clinical trials [20]. This scoring system, also called NAFLD Activity Score (NAS), unifies important features of NASH into an activity score or a "grade". This grade can range from a score 0 to 8, which consists of the summation of individual scores for steatosis (0-3), lobular inflammation (0-3), and hepatocellular ballooning (0-2). NAS of 1 or 2 indicates no NASH, while a NAS score of 5-8 corresponds to definite NASH. Activity scores 3 and 4 are noted as borderline cases of NASH, as these scores do not fulfill the pathologists' criteria for definite NASH. The scoring system was extended by a fibrosis score or the "stage" of disease, which reflects the unique patterns of fibrosis that can occur in NASH.

Despite the widespread use of NAS in preclinical NAFLD models, this scoring system has never been validated for experimental rodent samples. Therefore, a generic NAFLD grading system for preclinical (mouse) models has been developed based on the human NAS scoring system [23]. Furthermore, with this scoring method for rodents, the individual components of NASH (e.g. number of inflammatory cells) are analyzed on a continuous scale making it possible to investigate more subtle effects of treatments.

1.2 Etiology of disease

Although obesity and insulin resistance has been established as risk factors for NAFLD, the underlying mechanisms that contribute to disease progression from simple steatosis to NASH remain unclear.

The initial stage of NAFLD involves accumulation of fat, predominantly triglycerides, in the liver. The accumulation of fat may be the result of an imbalance between fatty acid transport, synthesis and oxidation or a combination of these factors. Originally, the pathogenesis of NASH was conceptualized as a disease of 'two-hits': the 'first hit', hepatic fat accumulation, sensitized the liver to a 'second hit' which caused tissue injury, inflammation and fibrosis [24]. A number of factors, including pro-inflammatory cytokines, endotoxins, adipokines, mitochondrial dysfunction, oxidative stress and subsequent lipid peroxidation, have been proposed as the second hit [25]. However, inflammatory cytokines such as IL-1β or endotoxin (LPS) that were superimposed on a high-fat diet (HFD) for several weeks failed to induce NASH in experimental models of disease, while metabolic triggers of inflammation (e.g. cholesterol, carbohydrates) caused NASH [26]. However, it is thought that the etiology of NASH is a more complex process and may involve specific metabolic factors, i.e. lipids, that trigger liver injury and disease progression.

The involvement of lipids in the pathogenesis of NASH has led to the concept of 'lipotoxicity', which implies that exposure to, or accumulation of, certain lipids within hepatic cells may directly cause cellular toxicity or act in a pro-inflammatory manner [27]. NASH is associated with two defects that can lead to lipotoxicity: (1) increased delivery of free fatty acids (FFA) to the liver caused by uninhibited lipolysis in insulin-resistant white adipose tissue [28]; and (2) the formation of cholesterol crystals within the lipid droplets of steatotic hepatocytes that can cause lipotoxic injury within these cells [27,29]. The role of white adipose tissue and cholesterol in NASH will be discussed in more detail in the following sections.

1.2.1 The role of white adipose tissue in liver pathology

The principal function of white adipose tissue (WAT) is to store and release fat in response to energy needs. In obesity, WAT mass is expanding in response to excess energy. However, this expansion may be limited, resulting in lipid accumulation in

other organs throughout the body (ectopic fat) [30]. It is hypothesized that ectopic hepatic fat accumulation observed in NAFLD could be due to increased delivery of free fatty acids (FFA) from adipose tissue. In support of this, studies in humans have demonstrated that increased FFA delivery from WAT is a significant source for lipids in the liver [28,31]. Excess FFA, rather than triglyceride accumulation, may result in lipotoxicity in the liver by activating inflammatory and oxidative stress related pathways [32]. Saturated fatty acids, such as palmitic acid, are poorly incorporated into triglycerides and have been shown to cause apoptosis in hepatocytes in vitro [33,34]. By contrast, specific monounsaturated fatty acids (e.g. oleic acid) are thought to have a protective role against palmitic acid-induced apoptosis by promoting incorporation of palmitic acid into triglycerides [33]. When triglyceride accumulation is impaired, free fatty acids may no longer be safely incorporated into triglyceride pools, leading the buildup of lipotoxic metabolites that can cause liver injury and trigger NASH development [32,35].

In insulin-resistant patients, FFA plasma levels are often found to be elevated possibly due to uncontrolled lipolysis in WAT and lead to increased FA flux to the liver [36]. A key mechanism in the pathogenesis of obesity-associated insulin resistance relates to WAT inflammation, which has been well demonstrated in experimental and human studies (reviewed in e.g. [37]). This WAT inflammation is characterized by infiltration of adipose tissue macrophages [38]. These infiltrating macrophages may secrete inflammatory mediators, including the pro-inflammatory cytokine Tumour-Necrosis Factor-alpha (TNFα), which contributes to a local chronic inflammatory state characterized by impaired fat deposition and increased lipolysis in WAT [39].

