Lysosomes 'in control'

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Lysosomes 'in control': where lipids meet
inflammation in metabolic syndrome

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Lysosomes ‘in control’: where lipids meet inflammation in metabolic syndrome

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
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The truth. It is a beautiful and terrible thing, and should therefore be treated with great caution.

A.P.W.B. Dumbledore

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Chapter 1

General introduction

Metabolic syndrome

Metabolic syndrome (MetS) comprises a constellation of components that identify centrally obese patients at increased risk for developing cardiovascular diseases (CVD) and diabetes mellitus type 2 (T2D). Despite controversy concerning the precise definition for this complex disorder, components of MetS that coincide are central obesity, insulin resistance (increased plasma fasting glucose), hypertension and dyslipidemia (increased plasma triglycerides and reduced plasma high-density lipoproteins (HDL)) with a central focus on the waist circumference (Figure 1.1).¹ Though other abnormalities (such as a chronic pro-inflammatory state) have been associated with MetS, the presence of three of the five previously mentioned components is considered sufficient for a diagnosis of this syndrome.² Current estimates identify over 600 million adult individuals worldwide as obese,³ clarifying the magnitude of this epidemic.

Figure 1.1 Development of metabolic syndrome (MetS). MetS is a complex metabolic disorder caused by a combination of genetic and environmental risk factors. As a consequence, certain metabolic disturbances arise, which predispose MetS patients to develop chronic diseases such as cardiovascular diseases, diabetes mellitus type II and cancer.

The large variation in susceptibility and age of onset in individuals with a similar risk profile, suggests a contribution of both genetic and environmental factors as well as an interaction between them in eliciting MetS.⁴ While the contribution of smoking, physical inactivity and a diet high in fats and carbohydrates is well established for inducing the aforementioned metabolic components, a family history of insulin resistance, hypertension and heart disease also increases the chance of an individual to develop the syndrome.⁵ Furthermore, perturbations in the fetal/developmental environment as well as a chronic exposure to stress have been implicated in assisting the development of MetS.⁶ Relevantly, next to CVD and T2D, individuals suffering from MetS are also at increased risk for developing other chronic diseases such as cancer, underscoring the enormous challenge health care is currently facing.

Besides the vast increase in the incidence of obesity in adults, it is important to mention the staggering increase in childhood obesity.^{7,8} Childhood MetS definitions use criteria deduced from the adult MetS definition, while the concept of MetS in children (i.e. the risk of developing cardiovascular diseases and diabetes mellitus type II) has not yet been validated.⁶ Yet, children suffering from MetS which persists until adulthood have a 4-fold higher likelihood to develop CVD and T2D,^{9,10} suggesting that the adult definition of MetS also has some relevance for children.

As the liver holds a key position in the regulation of energy metabolism, it is considered as one of the first organs to be affected during the emergence of the MetS. Therefore, one part of this thesis will focus on the manifestation of the MetS in the liver, referred to as fatty liver diseases.

The liver at the center of metabolism

Pathophysiology of the liver

The liver is a vital organ, located in the upper right quadrant of the abdomen consisting of four lobes (left, right, quadrate and caudate lobe). Microscopically, the liver is recognized by its fine and unique structure of liver cell plates surrounded by sinusoids and is organized in lobules (Figure 1.2), which maximally support liver function, namely synthesis, degradation and storage of a variety of compounds including fats, carbohydrates, proteins and toxic compounds. In contrast to other organs, the liver receives its blood supply from two sources: oxygen-rich arterial blood via the hepatic artery (25% of total blood flow of liver) and nutrient-rich blood via the portal vein, which drains the stomach, intestines, pancreas and spleen. Therefore, the liver is located at the crossroads of the digestive tract and the rest of the body, maintaining the body's metabolic (among others) homeostasis.^{11,12}

Histologically, the liver is divided into two groups of cells: parenchymal cells (hepatocytes) and non-parenchymal cells (sinusoidal endothelial cells, stellate cells,

Kupffer cells (KCs), lymphocytes and cholangiocytes). Whereas hepatocytes are considered as the 'functional liver cells', making up to 85% of the total liver mass, the non-parenchymal cells have more specific functions related to immunity (lymphocytes), fibrogenesis (stellate cells) and bile secretion (cholangiocytes). Additionally, blood and hepatocytes are only separated from each other by a single fenestrated layer of endothelial cells, creating an optimal environment for rapid exchange of metabolites (Figure 1.2).^{11,12} To protect the liver from pathogenic invasion, KCs, the liver-resident macrophages, patrol over the sinusoidal endothelial layer. The KC is therefore an essential cell type for the clearance of exogenous and endogenous antigens, which can induce liver damage. Upon recognition of an antigen, KCs are activated, leading to the secretion of inflammatory mediators, which in turn attract other immune cells required to support clearance of the antigen. This entire inflammatory process has to be meticulously regulated in order to prevent escalation of a detrimental inflammatory response.

In circumstances where the antigen cannot be removed (due to a defect in certain immune cells or due to a persistent insult by the antigen) the benign acute inflammatory response will result in a chronic, tissue-destructing inflammatory response. In this severe situation, an incessant recruitment of immune cells is combined with a reduced clearance of immune cells from the liver. In the long term, this chronic inflammatory response will lead to irreversible liver damage. Therefore, the infiltration of immune cells from the circulation into the liver represents a key step in the transition towards a detrimental inflammatory response.¹³ This thesis will primarily provide additional insight into the contribution of hepatic macrophages to this detrimental hepatic inflammatory response.

Figure 1.2 Microscopic structure of the liver. Hepatocytes form a unique structure, serving the exchange, storage and breakdown of compounds (i.e. nutrients, xenobiotics) in the body. While the rapid exchange of compounds in the liver is achieved with the help of the fenestrated endothelial sheet (=uptake), the ordered system of bile canaliculi enables the drainage of bile into the bile bladder (=efflux). (Figure taken from *Molecular Biology of the Cell* (© Garland Science 2008))

The liver as the hub of lipid metabolism

Functionally, the liver plays a pivotal role in a range of physiological systems including detoxification (drugs, alcohol), immunity, digestion (via production of bile acids), hemostasis, plasma osmotic pressure and serves as a biological sink for vitamins and essential metals.¹² Additionally, as previously mentioned, a key characteristic of the liver is its central position in the regulation of systemic metabolism. Besides the maintenance of plasma glucose concentrations and its essential role in protein metabolism, the liver has also a key role in the regulation of lipid metabolism.

Lipids are a group of hydrophobic compounds that are generally found compartmentalized (i.e. lipid droplets of triglycerides in adipocytes) and serve as a major energy source of the body. Other lipid-related functions range from regulatory functions (i.e. hormones; bile acid precursors) to serving as structural component of the cell (i.e. phospholipids and cholesterol). It is therefore of critical importance that all cells in the body are assured of an appropriate supply of lipids. This supply is ensured by the distribution of lipids over the body by means of lipoproteins, which serve as lipid-carriers through the hydrophilic bloodstream.¹⁴

Plasma lipoproteins are macromolecular complexes of lipids and specific proteins, the latter functioning as recognition site for cell-surface receptors and serving as activators for enzymes. Based on lipid and protein composition, site of origin, size and density, lipoproteins are categorized in chylomicrons, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). A general overview of lipoprotein and lipid metabolism is provided in Figure 1.3. In summary, chylomicrons receive dietary fats from the enterocytes of the small intestine in the form of triglycerides and cholesterol. Passing from the lymph to the blood, chylomicrons enter the circulation where they interact with the capillary endothelium of peripheral tissues (including adipose tissue). Digestion of triglycerides then leads to the release of free fatty acids (FFA) in the peripheral tissue. This triglyceride-depleted, cholesterol-rich 'remnant' chylomicron (CMR) travels to the liver, where it enters hepatocytes via the LDLR-related receptor (LRP1). The liver subsequently secretes VLDL particles to distribute triglycerides and cholesterol to peripheral tissues, and thereby serves as a lipid 'shuttle'. Peripheral tissues then either store those fats or use them as immediate energy source. Then, lipoprotein lipase (LPL) activity reduces the size of VLDL particles, turning them into intermediate-density lipoprotein (IDL) or even smaller LDLs. Receptor-mediated endocytosis via the LDL receptor (LDLR) then leads to the uptake of LDL particles by the liver or extrahepatic tissues, resulting in the deposition of (mainly) cholesterol. Finally, the essential HDL particle transports lipids from peripheral tissues to the liver. HDL particles, whether secreted by the liver or by enterocytes, are initially cholesterol-free. Only after contact with extrahepatic tissues (such as skeletal muscle and adipose tissues), they will extract cholesterol from the plasma membranes and promote cholesterol efflux via the ATP-binding cassette transporter A1 (ABCA1) and G1

(ABCG1) from these cells. After esterification of cholesterol, the HDL particle is remodeled by cholesterol ester transfer protein (CETP) and endothelial lipase (LIPG). Afterwards, HDL enters the hepatocytes via the scavenger receptor class B type I (SRB1).

Figure 1.3 Overview of lipoprotein metabolism. CM: chylomicrons; LPL: lipoprotein lipase; FFA: free fatty acids; CMR: chylomicron remnants; LRP1: LDL-related receptor 1; TG: triglyceride; C: cholesterol; VLDL: very-low-density lipoprotein; IDL: intermediate-density lipoprotein; LDL: low-density lipoprotein; LDLR: low-density lipoprotein receptor; HDL: high-density lipoprotein; ABCA1: ATP-binding cassette transporter A1; ABCG1: ATP-binding cassette transporter G1; CETP: cholesterol ester transfer protein; LIPG: endothelial lipase; SRB1: scavenger receptor class B type I. (Figures are adapted from Servier Medical Art)

This elegant lipid transport system together with the highly-coordinated intracellular signaling cascades which regulate *de novo* triglyceride and cholesterol synthesis are essential to maintain lipid homeostasis in the human body.¹⁵ Deficiencies in one of these components or imbalances of lipid intake/excretion can therefore lead to clinical problems. An example of such an imbalance is the intake of lipid-rich foods, or general overnutrition, two characteristics associated with obesity. As a result, plasma lipoprotein profiles shift towards elevated LDL and reduced HDL levels, which is then referred to as dyslipidemia.¹⁶⁻¹⁸ Consequently, excess triglycerides and cholesterol accumulate in non-fat storing organs such as the liver, leading to non-alcoholic fatty liver disease (NAFLD).¹⁹

Non-alcoholic steatohepatitis (NASH)

NALFD is defined as the presence of $\geq 5\%$ hepatic steatosis (defined as macrovesicular fat in hepatocytes) in the absence of competing liver disease etiologies (i.e. viral hepatitis) and significant alcohol consumption (>21 drinks/week in men and >14 drinks/week in women).²⁰ The term NAFLD covers a disease spectrum ranging from isolated hepatic steatosis (referred to as non-alcoholic fatty liver (NAFL)) to non-alcoholic steatohepatitis (NASH), which differs from the former by the additional presence of inflammation with or without fibrosis.²¹

Though the presence of NAFL is generally considered benign for the liver itself, patients suffering from NAFL are at increased risk for cardiac-related death. Therefore, NAFL patients should be monitored even at an early stage of the disease.²² For the liver itself, the transition from NAFL to NASH is more important as this transition predisposes the patient to develop cirrhosis and hepatocellular carcinoma, underscoring the importance of hepatic inflammation in NAFLD.^{19,23,24} Relevantly, NAFL patients showing features of T2D and hyperlipidemia (two core characteristics of the MetS) are at increased risk to develop NASH.^{19,25} However, despite associations between those pathological characteristics, clinicians still face a major challenge in diagnosing and treating NASH as the exact underlying mechanisms triggering hepatic inflammation are still unknown.²⁴ Recent calculations have predicted an annual economic burden for NASH of \$ 7 billion in the United States, £ 401 million in the United Kingdom and € 2.2 billion in Germany, France and Italy combined, emphasizing the gigantic pressure of this disease on health care institutions.²⁶

Epidemiology

Though the exact number remains unknown, increasing epidemiological data indicate that 25% of the global population suffers from NAFLD, with the highest prevalence in South America (31%) and the Middle East (32%).²⁶ Of all NALFD patients, it is estimated that one third also suffers from NASH.²⁷ Based on data retrieved from the National Health and Nutrition Examination Survey III (NHANES III), strong risk factors predicting the development of NASH include visceral obesity (88.7%), hypercholesterolemia (90.2%) and hypertension (45.5%)²⁸. Relevantly, a steady upward trend in NALFD prevalence was previously described, indicating NAFLD as a disease on the rise.²⁶ With regard to age, emerging data describe a staggering increase of NAFLD (including NASH) in children. Based on the combination of several studies, Loomba *et al.* estimated that NAFLD prevalence in adolescents is at least 3%,²⁹ increasing to 53% in obese children.³⁰ Especially worrisome are the findings that cardiovascular risk and morbidity as well as the risk to develop cancer and liver disease in children and adolescents are correlated with the diagnosis of a fatty liver.²⁹

Diagnosis

Though some patients experience non-specific symptoms such as fatigue and irritation of the right upper quadrant, the majority of NAFL and NASH patients are asymptomatic, impeding a fast and accurate diagnosis.²⁴ Indeed, NAFL and NASH diagnosis are often only made coincidentally when the patient is examined for unrelated symptoms.

The golden standard currently used to diagnose NASH is the histological examination of a liver biopsy by a trained pathologist.^{31,32} However, important limitations for this invasive approach are patient discomfort, risk of bleeding and possibly even death.³³ The use of a liver biopsy for NASH diagnosis is also questioned by sampling error (NASH lesions are distributed in a dispersed manner and only a small amount of liver tissue is sampled during a biopsy).^{32,34} Furthermore, Loomba *et al.* suggested that scoring of a liver biopsy should rather be viewed as a method to assess treatment responses rather than a diagnostic tool.²⁹ As such, the need for accurate, non-invasive tools to diagnose NASH is essential.³⁵

Plasma levels of the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST), indicative for liver cell damage, have been associated with NAFLD disease progression.³⁶ However, their value as a diagnostic NASH marker is highly debated due to lack of specificity³⁷ and high variability between patients.³⁸ Moreover, using these markers as sole diagnostic tool was shown to underestimate the amount of fatty liver patients.¹⁹ An alternative marker that has been tested is cytokeratin 18 (CK-18), a breakdown product generated during caspase 3-mediated apoptosis of hepatocytes.^{39,40} However, Cusi *et al.* observed a limited value for this marker in diagnosing NASH due to low sensitivity rates.⁴¹ As such, the use of CK-18 for NASH diagnosis needs to be further validated in other cohorts before implementation in the clinic can be fully supported.⁴²

Next to the use of plasma markers, several imaging methods have also been tested as tools for NASH diagnosis. Ultrasound elastography,⁴³ controlled attenuation parameter (CAP),⁴⁴ computed tomography (CT),⁴⁵ magnetic resonance imaging (MRI)⁴⁶ and the FibroScan⁴⁷ are non-invasive imaging approaches that assess fat and/or fibrosis in the liver. However, no imaging method is able to assess the level of inflammation in the liver, indicating that there is no accurate imaging method available to diagnose NASH. Together, despite the necessary and valuable efforts that have already been made to find non-invasive diagnostic tools for NASH, a simple straightforward approach to distinguish NASH from NAFL patients is still non-existent. Therefore, advances in the efficiency of NASH diagnosis are highly desirable.

Therapy

As the exact etiology for the development of NASH is unknown, specific therapeutic tools are lacking. To date, general lifestyle interventions (aimed at caloric restriction)

resulting in mild weight loss are the standard prescribed therapy for NASH patients.^{48,49} In spite of promising results, an issue that is currently discussed, is whether there is a certain threshold of histological severity above which mild weight loss no longer improves NASH.⁵⁰ Alternatively, it has to be noted that spectacular histological improvements have been observed after massive weight loss following bariatric surgery.⁵¹ However, drawbacks for this invasive procedure are severe complications and high costs.⁵²

Therefore, next to the general approach of caloric restriction, more liver-specific therapeutic options are necessary to overcome NASH. Currently, the only curative treatment option for NASH is replacing the liver by a healthy donor liver (followed by persistent lifestyle changes).²³ However, the shortage of available donor organs urges health care systems to develop alternative therapeutic tools to overcome NASH.⁵³ Given the associations between NASH and a plethora of pathological processes (i.e. oxidative stress, altered innate immunity, lipotoxicity, ...), interventions specifically aimed at improving these processes should theoretically ameliorate or even cure NASH. However, though multiple agents are currently under investigation,^{54,55} there are no approved pharmacological therapies for NASH yet.⁵⁶

In order to provide additional mechanistic insight into the pathogenesis of NASH, the establishment of an animal model that mimics human NASH is essential to serve as a basis for the development of novel diagnostic and therapeutic tools for this disease.

Hyperlipidemic *Ldlr*^{-/-} mice as model to investigate the onset of NASH

Though considerable advances have been made in developing animal models to investigate NASH, there is still no *in vivo* model available that perfectly mimics human NASH. An ideal mouse model should represent all features of human NASH, including liver-specific (lipid accumulation in liver, liver inflammation ...) and systemic (high lipid levels ...) characteristics. However, current NASH mouse models only mimic several features similar to human NASH. The leptin deficient *ob/ob* mouse model, for example, mimics obesity-related liver disease, but requires an additional stimulus to induce hepatic inflammation.⁵⁷ Exposing regular C57BL/6 mice on a methionine and choline-deficient (MCD) diet results in advanced stages of NASH with severe inflammation and fibrosis; however, this diet leads to significant weight loss and reduced plasma triglyceride levels, features not found in man.⁵⁸ Additionally, another factor complicating the translation of mechanistic insight from animal models to humans is the heterogeneity of human NAFLD (and NASH), presenting with many different phenotypes that have overlapping and non-overlapping elements.⁵⁹ Therefore,

additional models are necessary to represent a complete overview of the mechanisms governing NASH.

Hyperlipidemic mice receiving a high fat diet were shown to suffer from hepatic inflammation,⁶⁰ and therefore reflect the strong associations between NASH and the intake of a high fat diet and hyperlipidemia found in man.²⁸ In line, mice lacking the low-density lipoprotein receptor (*Ldlr*^{-/-}) were previously described by us and others as model for early stages of NASH.^{61,62} Compared to other mouse models, *Ldlr*^{-/-} mice on a high-fat, high-cholesterol diet (HFC) also show a human-like plasma lipoprotein profile.⁶² Relevantly, the loss of a functional LDL receptor in humans (then referred to as familial hypercholesterolemia) is associated with the development of NAFLD.^{63,64} Therefore, *Ldlr*^{-/-} mice given a HFC diet are considered a valuable model to investigate the onset of hepatic inflammation in NASH.⁶¹

The cell's cleaning-machinery at the center of inflammatory signaling

Lysosomal lipid storage in hepatic macrophages is associated with hepatic inflammation

Previous studies have indicated an essential role for hepatic macrophages in the induction of NASH.^{65,66} Similar to the formation of foamy macrophages in atherosclerosis, we have shown that scavenger receptor-mediated uptake of modified lipoproteins by hepatic macrophages also induces inflammation in the liver.^{67,68} However, while a deficiency of scavenger receptors in macrophages reduced hepatic inflammation in mice, the size of foamy hepatic macrophages did not change, indicating that the amount of accumulated lipids was similar.⁶⁷ As such, these studies yielded two important observations: 1) not all types of lipoproteins, but specifically scavenger receptor-internalized modified lipoproteins contribute to hepatic inflammation and 2) the total amount of lipids that accumulate in macrophages does not predict hepatic inflammation. In response to the first observation, we and others identified the modified lipoprotein oxidized low-density lipoprotein (oxLDL) as an essential determinant for hepatic inflammation.⁶⁹⁻⁷² Furthermore, these studies demonstrated that oxLDL-induced hepatic inflammation was accompanied by low levels of cytoplasmic lipids and large, swollen lysosomes in hepatic macrophages. In contrast, when inflammation was low, lipids tended to accumulate in the cytoplasm instead of the lysosomes^{68,73} (Figure 1.4). In line with this observation, lipids derived from LDL particles were efficiently cleared from lysosomes, whereas oxLDL-derived lipids were not and accumulated in the lysosomes instead.⁷⁴ Our findings show an association between hepatic inflammation and lysosomal accumulation of lipids, likely derived from oxLDL particles, in hepatic macrophages. Whether lysosomal lipid storage

in hepatic macrophages also causes hepatic inflammation had not been proven yet and is a question that is now addressed in **chapter 4** of this thesis.