Although obesity is strongly associated with metabolic complications, the distribution of fat appears more important than the total amount of fat mass per se. Increased intra-abdominal fat, but not subcutaneous fat, has been positively associated with insulin resistance [40] and contributes to increased FFA flux towards the liver in insulin-resistant individuals [31]. Furthermore, intra-abdominal fat mass strongly correlates with liver inflammation and fibrosis, whereas the amount of subcutaneous fat is not associated with histological changes in the liver [40].

Not only the distribution of fat mass in particular WAT depots may be of importance in NASH development, evidence also points to a role of the inflammatory state of WAT [41]. For instance, longitudinal studies in rodents demonstrated that HFD-induced expression of inflammatory genes in WAT precedes the development of NASH in obesity [42,43]. Furthermore, Cancello and colleagues [44] have shown that obese humans with inflamed intra-abdominal WAT have more fibro-inflammatory lesions in the liver than equally obese subjects without WAT inflammation. It is postulated that inflammation in WAT results in increased production of pro-inflammatory cytokines and adipokines (e.g. TNFα, leptin) and decreased production of protective adipokines (adiponectin) [45]. This imbalance in adipokines is thought to contribute to the development of NASH. Although evidence suggests that WAT constitutes an important source of inflammation in NAFLD, experimental proof for a causal role of WAT in NASH is still lacking.

1.2.2 The role of dietary cholesterol in liver pathology

Growing evidence suggest that cholesterol is a critical factor in the development of NASH. Data from epidemiological studies link dietary cholesterol intake to the risk and severity of NAFLD [46,47]. In line with this, expression of inflammatory genes were increased in livers of mice fed a high-cholesterol diet (1% w/w) but not with a lower concentration (0.25% w/w) [48]. Other experimental studies have shown that dietary cholesterol negatively affect the balance between storage and oxidation of fatty acids in the liver [49] and can lead to oxidative stress and hepatic inflammation [50]. Furthermore, increased levels of hepatic free cholesterol are observed in experimental [29,51-53] and human NASH [29,54,55], while lowering of excess hepatic free cholesterol levels improved liver disease severity [27,53,56,57]. Mechanistic studies have shown that free cholesterol accumulation in the liver can promote inflammation and fibrogenesis through the activation of intracellular signaling pathways in hepatic resident macrophages (Kupffer cells) [27] and hepatic stellate cells [58]. However, other studies in mice using dietary cholesterol have shown that hepatic inflammation can develop without steatosis [52] and obesity [59]. Furthermore, cholesterol-induced hepatic inflammation did not contribute to the development of insulin resistance in male LDLr-/-mice [60]

and addition of cholesterol to a high-fat diet (HFD) can protect from HFD-induced insulin resistance in mice (Hanemaaijer, Pieterman unpublished results). These data suggest that dietary cholesterol contributes to NASH development that is independent of insulin resistance.

1.2.3 Inflammatory processes in liver during NASH

Chronic inflammation in the liver is critical in the progression of NAFLD. Activation of the innate immune system – the body's rapid first-line defense against pathogens – is a key component in initiating and for sustaining inflammation in the liver [61]. Innate immune cells recognize pathogen invasion with intracellular or surface-expressed pattern recognition receptors (PRRs) by detecting pathogen-associated molecular patterns (PAMPs). These PRRs are also able to detect endogenous damage and stress signals through damage-associated molecular patterns (DAMPS). Importantly, the DAMP-associated immune response occurs in absence of pathogens and is referred to as 'sterile inflammation', which can occur in all tissues in response to injury and cellular damage. It is thought that metabolic overload (i.e. surplus of energy or macronutrients) triggers this 'sterile' or so-called 'metabolic inflammation' in metabolic diseases. However, underlying mechanisms controlling the inflammatory processes in NASH development remain poorly understood.

Kupffer cells are the primary sensors of PAMPs and DAMPs and considered key players in the pathogenesis of NASH, as depletion of these cells in mice results in resistance to develop hepatic steatosis, inflammation and fibrosis [62,63]. Specifically, the dysregulation between pro-inflammatory macrophages (M1) and anti-inflammatory macrophages (M2) is emerging as a central mechanism driving inflammation [64]. During NASH development, activation of Kupffer cells by inflammatory factors (e.g. FFA) may shift their phenotype towards a pro-inflammatory M1 state. Activation of Kupffer cells may also govern the recruitment of blood–derived monocytes/macrophages during NASH. Both, Kupffer cells and recruited macrophages enhance local inflammation and produce inflammatory mediators (e.g. TNFα, IL-1β) which, in turn, can further stimulate hepatocytes and stellate cells to induce steatosis and fibrosis, respectively [65].

Evidence suggests that recruitment of macrophages into the liver is primarily promoted by monocyte chemotactic protein-1 (MCP-1), a chemokine that is upregulated in livers of NASH patients [66]. In turn, MCP-1 drives the recruitment of C–C chemokine receptor 2 (CCR2) expressing monocytes into the liver. Genetic deletion of MCP-1 or CCR2 has been shown to reduce steatosis and macrophage infiltration in livers of mice [67,68], suggesting that interventions directed at CCR2 can represent an potential target for the treatment of NASH.

Inflammasomes have emerged as an important component of Kupffer cell activation and NAFLD progression. The inflammasome is a protein complex that consists of an intracellular sensor molecule (the NLR), an adaptor sensor protein (ASC) and the effector protein caspase-1 [69]. One of the best characterized inflammasomes is the NLR family pyrin domain-containing 3 (NLRP3) inflammasome. NLRP3 complex becomes activated in response to PAMPs or DAMPs, which results in the maturation of pro-caspase-1 into activated caspase-1. Activated caspase-1 cleaves the precursors of pro-inflammatory cytokines IL-1β and IL-18 into their biologically active counterparts, which are then readily secreted from the cells and initiate an inflammatory response [69]. Experimental studies have shown that knockdown of NLRP3-associated genes (i.e. ASC, NLRP3 or Caspase-1) in HFD-fed mice attenuates obesity-associated inflammation and reduces the development of metabolic complications, including insulin resistance [70,71] and NAFLD [72], suggesting that the NLRP3 inflammasome constitutes a potential target for therapeutic intervention in NASH.

1.3 NAFLD and CVD

Accumulating evidence suggest that patients with NAFLD have a 2-fold higher risk in developing CVD [5-7]. In line with this, NAFLD is associated with atherosclerosis development [73], the underlying pathology of CVD. However, the biological mechanisms linking NAFLD and CVD are still poorly understood.

Patients with NAFLD frequently have a disturbed lipid profile characterized by high triglyceride levels, increased (very) low-density lipoprotein ((V)LDL) cholesterol levels and decreased levels HDL cholesterol [5]. This unfavorable lipid profile drives atherosclerosis development and can, for instance, be the result of increased

hepatic production of triglyceride-rich VLDL to reduce metabolic overload in the liver [74].

Atherosclerosis is increasingly being considered an inflammatory disease in which the liver is thought to be a central mediator in the regulation of inflammation. Support for an atherogenic role of liver inflammation in humans comes from the observation that CVD risk is greater in NASH patients compared to subjects with simple steatosis [75]. Several studies suggest that increased production of pro-inflammatory factors by the liver play an important role in the pathogenesis of atherosclerosis (reviewed in [76,77]). Among them are markers of systemic inflammation, such as cytokines (e.g. IL-6 and TNFα) and acute-phase proteins (e.g. Serum Amyloid A (SAA), fibrinogen).

1.4 Treatments of NAFLD

1.4.1 Lifestyle intervention

Despite its prevalence, treatment options for NAFLD are limited. The recommended mainstay treatment for the majority of NAFLD patients is lifestyle modification (diet and/or increased physical activity) to induce weight loss. Recent studies have shown that reduced energy intake and increased physical activity induces weight loss and improves insulin resistance, liver enzymes, and hepatic fat content [78,79]. Lifestyle modification can also result in improved NAS score in NASH patients [80-82]. More specifically, these studies showed a significant reduction in steatosis grade, but most of them concluded that 7% to 10% weight loss is required for the improvement of hepatic inflammation, hepatocellular ballooning [81,82] and fibrosis [83].

Exercise alone can also improve hepatic fat content and insulin resistance in obese patients [84]. In addition, performing exercise has shown to reduce the likelihood of having NASH by a third [85], but whether exercise affects liver histopathology in NAFLD patients has not been reported so far.

It is important to note that weight loss is seldom maintained in many patients, because (low-caloric) diets and/or physical exercise are often discontinued [86]. In patients who failed to implement lifestyle changes, pharmacological treatments directed at improving NASH might be necessary.

1.4.2 Insulin sensitizers

Current pharmacological treatments of NAFLD aim at modifying risk factors. Insulin resistance is closely associated with NASH, therefore most therapeutic trials have focused on the effect of insulin sensitizers. In particular, the oral antidiabetic drugs thiazolidinediones (TZDs) have been intensively studied in patients with NASH [87,88].

An open label trial of rosiglitazone in 26 biopsy-proven NASH patients [89] and two placebo-controlled trials with pioglitazone [89,90] demonstrated improvements in liver enzymes as well as NAS score during 48 weeks of treatment. A recent meta-analysis of 4 randomized, placebo-controlled clinical trials also confirmed that both, rosiglitazone and pioglitazone significantly improve steatosis, ballooning, lobular inflammation while the effects on fibrosis were less clear [91]. However, the underlying mechanisms mediating the beneficial effects of TZDs in NASH development remain unclear.

TZDs improve insulin sensitivity by acting as selective agonists of the nuclear peroxisome proliferator-activated receptor (PPAR)-γ [92]. PPARγ is predominantly expressed in adipose tissue where it controls inflammatory and metabolic processes [37], suggesting that TZDs exert their hepatoprotective effects via WAT.

The side effects of TZDs however are of great concern, in particular weight gain, which tend to persist after discontinuation of the treatment [86]. Furthermore, the long-term safety of glitazones has been debated concerning the increased risk for cardiovascular events [93]. Hence, rosiglitazone was withdrawn from the market in September 2010. In November 2013, the U.S. Food and Drug Administration (FDA) has lifted its earlier restrictions for the use of rosiglitazone, as recent data did not show increased risk of heart attack compared to the standard type 2 diabetes medicines, such as metformin [94].