Figure 1.4 Lysosomal lipid accumulation in hepatic macrophages of *Ldlr*^{-/-} mice correlates with hepatic inflammation. We previously demonstrated by electron microscopy that livers with high levels of inflammation are characterized by lysosomal storage of lipids in hepatic macrophages (*left panel*). Lipid accumulation in the cytoplasm of the hepatic macrophage was associated with low hepatic inflammation (*right panel*). HM, hepatic macrophage; He, hepatocyte; orange lines surround hepatic macrophage; blue arrows indicate the accumulation of lipids in lysosomes (*left panel*) or cytoplasm (*right panel*).

Lysosomal function and its role in lipid metabolism and inflammation

To enable lysosomes to degrade exogenous (i.e. lipids, carbohydrates, DNA) and endogenous (i.e. during autophagy) material, they are equipped with catabolic enzymes that are active at an acidic pH. The acidic pH in the lysosomal lumen is generated by the action of the vacuolar-type H⁺-ATPase (V-ATPase), located at the limiting membrane of the lysosome.⁷⁵ In addition to their key role in intracellular digestion, increasing evidence assigns other important regulatory functions to lysosomes and associated enzymes. One of these regulatory functions is illustrated by the ability of the cell to discharge the lysosomal luminal content extracellularly (also termed extracellular vesicles (EVs)),⁷⁶ thereby regulating bone resorption,⁷⁷ hormonal homeostasis,⁷⁸ energy metabolism⁷⁹ and plasma membrane repair.⁸⁰ Relevantly, lysosomal exocytosis is also presumed as a mechanism to prevent the accumulation of intracellular debris (= to serve as protective physiological mechanism).⁸¹ However, when compounds (such as lipids) accumulate in lysosomes, lysosomal dysfunction is induced which leads to disturbances in lysosomal trafficking.⁸² As the regulation of EVs is beyond the scope of this PhD thesis but might be helpful in understanding certain parts of it, we like to refer to several excellent reports explaining these processes in detail.⁸³⁻⁸⁵

Besides their ability to influence extracellular processes by excreting their luminal content, the endo-lysosomal compartment (of which the lysosome is the terminal stage) plays an essential role in lipid metabolism. LDL-derived cholesteryl esters (CEs) are internalized by means of LDL receptor-mediated endocytosis, eventually reaching the lysosome where the action of lysosomal acid lipase (LAL) generates free unesterified cholesterol (FC)⁸⁶ (Figure 1.5). Lysosomal efflux of this FC to the cytoplasm is regulated by the Niemann-Pick disease type C1 (NPC1) and NPC2 proteins.^{87,88} Though the mechanism by which both proteins exert their function on cholesterol transport remains unclear, it is accepted that the NPC2 protein serves as a guide to transport FC to NPC1. Subsequently, NPC1 will govern the export of FC to the cytoplasm, where cholesterol is stored in cytoplasmic lipid droplets in the form of CEs or is excreted by efflux mechanisms.⁸⁹ Mutations in either of the two NPC proteins result in the severe lysosomal lipid storage disorder Niemann-Pick type C, underlining the key role of both proteins in cholesterol metabolism.⁹⁰

Figure 1.5 Intracellular cholesterol metabolism. After LDL receptor-mediated endocytosis, cholesteryl esters (CEs) are directed towards the lysosome, where they are hydrolyzed by lysosomal acid lipase (LAL), generating free unesterified cholesterol (FC). By guidance of the NPC2 protein, FC is subsequently transported to the cytoplasm via the lysosomal membrane protein NPC1. Afterwards cholesterol is stored in cytoplasmic lipid droplets as CEs or excreted by efflux mechanisms. Dashed line indicates movement; full line indicates conversion. LDL, low-density lipoprotein; CE, cholesteryl ester; FC, free unesterified cholesterol; LAL, lysosomal acid lipase; NPC2, Niemann-Pick type C2. (Figures are adapted from Servier Medical Art)

Finally, the lysosomal enzymes cathepsin B and D have previously been linked to processes related to apoptosis and inflammasome activation, implying their potential role in inflammatory signaling.^{91,92} Moreover, lysosomal dysfunction impairs autophagy, a process that mediates the self-degradation of cellular components.⁹³ As multiple studies have shown the tight link between autophagy and inflammation,⁹⁴⁻⁹⁶ lysosomal dysfunction can affect the inflammatory response by impairing autophagy. Altogether, these findings present a clear relation between lipid metabolism, lysosomes and inflammation. The first part of this thesis aims to investigate the potential involvement of lysosomes to the metabolic inflammatory response in the liver, and to explore its potential as a diagnostic and therapeutic target in the context of NASH (**Chapters 2, 4, 5, 6 and 7**).

The gut microbiota regulates pathophysiological processes

Findings obtained in the last decade have pointed towards an essential metabolic role for the micro-organisms residing in the human gastro-intestinal system, also referred to as the gut microbiota. The gut microbiota consists of bacteria, viruses, fungi, archaea and protozoa rendering the gastro-intestinal tract the most densely colonized microbial community of the human body, with up to 10^{14} microbial entities.^{97,98} Relevantly, the composition of the gut microbiota is subject to constant fluctuations in time within (intra-) and between (inter-) individuals. Variables responsible for compositional alterations include the host's genome^{99,100} and environmental factors such as diet, antibiotics and lifestyle.¹⁰¹⁻¹⁰³ Subsequently, shifts in the composition of gut microbiota have been shown to influence physiological processes such as lipid and lipoprotein metabolism,¹⁰⁴ glucose metabolism¹⁰⁵ and immunity¹⁰⁶ (Figure 1.6). Accordingly, alterations in the composition, defined as dysbiosis, might be at the basis of diseases such as obesity, suggesting a central role for gut microbiota in inducing pathologies.^{107,108} For detailed explanations regarding the exact mechanisms that play a role in dysbiosis (gut barrier integrity, gut permeability) and associated metabolic pathologies, we refer to chapter 3.

As previously mentioned, the liver receives approximately 75% of its blood supply from the intestines via the portal circulation and 25% from a branch of the aorta, and is therefore strategically located between the 'contaminated or unsterile' bowel and the 'sterile' systemic circulation, serving as a biological filter.^{109,110} However, in spite of its protective function as a first-line defense against systemic contamination, the liver itself is in a very delicate position upon dysbiosis, being the first organ (excluding the intestine) exposed to potentially detrimental effects of the gut microbiota (and their associated products/toxins).¹¹¹ Furthermore, the intimate connection between the liver and the gastro-intestinal system, referred to as the gut-liver axis,¹¹² suggests a role for

the gut microbiota in liver diseases such as NASH. However, information on the exact mechanisms by which the gut microbiota influences metabolic disorders is relatively scarce. More studies are necessary to completely understand the cause and consequences of shifts in certain gut microbial communities and whether or not these shifts impact the health status of an individual. Therefore, the second part of this thesis elaborates on the cause and consequences of shifts in the gut microbiota composition in MetS, which can be applied to NASH (**Chapters 2, 3 and 8**).

Figure 1.6 Gut microbiota composition influences physiological processes. The gut microbiota constitutes a community of micro-organisms present in the gastro-intestinal tract and consists of bacteria, viruses, fungi, archaea and protozoa. This composition is influenced by the host's genetics and by factors derived from the environment. These micro-organisms and the products they produce contribute to physiological processes such as metabolism and immunity (among others).

Thesis aim and outline

The absence of specific knowledge concerning the underlying mechanisms mediating hepatic inflammation in NASH leaves clinicians without effective therapeutic and diagnostic tools to help patients. Therefore, by building on the findings previously described by this research group, this thesis aims to give translational insight into the underlying mechanisms leading to NASH. Specifically, a novel non-invasive tool to diagnose NASH and therapeutic targets to combat NASH were investigated. The results of this thesis provide a solid translational platform to investigate and apply our findings in a clinical setting. Finally, the identified underlying mechanisms were also linked to changes in gut microbiota, providing an additional diagnostic/therapeutic target for metabolic disorders such as NASH.

Chapter 2 describes the relation between oxLDL and hepatic inflammation and specifically addresses lysosome- and gut microbiota-based mechanisms by which oxLDL can induce NASH. **Chapter 3** provides extensive information on the role of the gut microbiota in maintaining metabolic homeostasis. Moreover, the interplay between the diet, the innate immune system and the composition of the gut microbiota is highlighted. **Chapter 4** explores the causal contribution of lysosomal lipid accumulation in macrophages in diet-induced hepatic inflammation. To evaluate the involvement of lysosomal lipid storage in blood-derived hepatic macrophages, *Ldlr*^{-/-} mice were transplanted with *Npc1*^{mut} bone marrow. Additionally, chapter 4 also demonstrates the role of oxLDL in mediating lysosomal lipid-induced hepatic inflammation. In **chapter 5**, the predictive value of plasma cathepsin D as a tool to distinguish NASH from steatosis was evaluated in a biopsy-proven pediatric cohort. Subsequently, **chapter 6** describes plasma cathepsin D levels in relation to NASH in three adult NASH cohorts and in one adult NASH cohort in which patients underwent bariatric surgery. In **chapter 7**, the function of cathepsin D was investigated in diet-induced hepatic inflammation. To explore this, *Ldlr*^{-/-} mice were administered an inhibitor for aspartyl proteases and fed a HFC diet for three weeks. The results of chapter 7 provide a translational basis for targeting cathepsin D in NASH. **Chapter 8** illustrates the impact of a hematopoietic NPC1 mutation on the gut microbiota of *Ldlr*^{-/-} mice, emphasizing the link between lipid metabolism and gut microbiota composition in MetS. Finally, **chapter 9** discusses the most important findings of this thesis, puts them into perspective relative to its research field(s) and provides information regarding their value for clinicians.

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Chapter 2

Oxidized LDL at the crossroads of immunity in non-alcoholic steatohepatitis

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Abstract

Non-alcoholic steatohepatitis (NASH) is viewed as the hepatic manifestation of the metabolic syndrome and is a condition hallmarked by lipid accumulation in the liver (steatosis) along with inflammation (hepatitis). Currently, the etiology and mechanisms leading to obesity-induced liver inflammation are not clear and, as a consequence, strategies to diagnose or treat NASH in an accurate manner do not exist. In the current review, we put forward the concept of oxidized lipids as a significant risk factor for NASH. We will focus on the contribution of the different types of oxidized lipids as part of the oxidized low-density lipoprotein (oxLDL) to the hepatic inflammatory response. Furthermore, we will elaborate on the underlying mechanisms linking oxLDL to inflammatory responses in the liver and on how these cascades can be used as therapeutic targets to combat NASH.

Introduction

In parallel to the steep increase of obesity and type 2 diabetes, non-alcoholic fatty liver disease (NAFLD) is currently the most common liver disease worldwide¹ and increasingly recognized as the hepatic manifestation of the metabolic syndrome (MetS).^{2,3} NAFLD comprises a spectrum of liver disorders ranging from simple lipid accumulation in the liver (steatosis) to fibrosis and cirrhosis, which will eventually lead to liver failure and death.⁴ A key step in developing such advanced liver diseases is the progression of steatosis to non-alcoholic steatohepatitis (NASH), which is characterized by the combination of steatosis and inflammation in the liver.⁵ Furthermore, the presence of fibrosis in the liver is nowadays considered as a key symptom of active NASH, which is characterized by diminished long-term prognosis.⁶

As NAFLD is closely related to the presence of obesity and MetS, aberrations in lipid metabolism are one of the major hallmarks of this disease.⁷ Observed lipid deviations in NAFLD patients include elevated plasma and liver levels of triglycerides (TGs), low plasma high-density lipoproteins (HDL) and elevated low-density lipoproteins (LDL) and total cholesterol levels.^{4,8,9} Apart from abnormalities in lipid metabolism, another important aspect of NASH is the presence of oxidative stress.¹⁰ The increased generation of reactive oxygen species (ROS) combined with an impaired antioxidant defense creates a condition which is referred to as oxidative stress. Indeed, molecular components associated with oxidative stress have been demonstrated to be highly reactive with surrounding tissues, inducing tissue injury.¹¹

Considering that disturbances in lipid metabolism and oxidative stress make up two important aspects of NASH, increasing evidence indicates an important role for oxLDL-derived oxidized lipids in the pathogenesis of NASH. In this review, we aim to give an overview of the current data linking oxidized lipids to NASH and explain the mechanisms that lead to and are affected by lipid oxidation. Additionally, we will discuss and evaluate the potential clinical benefits of targeting oxidized lipids in NASH.

Liver lipids and related oxidation processes

Different types of lipids are proposed to influence the disease spectrum of NAFLD. First, the accumulation of TGs determines the histological appearance of a steatotic liver. Structurally, TGs are created by the fusion of a glycerol backbone and three free fatty acids (FFAs) that attach to it (Figure 2.1A). Therefore, increases in hepatic TG levels can be derived from an elevated influx of FFAs into the liver from lipolysis of adipose tissue, increased hepatic synthesis, reduced lipid export from the liver and an excess dietary fat intake.¹² Second, in spite of the abundant presence of TGs (and as such fatty acids (FAs)) in the steatotic liver, it becomes evident that not triglycerides,

but rather cholesterol is an important lipid compound in NAFLD progression. Putting mice on a high-cholesterol diet was shown to induce hepatic inflammation while omitting dietary cholesterol from the diet prevented the appearance of hepatic inflammation.¹³⁻¹⁵ Moreover, also in humans specifically the cholesterol levels were found to associate with the level of hepatic inflammation.^{16,17} Together, these findings indicate that cholesterol is a significant risk factor in the onset of NASH.

Relevantly, hepatic accumulation of FFA has been associated with disturbances of cellular processes which are linked to inflammation such as protein unfolding,¹⁸ innate immunity,^{19,20} destabilization of lysosomal membranes,²¹ oxidative stress²² and mitochondrial function.²³ In contrast, it has been shown that accumulation of lipid compounds *per se* does not induce hepatic inflammation. Moreover, several findings even suggested that TG synthesis is protecting the liver from lipotoxicity by buffering FFA accumulation.^{24,25} Furthermore, authors have also argued the role of FAs in inducing hepatic lipotoxicity in NASH.^{26,27} Similarly, lipid-reducing drugs were shown not to improve NASH.^{28,29} Together, despite the strong correlation between dyslipidemia and NASH, it has become clear that disturbances in lipid metabolism cannot merely explain the progression towards the inflammatory response in the liver. Oxidative stress is an important process which has been shown to be involved in the progression from a benign fatty liver to NASH.^{30,31} Elevated ROS levels can be derived from a range of stimuli, including cellular stress, mitochondrial dysfunction, disturbances in antioxidants and other enzyme dysfunctions.³² Next to its physiological role in the regulation of signaling cascades, ROS are recognized as oxidizing agents that impair the structure of important components of the cell such as DNA, lipid membranes and proteins.³³ Therefore, given that both lipid metabolism and oxidative stress in the liver make up important aspects of NASH pathogenesis, it is not surprising that both processes are intertwined, creating inflammatory oxidized lipids affecting the liver.

One of the best-studied examples of an oxidized lipoprotein associated with inflammation is oxidized LDL (oxLDL). Though initially associated with atherosclerosis,³⁴ oxLDL has recently been associated with inflammatory processes in NASH.^{35,36} In both the arteries and the liver, the uptake of oxLDL by macrophages was shown to be an important player in generating a detrimental inflammatory response.³⁷

Concerning oxLDL, an important footnote should be stated. Initially, oxLDL was described as an LDL particle containing protein components that are modified due to oxidation reactions. As a result of these reactions, a net negative charge could be observed on this particle, making it prone to recognition by macrophages.^{38,39} However, it became clear that in contrast to the protein modifications, it is more likely that the oxidation of lipids, present inside the lipoprotein particle, determines the pro-inflammatory phenotype observed in oxLDL-loaded macrophages.⁴⁰ Firstly, phospholipid oxidation products were shown to act pro-inflammatory.⁴¹ Next, fatty acids, both in the ester and free form, are prone to interact with free radicals (Figure

2.1A). However, despite the formation of the highly reactive malondialdehyde (MDA) product (Figure 2.1A), oxidation of polyunsaturated fatty acids (PUFAs) is considered as a protective mechanism to prevent oxidation of sterol-like structures, then referred to as cholesterol oxidation products or oxysterols.⁴² Indeed, ROS-induced oxidation of sterol-like structures has been associated with inflammatory processes in the liver, arteries and central nervous system.⁴³⁻⁴⁵

Of note, a clear distinction (both structurally and biologically) has to be made between ROS-mediated oxidation and enzymatic oxidation of cholesterol. ROS-mediated oxidation (or autooxidation) of cholesterol induces ring-oxidation products, which are generally considered to be created in a non-enzymatic manner (Figure 2.1B, *upper panel*). In contrast, cholesterol also undergoes enzymatic oxidation, thereby creating side-chain oxidation products (Figure 2.1B, *lower panel*). This hydroxylation reaction is mediated by cytochrome P450 enzymes and is necessary to create the intermediates for bile acids and steroid hormones.⁴⁶ Also, in contrast to the pro-inflammatory role of ROS-generated products, cholesterol oxidation products generated via enzymatic reactions were shown to act anti-inflammatory. Via subcutaneous injections into a hyperlipidemic mouse model, 27-hydroxycholesterol (27HC), an intermediate in bile acid synthesis, was shown to reduce hepatic inflammation.⁴⁷ Additionally, hematopoietic overexpression of CYP27, the enzyme which generates 27HC, led to a reduction in hepatic inflammation independent of plasma 27HC levels.⁴⁸ Furthermore, 25-hydroxycholesterol (25HC) was recently shown to decrease macrophage-induced inflammation via regulating inflammasome activation.⁴⁹ Finally, estrogens have also been linked to anti-inflammatory immune responses.⁵⁰

Compounds that are structurally and functionally similar to cholesterol (and therefore also subject to oxidation reactions) are plant sterols. Via incorporation in oils, cereals, fruits and nuts (among others), plant sterols make up about 20% of the total amount of sterols consumed in the Western-type diet, equaling 300 mg/day.⁵¹ Relevantly, though plant sterols cannot be synthesized from any precursor inside the body,⁵² important biological functions have been assigned to these steroid complexes such as their blood cholesterol-lowering potential and their ability to reduce cardiovascular risk.^{53,54} However, analogous to cholesterol, plant sterols are also prone to undergo oxidation reactions, leading to the generation of plant sterol oxidation products (POPs) or oxyphytosterols.⁵⁵ The formation of these oxyphytosterols can be achieved by uptake from the diet itself or via enzymatic reactions or radical autooxidation of absorbed plant sterols, with the latter being the main source.⁵⁶ Several inferential data have suggested the contribution of POPs to inflammatory processes in disorders such as atherosclerosis.⁵⁷ However, due to the lack of knowledge concerning oxyphytosterol metabolism and inconclusive results obtained with them, the exact effects of POPs on inflammatory responses *in vivo* and in humans is still under debate.⁵³

A

B

Figure 2.1 The formation of oxidized lipids. (A) Triglycerides are synthesized by the condensation of a glycerol molecule with three free fatty acids. Next, via lipid peroxidation, fatty acids can be oxidized, generating the highly reactive products malondialdehyde and 4-hydroxynonenal. (B) Oxidation of cholesterol gives rise to two types of products: ring oxidation (or autoxidation) cholesterol products (*upper panel*) and side-chain oxidation cholesterol products (*lower panel*). Generally, autoxidation products are generated via direct interaction with ROS, whereas side-chain oxidation is mostly accomplished via enzymatic oxidation reactions. Besides structural differences, both types of cholesterol oxidation products also differ in their effect on inflammation (pro-inflammatory vs. anti-inflammatory resp.). Whereas ring oxidation products have been associated with pro-inflammatory properties, mainly anti-inflammatory functions have been assigned to side-chain cholesterol products such as 25-hydroxycholesterol, the bile acid derivative 27-hydroxycholesterol and the estrogen-derived hormones estriol and estradiol.