1.4.3 Nutritional interventions

Epidemiological studies show that diet is an important determinant for the risk of both NAFLD and associated comorbidities [95-97]. In particular the intake of saturated fatty acids (SFA) is associated with a greater risk of NAFLD [98]. Other studies in patients further support this association, reporting that NAFLD patients

have a higher intake of SFA, fructose and cholesterol with lowered consumption of polyunsaturated fatty acids, fibers, and antioxidants [46,98]. Although it is thought that caloric restriction is most important for improvement of NAFLD [19,99], evidence suggest that modulating the composition of the diet can also be of importance [100]. Data from a recent randomized controlled trial showed that a Mediterranean diet, which is rich in monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA), can reduce liver fat content and improve hepatic insulin resistance, independent of the observed weight loss effect [101]. Supplementation of MUFA and/or PUFA is currently investigated as a potential treatment against NAFLD [100,102,103]. Increased MUFA intake, particularly as a replacement for SFA, is associated with decreased insulin resistance and hepatic steatosis [101]. Others have shown that omega-3 PUFA administration to patients with NAFLD can have beneficial effects on liver enzymes and hepatic steatosis [104,105] and may also improve hepatic inflammation and fibrosis [105]. Despite these encouraging data about the efficacy of PUFAs as a treatment of NAFLD in humans, they have been limited by small sample sizes, lack of randomization or placebo arms. Hence, further studies are needed to assess the feasibility and benefits of such alimentary interventions. Nevertheless, these data suggest that a switch in the type of fat consumed or supplementation of specific fatty acids could be of interest as a treatment to reduce metabolic complications.

1.5 Outline of this thesis

NAFLD is a complex disease, in which the origin and molecular mechanisms controlling the progression of simple steatosis to NASH remain poorly understood. The aim of this thesis is to provide more insight in the mechanisms underlying NAFLD progression, focusing on the role of WAT and specific aspects that can trigger metabolic inflammation.

The first part of this thesis focuses on the link between WAT and liver, in which we studied the potential role of WAT in NASH development (**Chapter 2**). As obesity-induced inflammation in WAT is thought to be critical in NASH development, we first examined the sequence of inflammatory events in different WAT depots and liver in a time-course experiment in context of diet-induced obesity. In a subsequent

experiment, we examined whether WAT is causally involved in NASH development by surgical removal of a specific inflamed WAT depot. As WAT may constitute a new target for the treatment of NAFLD, we next examined whether intervention in WAT inflammation with rosiglitazone (a PPARγ activator) would attenuate NAFLD development (**Chapter 3**).

Chapter 4 and **chapter 5** focused on interventions directed at specific mediators in metabolic inflammation to study whether these interventions can attenuate the development of NAFLD. More specifically, we studied the therapeutic effect of a CCR2 inhibitor (**chapter 4**) and inflammasome inhibition, using a caspase-1 inhibitor, (**chapter 5**) on NAFLD development in context of manifest insulin resistance and WAT inflammation.

In **chapter 6**, we examined the potential of a distinct nutritional strategy to prevent the development of NAFLD, by changing the macronutrient composition of the diet to reduce metabolic overload. We investigated whether 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.

Metabolic overload results in the increased fat deposition within the liver, however it is unclear whether a distinct type of fat storage i.e. macrovesicular or microvesicular steatosis, contributes to NAFLD progression. Therefore, 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 that were also used in the previous chapters. Finally, the results obtained in the studies described herein and their implications are discussed in **chapter 8**.

REFERENCES

1. Hurt RT, Kulisek C, Buchanan LA, McClave SA. The obesity epidemic: challenges, health initiatives, and implications for gastroenterologists. Gastroenterology & hepatology 2010;6:780-792.
2. Marchesini G, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini R, et al. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. Hepatology 2003;37:917-923.
3. Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, et al. The diagnosis and management of non-alcoholic fatty liver disease: Practice guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. The American journal of gastroenterology 2012;107:811-826.
4. Tiniakos DG, Vos MB, Brunt EM. Nonalcoholic fatty liver disease: pathology and pathogenesis. Annual review of pathology 2010;5:145-171.
5. Bhatia LS, Curzen NP, Calder PC, Byrne CD. Non-alcoholic fatty liver disease: a new and important cardiovascular risk factor? European heart journal 2012;33:1190-1200.
6. Armstrong MJ, Adams LA, Canbay A, Syn WK. Extrahepatic complications of nonalcoholic fatty liver disease. Hepatology 2014;59:1174-1197.
7. Adams LA, Lymp JF, St Sauver J, Sanderson SO, Lindor KD, Feldstein A, et al. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. Gastroenterology 2005;129:113-121.
8. Angulo P. Long-term mortality in nonalcoholic fatty liver disease: is liver histology of any prognostic significance? Hepatology 2010;51:373-375.
9. Williams CD, Stengel J, Asike MI, Torres DM, Shaw J, Contreras M, et al. Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. Gastroenterology 2011;140:124-131.
10. Loomba R, Sanyal AJ. The global NAFLD epidemic. Nature reviews Gastroenterology & hepatology 2013;10:686-690.
11. Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. Alimentary pharmacology & therapeutics 2011;34:274-285.
12. Rinella ME. Nonalcoholic fatty liver disease: a systematic review. Jama 2015;313:2263-2273.
13. Armstrong MJ, Houlihan DD, Bentham L, Shaw JC, Cramb R, Olliff S, et al. Presence and severity of non-alcoholic fatty liver disease in a large prospective primary care cohort. Journal of hepatology 2012;56:234-240.
14. Kotronen A, Westerbacka J, Bergholm R, Pietilainen KH, Yki-Jarvinen H. Liver fat in the metabolic syndrome. The Journal of clinical endocrinology and metabolism 2007;92:3490-3497.
15. Mofrad P, Contos MJ, Haque M, Sargeant C, Fisher RA, Luketic VA, et al. Clinical and histologic spectrum of nonalcoholic fatty liver disease associated with normal ALT values. Hepatology 2003;37:1286-1292.
16. Wieckowska A, McCullough AJ, Feldstein AE. Noninvasive diagnosis and monitoring of nonalcoholic steatohepatitis: present and future. Hepatology 2007;46:582-589.
17. Dam-Larsen S, Franzmann M, Andersen IB, Christoffersen P, Jensen LB, Sorensen TI, et al. Long term prognosis of fatty liver: risk of chronic liver disease and death. Gut 2004;53:750-755.

18. Ekstedt M, Hagstrom H, Nasr P, Fredrikson M, Stal P, Kechagias S, et al. Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up. Hepatology 2015;61:1547-1554.
19. Green CJ, Hodson L. The influence of dietary fat on liver fat accumulation. Nutrients 2014;6:5018-5033.
20. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41:1313-1321.
21. Caldwell S, Ikura Y, Dias D, Isomoto K, Yabu A, Moskaluk C, et al. Hepatocellular ballooning in NASH. Journal of hepatology 2010;53:719-723.
22. Farrell GC, van Rooyen D, Gan L, Chitturi S. NASH is an Inflammatory Disorder: Pathogenic, Prognostic and Therapeutic Implications. Gut and liver 2012;6:149-171.
23. Liang W, Menke AL, Driessen A, Koek GH, Lindeman JH, Stoop R, et al. Establishment of a general NAFLD scoring system for rodent models and comparison to human liver pathology. PloS one 2014;9:e115922.
24. Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology 1998;114:842-845.
25. Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. Hepatology 2010;52:1836-1846.
26. Liang W, Lindeman JH, Menke AL, Koonen DP, Morrison M, Havekes LM, et al. Metabolically induced liver inflammation leads to NASH and differs from LPS- or IL-1beta-induced chronic inflammation. Laboratory investigation; a journal of technical methods and pathology 2014;94:491-502.
27. Ioannou GN, Van Rooyen DM, Savard C, Haigh WG, Yeh MM, Teoh NC, et al. Cholesterol-lowering drugs cause dissolution of cholesterol crystals and disperse Kupffer cell crown-like structures during resolution of NASH. Journal of lipid research 2015;56:277-285.
28. Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. The Journal of clinical investigation 2005;115:1343-1351.
29. Ioannou GN, Haigh WG, Thorning D, Savard C. Hepatic cholesterol crystals and crown-like structures distinguish NASH from simple steatosis. Journal of lipid research 2013;54:1326-1334.
30. Virtue S, Vidal-Puig A. It's not how fat you are, it's what you do with it that counts. PLoS Biol 2008;6:e237.
31. Nielsen S, Guo Z, Johnson CM, Hensrud DD, Jensen MD. Splanchnic lipolysis in human obesity. The Journal of clinical investigation 2004;113:1582-1588.
32. Neuschwander-Tetri BA. Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites. Hepatology 2010;52:774-788.
33. Listenberger LL, Han X, Lewis SE, Cases S, Farese RV, Jr., Ory DS, et al. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. Proceedings of the National Academy of Sciences of the United States of America 2003;100:3077-3082.
34. Wei Y, Wang D, Topczewski F, Pagliassotti MJ. Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells. American journal of physiology Endocrinology and metabolism 2006;291:E275-281.
35. Sahini N, Borlak J. Recent insights into the molecular pathophysiology of lipid droplet formation in hepatocytes. Progress in lipid research 2014;54:86-112.
36. Lomonaco R, Ortiz-Lopez C, Orsak B, Webb A, Hardies J, Darland C, et al. Effect of adipose tissue insulin resistance on metabolic parameters and liver histology in obese patients with nonalcoholic fatty liver disease. Hepatology 2012;55:1389-1397.