Despite mainly being linked to cardiovascular disorders, well-designed studies demonstrated the accumulation of oxyphytosterols in a variety of tissues with the liver accumulating the highest concentration of these oxidized lipids.^{58,59} Therefore, regardless of their unknown functions, oxyphytosterols might contribute to inflammatory processes in the liver, which justifies further research of these compounds in the context of NASH. In summary, it is important to identify which lipids have undergone which type of oxidation reaction in order to assess the possible associated detrimental effects.

Relevantly, though both intra- and extracellular-derived oxidized lipids contribute to the inflammatory response in the liver, their relative contribution has, to our knowledge, not been investigated yet. However, crosstalk between intra- and extracellular oxidation mechanisms has been implicated,⁶⁰ thereby implying that both intra- and extracellular oxidized lipids likely assist each other in inflammatory processes.

Biological evidence linking oxidized lipids to NASH

Oxidized lipids related to the oxLDL particle have been increasingly associated with inflammatory diseases such as atherosclerosis,³⁴ rheumatoid arthritis⁶¹ and neurodegenerative disorders.^{62,63} Being one of the central organs regulating whole body lipid metabolism, the liver needs to withstand the largest portion of detrimental oxidized lipids and might therefore be the organ that is most affected by these deleterious lipids. In this section, we provide a summary of the described effects of oxLDL-related oxidized lipids on liver cells and their contribution to hepatic inflammation and fibrosis.

Evidences linking oxLDL to hepatic inflammation and fibrosis at cellular level

Several observations have supported the central role of oxLDL in hepatic inflammatory and fibrotic responses. Though the major detrimental effects of oxLDL are mediated via the macrophages of the liver (the Kupffer cells (KCs)), also other liver cell types are directly and indirectly affected. Here, we give an overview of the effects of oxLDL on different liver cell types, leading to hepatic inflammatory and fibrotic responses.

Perhaps the strongest evidence that oxLDL is involved in NASH was shown by the recent development of a mouse model for NASH that was based on the combination of a high fat diet (HFD) and intravenous injections of oxLDL,⁶⁴ Mice injected with oxLDL and fed an HFD diet displayed hepatic steatosis, fibrosis, dyslipidemia, intense inflammation and hepatic injury with inflammatory cell infiltration compared to mice only receiving the HFD. More specifically, the authors indicated that the oxLDL-

mediated hepatic inflammatory response observed in this model was derived from hepatocytes and hepatic macrophages.

Indeed, KCs have been increasingly associated with the pathogenesis of NASH,⁶⁵⁻⁶⁷ In livers of hyperlipidemic mice, the formation of bloated foamy KCs was observed which was associated with hepatic inflammation,¹³ Isolated KCs from HFD-fed mice contained increased cholesterol levels, which were associated with a pro-inflammatory phenotype.⁶⁸ Comparable to foamy KCs, the inflammatory process underlying oxLDL-induced foam cell formation is a well-established concept in the field of atherosclerosis.³⁴ Next to inducing inflammation, oxLDL-uptake by KCs was also associated with increased hepatic gene expression of the pro-fibrotic genes collagen type 1, alpha 1 (*Col1a1*) and transforming growth factor beta (*Tgf-β*).^{35,36} Therefore, oxLDL-induced activation of KCs can be considered as a mechanism by which inflammation and fibrosis arise in NASH (Figure 2.2).

Internalization of modified lipids in macrophages was shown to be mediated by the scavenger receptors (SRs) scavenger receptor A (SR-A) and cluster of differentiation 36 (CD36).⁶⁹ Though CD36 has a greater affinity than SR-A to bind oxLDL,⁷⁰ both receptors are involved in its internalization as protein levels of both receptors were increased after oxLDL incubation in macrophages.^{64,71} Furthermore, hyperlipidemic mice containing a hematopoietic deletion of both SR-A and/or CD36 resulted in reduced levels of hepatic inflammation and fibrosis, indicating that SR-mediated internalization of modified lipids by KCs is an important trigger to develop NASH.³⁶

Though KCs seem to be the most important liver cells in mediating the oxLDL-induced effects on hepatic inflammation, also other liver cell types are exposed to oxLDL and contribute at least partly to the inflammatory and fibrotic response in the liver. Being the most abundant liver cell, the hepatocyte is a crucial cell type for the regulation of general liver metabolism.⁷² Currently, oxLDL-mediated hepatocyte damage has mainly been explained as a secondary effect of KC activation, whereby the KC directly communicates with the hepatocyte.⁷³ However, especially because hepatocytes are responsive to elevated circulating oxLDL levels by upregulating CD36 expression,^{64,74} it is likely that oxLDL also mediates certain pro-inflammatory effects directly via the hepatocyte. In agreement with this view, by activation of the apoptotic Fas signaling pathway, hepatocyte apoptosis was demonstrated to play an important role in NASH.⁷⁵ Taking into consideration that oxidized lipids were previously found inside apoptotic cells,⁷⁶ oxLDL-induced hepatocyte apoptosis is a possible mechanism to trigger hepatic inflammation (Figure 2.2). Also, lipid peroxidation of F₂-isoprostanes in hepatocytes mediated activation of stellate cells, thereby leading to hepatic fibrosis.⁷⁷ Indeed, hepatic stellate cells (HSCs) have been established as the central hepatic cell in inducing a fibrotic response in the liver.⁷⁸ After a deleterious stimulus that induces liver injury, HSCs are activated, thereby secreting collagens and other matrix proteins that are deposited in fibrosis.⁷⁹ Next to cellular injury, CD36- and lectin-like oxLDL receptor 1 (LOX-1)-mediated internalization of oxLDL in HSCs was shown to stimulate the

synthesis of extracellular matrix, proving oxLDL as a harmful and pro-fibrogenic factor^{79,80} (Figure 2.2).

Figure 2.2 Overview of oxLDL-induced cellular mechanisms resulting in NASH. OxLDL-induced NASH can be mediated via different mechanisms distributed over several hepatic cell types. By internalizing oxLDL via the scavenger receptors CD36 and SR-A, inflammasome activation and/or impairment of the autophagy process will enable the Kupffer cell to trigger hepatic inflammation (*Top left panel*). Next, uptake of oxLDL by the hepatocyte leads to activation of the inflammasome and induces hepatocyte apoptosis, two processes that induce inflammation (*Top right panel*). Third, oxLDL internalization via CD36 and LOX-1 is able to activate the HSC, leading to a pro-fibrotic response (*Lower right panel*). Finally, LOX-1 is also able to incorporate oxLDL inside the LSEC, leading to NF-κB activation, endothelial injury, defenestration (*Lower left panel*). However, oxLDL-mediated hepatic inflammatory responses mediated by LSECs *in vivo* have not yet been investigated. Additionally, intercellular mechanisms (hepatic cell type affecting another hepatic cell type, e.g. oxLDL activation of Kupffer cell will affect hepatocyte) will also contribute to the development of NASH. CD36, cluster of differentiation 36; SR-A, scavenger receptor A; LOX-1, lectin-like oxLDL receptor 1; HSC, hepatic stellate cell; LSEC, liver sinusoidal endothelial cell.

Besides KCs, hepatocytes and HSCs, another liver cell type which has been shown to be affected by oxLDL is the sinusoidal endothelial cell.⁸¹ Together with KCs, liver sinusoidal endothelial cells (LSECs) constitute the largest scavenger cell system in the body, enabling them to internalize oxLDL.⁸² Moreover, LSEC dysfunction leading to defenestration has been shown to precede liver inflammation and fibrosis. This implies

that LSECs are potentially able to mediate oxLDL-induced inflammation and fibrosis.^{83,84} In contrast to KCs, oxLDL-uptake by LSECs is mainly mediated via LOX-1.⁸¹ Besides facilitating oxLDL-induced endothelial injury and defenestration of LSECs *in vitro*, LOX-1 was also demonstrated to directly induce inflammation in the context of atherosclerosis.⁸⁵ Nevertheless, *in vivo* evidence for oxLDL-induced defenestration remains scarce and should be further investigated. Overall, oxLDL influences several hepatic cell types that set different intracellular and intercellular cascades in motion, leading to the development of NASH. As such, oxLDL is a deleterious lipid that causes cellular injury in the liver by affecting different hepatic cell types in a direct and indirect way (Figure 2.2).

Of note, an experimental approach to prevent the uptake of oxLDL from every liver cell is by increasing oxLDL-specific antibodies. The concept of directing an immune response specifically to oxLDL is based on the presence of so-called oxidation-specific epitopes (OSEs) at the surface of the oxLDL particle. OSEs are products of lipid peroxidation and include the expression of the highly immunogenic products MDA and acetaldehyde-adducted products (MAA), oxidized cardiolipin, phosphocholine (PC) and 4-hydroxynonenal (4-HNE).⁸⁶ As such, boosting an immune response specifically to the PC-epitope of oxLDL was shown to reduce atherosclerosis⁸⁷ and NASH³⁵ *in vivo* and was associated with less foamy macrophages. Therefore, an attractive way to prevent the aforementioned deleterious effects of oxLDL-derived oxidized lipids on liver cells is the increase of circulating antibodies specifically directed to oxLDL.

Oxidized lipids in human NASH

Next to evidence linking oxLDL to hepatic inflammation and fibrosis at cellular level, human studies have incontrovertibly confirmed the importance of oxidative stress, and more specifically, the central role of oxLDL-derived oxidized lipids to NASH. First, human studies have identified a disturbed oxidative plasma profile in NASH patients, resulting in a pro-oxidant state.^{88,89} In line with this increase of oxidative stress in NASH patients, systemic lipid peroxidation products were elevated in NASH patients.^{90,91} Specifically MDA, a product derived from the oxidation of PUFAs, was shown to be increased in NASH patients.⁹¹⁻⁹³ In line, higher antibody titers of MDA-derived adducts were associated with increased severity of lobular inflammation and a 13-fold increased risk of developing NASH, indicated by an NAFLD activity score of ≥ 5 in a biopsy-proven pediatric NASH cohort.⁹⁴

Additionally, other FA-derived oxidation products have been associated with NASH. By performing high-sensitivity mass spectrometry on the plasma of biopsy-proven NASH patients, it was found that a subset of oxidation products, derived from the PUFA linoleic acid, could distinguish NASH from steatotic and healthy patients.⁹⁵ Moreover, elevated levels of these oxidized fatty acids correlated with hepatic histologic

inflammatory grading and were independent of other metabolic parameters, such as diabetes, obesity or hypertension.

In line, hepatic oxidative DNA damage was increased in the livers of particularly NASH patients, as measured by 8-oxo-2'-deoxyguanosine levels.⁹⁶ Likewise, an increase of the oxidant-generating enzyme myeloperoxidase was detected both in the liver and in the plasma of obese NASH patients.⁹⁷

In spite of the substantial evidence showing increased oxidative stress and the likewise elevation of oxidized fatty acids in NASH patient, it is not yet known to what extent oxLDL itself contributes to this process (and the generation of these oxidized fatty acids). Taking all the current information regarding oxidative stress and disturbed lipid metabolism in NASH into consideration, the specific contribution of oxLDL to NASH should definitely be investigated in more depth, starting by measuring oxLDL levels both in plasma and liver of NASH patients.

Underlying cascades linking oxidized lipids to NASH

OxLDL is generally accepted as a toxic compound that is able to cause detrimental inflammatory responses in the liver and other related organs. Mediation of these oxLDL-induced inflammatory responses is accomplished via several underlying mechanisms. In this section, we will discuss the specific underlying mechanisms that link oxLDL to hepatic inflammation.

Intralysosomal oxLDL as the trigger for downstream inflammatory processes

A central cell organelle for the regulation of metabolic and inflammatory responses is the endo-/lysosomal compartment.⁹⁸ Though other important functions have been assigned to lysosomes, its most established function is to degrade extracellularly-derived content⁹⁹. Therefore, impairments in the degradative capacity of lysosomes directly lead to major disturbances in cellular physiology, thereby causing inflammatory responses.

Preventing the uptake of oxLDL by KCs resulted in a decreased hepatic inflammatory response that was correlated with reduced levels of oxidized cholesterol inside the lysosomes.³⁵ Also, Yancey *et al.* observed that the accumulation of oxidized cholesterol inside the lysosome is highly resistant to removal, suggesting that storage of oxidized cholesterol inside lysosomes is a crucial process in inducing inflammatory responses.^{100,101} Besides accumulation of extracellularly-derived oxidized cholesterol inside lysosomes, Wen and Leake suggested that oxidation of cholesterol can also occur inside the lysosome itself.¹⁰² They found that specifically the highly toxic ring-oxidation products of cholesterol, 7-ketocholesterol and 7 β -hydroxycholesterol were created inside the lysosomes of J774 macrophages after incubation with non-oxidized

aggregated LDL. These data confirm the previously mentioned view of aiming at oxidation processes in order to prevent inflammatory responses.

A first mechanism by which intralysosomal oxLDL storage is linked to an inflammatory response is via mediating apoptosis (Figure 2.2). By damaging the lysosomal membrane, oxLDL caused the release of acidic lysosomal enzymes (cathepsin L and D) in human macrophages to the cytoplasm.¹⁰³ The relocation of cathepsin B to the cytoplasm was shown to trigger hepatocyte apoptosis by promoting the mitochondrial release of cytochrome c.¹⁰⁴ As hepatocyte apoptosis was demonstrated to play an important role in NASH,⁷⁵ the transfer of cathepsins from the lysosomal compartment to the cytoplasm of hepatocytes might be an important mechanism underlying hepatic inflammation.

Next to mediating apoptosis, cytosolic cathepsins can also activate inflammasomes¹⁰⁵ (Figure 2.2). Inflammasomes are a group of cytosolic protein complexes, which govern the activation of the serine protease caspase-1 (CASP-1), leading to the secretion of the pro-inflammatory cytokines IL-1 β and IL-18.¹⁰⁶ Of the range of stimuli that are currently known to induce inflammasome activation, two important signals are ROS¹⁰⁷ and cholesterol crystals.¹⁰⁸ The generation of increased ROS levels during cellular stress or cell death was demonstrated to initiate the detachment of thioredoxin-interacting protein (TXNIP) from thioredoxin, thereby empowering activation of the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome.¹⁰⁷ Notably, Sheedy *et al.* showed that the CD36-mediated uptake of oxLDL, among other ligands, coordinated activation of the NLRP3 inflammasome, thereby directly linking the oxidation of lipids to the formation of cholesterol crystals. Furthermore, different mouse models have confirmed the contribution of cholesterol crystal-mediated activation of the inflammasome in the context of NASH.^{47,109} In line with this view, plasma interleukin-1 receptor antagonist has been proposed as a useful tool to detect NASH.^{110,111} Additionally, compared to steatotic livers, livers from NASH patients displayed cholesterol crystals, which were resolved after therapeutic intervention with cholesterol-reducing agents.¹¹² Although the exact origin of cholesterol crystal formation is unclear,¹¹³ internalization of oxLDL leading to intracellular crystal formation appears to be an attractive concept by which inflammasome activation induces hepatic inflammation.

Another physiological process that is affected by oxLDL-induced lysosomal dysfunction is autophagy¹¹⁴ (Figure 2.2). Autophagy is a process of self-degradation of cellular components in which autophagosomes insulate cell organelles or cytosolic portions and fuse with lysosomes in order to degrade their content.¹¹⁵ Functionally, by producing energy, autophagy maintains cellular energy level under starvation conditions but is also involved in regulating homeostasis via degradation and resynthesis of damaged components.¹¹⁶ Accumulation of oxLDL inside lysosomes can lead to lysosomal dysfunction, thereby impairing autophagy.¹¹⁷ Additionally, it was demonstrated that cholesterol abnormalities in lysosomal storage disorders disturb key

components of the fusion machinery between autophagosomes and lysosomes.¹¹⁸ Relevantly, several studies have demonstrated the protective function of autophagy in macrophage-induced inflammation in atherosclerosis.¹¹⁹ In autophagy-deficient hyperlipidemic mice, elevated levels of apoptosis and oxidative stress in lesional macrophages combined with aggravated lesional efferocytosis were detected, implying a protective role for autophagy in atherosclerosis.¹¹⁹ In line, others showed that autophagy proteins have been shown to negatively regulate the inflammasome.¹²⁰ Also, *Atg7*-deficient mouse macrophages produced higher levels of mature IL-18 and IL-1 β after endotoxin-induced stimulation of TLR4. Furthermore, these researchers found increased serum IL-18 and IL-1 β cytokine levels in mouse chimeras engrafted with *Atg16/1*^{-/-} fetal liver hematopoietic progenitors, suggesting a direct inverse link between autophagy and the inflammasome.¹²¹ In contrast, oxLDL-mediated induction of autophagy was recently shown to induce endothelial injury in ApoE^{-/-} mice, suggesting a harmful role for autophagy in atherosclerosis.¹²² Concerning the liver, autophagy may play an important role in preventing the development of NAFLD. Inhibition of autophagy in cultured hepatocytes and mouse liver increased TG storage in lipid droplets, showing a crucial role for autophagy in maintaining hepatic lipid levels.¹¹⁴ Besides the regulation of lipid content, another mechanism by which autophagy protects the liver from damage is via oxidative stress regulation.¹²³ The antioxidant effect of Sestrins (Sesns) was shown to be dependent of the autophagic substrate p62, which prevented oxidative liver damage.¹²³ Also, liver-specific deletion of *Atg5* resulted in increased levels of oxidative stress in tumor cells, confirming the link between autophagy and oxidative stress regulation.¹²⁴ Together, a large amount of research has been performed to explain the underlying mechanisms linking autophagy to inflammation and oxidative stress levels. However, despite the inferential evidence for the protective role of autophagy in regulating inflammation, other studies imply its contribution to inflammation. It is therefore necessary to further investigate the exact underlying mechanisms by which autophagy exerts its function in order to assign specific conclusions to its working mechanisms, which may lead to novel therapeutic targets.

Intestinal dysbiosis as a regulator of oxidative state

A newly recognized system by which oxidation processes are influenced is via changes in composition and function of the microbial community inside the gut, also known as the gut microbiota. In the last decade, there is growing recognition for the concept of humans being supraorganisms consisting of human- and microbial-derived cells, thereby carrying two different genomes: our own inherited genome (~ 23 000 genes) and microbiota-related genome, referred to as the microbiome (~ 3 million genes).¹²⁵ As such, an explosive amount of research has been performed on the composition of the gut microbiome and its influence on disorders associated with metabolism (e.g. of

lipids), oxidative status and inflammation (among many others).¹²⁶⁻¹²⁸ As the gut-liver axis is increasingly recognized as a central concept of metabolism, we will highlight several findings that link the gut microbiota to lipid and oxidation metabolism in the context of NASH.