37. Shoelson SE, Lee J, Goldfine AB. Inflammation and insulin resistance. The Journal of clinical investigation 2006;116:1793-1801.
38. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. The Journal of clinical investigation 2003;112:1796-1808.
39. Olefsky JM, Glass CK. Macrophages, inflammation, and insulin resistance. Annual review of physiology 2010;72:219-246.
40. van der Poorten D, Milner KL, Hui J, Hodge A, Trenell MI, Kench JG, et al. Visceral fat: a key mediator of steatohepatitis in metabolic liver disease. Hepatology 2008;48:449-457.
41. Mirza MS. Obesity, Visceral Fat, and NAFLD: Querying the Role of Adipokines in the Progression of Nonalcoholic Fatty Liver Disease. ISRN gastroenterology 2011;2011:592404.
42. Duval C, Thissen U, Keshtkar S, Accart B, Stienstra R, Boekschoten MV, et al. Adipose tissue dysfunction signals progression of hepatic steatosis towards nonalcoholic steatohepatitis in C57BL/6 mice. Diabetes 2010;59:3181-3191.
43. Liang W, Tonini G, Mulder P, Kelder T, van Erk M, van den Hoek AM, et al. Coordinated and interactive expression of genes of lipid metabolism and inflammation in adipose tissue and liver during metabolic overload. PloS one 2013;8:e75290.
44. Cancello R, Tordjman J, Poitou C, Guilhem G, Bouillot JL, Hugol D, et al. Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity. Diabetes 2006;55:1554-1561.
45. Tilg H, Moschen AR. Adipocytokines: mediators linking adipose tissue, inflammation and immunity. Nature reviews Immunology 2006;6:772-783.
46. Musso G, Gambino R, De Michieli F, Cassader M, Rizzetto M, Durazzo M, et al. Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis. Hepatology 2003;37:909-916.
47. Yasutake K, Nakamuta M, Shima Y, Ohyama A, Masuda K, Haruta N, et al. Nutritional investigation of non-obese patients with non-alcoholic fatty liver disease: the significance of dietary cholesterol. Scandinavian journal of gastroenterology 2009;44:471-477.
48. Kleemann R, Verschuren L, van Erk MJ, Nikolsky Y, Cnubben NH, Verheij ER, et al. Atherosclerosis and liver inflammation induced by increased dietary cholesterol intake: a combined transcriptomics and metabolomics analysis. Genome biology 2007;8:R200.
49. Kalaany NY, Gauthier KC, Zavacki AM, Mammen PP, Kitazume T, Peterson JA, et al. LXRs regulate the balance between fat storage and oxidation. Cell metabolism 2005;1:231-244.
50. Subramanian S, Goodspeed L, Wang S, Kim J, Zeng L, Ioannou GN, et al. Dietary cholesterol exacerbates hepatic steatosis and inflammation in obese LDL receptor-deficient mice. Journal of lipid research 2011;52:1626-1635.
51. Van Rooyen DM, Larter CZ, Haigh WG, Yeh MM, Ioannou G, Kuver R, et al. Hepatic free cholesterol accumulates in obese, diabetic mice and causes nonalcoholic steatohepatitis. Gastroenterology 2011;141:1393-1403, 1403 e1391-1395.
52. Wouters K, van Gorp PJ, Bieghs V, Gijbels MJ, Duimel H, Lutjohann D, et al. Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis. Hepatology 2008;48:474-486.
53. Morrison MC, Liang W, Mulder P, Verschuren L, Pieterman E, Toet K, et al. Mirtoselect, an anthocyanin-rich bilberry extract, attenuates non-alcoholic steatohepatitis and associated fibrosis in ApoE(*)3Leiden mice. Journal of hepatology 2015;62:1180-1186.