A key concept by which influences of the gut microbiota on host metabolism are currently explained is by perturbation of the intestinal integrity, leading to increased intestinal permeability causing endotoxin translocation to the circulatory system.^{129,130}

Though there are many functions by which certain intestinal-derived bacterial strains can affect liver physiology, here we specifically focus on their potential to disturb processes related to oxidative stress, lipid peroxidation, inflammation and fibrosis in the liver.

Several independent studies have suggested the association between gut microbiota, increased levels of oxidized lipids and NAFLD severity. First, in a pediatric NASH cohort alcohol-induced gut permeability was postulated as a mechanism by which the liver is increasingly exposed to ROS.¹³¹ Specifically, the authors suggested that due to the enrichment of alcohol-producing bacteria (i.e. *E.Coli*) higher ROS levels perturb the intestinal barrier, leading to gut permeability. Additionally, the increase in ROS production resulted in the oxidation of compounds associated with LDL, thereby enhancing liver inflammation. A similar result, which is in line with this hypothesis, was obtained by Boursier *et al.* who showed that *Ruminococcus*, which are able to produce alcohol,¹³² were capable of distinguishing between three distinct subgroups with NALFD severity.¹³³ Other studies postulated that increased bacterial degradation of pyruvate, a product generated during the breakdown of carbohydrates, resulted in marked elevations in oxidative stress levels.^{134,135} Indeed, increased levels of lipid peroxidation and liver inflammation in mice on a chronic fructose diet were partly explained by increased intestinal permeability, linking intestinal dysbiosis to disturbances in oxidative stress and liver inflammation.¹³⁶ Relevantly, microbial hydroxylation reactions of steroid substrates such as cholesterol were shown to produce oxidized derivatives, which can readily translocate the intestinal barrier.¹³⁷ In addition to their effect on inflammation, the composition of the microbiota has also been associated with liver fibrosis.¹³⁸ For detailed description concerning the link between gut microbiota and liver fibrosis, we refer to Seki and Schnabl¹³⁸ and Brenner *et al.*¹³⁹

Another aspect by which gut microbiota have been linked to the oxidative status of the host is via its ability to regulate host amino acid and glutathione metabolism.¹²⁸ By comparing conventionally raised (CONV-R) mice with germ-free (GF) mice, Mardinoglu *et al.* found that *de novo* synthesis of the antioxidant glutathione (GSH) was decreased in the small intestine of CONV-R mice compared to GF mice due to restricted availability of glycine. However, likely to rebalance oxidative stress levels, the expression of glutathione reductase (Gsr), the enzyme responsible for the generation of GSH, was higher in three separate small intestinal segments of CONV-R mice

compared to GF mice. These results indicate that the gut microbiota is involved in the preservation of oxidative stress levels. Another recently published study by Cai *et al.* confirmed this link between antioxidants and gut microbiota composition in mice.¹⁴⁰ In contrast to the gut microbiota influencing the redox state of the host, some evidence also suggests that the redox state itself can affect gut microbiota composition.¹⁴¹ Although increasing efforts are being made, one of the pitfalls of microbiota-related research is the lack of functional understanding.¹⁴² Additionally, studies comparing the bacterial taxonomy of NAFLD with NASH patients and their relation to oxidation are ambiguous and sometimes contradictory (for a broad overview of all studies performed so far, we refer to Schnabl *et al.*¹⁴³). This contradiction can be explained by the fact that a similar disease (i.e. NASH) between patients of different ethnicity/age is associated with a different microbial profile, as the microbiota are majorly influenced by both genetic and environmental factors.¹²⁷ Other explanations include a small number of subjects and differences in techniques used. Together, elucidation of the exact mechanisms linking gut microbiota and oxidized lipids to NAFLD and its severity is necessary to enhance our understanding of the pathogenesis, thereby providing new therapeutic targets to tackle this health problem.

Clinical perspectives/implications

At present, there is a lack of well-defined effective diagnostic and therapeutic approaches for NASH. Concerning NASH diagnosis, histological assessment of a liver biopsy is considered the golden standard.¹⁴⁴ However, due to its side effects, other non-invasive approaches to diagnose NASH are necessary.¹⁴⁴ While the currently applied markers in the clinic alanine aminotransferase (ALT) and aspartate aminotransferase (AST) lack specificity and sensitivity,¹⁴⁵ other promising plasma biomarkers such as cytokeratin-18¹⁴⁶ and cathepsin D¹⁴⁷ need further investigation before implementation in the clinic.

Regarding therapeutic approaches to combat NASH, there is no effective therapy available that specifically reduces hepatic inflammation. As NAFLD is considered the hepatic manifestation of the MetS, most NASH patients generally meet the criteria for the MetS, i.e., being obese, insulin resistant, hypertensive and hyperlipidemic.⁴ Therefore, though difficult to adhere to this therapy, the most suitable instruction for reducing NASH aims at lifestyle alterations, such as changing nutritional habits and increasing physical activity.

Altogether, non-invasive tests are necessary to diagnose NASH at early stages of the pathogenesis to allow opportunities to prevent further progression towards severe and irreversible liver damage. Moreover, there is a critical demand for novel and safe therapeutic approaches for NASH that will set the stage for a definite reduction in hepatic inflammation (Figure 2.3).

Figure 2.3 Schematic diagram of currently tested therapeutic approaches against oxLDL-induced NASH. OxLDL is an important risk factor for the development NASH. Two important processes that are at the basis of oxLDL formation are increased circulating lipid levels (which automatically increase the risk of oxLDL formation) and intestinal dysbiosis (which influences oxidation status and circulating lipid levels). The figure provides an overview of the currently investigated therapeutic approaches to prevent oxLDL exerting its pro-inflammatory and pro-fibrotic effects on the liver. OSE, oxidation-specific epitopes; PUFA, polyunsaturated fatty acids; PCSK9, pro-protein convertase subtilisin/kexin type 9

Lowering lipids to prevent their oxidation

Circulating LDL cholesterol levels have been shown to be directly proportional to the amount of circulating oxLDL levels,¹⁴⁸ implying that reducing plasma LDL cholesterol levels will result in a decrease of plasma oxLDL. Though not with the idea to reduce oxLDL but rather lipid levels in general, lipid-lowering agents have been extensively tested in NASH patients. In spite of its potential to drastically reduce lipid levels, the use of fibrates or statins in the clinic is still controversial for treating NASH.¹⁴⁹⁻¹⁵¹ With

respect to their effects on oxidation processes, both statins and fibrates have been acknowledged to influence oxidative stress levels (among other processes).^{152,153} Moreover, reduced levels of plasma oxLDL have been observed in statin-treated NASH patients, suggesting a beneficial role for statins in reducing plasma oxLDL levels.¹⁵⁴ However, several studies have shown reductions of the mitochondrial antioxidant coenzyme Q10 upon statin treatment, thereby promoting oxidation intracellularly.¹⁵⁵⁻¹⁵⁷ Also for fibrates, increased expression of markers related to peroxisomal and mitochondrial β -oxidation were observed, indicating increased intracellular oxidative stress upon this treatment.¹⁵⁰ Hence, despite having the potential to reduce plasma oxLDL levels, tissue-specific adverse effects of lipid-reducing agents should be taken into account when using these drugs in the clinic.

Next to general lipid-reducing agents, other treatments are known to be more selective, such as lowering levels of small-dense LDL (sdLDL) particles. Those treatments include niacin, dietary carbohydrate restriction and purified eicosapentaenoic acid (EPA) supplementation. SdLDL particles are an important substrate for oxidation in plasma, and as such, can indirectly affect plasma oxLDL levels. So far, EPA and dietary carbohydrate restriction have been shown to be successful in reducing biopsy-proven hepatic inflammation in small pilot studies.^{158,159} Additionally, niacin improved diet-induced NASH in rodents and should be tested in NASH patients in the future.¹⁶⁰ Altogether, lowering sdLDL levels is an attractive approach to decrease oxLDL levels and thereby reducing NASH.

Another rather new approach to reduce specifically cholesterol levels is by blocking the absorption of cholesterol in the intestine. Ezetimibe is one such pharmacological compound that inhibits intestinal cholesterol uptake by binding to the Niemann-Pick C1-like 1 sterol transporter in the membrane of the enterocyte brush border.¹⁶¹ However, treating NASH patients with ezetimibe did not reduce liver fat nor lobular inflammation.¹⁶² Besides ezetimibe, another way to reduce cholesterol concentrations can be achieved by aiming at pro-protein convertase subtilisin/kexin type 9 (PCSK9), a protein secreted by hepatocytes that governs LDL receptor breakdown. Injecting patients with alirocumab or evolocumab, two antibodies directed against circulating PCSK9, resulted in a 62% and respectively 56% reduction in circulating LDL cholesterol levels.¹⁶³ Though reducing cholesterol levels will probably lead to a decreased formation of oxidized cholesterol derivatives and improve NASH pathology, the effect of both strategies on oxidized lipid derivative levels has not been investigated to our knowledge.

Besides reducing lipid levels by means of pharmacological intervention, another more obvious way to accomplish this beneficial effect is via dietary intervention. Apart from changing dietary habits in general, a relatively new approach is to change the composition of the diet by the addition of specific food or food components (so-called functional foods). The beneficial potential of functional foods was first exemplified

when addition of plant sterol and plant stanol esters reduced serum total and LDL cholesterol concentrations¹⁶⁴. However, despite promising results in mice,¹⁶⁵ no human data are currently available about the potential of plant sterol and stanol esters in NASH. Additionally, the possibility of sterols undergoing oxidation can be a major drawback for the use of dietary sterol esters in these patients and should therefore be considered carefully. In contrast, plant stanol esters do not contain a double bond in their ring structure, preventing them from becoming oxidized.¹⁶⁶ Therefore, the addition of plant stanol esters to the diet might be preferred above their sterol relatives.

In summary, despite its association with NAFLD severity, therapeutic options aimed at reducing lipid levels do not seem to improve hepatic inflammation and outcome. Though more well-designed human intervention studies examining the effect of these lipid-lowering agents are necessary to truly understand their effects on oxidized lipids,¹⁶⁷ it appears that new therapies, targeting other underlying processes (such as oxidation) are necessary to overcome NASH.

Plasma oxLDL and OSE-antibodies

The identification that oxidized lipids as part of oxLDL play a significant role in hepatic inflammation opens new avenues for novel therapeutic and non-invasive diagnostic methods to detect NASH.

First, measuring circulating oxLDL levels can be a valid option for NASH diagnosis, but also a tool to select patients who are at increased risk to develop NASH. So far, only Chalasani *et al.* showed increased oxLDL levels in the plasma of biopsy-proven NASH patients compared to BMI-matched controls⁹¹ and thus confirmation is needed. To evaluate the prognostic value of plasma oxLDL for the non-invasive detection of NASH, the evaluation of plasma oxLDL needs to be performed in larger, well-defined NASH cohorts. Despite a growing interest in measuring plasma oxLDL and the increased development of several enzyme-linked immunosorbent assays designed for oxLDL detection, oxLDL values are difficult to detect in the blood and there seems to be a considerable variability between the different assays. As a result, none of these tested assays have been approved for clinical practice.¹⁶⁸ Therefore, one robust and standardized assay for routine use is needed to validate the potential of plasma oxLDL as a non-invasive marker for NASH.

Next to the direct measurement of plasma oxLDL, other indirect ways can also give an indication of disturbances in plasma oxLDL levels. Emerging evidence particularly demonstrated that natural antibodies can bind specifically to OSEs present on the oxLDL particle. Indeed, several groups have shown a correlation between plasma anti-oxLDL antibodies and atherosclerosis.^{169,170} While IgG autoantibodies have been positively associated with cardiovascular disease (CVD),¹⁶⁹ IgM autoantibodies were shown to be protective and are therefore negatively correlated with CVD.¹⁷¹ In line

with this inverse correlation for IgMs in CVD, our group confirmed that plasma IgM anti-oxLDL antibodies negatively correlate with hepatic inflammation (unpublished data). Also, Albano *et al.* found an association between specific antibodies directed to lipid peroxidation products and NAFLD severity, including advanced fibrosis.³⁰ In this view, anti-oxLDL antibodies can potentially be used as a diagnostic tool for NASH detection. However, it is important to note that the amount of anti-oxLDL antibodies naturally vary between people and can change over time.¹⁷² Additionally, there is molecular mimicry between OSEs of oxLDL and epitopes located on infectious agents, suggesting that exposure to pathogens may influence the production of anti-oxLDL antibodies.¹⁷² Therefore, the use of plasma anti-oxLDL antibodies as marker for NASH diagnosis needs to be further investigated and confirmed in other studies before it can be implemented in the clinic.

Besides the application of plasma anti-oxLDL antibodies as diagnostic tools for NASH, increasing their levels also holds therapeutic promise as these antibodies can prevent the uptake of oxLDL. Besides inducing an immune response against the PC-epitope, as aforementioned, immunization strategies against other OSEs, such as MDA and MAA also conferred protection against inflammation in atherosclerosis.^{173,174} However, while these methods were successful in reducing atherosclerosis, applications of these immunization strategies in the context of NASH are lacking and should therefore be further investigated.

Antioxidants

As the oxidation of lipids has been highlighted as an important risk factor for the development of NASH, a plausible therapeutic approach to counter NASH development involves rebalancing the oxidation process. To manage this re-equilibrium of oxidation, pro-oxidant mechanisms can be reduced and/or antioxidant mechanisms can be increased, with the latter being the most applied approach. In relation to its effect on oxLDL, antioxidants have been shown to decrease oxLDL levels after administration of the antioxidant cumin¹⁷⁵ and the macrophage-generated antioxidant 7,8-dihydroneopterin,¹⁷⁶ proving antioxidants as a proper approach to decrease oxLDL.

Next, vitamin E therapy, well-known for its antioxidant properties, was evaluated in several human randomized controlled trials and open-label studies.¹⁷⁷⁻¹⁷⁹ The largest study was performed by Sanyal *et al.* which compared vitamin E to placebo in a biopsy-proven NASH population selected for being non-diabetic and non-cirrhotic patients.¹⁷⁷ Patients that were assigned for 24 months to vitamin E treatment demonstrated improved steatosis and inflammation compared to those receiving placebo. Similarly, these findings were confirmed in a study of smaller sample size, which demonstrated improved hepatocellular ballooning in the liver, i.e. an improvement in scoring for the severity of ballooning injury is defined by enlarged hepatocytes that exhibit a nucleus located in the central, rarefied cytoplasm and changes in the cytoskeleton,^{180,181} and

NASH activity after vitamin E treatment in NASH patients.¹⁷⁹ From a mechanistic point of view, it is not yet clear how vitamin E exert its function on NASH. While the predominant view considers vitamin E as a fat-soluble antioxidant that blocks the propagation of lipid oxidation and generation of ROS,¹⁸² others have pointed to its potential to modulate the activity of certain signaling enzymes.¹⁸³ Also, information regarding the link between vitamin E and oxLDL is currently lacking. Therefore, further research on the exact mechanism of action of vitamin E in NASH is indispensable. Additionally, red flags have been raised concerning the safety of vitamin E, since studies reported an increased risk for mortality,¹⁸⁴ the development of hemorrhagic stroke¹⁸⁵ and prostate cancer.¹⁸⁶

While the strongest evidence for the use of antioxidants in NASH is available on vitamin E, other compounds that are considered to possess antioxidant properties including pentoxifylline,¹⁸⁷ betaine¹⁸⁸ and L-carnitine¹⁸⁹ are not potent antioxidants *in vitro*. In addition, plasmalogens were recognized as relevant antioxidant lipids that protect against oxidation of lipoproteins. Reduced levels of plasmalogens were found in the plasma of steatotic and NASH patients, compared to healthy individuals.^{190,191} In contrast, other studies detected elevated levels of the plasmalogens in the plasma of patients with steatosis, NASH and cirrhosis as compared to normal liver subjects.^{191,192} Therefore, though ambiguously, plasmalogens have been associated with NAFLD severity, emphasizing the role of oxidative stress in NAFLD severity. However, more research is warranted to fully elucidate their function in NASH.

Altogether, there is increasing evidence claiming antioxidants as a suitable approach to treat NASH. However, validation of the obtained results combined with the assessment of potential side effects is necessary. Likewise, antioxidant therapies were often shown to be unsuccessful, partly due to issues related to dosage, duration and lack of insight in intracellular signaling.¹⁹³ Therefore, more and larger therapeutic clinical trials including histological confirmation of NASH diagnosis should be conducted in the future.

Modulation of the gut microbiome

By identifying the gut microbiota as a new organ that interacts with the host's physiology, experts have gained increasing interest in using this 'internal ecosystem' as a tool and/or as a target in a range of diseases including NASH.

Though being rather hypothetical at this moment, from a diagnostic point of view, the collection of fecal samples of a patient to use for diagnostic purposes (or assessing NAFLD stage) is straightforward, non-invasive and easy and has therefore potential to be implemented in the clinic as compared to the use of the liver biopsy. However, as previously mentioned, despite an established association between gut microbiota dysbiosis and NAFLD/NASH, analysis of fecal microbiota of NAFLD patients have led to

variable results.^{131,133,194} Thus, using fecal material as an approach for assessing the pathological state of the liver is at this point irrelevant.

Regarding therapeutic approaches, it appears that manipulation of the gut microbiota can assist in counteracting the disturbed metabolic processes in the liver. Due to the increasing interest in the gut microbiome (and the exceptional results associated with it), major advances have been made in modulating the microbiota.

A strategy by which composition or metabolic function (such as oxidation processes) of gut microbiota can be modulated is via specific dietary approaches. One such approach consists of the administration of prebiotics to the diet. Prebiotics are a group of nondigestible fibers that are fermented by colonic microbiota and enhance the growth of beneficial commensal organisms. Concerning their modulating capacity on the gut microbiota, the prebiotics oligofructose,¹⁹⁵ galacto-oligosaccharides¹⁹⁶ and inulin¹⁹⁷ have been shown to possess antioxidant capacities. However, despite its potential to control oxidative processes, more research is necessary to fully understand the exact mechanisms by which these compounds affect the gut microbiota and related metabolic processes such as oxidative stress. Another functional food that is considered to promote gut microbiota are polyphenols. Polyphenols are a class of secondary plant metabolites well known for their antioxidant capacities.¹⁹⁸ However, recent studies also demonstrated an effect on the health-promoting bifidobacteria and lactobacilli strains, thereby possibly regulating the host's oxidation status.¹⁹⁹

An alternative method to influence gut microbiota is by using probiotics. Probiotics are live microorganisms that, when administered, provide a substantial health benefit to the host.²⁰⁰ Delivering *Lactobacillus acidophilus* ATCC 4356 to ApoE^{-/-} mice attenuated atherosclerotic lesion progression in a dose-dependent manner by modulation of oxidative stress (lower oxLDL and higher superoxide dismutase levels) and inflammation.²⁰¹ Also, *Bifidobacterium longum* was shown to play a central role in controlling the level of oxidative damage *in vitro* and *in vivo*, underscoring the importance of gut microbiota in controlling oxidative processes.²⁰²

Finally, a new method to change systemic oxidation status by affecting the function (and inevitably also composition) of gut microbiota is by means of fecal microbiota transplantation (FMT). FMT is a technique in which fecal-derived intestinal microbiota from a donor individual are administered to the intestinal tract of a recipient.¹⁴² Concerning NASH, Henao-Mejia *et al.* showed that alteration of the intestinal microbiota composition contributed to the progression of murine steatohepatitis and clinical trials are currently ongoing to validate this technique in humans.²⁰³ However, together with transferring the beneficial characteristics of the donor's intestinal microbiota, transmission of detrimental pathogens to the recipient is likely as well.²⁰⁴ It is therefore not surprising that accurate and careful screening of donors is crucial in order to create a beneficial effect in the recipient.¹⁴² Furthermore, it is unknown how FMT might interfere with viruses, protozoa and fungi present in the intestinal tract, nor is it clear how long it takes for one FMT intervention to show a beneficial outcome for

the recipient or what exactly constitutes a ‘healthy’ microbiome.²⁰⁵ Hence, partially due to being in its infancy, functional insight into the mechanisms that are affected by this therapy is highly necessary before it can be approved for clinical use.