54. Puri P, Baillie RA, Wiest MM, Mirshahi F, Choudhury J, Cheung O, et al. A lipidomic analysis of nonalcoholic fatty liver disease. Hepatology 2007;46:1081-1090.
55. Caballero F, Fernandez A, De Lacy AM, Fernandez-Checa JC, Caballeria J, Garcia-Ruiz C. Enhanced free cholesterol, SREBP-2 and StAR expression in human NASH. Journal of hepatology 2009;50:789-796.
56. Zheng S, Hoos L, Cook J, Tetzloff G, Davis H, Jr., van Heek M, et al. Ezetimibe improves high fat and cholesterol diet-induced non-alcoholic fatty liver disease in mice. Eur J Pharmacol 2008;584:118-124.
57. Wielinga PY, Yakala GK, Heeringa P, Kleemann R, Kooistra T. Beneficial effects of alternate dietary regimen on liver inflammation, atherosclerosis and renal activation. PloS one 2011;6:e18432.
58. Teratani T, Tomita K, Suzuki T, Oshikawa T, Yokoyama H, Shimamura K, et al. A high-cholesterol diet exacerbates liver fibrosis in mice via accumulation of free cholesterol in hepatic stellate cells. Gastroenterology 2012;142:152-164 e110.
59. Sumiyoshi M, Sakanaka M, Kimura Y. Chronic intake of a high-cholesterol diet resulted in hepatic steatosis, focal nodular hyperplasia and fibrosis in non-obese mice. The British journal of nutrition 2010;103:378-385.
60. Funke A, Schreurs M, Aparicio-Vergara M, Sheedfar F, Gruben N, Kloosterhuis NJ, et al. Cholesterol-induced hepatic inflammation does not contribute to the development of insulin resistance in male LDL receptor knockout mice. Atherosclerosis 2014;232:390-396.
61. Kubes P, Mehal WZ. Sterile inflammation in the liver. Gastroenterology 2012;143:1158-1172.
62. Huang W, Metlakunta A, Dedousis N, Zhang P, Sipula I, Dube JJ, et al. Depletion of liver Kupffer cells prevents the development of diet-induced hepatic steatosis and insulin resistance. Diabetes 2010;59:347-357.
63. Neyrinck AM, Cani PD, Dewulf EM, De Backer F, Bindels LB, Delzenne NM. Critical role of Kupffer cells in the management of diet-induced diabetes and obesity. Biochem Biophys Res Commun 2009;385:351-356.
64. Marra F, Lotersztajn S. Pathophysiology of NASH: perspectives for a targeted treatment. Current pharmaceutical design 2013;19:5250-5269.
65. Ramadori G, Armbrust T. Cytokines in the liver. European journal of gastroenterology & hepatology 2001;13:777-784.
66. Haukeland JW, Damas JK, Konopski Z, Loberg EM, Haaland T, Goverud I, et al. Systemic inflammation in nonalcoholic fatty liver disease is characterized by elevated levels of CCL2. Journal of hepatology 2006;44:1167-1174.
67. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, et al. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. The Journal of clinical investigation 2006;116:1494-1505.
68. Miura K, Yang L, van Rooijen N, Ohnishi H, Seki E. Hepatic recruitment of macrophages promotes nonalcoholic steatohepatitis through CCR2. American journal of physiology Gastrointestinal and liver physiology 2012;302:G1310-1321.
69. Guo H, Callaway JB, Ting JP. Inflammasomes: mechanism of action, role in disease, and therapeutics. Nat Med 2015;21:677-687.
70. Vandanmagsar B, Youm YH, Ravussin A, Galgani JE, Stadler K, Mynatt RL, et al. The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. Nat Med 2011;17:179-188.
71. Stienstra R, van Diepen JA, Tack CJ, Zaki MH, van de Veerdonk FL, Perera D, et al. Inflammasome is a central player in the induction of obesity and insulin resistance. Proceedings of the National Academy of Sciences of the United States of America 2011;108:15324-15329.

72. Henao-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, et al. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature 2012;482:179-185.
73. Brea A, Mosquera D, Martin E, Arizti A, Cordero JL, Ros E. Nonalcoholic fatty liver disease is associated with carotid atherosclerosis: a case-control study. Arterioscler Thromb Vasc Biol 2005;25:1045-1050.
74. Fabbrini E, Sullivan S, Klein S. Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. Hepatology 2010;51:679-689.
75. Targher G, Bertolini L, Padovani R, Rodella S, Zoppini G, Zenari L, et al. Relations between carotid artery wall thickness and liver histology in subjects with nonalcoholic fatty liver disease. Diabetes care 2006;29:1325-1330.
76. Blake GJ, Ridker PM. Novel clinical markers of vascular wall inflammation. Circ Res 2001;89:763-771.
77. Targher G, Marra F, Marchesini G. Increased risk of cardiovascular disease in non-alcoholic fatty liver disease: causal effect or epiphenomenon? Diabetologia 2008;51:1947-1953.
78. Petersen KF, Dufour S, Befroy D, Lehrke M, Hendler RE, Shulman GI. Reversal of nonalcoholic hepatic steatosis, hepatic insulin resistance, and hyperglycemia by moderate weight reduction in patients with type 2 diabetes. Diabetes 2005;54:603-608.
79. Lazo M, Solga SF, Horska A, Bonekamp S, Diehl AM, Brancati FL, et al. Effect of a 12-month intensive lifestyle intervention on hepatic steatosis in adults with type 2 diabetes. Diabetes care 2010;33:2156-2163.
80. Eckard C, Cole R, Lockwood J, Torres DM, Williams CD, Shaw JC, et al. Prospective histopathologic evaluation of lifestyle modification in nonalcoholic fatty liver disease: a randomized trial. Therap Adv Gastroenterol 2013;6:249-259.
81. Huang MA, Greenson JK, Chao C, Anderson L, Peterman D, Jacobson J, et al. One-year intense nutritional counseling results in histological improvement in patients with non-alcoholic steatohepatitis: a pilot study. The American journal of gastroenterology 2005;100:1072-1081.
82. Vilar-Gomez E, Martinez-Perez Y, Calzadilla-Bertot L, Torres-Gonzalez A, Gra-Oramas B, Gonzalez-Fabian L, et al. Weight Loss Through Lifestyle Modification Significantly Reduces Features of Nonalcoholic Steatohepatitis. Gastroenterology 2015;149:367-378 e365; quiz e314-365.
83. Bacchi E, Negri C, Targher G, Faccioli N, Lanza M, Zoppini G, et al. Both resistance training and aerobic training reduce hepatic fat content in type 2 diabetic subjects with nonalcoholic fatty liver disease (the RAED2 Randomized Trial). Hepatology 2013;58:1287-1295.
84. Kistler KD, Brunt EM, Clark JM, Diehl AM, Sallis JF, Schwimmer JB, et al. Physical activity recommendations, exercise intensity, and histological severity of nonalcoholic fatty liver disease. The American journal of gastroenterology 2011;106:460-468; quiz 469.
85. Ratziu V, Goodman Z, Sanyal A. Current efforts and trends in the treatment of NASH. Journal of hepatology 2015;62:S65-75.
86. Ratziu V. Pharmacological agents for NASH. Nature reviews Gastroenterology & hepatology 2013;10:676-685.
87. Cusi K. Role of obesity and lipotoxicity in the development of nonalcoholic steatohepatitis: pathophysiology and clinical implications. Gastroenterology 2012;142:711-725 e716.
88. Neuschwander-Tetri BA, Brunt EM, Wehmeier KR, Oliver D, Bacon BR. Improved nonalcoholic steatohepatitis after 48 weeks of treatment with the PPAR-gamma ligand rosiglitazone. Hepatology 2003;38:1008-1017.