Emerging therapies

To conclude this section, we will shortly elaborate on promising therapies that are currently being investigated as a treatment for NASH and assess their potential to affect oxLDL levels. Firstly, the farnesoid X receptor (FXR)-agonist obeticholic acid (OCA) is a semi-synthetic bile acid derivative that is known to increase β -oxidation and has recently generated promising results as NASH therapeutic.^{206,207} OCA was shown to ameliorate a range of metabolic metrics, including hepatic steatosis, glucose tolerance, inflammation and hepatic fibrosis.²⁰⁸ Though no direct effects on oxLDL levels have been measured, the beneficial effects of OCA in NASH are in line with our previously mentioned anti-inflammatory effects of cholesterol products generated via enzymatic oxidation reactions. Another promising compound that has recently gained attention is the fibrate elafibranor which is known to increase fatty acid oxidation. Elafibranor is a PPAR α/δ -agonist that showed hepatoprotective effects by reducing steatosis, inflammation and fibrosis in different NASH rodent models.²⁰⁹ Translating these findings to the clinic, Ratziu *et al.* recently demonstrated elafibranor to induce resolution of NASH in patients, proving it to be a very promising new therapeutic.¹⁵¹ However, no effects on oxidative stress were defined in both studies investigating elafibranor. Finally, simtuzumab, a monoclonal antibody that binds to the extracellular matrix-modifying enzyme lysyl oxidase-like-2 (LOXL2), which mediates the post-translational oxidative deamination of lysine residues on target proteins, was shown to reduce inflammatory cytokine levels and TGF- β pathway signaling in steatohepatic livers.²¹⁰ However, no direct effects on oxidative stress or on oxLDL levels have been measured so far. Together, while accumulating studies indicate a positive effect of these therapies on NASH, their effect on oxLDL levels is at this point rather controversial. As such, additional results need to be generated to assess the link between oxLDL to these treatments.”

Conclusions

Despite the strong correlation between disturbances in lipid and lipoprotein metabolism in NASH, it becomes clear that dyslipidemia cannot merely explain the progression towards the hepatic inflammatory state of this disorder. Oxidized lipids present inside the oxLDL particle have been known to induce cellular damage by interfering with physiological processes leading to inflammatory responses. Lately, the deteriorating capacity of these oxidized lipids was also linked to processes in the liver,

showing clear associations between liver inflammation and the level of these oxidized lipids. Increasing evidence indicates that the detrimental effects of oxidized lipids are dependent on the type of oxidation that takes place as well as the lipid structure that is subject to a certain oxidation reaction, creating a range of oxidized lipids each having unique biological or/and pathological functions. The finding that oxidized lipids are an important risk factor for NASH opens new avenues for diagnosis and therapy against hepatic inflammation. Altogether, we suggest that studies concerning NASH should elaborate on the interplay between oxidative stress and lipids. Moreover, instead of focusing on the oxLDL particle as a whole, future research should specifically aim at elucidating the composition of oxidized lipids inside oxLDL (FA- or cholesterol-derived) and investigate how these relate to inflammatory responses in the liver and related metabolic tissues.

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Chapter 3

The immunity-diet-microbiota axis in the development of metabolic syndrome

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Abstract

Purpose of review

Recent evidence demonstrates that the gut-microbiota can be considered as one of the major factors causing metabolic and cardiovascular diseases.

Recent findings

Pattern recognition receptors as well as antimicrobial peptides are a key factor in controlling the intestinal microbiota composition. Deficiencies in these genes lead to changes in the composition of the gut-microbiota, causing leakage of endotoxins into the circulation, and the development of low-grade chronic inflammation and insulin resistance. Dietary composition can also affect the microbiota: a diet rich in saturated fats allows the expansion of pathobionts that damage the intestinal epithelial cell layer and compromise its barrier function. In contrast, a diet high in fiber supports the microbiota to produce short-chain fatty acids, thereby promoting energy expenditure and protecting against inflammation and insulin resistance.

Summary

The interactions between the microbiota, innate immunity, and diet play an important role in controlling metabolic homeostasis. A properly functioning innate immune system, combined with a low-fat and high-fiber diet, is important in preventing dysbiosis and reducing the susceptibility to developing the metabolic syndrome and its associated cardiovascular diseases.

Introduction

Recent evidence points to an important role for the microbiota in the development of the metabolic syndrome. The link between the microbiota and host metabolism was initially discovered by Jeffrey Gordon's group, which showed that germfree mice are not susceptible to developing diet-induced obesity.¹ Conventionalization of germfree mice by the microbiota of lean mice led to an increase in adiposity and, strikingly, this effect was significantly stronger if the microbiota were derived from obese mice, demonstrating a causal role of microbiota composition on energy metabolism, and eventually leading to obesity. Microbiota analysis has shown that obese mice have a higher ratio of the *firmicutes:bacteroidetes* phyla and this composition has a greater capacity to harvest energy from the diet.² This means that the composition of the microbiota changes during obesity and that this change also contributes to the further development of obesity.

To study whether the microbiota composition also affects host metabolism in man, Ridaura et al performed an elegant study in which they transplanted the gut microbiota of human twins discordant for obesity into germfree mice. The mice that received microbiota from obese human subjects had a significantly increased body mass and adiposity compared to mice receiving microbiota from lean subjects,³ supporting that the microbiota contribute to development of obesity. Differences in microbiota composition and function have also been linked to the more advanced stages of metabolic syndrome, such as type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD), and cardiovascular diseases (CVD).³⁻⁶ A change in microbiota composition is not merely associated with disease, but also appears to play a causal role in the development of the metabolic syndrome in humans, since transplanting the microbiota of healthy individuals into subjects diagnosed with metabolic syndrome led to an increased insulin sensitivity, thereby improving the metabolic phenotype of these patients.⁷ Thus, the microbiota are an important factor in the development of the metabolic syndrome, although we are only just starting to understand how the microbiota influences the development of metabolic diseases. Microbiota composition is controlled by the mucosal immune system and is strongly influenced by dietary consumption. If the microbiota is not well maintained, this can lead to leakage of bacterial cell wall components into the circulation, which can contribute to low-grade systemic inflammation, and consequently to metabolic syndrome. The microbiota can also influence the development of metabolic syndrome through the production of metabolites, such as short chain fatty acids, which can directly affect the host metabolism. In this review we will discuss the importance of the microbiota in the development of metabolic syndrome and how microbiota composition and its function is controlled by the mucosal immune system and affected by dietary intake.

Microbial control by innate immunity

The mucosal immune system is delicately balanced between tolerating commensal bacteria and eliminating pathogens. The first line of defense in the mucosal immune system is the physical barrier, consisting of epithelial cells kept together by tight junctions and the mucus layer lying on top of the epithelial cells. Goblet cells produce the mucus overlying the epithelial cells, physically separating the bacteria from the intestinal epithelial layer. The Paneth cells excrete antimicrobial peptides such as RegIIIy and alpha-defensins into this mucus layer. These peptides are important in preventing bacteria that are present naturally in the gut lumen from penetrating the mucus layer and adhering to the intestinal cell wall (Figure 3.1).

Figure 3.1 Microbiota control by immunity is essential to prevent dysbiosis and endotoxemia. **1a)** The epithelial lining of the intestine is mainly formed by enterocytes, which are linked together by tight junctions. Goblet cells produce the mucus layer physically separating the microbiota, whereas Paneth cells excrete antimicrobial peptides such as RegIIIy into this layer upon stimulation of the IL22 receptor by IL-22. This prevents bacteria from the microbiota to infiltrate the intestinal epithelial cell layer. Intracellular and extracellular PRRs recognize endotoxins, binding of endotoxins to PRRs on enterocytes stimulates production of antimicrobial peptides and formation of tight junctions. **1b)** The intracellular PRR NLRP3 plays an important role in shaping the microbiota composition. Disruption of NLRP3 function leads to dysbiosis, characterized by increased abundance of *prevotellaceae* and *TM7*, whereas abundance of *Lactobacillus* is decreased. **1c)** Also extracellular PRRs are important in controlling microbiota composition. Disruption of TLR2 leads to dysbiosis, characterized by an increased *firmicutes:bacteroidetes* ratio. **1d)** Disruption of stimulation of the IL22 receptor by IL-22 leads to decreased production of RegIIIy. Consequently bacteria from the microbiota can infiltrate the intestinal epithelial cell layer. Disturbance of NLRP3, TLR2 or IL22R function, leads to increased permeability due to disrupted tight junctions. Consequently endotoxins can leak through the intestinal epithelial cell layer into the systemic circulation, causing endotoxemia and contributing to low-grade systemic inflammation, which leads to development of the metabolic syndrome

Antimicrobial peptides

It has recently been shown that REGIIIy^{-/-} mice have increased numbers of mucosa-associated-bacteria, which leads to the development of low-grade intestinal inflammation.^{8,9} RegIIIy is secreted by epithelial and Paneth cells under stimulation of the cytokine IL-22, thus IL-22 plays an important role in protecting the mucosal intestinal barrier (Figure 3.1). IL-22R knockout mice show aberrant mucosal immunity and develop metabolic syndrome, characterized by an increase in bodyweight,

hyperglycemia, insulin resistance, and decreased glucose tolerance. Strikingly, on administration of IL-22-Fc, which stimulates IL-22R, mice on a high fat diet (HFD) show reduced bodyweight, smaller fat pads, and better insulin sensitivity and glucose tolerance. Thus, mucosal protection by IL-22 plays an important role in protecting the host against metabolic syndrome.¹⁰ Protection by IL-22 can likely be explained by the reduced infiltration of bacteria in the intestinal epithelial cell layer, thus preventing low-grade inflammation of the intestine and maintaining intestinal integrity. Maintenance of intestinal integrity is important to prevent translocation of bacteria or endotoxins from the gut lumen into the systemic circulation. Translocation of endotoxins (metabolic endotoxemia) into the systemic circulation leads to weight gain, higher insulin resistance and lower glucose tolerance.¹¹ Endotoxemia has also long been associated with the development of atherosclerosis in humans and increased production of endotoxins has been associated with symptomatic atherosclerosis in humans.⁵ Altogether, leakage of endotoxins from the gut lumen into the systemic circulation contributes to the development of metabolic syndrome and atherosclerosis (Figure 3.1).

In conclusion, a reduced control of microbiota localization by antimicrobial peptides could lead to more leakage of endotoxins into the circulation leading to inflammation in the liver, adipose tissue, and arteries.

Pattern recognition receptors

In addition to the physical features of the immune system preventing the translocation of bacteria, pattern recognition receptors (PRR) play an important role in protecting the human body from bacterial invasion by recognizing pathogen-associated molecular patterns (PAMPS), such as endotoxins. There are two major forms of PRRs: extracellular PRRs mainly consisting of the Toll-like receptor family (TLR) and intracellular PRRs, such as NOD-like receptors. Both types of PRRs have been shown to be important in controlling the microbiota composition and can thus affect the development of metabolic syndrome. TLRs as well as NOD-like receptors are expressed in the intestinal epithelial cells and are important for their proliferation, IgA production, tight junction formation, and the production of the antimicrobial peptides discussed earlier. This means the expression of PRRs in intestinal epithelial cells can play an important role in preventing a leaky gut.^{12,13} PRRs are also abundantly expressed on innate immune cells, such as macrophages and dendritic cells, where they trigger phagocytosis of bacteria and the expression of cytokines and co-stimulatory molecules, which trigger the adaptive immunity.¹⁴ Thus, PRRs in the intestine are important in controlling the gut microbiota and preventing translocation of bacteria into the systemic circulation.

Extracellular pattern recognition receptors

Germfree TLR2 knockout mice have been shown to be resistant to diet-induced insulin resistance, but they show a more severe development of metabolic syndrome upon colonization with microbiota. Their microbiota composition is changed showing a three-fold increase in *firmicutes* and a decrease in *bacteroidetes*. This change in composition leads to increased translocation of PAMPs, such as lipopolysaccharide into the circulation, causing subclinical inflammation and eventually insulin resistance, glucose intolerance, and obesity (Figure 3.1). Strikingly, the metabolic phenotype is transferable to wild-type mice, by microbiota transfer, demonstrating that a lack of microbiota control is responsible for the observed phenotype.¹⁵ Lack of microbial control in TLR5 knockout mice also leads to the metabolic syndrome developing under the influence of a HFD.¹⁶ There are thus several lines of evidence showing that TLRs play an important role in controlling the microbiota composition and preventing metabolic diseases.

NOD-like receptors

Inflammasomes belong to the NOD-like receptor (NLR) family and are important intracellular PRRs that play an important role in mucosal defense. Upon recognition of danger signals by an NLR (e.g. NLRP3), a multiprotein complex is formed with ASC and caspase 1, and together these are named inflammasomes. On formation of the inflammasome, caspase 1 is activated and pro-IL-1 β and pro-IL-18 are post-translationally processed into their active isoforms IL-1 β and IL-18. Disruption of the NLRP3 inflammasome function has been shown to influence the development of NAFLD.

The NLRP3 inflammasome influences the microbiota composition as well as the localization of the microbiota. NLRP3^{-/-} mice have increased numbers of *prevotellaceae* and the bacterial phylum TM7, while having a reduced abundance of the *lactobacillus* genus (Figure 3.1).⁴ This change in microbiota composition leads to an increased severity of NAFLD under the influence of a methionine-choline-deficient diet. Increased severity of NAFLD is triggered by translocation of endotoxins into the systemic circulation, which activate TLR2 and TLR9 in the liver and consequently upregulate TNF- α and increase inflammation in the liver. Increased development of NAFLD depends on the inability of NLRP3^{-/-} mice to process pro-IL-18 into IL-18, since IL-18^{-/-} mice also showed a more severe development of NAFLD, which could be transferred to wild-type mice by microbiota transplantation.⁴ It is possible that the more severe development of NAFLD due to the translocation of endotoxins into the systemic circulation could be explained by the protective function that IL-18 exhibits against mucosal challenges or disruption of the intestinal mucosa.¹⁷

NLRP3 does not merely play a role in controlling the microbiota composition but its expression in the intestinal epithelial cells is also important in preventing infiltration of the mucosal barrier by bacteria from the microbiota.¹³

In conclusion, intracellular PRRs could have an important function in the protection against NAFLD, as highlighted by the function of NLRP3 in controlling the microbiota composition and localization in the intestine. Consequently, NLRP3 acts in preventing translocation of bacteria and endotoxins that can lead to low-grade systemic inflammation, which contributes to NAFLD development. Whether other intracellular PRRs also have a protective role against NAFLD and whether they are also important for protection against T2D and CVD still needs to be established.

Diet-microbiota interactions in the development of metabolic syndrome

In addition to mucosal immunity, the diet strongly influences microbiota composition and function.^{18,19} Two general changes can be observed on consumption of a high-fat, low-fiber diet. First, pathobionts can expand during consumption of a HFD, and second, the abundance of protective bacteria, such as producers of short chain fatty acids, declines¹⁹⁻²¹ (discussed later).

Pathobiont expansion under influence of dietary components

Decreased protection against pathobionts during consumption of a HFD is exemplified by the increased infiltration of *adherent-invasive Escherichia Coli* into the intestinal epithelial cell layer. This infiltration leads to less mucus thickness, and more intestinal inflammation and permeability (Figure 3.2).²²

Furthermore, expansion of pathobionts has been shown in humans with a diet rich in animal fats leading to expansion of the sulfate-reducing bacterium *Bilophilia Wadsworthia* (Figure 3.2).^{19,21} Increased abundance of *Bilophilia Wadsworthia* in the microbiota has been associated with overall adiposity, dyslipidemia,⁶ and T2D and it triggers intestinal inflammation.^{21,23} This inflammation could possibly be explained by the production of H₂S by *Bilophilia Wadsworthia*, which is toxic to host cells.²⁴ In addition, H₂S deprives the enterocytes of energy by inhibiting oxidation of the most abundant energy source, butyrate.²⁵ Hence, increased infiltration of the intestinal epithelial layer, or production of toxic metabolites by pathobionts, can damage the intestinal epithelial layer and provide yet another cause of translocation of endotoxins or bacteria from the gut lumen into the systemic circulation, thereby contributing to the metabolic syndrome.

Figure 3.2 High fat diet induces pathobiont expansion. The intestinal epithelial layer protects against invasion of bacteria or endotoxins into the systemic circulation during consumption of a chow diet (left hand side of the figure). Consumption of a HFD leads to expansion of the pathobionts *AIEC* and *Bilophilia Wadsworthia*. *AIEC* infiltrate the intestinal epithelial layer, causing decreased mucus thickness, inflammation and increased permeability. Expansion of *Bilophilia Wadsworthia* leads to increased production of H₂S. H₂S is toxic to host cells and can possibly cause intestinal inflammation (dotted arrow). H₂S can disturb the intestinal epithelial cell layer also by inhibiting the oxidation of butyrate by enterocytes, leading to decreased energy availability (right hand side of the figure).

Modulation of dietary-derived choline by the microbiota promotes CVD

Modulation of dietary-derived choline, phosphatidylcholine or L-carnitine by the microbiota directly contributes to development of CVD. The microbiota converts these dietary components into tri-methyl-amine (TMA) by the CutC gene cluster present in the microbiota.^{26,27} TMA is taken up by the host and metabolized into tri-methylamine-N-oxide (TMAO) by flavin mono-oxygenase 3 in the liver. Increased levels of TMAO are associated with major cardiovascular events in humans and a causal role was demonstrated by promoting atherosclerosis in mice upon ingestion of TMAO.²⁶⁻²⁹ Altogether, conversion of dietary components by the gut microbiota leads to production of TMAO, which then promotes CVD.

Function of short chain fatty acids on host metabolism

The beneficial effects of “healthy” fiber-rich diets, which influence microbiota composition, are now better understood.^{30,31} The microbiota of children consuming a high fiber diet have a lower *firmicutes:bacteroidetes* ratio. Furthermore the microbiota has an increased capacity to degrade dietary fibers, leading to the production of short chain fatty acids (SCFA), such as acetate, propionate and butyrate.³⁰ Human studies show a protective role for SCFA against metabolic syndrome, T2D and atherosclerosis.⁵⁻⁷

The capacity of the microbiota to produce butyrate is negatively correlated with C-reactive protein levels in patients with atherosclerosis.⁵ In addition, a decrease in butyrate production was found in T2D patients.⁶ Causality for butyrate having a protective role against the development of metabolic diseases was demonstrated in a mouse study that showed that administering butyrate to mice on a HFD prevented the development of obesity and insulin resistance.³²

The mode of action for the protective role of SCFA against metabolic syndrome is mainly through binding to GPR41 and GPR43. These G-coupled receptors are widely expressed, including in tissues with an immunological function such as the spleen, lymph nodes, and bone marrow, as well as in metabolic tissues such as the large intestine, adipose tissue, and pancreas.³³⁻³⁶ Binding of propionate to GPR41 in sympathetic neurons leads to an increased heart rate and oxygen consumption, causing higher energy expenditure (Figure 3.3).³⁷ Energy uptake is also modulated by SCFA. The SCFA propionate and butyrate stimulate intestinal gluconeogenesis and has beneficial metabolic effects, including the prevention of obesity and improved glucose and insulin tolerance. Butyrate directly stimulates intestinal gluconeogenesis, whereas the effect of propionate is dependent on gut-brain neural signaling via GPR41.³⁸ Furthermore, SCFA stimulate adipocytes to produce leptin, which is known to improve insulin sensitivity as well as to induce satiety, thereby controlling energy intake as well as uptake.^{39,40} Moreover, SCFA also modulate glucose metabolism by stimulating L-cells in the intestine via GPR43, leading to the production of glucagon-like peptide 1 (GLP-1) and thereby improving glucose tolerance (Figure 3.3).⁴¹ The importance of GPR43 in the signaling of SCFA was elegantly demonstrated by Kimura et al, who showed that GPR43^{-/-} mice developed increased obesity, adiposity, white adipose tissue inflammation, and decreased glucose tolerance.⁴² Whereas overexpression of GPR43 restricted to the adipose tissue suppresses insulin signaling in adipocytes, thereby preventing fat accumulation and metabolic dysfunction.⁴² In conclusion, SCFA are protective against metabolic syndrome by their modulation of the host metabolism via GPR41 and GPR43.

Figure 3.3 SCFA are protective against development of metabolic syndrome. Fibers are converted by the microbiota to SCFA. Production of SCFA stimulates L-cells in the intestine via GPR43 to produce GLP-1, this leads to increased glucose tolerance. In addition, SCFA can directly stimulate intestinal gluconeogenesis (IGN), either by direct stimulation of IGN via an unknown mechanism or by stimulation of IGN dependent on the gut-brain axis. Increased IGN leads to decreased obesity, increased glucose tolerance and insulin sensitivity. Next to protective effects in the intestine, SCFA are also taken up into the circulation where stimulation of GPR41 or GPR43 in adipose tissue leads to increased production of leptin and thereby increased insulin sensitivity. Furthermore SCFA can also stimulate sympathetic neurons leading to an increased heart rate and consequently increased energy expenditure.

Bile acid modulation

A well-established role for the gut microbiota is the metabolism of bile acids [see review by Jones et al].⁴³ Primary bile salts are deconjugated by bile salt hydrolases, which are present in multiple gram-positive bacteria.⁴⁴ Recently the physiological role of bile salt hydrolase (BSH) by bacteria was demonstrated: the expression of bacterial BSH was found to reduce weight gain, and lower plasma cholesterol and hepatic triglycerides levels.⁴⁵ In addition, the microbiota affect the composition of the bile acid pool by 7- α /7- β dehydroxylation, desulfation and dehydrogenation, leading to the

formation of secondary bile acids, such as deoxycholic acid, lithocholic acid, and tauro-beta-muricholic acid.⁴³ Bile acids have been shown to be important regulators of metabolic signaling via the Farnesoid X receptor (FXR) and the G-coupled protein receptor, TGR5.⁴⁶ Stimulation of FXR improves the glucose and lipid metabolism, leading to increased insulin sensitivity and decreased steatosis in the liver. Activation of TGR5 in the intestine by bile acids leads to secretion of GLP-1, thereby improving glucose tolerance and insulin sensitivity. FXR is activated by bile acids with the following potency: chenodeoxycholic acid > deoxycholic acid > lithocholic acid >> cholic acid, whereas TGR5 is activated as follows: lithocholic acid > deoxycholic acid > chenodeoxycholic acid > cholic acid.⁴⁶ Next to the agonistic effect of bile acids on FXR, Tauro β -muricholate (T β MCA) has recently been reported to be an antagonist of FXR. In mice the microbiota reduces T β MCA levels, leading to increased activation of FXR in the intestine and excretion of FGF15, which is transported to the liver and inhibits bile acids synthesis.⁴⁷ Although the potency of multiple bile acids in stimulating FXR and TGR5 are known, it is not yet fully understood how changes in the composition of the bile acid pool affect the metabolism. It should be noted that bile acid metabolism differs considerably between mice and humans. The human bile acid pool is much more hydrophobic. Hence, data for mice cannot be translated easily to the human situation. Yet, both in humans and mice the microbiota are important for regulating the bile acid composition, and can thereby regulate the metabolism via FXR and TGR5. Which members of the bacterial community are responsible for the many different modifications of bile acids, as well as the effect of the bile acid pool composition on metabolism is not yet well understood and this would be an interesting target for future research.

Conclusion

There is no doubt that the intestinal microbiota plays an important role in host metabolism and that dysbiosis is a strong risk factor in the development of metabolic syndrome and CVD. We are just starting to understand how the host's immune system and dietary intake can influence the microbiota composition and function. Technological advances include lower sequencing costs, which enables the generation of meta-genomic data to identify individual species and specific functions, such as the presence of the CutC gene cluster. The strain-specific information is crucial to gaining a better understanding of the complex interactions between the different members in the microbiota and their cross-talk with the host in relation to metabolic dysfunction. This could lead to the identification of new biomarkers for metabolic and other diseases. There is furthermore an urgent need for meta-genomic data from prospective cohort studies that include multiple layers of information, such as dietary intake and metabolic data (stool, plasma). The causal roles of newly identified pathobionts or the functions of specific members of the microbiota found in mice or

human studies need to be validated in germfree and gnotobiotic mouse models, as well as in human intervention studies with individual bacterial strains. Ultimately, better insight into the immunity-diet-microbiota axis is expected to reveal new targets for intervention and an enormous potential for the prevention of cardiovascular disease.

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Chapter 4

Blood-derived macrophages prone to accumulate
lysosomal lipids trigger oxLDL-dependent murine
hepatic inflammation

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Abstract

Despite the consistent rise of non-alcoholic steatohepatitis (NASH) worldwide, the mechanisms that govern the inflammatory aspect of this disease remain unknown. Previous research showed an association between hepatic inflammation and lysosomal lipid accumulation in blood-derived hepatic macrophages. Additionally, in vitro findings indicated that lipids, specifically derived from the oxidized low-density lipoprotein (oxLDL) particle, are resistant to removal from lysosomes. On this basis, we investigated whether lysosomal lipid accumulation in blood-derived hepatic macrophages is causally linked to hepatic inflammation and assessed to what extent increasing anti-oxLDL IgM autoantibodies can affect this mechanism. By creating a proof-of-concept mouse model, we demonstrate a causal role for lysosomal lipids in blood-derived hepatic macrophages in mediating hepatic inflammation and initiation of fibrosis. Furthermore, our findings show that increasing anti-oxLDL IgM autoantibody levels reduces inflammation. Hence, therapies aimed at improving lipid-induced lysosomal dysfunction and blocking oxLDL-formation deserve further investigation in the context of NASH.

Introduction

Analogous to the steep rise of obesity and diabetes, the prevalence of non-alcoholic fatty liver disease (NAFLD) is currently estimated at 25% to 45% in the general population.^{1,2} NAFLD encompasses a spectrum of liver diseases that are histologically categorized in nonalcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH).³ Whereas hepatic steatosis without hepatic injury is referred to as NAFL, NASH is defined by a conjunction of steatosis and inflammation, which presents with or without fibrosis.³ Though the development of inflammation paves the way for advanced liver diseases, the mechanisms underlying the hepatic inflammatory response are largely unknown. As this lack of mechanistic understanding is a key antecedent for the lack of well-defined effective therapies, it is of utmost importance to improve the knowledge regarding the mechanisms triggering hepatic inflammation.

Previous research from our group indicated an association between hepatic inflammation and lysosomal lipid accumulation inside resident Kupffer cells (KCs) as well as in blood-derived macrophages.⁴⁻⁶ This relation was confirmed by the presence of cholesterol-accumulating KCs in livers of NASH patients, bolstering the notion of lysosomal lipid storage in hepatic macrophages as a potential mechanism for NASH.⁷ Also, it has been shown that incubating macrophages with oxLDL *in vitro* results in the lysosomal accumulation of cholesteryl esters (CEs) and free cholesterol, suggesting a direct role for oxLDL in mediating lysosomal lipid-induced inflammation.^{8,9} Of note, excessive accumulation of lipids in the lysosomal compartment of cells also occurs in the context of Niemann-Pick type C1 (NPC1) disease. Characterized by hepatosplenomegaly, foam cell formation and hepatic inflammation, NPC1 patients exhibit features resembling NASH.¹⁰ Therefore, it becomes evident that the pathology of lysosomal lipid accumulation is not limited to lysosomal lipid storage diseases such as NPC1, but also plays a role in other lipid-associated inflammatory diseases such as NASH.

Previously, it has been demonstrated that oxLDL and the bacterium *Streptococcus pneumoniae* exhibit molecular mimicry for the phosphorylcholine (PC) epitope, a major target for naturally occurring immunoglobulin M (IgM) antibodies. Immunizing mice with heat-killed *Streptococcus pneumoniae* has been shown to increase anti-oxLDL IgM autoantibodies and to reduce atherosclerotic lesion formation¹¹ and hepatic inflammation⁵ in low-density lipoprotein receptor knockout (*Ldlr*^{-/-}) mice. Unlike acetylated LDL (acLDL) and native LDL, injection of oxLDL in hyperlipidemic mice showed increased lysosomal lipid accumulation in hepatic macrophages, corroborating the detrimental effect of oxLDL in promoting inflammation by disturbing the physiology of the lysosome.⁶ Altogether, several studies indicate that accumulation of oxLDL and associated lipids in lysosomes of macrophages is associated with increased

inflammation. However, none of these studies provide specific evidence that lysosomal storage of lipids in hepatic macrophages can be a mechanistic trigger for inflammation in NASH. Moreover, to what extent oxLDL contributes to lysosomal lipid accumulation-induced hepatic inflammation has to our knowledge never been investigated.

Here, we investigated whether lysosomal lipid accumulation in blood-derived hepatic macrophages is a mechanistic trigger for hepatic inflammation and assessed to what extent anti-oxLDL IgM autoantibodies can affect this mechanism. For this purpose, lysosomal lipid accumulation in blood-derived hepatic macrophages was generated by transplanting bone marrow of *Npc1*-mutant (*Npc1^{mut}*) or wildtype (*Npc1^{wt}*) mice into *Ldlr^{-/-}* mice on a high-fat, high-cholesterol (HFC) diet for 12 weeks. To investigate the specific contribution of oxLDL on lysosomal lipid-induced hepatic inflammation, mice were immunized with heat-killed *Streptococcus pneumoniae*.^{5,11} This study describes lysosomal lipid accumulation in blood-derived hepatic macrophages as a novel mechanism that triggers hepatic inflammation. Moreover, our findings suggest a role for oxLDL in mediating lysosomal lipid-induced hepatic inflammation.

Materials and methods

Mice, bone marrow transplantation, immunization, and diet

Niemann-Pick type C1^{nih} mutant (*Npc1^{mut}*) mice (a kind gift from Prof. Dr. Lieberman from University of Michigan Medical School) were backcrossed into a C57BL/6 background for more than 10 generations. *Npc1^{mut}* and *Ldlr^{-/-}* mice were housed under standard conditions and had access to food and water ad libitum. Experiments were performed according to Dutch regulations and approved by the Committee for Animal Welfare of Maastricht University.

To generate myeloid *Npc1^{mut}* deficient *Ldlr^{-/-}* mice, bone marrow transplantations were performed. Twenty-two week-old female *Ldlr^{-/-}* mice received antibiotic water containing neomycin (100 mg/l; Gibco, Breda, the Netherlands) and 6*10⁴ U/l polymycin B sulphate (Gibco, Breda, the Netherlands) one week before and four weeks after irradiation. One day before and on the day of the transplantation, *Ldlr^{-/-}* mice were lethally irradiated with 6 Gray of γ -radiation, thus receiving 12 Gray in total. Lethally irradiated *Ldlr^{-/-}* mice were then injected with 1*10⁷ bone marrow cells donated from either *Npc1^{mut}* mice or wildtype littermate controls (*Npc1^{wt}*). In order to fully ensure bone marrow replacement, mice had a nine week recovery period. After nine weeks of recovery, transplanted (-tp) mice received a high-fed, high-cholesterol (HFC) diet, containing 21% butter and 0.2% cholesterol (diet 1635; Scientific Animal Food and Engineering, Villemoissons-sur-Orge, France) for 12 weeks. Five weeks after bone marrow transplantation, mice were divided into three groups. One group

received the equivalent of 10^8 colony-forming units of heat-killed R36A (unencapsulated *Streptococcus pneumoniae*) emulsified in 200 μ l sterile 0.9% NaCl for the primary subcutaneous immunization. Subsequently, two intraperitoneal booster immunizations were administered every two weeks. The two other control groups received an 0.9% NaCl injection only. From the start of the diet, intraperitoneal booster immunizations were administered every three weeks. An overall overview of the experimental set-up is depicted in Supplementary Figure S4.4.

Liver tissue was isolated and snap-frozen in liquid nitrogen and stored at -80°C or fixed in 4% formaldehyde/PBS. The biochemical determination of plasma cholesterol and liver triglyceride levels, electron microscopy, RNA isolation, complementary DNA synthesis, quantitative polymerase chain reaction and auto-antibody titers against anti-oxLDL IgM antibodies are described extensively.^{4,5,12-15} Liver cholesterol levels were quantified as described previously.¹⁶

Bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDMs) were isolated from the tibiae and femurs of wildtype and *Npc1*^{mut} mice. Cells were cultured in RPMI-1640 (GIBCO Invitrogen, Breda, the Netherlands) with 10% heat-inactivated fetal calf serum (Bodinco B.V. Alkmaar, the Netherlands), penicillin (100 U/ml), streptomycin (100 μ g/ml) and L-glutamine 2 mM (all GIBCO Invitrogen, Breda, the Netherlands), supplemented with 20% L929-conditioned medium (LCM) for 8–9 days to generate BMDMs. After attachment, *Npc1*^{mut} macrophages were seeded at 350,000 cells per well in 24-well plates and incubated for 24h with oxLDL (25 μ g/ml; Alfa Aesar: J65591, Wardhill, MA, USA), with or without anti-oxLDL EO6 antibodies (Avanti Polar Lipids, Alabaster, AL, USA). Wildtype macrophages underwent a similar procedure, but did not receive the EO6 antibodies. Then cells were washed and stimulated with lipopolysaccharide (LPS; 100 ng/ml) for 3h (wildtype cells) or 4h (*Npc1*^{mut} cells). Finally, supernatant was collected for protein measurements and cells were lysed for mRNA expression analysis.

Statistical analysis

Data were statistically analyzed by performing two-tailed non-paired *t*-tests using GraphPad Prism, version 6.0 for Windows. Data were expressed as the mean $\pm$ SEM and considered significant at $p < 0.05$. *, ** and *** indicate $p < 0.05$, 0.01 and 0.001 respectively. Additional explanation is provided in *Supplementary Information*.

Results

Lysosomal lipid accumulation in blood-derived hepatic macrophages results in a severe hepatic pathological phenotype

To ensure successful bone marrow replacement, bone marrow efficiency was assessed. Transplantation of both wildtype and *Npc1^{mut}* bone marrow approximated an efficiency of 90% (Supplementary Table S4.1), proving the bone marrow transplantation successful.

Furthermore, to confirm the successful transplantation at the microscopic level, hepatic tissues were subjected to electron microscopy analysis. As pointed out by the lower magnification, livers from *Npc1^{mut}*-tp mice demonstrated dense clusters of macrophages which resembled granuloma-like structures. These structures were absent in *Npc1^{wt}*-tp mice, indicating that these structures are related to the NPC1 mutation (Figure 4.1A). Also, the majority of resident KCs were located adjacent to the large granulomas, containing various numbers of small lipid inclusions (resembling cholesterol crystals (Supplementary Figure S4.1)). No detectable differences in phenotype between non-immunized and immunized *Npc1^{mut}*-tp mice were observed.

Relevantly, these granuloma structures were also visible at higher microscopic magnification by H&E staining (Supplementary Figure S4.2). Also, relative liver and spleen weights were dramatically increased in mice transplanted with *Npc1^{mut}* bone marrow compared to *Npc1^{wt}*-tp mice, confirming the severe pathological phenotype (Supplementary Figure S4.2). After immunization, both relative liver and spleen weight decreased in immunized *Npc1^{mut}*-tp compared to control-treated *Npc1^{mut}*-tp, suggesting that inhibition of oxLDL uptake by macrophages ameliorates the pathological phenotype.

Increased anti-oxLDL IgM autoantibody titers after heat-killed pneumococci immunization

To determine whether immunization with heat-killed *Streptococcus pneumoniae* was performed successfully, IgM autoantibody levels were measured in the plasma. Immunization with heat-killed pneumococci resulted in an increase of plasma IgM antibodies of the EO6/T15 idiotype (Figure 4.1B), which bind oxLDL by specifically recognizing the phosphorylcholine epitope.¹⁷ In line, increased IgM antibodies against copper-oxidized LDL (Cu-oxLDL) were detected in immunized mice compared to control mice (Figure 4.1C). Thus, immunization with heat-killed pneumococci induced a modest anti-oxLDL IgM autoantibody production in *Npc1^{mut}*-tp mice, confirming successfulness of the immunization.

Figure 4.1 Hepatic phenotype of *Npc1*^{wt-tp} and *Npc1*^{mut-tp} mice and IgM autoantibody titers in plasma. (A) Representative electron microscopy pictures of resident (Kupffer cell) and bone marrow-derived macrophages of *Npc1*^{wt-tp} (scale bar 2 μ m) and control or immunized *Npc1*^{mut-tp} mice (scale bar 10 μ m). Area within the dashed line: *Npc1*^{mut} granuloma; K: Kupffer cell. (B-C) IgM EO6 antibodies (B) and IgM antibodies to copper-oxidized (CuOx)LDL (C) were measured in plasma of mice with or without immunization at a dilution of 1:100. Data are expressed as relative light units (RLU)/100ms. n=10-11 mice/group. Asterisks indicate significant difference from non-immunized *Npc1*^{mut-tp} mice by use of two-tailed unpaired *t* test. *** *p*<0.001. All error bars are SEM.

Disturbances in lipid metabolism are partly restored after elevation of anti-oxLDL IgM autoantibody levels

To determine the effect of lysosomal lipid accumulation in hepatic macrophages on lipid metabolism, we examined cholesterol and triglyceride levels in liver and plasma. Whereas hepatic cholesterol levels were elevated in *Npc1*^{mut-tp} mice compared to *Npc1*^{wt-tp} mice, immunization of *Npc1*^{mut-tp} mice decreased hepatic cholesterol, indicating improved hepatic cholesterol metabolism upon immunization (Figure 4.2A). In contrast, plasma cholesterol levels reduced by almost 50% in *Npc1*^{mut-tp} mice compared to *Npc1*^{wt-tp} mice, but did not differ between immunized and non-immunized *Npc1*^{mut-tp} mice (Figure 4.2B). Detailed investigation of the size of the hepatic granulomas by means of CD68 staining confirmed the presence of granulomas in *Npc1*^{mut-tp} mice, while being absent in *Npc1*^{wt-tp} mice (Figure 4.2C-D). Additionally, immunizing *Npc1*^{mut-tp} mice with heat-killed pneumococci resulted in a strong decrease of granuloma size, suggesting oxLDL as an important compound in disturbing cholesterol metabolism in this model (Figure 4.2C-D).

Furthermore, hepatic triglyceride levels were decreased in *Npc1^{mut}*-tp mice compared to *Npc1^{wt}*-tp mice and remained similar between immunized and control-treated *Npc1^{mut}*-tp mice (Figure 4.2E). Analogous to hepatic triglycerides, similar trends were observed in plasma triglyceride and free fatty acid levels (Figure 4.2F-G).