89. Belfort R, Harrison SA, Brown K, Darland C, Finch J, Hardies J, et al. A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. The New England journal of medicine 2006;355:2297-2307.
90. Promrat K, Lutchman G, Uwaifo GI, Freedman RJ, Soza A, Heller T, et al. A pilot study of pioglitazone treatment for nonalcoholic steatohepatitis. Hepatology 2004;39:188-196.
91. Boettcher E, Csako G, Pucino F, Wesley R, Loomba R. Meta-analysis: pioglitazone improves liver histology and fibrosis in patients with non-alcoholic steatohepatitis. Alimentary pharmacology & therapeutics 2012;35:66-75.
92. Yki-Jarvinen H. Thiazolidinediones. The New England journal of medicine 2004;351:1106-1118.
93. Nissen SE, Wolski K. Rosiglitazone revisited: an updated meta-analysis of risk for myocardial infarction and cardiovascular mortality. Archives of internal medicine 2010;170:1191-1201.
94. Tucker ME. FDA panel advises easing restrictions on rosiglitazone. Bmj 2013;346:f3769.
95. Jia Q, Xia Y, Zhang Q, Wu H, Du H, Liu L, et al. Dietary patterns are associated with prevalence of fatty liver disease in adults. European journal of clinical nutrition 2015;69:914-921.
96. Nettleton JA, Polak JF, Tracy R, Burke GL, Jacobs DR, Jr. Dietary patterns and incident cardiovascular disease in the Multi-Ethnic Study of Atherosclerosis. Am J Clin Nutr 2009;90:647-654.
97. Nettleton JA, Steffen LM, Ni H, Liu K, Jacobs DR, Jr. Dietary patterns and risk of incident type 2 diabetes in the Multi-Ethnic Study of Atherosclerosis (MESA). Diabetes care 2008;31:1777-1782.
98. Zelber-Sagi S, Nitzan-Kaluski D, Goldsmith R, Webb M, Blendis L, Halpern Z, et al. Long term nutritional intake and the risk for non-alcoholic fatty liver disease (NAFLD): a population based study. Journal of hepatology 2007;47:711-717.
99. Sacks FM, Bray GA, Carey VJ, Smith SR, Ryan DH, Anton SD, et al. Comparison of weight-loss diets with different compositions of fat, protein, and carbohydrates. The New England journal of medicine 2009;360:859-873.
100. McCarthy EM, Rinella ME. The role of diet and nutrient composition in nonalcoholic Fatty liver disease. Journal of the Academy of Nutrition and Dietetics 2012;112:401-409.
101. Ryan MC, Itsiopoulos C, Thodis T, Ward G, Trost N, Hofferberth S, et al. The Mediterranean diet improves hepatic steatosis and insulin sensitivity in individuals with non-alcoholic fatty liver disease. Journal of hepatology 2013;59:138-143.
102. Mouzaki M, Allard JP. The role of nutrients in the development, progression, and treatment of nonalcoholic fatty liver disease. Journal of clinical gastroenterology 2012;46:457-467.
103. Finelli C, Tarantino G. Is there any consensus as to what diet or lifestyle approach is the right one for NAFLD patients? Journal of gastrointestinal and liver diseases : JGLD 2012;21:293-302.
104. Capanni M, Calella F, Biagini MR, Genise S, Raimondi L, Bedogni G, et al. Prolonged n-3 polyunsaturated fatty acid supplementation ameliorates hepatic steatosis in patients with non-alcoholic fatty liver disease: a pilot study. Alimentary pharmacology & therapeutics 2006;23:1143-1151.
105. Tanaka N, Sano K, Horiuchi A, Tanaka E, Kiyosawa K, Aoyama T. Highly purified eicosapentaenoic acid treatment improves nonalcoholic steatohepatitis. Journal of clinical gastroenterology 2008;42:413-418.