To further define the influences on hepatic lipid metabolism, hepatic expression of genes involved in lipid homeostasis was examined. Compared to *Npc1^{wt}*-tp mice, *Npc1^{mut}*-tp mice showed increased gene expression levels of Cluster of differentiation 36 (*Cd36*), Scavenger receptor A (*Sr-a*), Niemann-Pick type C2 (*Npc2*) and ATP-binding cassette transporter G1 (*Abcg1*), confirming disturbance of lipid metabolism (Figure 4.2H). Furthermore, though no differences were observed in gene expression levels of the ATP-binding cassette transporter A1 (*Abca1*) (Figure 4.2H), expression levels of all other markers were reduced upon R36A immunization of *Npc1^{mut}*-tp mice, strengthening the importance of oxLDL to the disturbances in lipid metabolism in this model. Altogether, these results imply an important contribution of oxLDL to lysosomal lipid-induced disturbances in lipid metabolism.

OxLDL contributes to lysosomal lipid-induced hepatic inflammation

To determine whether lysosomal lipid accumulation in blood-derived macrophages is a trigger for hepatic inflammation, hepatic cryosections were stained for the inflammatory markers Mac-1 (infiltrated macrophages and neutrophils; against Cd11b) and NIMP (neutrophils). Number of cells expressing both inflammatory markers was increased in *Npc1^{mut}*-tp mice compared to *Npc1^{wt}*-tp mice, supporting our hypothesis that lysosomal lipid accumulation in blood-derived macrophages is a direct trigger for hepatic inflammation (Figure 4.3A-B). Additionally, increasing circulating anti-oxLDL IgM autoantibodies reduced Mac-1- and NIMP-positive cell levels (Figure 4.3A-C). These inflammatory findings were confirmed by performing hepatic gene expression analysis of the inflammatory markers integrin alpha M (*Itgam*), tumor necrosis factor alpha (*Tnfa*), interleukin 12 (*Il12*), CXC chemokine receptor-4 (*Cxcr4*), monocyte chemoattractant protein 1 (*Mcp1*), *Caspase-1* and CC chemokine receptor-2 (*Ccr2*) (Figure 4.3D). Relevantly, while single, just infiltrated macrophages were positive for Mac-1, macrophages present inside the large granulomas of the *Npc1^{mut}*-tp group were Mac-1-negative (Figure 4.3C). This observation revealed that blood-derived hepatic macrophages lose the Cd11b phenotypic marker over time.

Figure 4.2 **Lipid parameters.** (A-B) Hepatic and plasma cholesterol levels of *Npc1*^{wt-tp} and control-treated or immunized *Npc1*^{mut-tp} mice on HFC diet. (C) Quantification of CD68 staining by measuring CD68 positive area. (D) Representative histological pictures of the CD68 staining (200x magnification). (E-F) Hepatic and plasma triglyceride levels. (G) Plasma free fatty acids. (H) Hepatic gene expression analysis of *Cd36*, *Npc2* and *Abcg1*, *Sr-a* and *Abca1*. n=9-11 mice/group. Gene expression data are set relative to *Npc1*^{wt-tp} mice. Asterisks indicate significant difference from non-immunized *Npc1*^{wt-tp} and *Npc1*^{mut-tp} mice by use of two-tailed unpaired *t* test. *, ** and *** indicate *p*<0.05, 0.01 and 0.001 resp. All error bars are SEM. TC, total cholesterol; TTG, total triglycerides.

Figure 4.3 Parameters of hepatic inflammation. (A-B) Liver sections were stained for both infiltrating macrophages and neutrophils (Mac-1) and neutrophils solely (NIMP) and counted. (C) Representative images of the Mac-1 staining (magnification x200) after HFC feeding of Npc1^{wt}-tp and control-treated or immunized Npc1^{mut}-tp mice for 12 weeks. (D) Hepatic gene expression analysis of *Itgam*, *Tnfa*, *Il12*, *Cxcr4*, *Mcp1*, *Caspase-1* and *Ccr2*. Gene expression data were set relative to Npc1^{wt}-tp mice. n=9-11 mice/group. Asterisks indicate significant difference from non-immunized Npc1^{wt}-tp and Npc1^{mut}-tp mice by use of two-tailed unpaired t test. * p<0.05; ** p<0.01; *** p<0.001. All error bars are SEM.

To confirm the pro-inflammatory properties of oxLDL in bone marrow-derived macrophages (BMDMs), wildtype BMDMs were incubated with oxLDL for 24 hours, followed by 3 hour stimulation with lipopolysaccharide (LPS). Gene expression levels of the pro-inflammatory markers *Tnfa* and *Mcp1* were increased upon oxLDL incubation, confirming the pro-inflammatory effect of oxLDL in BMDMs (Supplementary Figure S4.3). Next, to explore the specific contribution of anti-oxLDL IgM autoantibodies to lysosomal lipid-induced inflammation in blood-derived macrophages, we isolated Npc1^{mut} BMDMs and stimulated these with oxLDL, in the absence or presence of the anti-oxLDL antibody EO6. In the presence of EO6 antibodies, Npc1^{mut} BMDMs stimulated with oxLDL demonstrated reduced inflammation, as indicated by reduced TNFα protein levels and reduced *Tnfa* and *Ccr2* gene expression (Figure 4.4).

Combining the *in vivo* and *in vitro* data, these results reveal for the first time that lysosomal lipid accumulation in blood-derived macrophages is a mechanistic trigger for lipid-induced hepatic inflammation. On top, these data identify an essential role for oxLDL in mediating these inflammatory effects.

Figure 4.4 **EO6-treatment reduced oxLDL-induced inflammation in *Npc1^{mut}* BMDMs.** TNFα protein levels (A) and gene expression of inflammatory-related genes *Tnfa* and *Ccr2* (B) after oxLDL loading of *Npc1^{mut}* BMDMs in absence or presence of anti-oxLDL EO6 antibodies. All data represent n=3 (triplicates) for each experimental group. Asterisks indicate significant difference from non-immunized *Npc1^{wt}-tp* and *Npc1^{mut}-tp* mice by use of two-tailed unpaired *t* test. * $p < 0.05$; *** $p < 0.001$. All error bars are SEM.

Increasing anti-oxLDL IgM autoantibodies attenuates initiation of hepatic fibrosis in mice carrying lysosomal lipid storing hepatic macrophages

As hepatic fibrosis is a key symptom of active NASH,¹⁸ we investigated whether the inflammatory trigger of lysosomal lipid accumulation in blood-derived hepatic macrophages can initiate hepatic fibrosis. Hepatic collagen levels, characterized by Sirius Red staining, were elevated in *Npc1^{mut}-tp* mice compared to *Npc1^{wt}-tp* mice (Figure 4.5A-B), as indicated by the increased collagen formation surrounding the granulomas (Figure 4.5B). R36A immunization of *Npc1^{mut}-tp* mice showed a trend towards a decrease in the level of fibrosis (Figure 4.5A). Furthermore, these histological findings were confirmed by gene expression levels of the fibrotic markers, transforming growth factor beta (*Tgf-β*) and tissue inhibitor of metalloproteinase-3 (*Timp3*) (Figure 4.5C). Altogether, these data suggest that oxLDL can initiate hepatic fibrosis by contributing to lysosomal lipid accumulation in blood-derived hepatic macrophages.

Discussion

Currently, the mechanisms underlying NASH are poorly understood, restricting the development of well-defined, effective therapies. Previously, an association was demonstrated between murine hepatic inflammation and lysosomal lipid accumulation in hepatic macrophages⁴⁻⁶. Here, we prove that lysosomal lipid accumulation in blood-derived hepatic macrophages is a direct trigger for hepatic inflammation and initiates fibrosis. Moreover, our results show that increasing anti-oxLDL IgM autoantibody levels improves inflammation and lipid metabolism, suggesting that oxLDL plays a key role in mediating hepatic inflammation by promoting lipid accumulation in lysosomes of

blood-derived hepatic macrophages. Therefore, this study provides additional insights into the inflammatory mechanisms driving NASH. Therapies aimed at improving lipid-induced lysosomal dysfunction and blocking the formation of oxLDL should therefore be investigated in the future.

Figure 4.5 Parameters of hepatic fibrosis. (A) Quantification of Sirius Red (collagen) staining. (B) Representative pictures of Sirius Red staining (original magnification, 100x) of *Npc1^{wt}-tp* mice and *Npc1^{mut}-tp* mice with or without immunization on an HFC diet for 3 months. (C) Gene expression analysis of the fibrosis markers, *Tgf-β* and *Timp3*. n=9-11 mice/group. Gene expression data are shown relative to *Npc1^{wt}-tp* mice. Asterisks indicate significant difference from non-immunized *Npc1^{wt}-tp* and *Npc1^{mut}-tp* mice by use of two-tailed unpaired t test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. All error bars are SEM.

Whereas initially viewed as the cell's degradation center, it has become increasingly clear that the lysosome constitutes a central role in regulating a plethora of physiological processes.¹⁹ Specifically, upon accumulation of macromolecules (including lipids), lysosomes gradually lose their proteolytic and regulatory function, leading to disturbances in processes related to apoptosis, autophagy, calcium homeostasis, protein folding and, above all, metabolism and inflammation.²⁰ Indeed, severely increased inflammation was observed in NPC1²¹ and Wolman²² disease patients, both lysosomal lipid storage disorders (LSDs), thereby linking lipid storage to inflammation. Likewise, previous studies by us and others have demonstrated an association between hepatic inflammation and lysosomal lipid accumulation.^{5,6,23} Building on this previous knowledge, we here demonstrate for the first time that lysosomal lipid storage in

blood-derived hepatic macrophages can be viewed as an actual trigger for hepatic inflammation and initiates fibrosis, two central features of NASH. Our data also imply that oxLDL contributes to this hepatic inflammatory mechanism. As such, we propose that lysosomal accumulation of oxLDL-derived lipids is a trigger for NASH.

The observation that elevations in anti-oxLDL IgM autoantibody levels could partly protect from the inflammatory phenotype observed in *Npc1^{mut}-tp Ldlr^{-/-}* mice suggests that an important fraction of lysosomal lipids in blood-derived hepatic macrophages originates from oxLDL particles. In line, while circulating levels of oxLDL were reported to represent 0.001% of native LDL in healthy individuals,²⁴ oxLDL levels were shown to increase up to 1.8% in patients with the metabolic syndrome.²⁵ Relevantly, 7 β -hydroxycholesterol and 7-ketocholesterol, the main cholesterol oxidation products present inside oxLDL particles,^{26,27} were previously linked to inflammatory processes, suggesting that cholesterol oxidation products contained in the oxLDL particle are responsible for eliciting inflammation.^{28,29} Also, oxLDL-induced formation of cholesterol crystals was demonstrated to induce lysosomal membrane permeabilization.³⁰⁻³² Lysosomal membrane permeabilization and the subsequent release of lysosomal enzymes into the cytoplasm is a known prerequisite for activation of the inflammasome,³³ increased apoptosis³⁴ and of necrotic cell death,³⁵ three pathways leading to increased hepatic inflammation. In line with these observations, blood-derived hepatic *Npc1^{mut}* macrophages exhibited ruptured membrane structures, suggesting that cholesterol oxidation products induced lysosomal permeabilization in these macrophages resulting in hepatic inflammation. However, more evidence is needed to prove the actual contribution of cholesterol oxidation products to lysosomal permeabilization. Furthermore, to what extent oxLDL levels in our mouse model are comparable to oxLDL levels in NASH patients is not completely clear. Nevertheless, as both NASH patients³⁶ and *Npc1^{-/-}-tp Ldlr^{-/-}* mice (described by Zhang *et al.*³⁷) show increased levels of plasma oxidized lipids, our mouse model can be considered a proper model to investigate the contribution of oxidized lipids to the human NASH situation. Oxidation of lipids were also shown to occur within lysosomes.³⁸ Therefore, it is likely that the immunized *Npc1^{mut}-tp* group still contains internally oxidized lipid products within lysosomes, which contribute to the hepatic inflammatory response.

Besides cholesterol oxidation products, other components present inside or in the surface monolayer of oxLDL particles have also been associated with inflammatory responses. For example, oxidized phospholipids and their highly reactive degradation product malondialdehyde, two lipid products identified on the surface of the oxLDL particle,^{39,40} have been identified in NASH patients.^{36,41,42} Furthermore, other lipids such as free cholesterol, cholesteryl esters, proteins and their oxidized derivatives have been identified in oxLDL.²⁹ Additionally, while next to oxidized lipids also other non-oxidized cholesterol products contribute to the observed pathology, the finding that

targeting specifically oxidized LDL leads to significant improvements in liver pathology provides evidence for an essential role for oxLDL in mediating lysosomal lipid-induced hepatic inflammation. Therefore, future research should aim to identify and provide detailed knowledge of the lipids specifically contained in oxLDL particles as this might lead to the identification of novel therapeutic targets for NASH.

Of note, besides oxLDL, also apoptotic cells expose the PC-epitope which is recognized by IgM autoantibodies described in this manuscript.⁴³ As increased apoptosis has often been associated with increased levels of hepatic inflammation,^{44,45} it is possible that elevations in IgM autoantibody levels reduced hepatic inflammation in our model via increased clearance of apoptotic cells, rather than preventing the uptake of oxLDL by hepatic macrophages.

The current study also describes that blood-derived hepatic macrophages, which exhibited lysosomal lipid accumulation due to a NPC1 mutation, lose the CD11b phenotypic marker (indicative for hematopoietic origin⁴⁶) and gain the CD68 phenotypic marker (indicative for resident KCs⁴⁶) after infiltration in the liver, suggesting that blood-derived macrophages have phenotypically switched into resident KCs. Additionally, embryonically-derived resident KCs displayed intracellular cholesterol crystal formation and engulfed the granulomas (which appeared to be the result of a clustering of blood-derived hepatic macrophages carrying the NPC1 mutation). Therefore, the inability to eliminate the inflammatory stimulus (which is derived from the granulomas created by blood-derived hepatic macrophages) in the liver is likely due to a combination of dysfunctional blood-derived hepatic macrophages as well as the lack of a functional repertoire of resident embryonically-derived KCs to overcome this insult. Indeed, under inflammatory conditions, blood-derived tissue-resident macrophages were shown to dominate the inflammatory response in tissues.⁴⁷ Therefore, the findings of the current study indicate that functional blood-derived hepatic macrophages are essential to overcome lipid challenges in the liver and suggest that improving the lysosomal function in macrophages (from hematopoietic or embryonic origin) can be beneficial for NASH.

Although a bone marrow-specific NPC1 mutation was used in this study, the phenomenon of lysosomal lipid accumulation in hepatic macrophages in NASH patients is not necessarily the result of a deficiency of the *Npc1* gene, but rather a consequence of a prolonged exposure of lipids to the liver. Furthermore, patients suffering from NASH traditionally show increased hepatic levels of cholesterol and triglycerides², while our model only showed elevations in hepatic cholesterol levels. Therefore, the mouse model described in this study should be considered a proof-of-concept mouse model for the involvement of lysosomal lipid storage in blood-derived hepatic

macrophages to hepatic inflammation and fibrosis rather than a mouse model exactly mimicking the human situation of NASH.

Conclusion

In conclusion, we demonstrate here for the first time that lipid accumulation in lysosomes of blood-derived hepatic macrophages is a key trigger of hepatic inflammation and mediates initiation of fibrosis. Furthermore, increasing anti-oxLDL IgM autoantibody levels ameliorated the pathological phenotype, suggesting a key role for oxLDL in this process. Hence, therapies aimed at improving lipid-induced lysosomal dysfunction and blocking the formation of oxLDL should be further investigated in the context of NASH and might be of relevance for other metabolic inflammatory disorders.

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Supplemental material

Figure S4.1 Hepatic phenotype of *Npc1^{mut}-tp* mice. The formation of cholesterol crystals was observed in Kupffer cells (indicated by the arrows) that engulfed the granulo-ma-like structures in *Npc1^{mut}-tp* mice.

Figure S4.2 Hepatosplenic pathological phenotype in *Npc1^{mut}-tp Ldlr^{-/-}* mice. (A) General histology of the liver by an HE staining of *Npc1^{wt}-tp* mice and non-immunized and immunized *Npc1^{mut}-tp* mice. Arrows indicate macrophage granulomas. (B) Relative liver and spleen weights. n=9-11 mice/group. Asterisks indicate significant difference from non-immunized *Npc1^{wt}-tp* and *Npc1^{mut}-tp* mice by use of two-tailed unpaired t test. * $p < 0.05$; *** $p < 0.001$. All error bars are SEM. R36A, unencapsulated *Streptococcus pneumoniae*.

Figure S4.3 Pro-inflammatory properties of oxLDL in BMDMs. Gene expression levels of the pro-inflammatory markers *Tnfα* and *Mcp1* after oxLDL loading of wildtype BMDMs. Data are the result of three independent experiments. Asterisks indicate significant difference from control-treated BMDMs by two-tailed unpaired t test. * $p < 0.05$; ** $p < 0.01$. All error bars are SEM.

Figure S4.4 Schematic overview of experimental set-up.

Table S4.1 Bone marrow efficiency of *Npc1*^{wt}- and *Npc1*^{mut}-tp *Ldlr*^{-/-} mice.

Genotype	% LDLR
<i>Npc1</i> ^{wt} -tp <i>Ldlr</i> ^{-/-} mice	90.47 ± 6.96
<i>Npc1</i> ^{mut} -tp <i>Ldlr</i> ^{-/-} mice	87.96 ± 2.21

Supplementary Materials and Methods

Bone marrow efficiency

In order to determine the chimerism in the transplanted mice, we used donor bone marrow that has an *Ldlr*^{WT} origin, whereas recipient bone has an *Ldlr*^{-/-} origin. Genomic DNA was isolated using the PureLink Genomic DNA (K182002; ThermoFisher Scientific).

A standard curve was generated by mixing DNA from *Ldlr*^{-/-} and *Ldlr*^{WT} bone marrow cells at different ratios. Chimerism was determined by quantifying the amount of *Ldlr*^{-/-} DNA in samples from 70 µL peripheral blood. To standardize for the amount of input DNA, the non-relevant p50 gene was quantified. Samples were assayed in duplicate on a 7900HT real-time PCR system by using 25 ng DNA, SensiMixTM Sybr & Fluorescein kit (QT615-05, Bioline), according to the manufacturer's instructions.

Ldlr^{-/-}-specific primers are forward 5'- GCTGCAACTCATCCATATGCA-3' and reverse 5'- GGAGTTGTTGACCTCGACTCTAGAG-3'. Forward and reverse p50-specific primers are 5'- ACCTGGGAATACTTCATGTGACTAA-3' and 5'-ACACCAGAAGTCCAGGATTATCAG-3', respectively. A standard curve was generated by plotting the mean threshold cycle (Ct) ΔCt (Ct p50 – Ct *Ldlr*^{-/-}) against the logarithm of the percentage *Ldlr*^{-/-} and calculation of a regression line. Chimerism was calculated from the percentage of *Ldlr*^{-/-} DNA in the blood samples (representing the remaining recipient bone marrow), determined by applying the mean ΔCt of the sample to the standard curve. The average efficiency of the bone marrow transplantation was approximately 90% (Table S4.1).

Preparation immunogen

For immunization, the heat-inactivated R36A strain of *Streptococcus pneumoniae* (Birmingham, Alabama) was used, still bearing the PC headgroup epitope similar to oxLDL. Colonies of the R36A strain were harvested at mid log phase after incubation at 37°C on blood agar plates and transferred to Todd-Hewitt plus 0.5% yeast broth. The mid log phase is characterized by an OD value of 0.425 to 0.45 at 600 nm. *S. pneumoniae* was heat-inactivated at 60°C for 30 minutes; afterwards no colonies of this suspension were detected on blood agar plates. For freezer stocks of strain R36A, small aliquots of *S. pneumoniae* at mid log density were harvested and suspended in Todd-Hewitt plus 80% sterile glycerol and stored at -80°C.

Liver histology

Frozen liver sections (7 µm) were fixed in acetone and subsequently blocked for endogenous peroxidase by incubation with 0.25% of 0.03% H₂O₂ for 5 minutes. Primary antibodies used were against infiltrated macrophages and neutrophils (rat-anti-mouse Mac-1 [M1/70]), neutrophils (rat-anti-mouse Ly6-C, clone NIMP-R14) (generous gift from Prof Heeringa, Groningen, The Netherlands) and Kupffer cells (KCs) (rat-anti-mouse CD68, clone FA11) (generous gift from Prof Gordon, Oxford, UK) was applied as color substrate and hematoxylin (4085.9002, Klinipath, Duiven, The Netherlands) for nuclear counterstaining. Sections were enclosed with Faramount aqueous mounting medium (S302580; DAKO, Glostrup, Denmark).

Paraffin-embedded liver sections (4 µm) were stained with Hematoxylin-Eosin (HE; Hematoxylin, 4085.9002; Klinipath, Duiven, The Netherlands; and Eosin, E4382; Sigma-

Aldrich) and Sirius red (Direct Red 80, 43665; Sigma-Aldrich). Pictures were taken with a Nikon digital camera DMX1200 and ACT-1 v2.63 software (Nikon Instruments Europe, Amstelveen, The Netherlands).

The CD68 positive area was quantified in 3 microscopical views of the liver by using Adobe Photoshop v. 9.0 in a blinded manner. The number of Mac1- and NIMP1-positive immune cells was counted in 6 microscopical views of each liver section (original magnification, 200×) in a blinded manner and was noted as cells/square millimeter. n=6-10 mice/group.

The Sirius Red positive area was quantified in 6 microscopical views of the liver by using Adobe Photoshop v. 9.0 in a blinded manner.

Mouse tumor necrosis factor enzyme-linked immunosorbent assay (ELISA)

The mouse tumor necrosis factor ELISA assay was performed on diluted supernatant from stimulated BMM (1:10) according to the manufacturer's instructions (88-7324-88; eBioscience, San Diego, CA, USA).

Chapter 5

Plasma cathepsin D levels: a novel tool to predict pediatric hepatic inflammation

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Abstract

Background and aims

Non-alcoholic steatohepatitis (NASH) is the most severe form of a hepatic condition known as non-alcoholic fatty liver disease (NAFLD). NASH is histologically characterized by hepatic fat accumulation, inflammation and ballooning, and eventually coupled with fibrosis, which in turn may progress to end-stage liver disease even in young individuals. Hence, there is a critical need for specific non-invasive markers to predict hepatic inflammation at an early age. We investigated if plasma levels of cathepsin D (CatD), a lysosomal protease, correlated with severity of liver inflammation in pediatric NAFLD.

Methods

Liver biopsies from children (n=96) with NAFLD were histologically evaluated according to the criteria of Kleiner (NAFLD activity score) and the Brunt's criteria. At the time of the liver biopsy, blood was taken and levels of CatD, alanine aminotransferase (ALT) and cytokeratin-18 (CK-18) were measured in plasma.

Results

Plasma CatD levels were significantly lower in subjects with liver inflammation compared to steatotic subjects. Furthermore, we found that CatD levels were gradually reduced and corresponded with increasing severity of liver inflammation, steatosis, hepatocellular ballooning and NAFLD activity score. CatD levels correlated with pediatric NAFLD disease progression better than ALT and CK-18. In particular, CatD showed a high diagnostic accuracy (ROC-AUC: 0.94) for the differentiation between steatosis and hepatic inflammation, and reached almost the maximum accuracy (ROC-AUC: 0.998) upon the addition of CK-18.

Conclusions

Plasma CatD holds a high diagnostic value to distinguish pediatric patients with hepatic inflammation from children with steatosis.

Introduction

The current obesity epidemic in children is paralleled by an increasing prevalence of non-alcoholic fatty liver disease (NAFLD), a condition characterized by early hepatic steatosis and by a more severe form known as non-alcoholic steatohepatitis (NASH). Key histologic components of NASH are steatosis, inflammation and hepatocellular ballooning.¹ Children with biopsy-proven NAFLD were followed for 20 years and demonstrated a shorter long-term survival. Some children evolved towards advanced fibrosis, others to cirrhosis and a 20-year old girl needed a liver transplantation due to end-stage liver disease, hereby showing the progressive nature of NAFLD even in young individuals.² Therefore, as NASH progresses to these more severe chronic liver conditions, its early detection is crucial to design adequate patient management and to reduce end-stage liver disease and overall mortality. Unfortunately, NASH cannot be identified without pursuing a liver biopsy, the reference standard for accurate staging and grading of NAFLD. Due to the invasiveness of this method, the high costs, the level of discomfort, sampling error and its risk for complications such as hemorrhage and bile peritonitis,³ liver biopsies, especially at a young age, should be replaced with non-invasive markers to detect NASH.

In addition to plasma alanine aminotransferase (ALT), a number of functionally diverse biomolecules could be used as potential markers for pediatric NASH, including several proteins that change their expression and stability during the development of steatosis, inflammation and ballooning.⁴ An extensively studied molecule is the caspase-cleaved cytokeratin-18 (CK-18), that, in addition to its ability to detect hepatocellular apoptosis, is also able to distinguish NASH from simple steatosis both in adults and children.^{5,6}

Previously, we demonstrated a clear and direct association between hepatic inflammation and lysosomal cholesterol accumulation inside Kupffer cells (KCs) of low-density lipoprotein receptor knockout (*Ldlr*^{-/-}) mice fed a high-fat, high-cholesterol diet.⁷⁻⁹ In line with our observation in mice, cholesterol-containing KCs were also demonstrated recently in livers of NASH patients.¹⁰ Further studies have shown that lysosomal cholesterol accumulation induces disturbances in lysosomal (enzyme) trafficking.^{11,12} In particular, cathepsins, the main class of lysosomal proteases, have been described to play an early role in inflammation.¹³ In line, recent evidence showed a disruption of hepatic cathepsin expression in NAFLD patients.¹⁴

We hypothesized that cathepsin D levels in plasma may be altered in subjects with NASH compared to subjects without NASH (i.e. steatosis). Therefore, the aim of the present study was to assess plasma levels of cathepsin D in children with different stages of NAFLD and to correlate these to histological criteria used for the diagnosis of NASH. Additionally, receiver operating characteristic (ROC) curves were plotted to test the diagnostic accuracy of cathepsin D.

Materials and methods

Sample collection for the present study was performed at the Hepato-Metabolic Department of Bambino Gesù Children's Hospital during the period September 2012–December 2013. The study design was conformed to the ethical guidelines of the Declaration of Helsinki (1975) and was performed according to the recommendations of the Ethics Committee of the Hospital.

We included consecutive children aged 3 to 12 years with an ultrasonographic diagnosis of NAFLD and persistently (≥ 6 months) elevated serum aminotransferases. The decision to biopsy NAFLD children was based on our previously published data, demonstrating a high prevalence of necro-inflammatory changes and fibrosis in children with ultrasonographic steatosis and persistently elevated liver enzymes.¹⁵ Blood samples were obtained just before the ultrasound-guided liver biopsy. All blood samples were originally processed to yield plasma and stored at -80°C . Exclusion criteria were hepatic virus infections (hepatitis A, B, C, cytomegalovirus; and Epstein–Barr virus), type I diabetes mellitus, excessive alcohol consumption (≥ 140 g/week), history of parenteral nutrition, and use of drugs known to induce steatosis (eg, valproate, amiodarone, or prednisone) or to affect body weight and carbohydrate metabolism. Autoimmune liver disease, metabolic liver disease, Wilson's disease, celiac disease, type II diabetes mellitus and alpha-1-antitrypsin deficiency were ruled out using standard clinical, laboratory, and histological criteria.^{16,17}

Liver histology

The clinical indication for liver biopsy was either to assess and better define the presence of NASH and the degree of fibrosis or to exclude other likely liver diseases.¹⁶ A Sonoline Omnia ultrasound machine (Siemens, Munich, Germany) equipped with a 5-MHz probe (5.0 C 50, Siemens) and a biopsy adaptor was employed.

The liver specimens of ≥ 15 mm length including at least 5–6 complete portal tracts were considered adequate for the purpose of the study. Biopsies were routinely processed (i.e. formalin-fixed and paraffin-embedded) and sections of liver tissue were stained with hematoxylin-eosin, Van Gieson, Periodic acid-Schiff diastase, and Prussian blue stain. Biopsies were evaluated by a single expert hepato-pathologist, who was unaware of the patient's clinical and laboratory data. To determine the intra-observer agreement, the pathologist scored the liver biopsies blindly twice and the weighted κ coefficients for different histological features were calculated.

The main histological features of NAFLD/NASH, including steatosis, inflammation, hepatocellular ballooning and fibrosis were scored using the NAFLD Clinical Research Network (CRN) criteria.¹⁸ Briefly, steatosis was graded on a 4-point scale: grade 0=steatosis involving $<5\%$ of hepatocytes; grade 1=steatosis involving up to 33% of hepatocytes; grade 2=steatosis involving 34–65% of hepatocytes; and grade

3=steatosis involving $\geq 66\%$ of hepatocytes. Lobular inflammation was graded on a 4-point scale: grade 0=no foci; grade 1= ≤ 2 foci per 200 $\times$ field; grade 2=2–4 foci per 200 $\times$ field; and grade 3= ≥ 4 foci per 200 $\times$ field. Hepatocellular ballooning was graded from 0 to 2: 0=none; 1=few balloon cells; and 2=many/prominent balloon cells. The stage of fibrosis was quantified using a 5-point scale: stage 0=no fibrosis; stage 1=perisinusoidal or periportal (1a=mild, zone 3, perisinusoidal; 1b=moderate, zone 3, perisinusoidal; 1c=portal/periportal); stage 2=perisinusoidal and portal/periportal; stage 3=bridging; and stage 4=cirrhosis. Features of steatosis, lobular inflammation, and hepatocellular ballooning were combined to obtain the NAFLD activity score (NAS score). The NAS score ranged from 1 to 7. As recently recommended by NASH CRN¹⁹, a microscopic diagnosis based on overall injury pattern (steatosis, hepatocellular ballooning, inflammation) as well as the presence of additional lesions (e.g. zonality of lesions, portal inflammation and fibrosis) has been assigned to each case. Accordingly, biopsies were subdivided into: steatosis, borderline NASH and definite NASH.¹⁹

Laboratory assessment

At the time of the liver biopsy, blood was taken for further analysis. In all patients, enrolled in the present study, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), triglycerides, total cholesterol, albumin levels, glucose tolerance and prothrombin time (international normalized ratio, INR) were evaluated using standard laboratory methods. Insulin resistance was calculated according to the Homeostatic Model Assessment-Insulin Resistance (HOMA-IR) derived from basal values of glucose and insulin as previously described.²⁰

Human cathepsin D enzyme-linked immunosorbent assay

Plasma samples were diluted and cathepsin D levels were determined by the cathepsin D enzyme-linked immunosorbent assay according to the manufacturers' protocol (Uscn Life Science Inc, Wuhan, China). The absorbance was measured on a Benchmark 550 microplate reader (Bio-rad). The detection limit ranges approximately from 46.88 to 3,000 pg/ml. Coefficients of variation (CV%) for intra- and inter assays are <10 and $<12\%$ respectively. Cathepsin D measurements were performed blinded to the histology findings of the study participants.

CK-18 level measurements

Part of plasma samples were used for quantitative determination of CK-18 levels by the M30-Apoptosense ELISA kit (PEVIVA) purchased from Li Starfish (Milan, Italy). All assays were performed in duplicates, and the absorbance was determined using a microplate reader (Molecular Bio-Rad, Milan, Italy).

Statistical analysis

The data were analysed by performing one-way analysis of variance (ANOVA) and posthoc Tukey's test. The data were expressed as mean $\pm$ SEM and considered significant at $p < 0.05$. In order to evaluate the performance of plasma cathepsin D for distinguishing steatosis vs. NASH patients, borderline NASH vs. NASH and, steatosis and borderline NASH vs. NASH, the sensitivity, specificity, predictive values and area under curve (AUC) were calculated using receiver operating characteristic (ROC) curve analysis. A statistical significance between two AUCs was evaluated by computing the Z-score, followed by calculating two-tailed p-values. $P < 0.05$ was considered significant. The analysis and the graphs were performed using GraphPad Prism 5 (version 5.03).

To assess the diagnostic accuracy of the combination CK-18 with cathepsin D, a logistic regression was fitted to steatosis, borderline NASH and NASH patients for both CK-18 and cathepsin D. Statistical ROC analyses and AUC presented for the combination CK-18 with cathepsin D were performed using the freely available program R²¹ and the publicly available libraries 'glm'²² and 'DiagnosisMed'.

Results

General characteristics of the pediatric population

Ninety-six children (mean age: 8.9 years, ranging from 3.3-12.1 years) with NAFLD were enrolled in the study. Of those ninety-six children, 56 were boys and 40 girls with a mean age of 9.3 and 8.4 years respectively. NASH was diagnosed in 27% (26 subjects) of the study population, while 51 subjects (53%) were diagnosed with borderline NASH and 19 subjects (20%) with steatosis (Table 5.1). All children were obese (BMI $> 95^{\text{th}}$ percentile) and no difference was observed in BMI percentile and waist circumference (WC) percentile between children with steatosis, borderline NASH or definite NASH (Table 5.2). No differences were found in insulin resistance, glucose tolerance, the lipid profile and coagulation profile (HOMA-IR, impaired glucose tolerance (IGT), cholesterol, triglycerides, albumin, and INR). Liver-specific parameters including ALT, AST and GGT were similar between all the groups. In response to NAFLD severity, a gradual significant decrease was detected for plasma cathepsin D, a lysosomal protease (steatosis vs. borderline NASH: $p = 0.0017$; steatosis vs. NASH: $p < 0.0001$; borderline NASH vs. NASH: $p < 0.0001$). In contrast, CK-18 increased in response to NAFLD severity and could differentiate steatosis from borderline NASH ($p = 0.0001$) and NASH ($p = 0.0003$). No difference in CK-18 levels was observed between borderline NASH and definite NASH ($p = 0.95$).

Table 5.1 Histological features of the pediatric subjects.

	Steatosis (n=19)	Borderline NASH (n=51)	NASH (n=26)
NAS	2.2 ± 0.3	3.9 ± 0.2	5.7 ± 0.1
Steatosis (%)			
5-33%	15 (78.9)	10 (19.6)	2 (7.7)
34-65%	3 (15.8)	5 (9.8)	5 (19.2)
≥66%	1 (5.3)	36 (70.6)	19 (73.1)
Inflammation (%)			
Grade 0	5 (26.3)	1 (2.0)	0
Grade 1	13 (68.4)	33 (64.7)	12 (46.2)
Grade 2	1 (5.3)	17 (33.3)	14 (53.8)
Ballooning (%)			
None	17 (89.5)	22 (43.1)	3 (11.5)
Few	1 (5.3)	24 (47.1)	15 (57.7)
Many	1 (5.3)	5 (9.8)	8 (30.8)
Fibrosis (%)			
Stage 0	10 (52.6)	19 (37.3)	7 (26.9)
Stage 1	8 (42.1)	26 (51.0)	16 (61.5)
Stage 2	1 (5.3)	5 (9.8)	0
Stage 3	0	1 (2.0)	3 (11.5)

NAS: NAFLD activity score. NAS is represented as mean±SEM.

Table 5.2 Clinical characteristics of the pediatric population.

	Steatosis(1) (n=19)	Borderline NASH(2) (n=51)	NASH(3) (n=26)	p-value between groups		
				1 vs. 2	1 vs. 3	2 vs. 3
Male (%)	12 (63.2)	29 (56.9)	15 (57.7)	0.886	0.930	0.997
Age (years)	10.0 ± 0.5	8.4 ± 0.3	9.2 ± 0.4	0.016	0.378	0.314
Weight (kg)	52.0 ± 2.6	45.2 ± 2.2	44.3 ± 2.0	0.155	0.149	0.958
Length (cm)	139.9 ± 2.8	132.4 ± 2.4	135.0 ± 2.7	0.158	0.531	0.753
BMI percentile	96.0 ± 0.8	96.0 ± 0.7	96.1 ± 0.8	1.000	0.995	0.996
WC percentile	83.6 ± 2.4	77.9 ± 1.4	82.9 ± 1.7	0.079	0.973	0.084
Obese (%)	19 (100)	47 (92.2)	24 (92.3)	0.458	0.551	1.000
Cholesterol (mg/dl)	167.3 ± 8.6	163.3 ± 5.1	153.3 ± 7.6	0.915	0.427	0.506
Triglycerides (mg/dl)	94.0 ± 8.4	111.1 ± 8.2	125.9 ± 21.9	0.664	0.328	0.685
ALT(U/l)	61.6 ± 7.4	83.7 ± 5.6	91.0 ± 15.3	0.258	0.152	0.830
AST (U/l)	46.7 ± 3.5	55.3 ± 2.6	57.4 ± 7.2	0.387	0.311	0.929
GGT (U/l)	26.0 ± 2.5	29.1 ± 2.7	30.0 ± 4.4	0.814	0.769	0.981
Cathepsin D (pg/ml)	32658 ± 3640	21428 ± 1753	8095 ± 784	0.002	<0.0001	<0.0001
CK-18 (U/l)	261.6 ± 24.5	350.6 ± 11.2	356.4 ± 6.6	0.0001	0.0003	0.950
Albumin (g/dl)	4.5 ± 0.1	4.5 ± 0.1	4.6 ± 0.1	0.866	0.668	0.869
INR	1.1 ± 0.05	1.2 ± 0.03	1.1 ± 0.04	0.838	0.997	0.853
IGT(%) /Prediabetes	0 (0)	9 (17.6)	4 (15.4)	0.137	0.297	0.959
HOMA-IR	2.7 ± 0.4	2.5 ± 0.3	2.4 ± 0.3	0.973	0.922	0.970

Data are represented as mean±SEM. Data was statistically analyzed by performing one-way ANOVA tests using SPSS software, version 22.0 (SPSS, Chicago, IL, USA). BMI: body mass index; WC: waist circumference; INR: international normalized ratio; CK-18: cytokeratin-18; IGT: impaired glucose tolerance; HOMA-IR: homeostasis model assessment of insulin resistance.

Significantly reduced levels of plasma cathepsin D correlate with pediatric NAFLD disease severity

First, we investigated whether plasma cathepsin D levels correlated with histological features of NAFLD. We found that plasma levels of cathepsin D decreased significantly in parallel with the increasing degree of NAFLD and the NAS score (Figure 5.1). Furthermore, in line with the increased presence of hepatocellular ballooning, a key histological feature of NASH, plasma cathepsin D declined. By examining the individual stages of NAFLD, we demonstrate that, unlike late-stage fibrosis, cathepsin D was reduced upon increasing degrees of steatosis and inflammation. Thus, plasma cathepsin D was significantly lowered in children with NASH compared to children with either steatosis or borderline NASH and was associated with early stages of NAFLD (i.e. steatosis and inflammation).

Figure 5.1 Cathepsin D in plasma of pediatric non-alcoholic fatty liver disease (NAFLD) subjects. Cathepsin D levels were analyzed in children with NAFLD divided by biopsy-proven steatosis, borderline non-alcoholic steatohepatitis (NASH), and NASH (light-grey bars) or divided upon the NAS score (dark-grey bars). The colored bars show plasma cathepsin D for the individual stages of NAFLD. Classification *Mild* indicates a stage 0 for hepatocellular ballooning, stage 1 for steatosis, and stage 0 for inflammation and fibrosis. Classification *Moderate* indicates stage 1 for hepatocellular ballooning, stage 2 for steatosis, and stage 1 for inflammation and fibrosis. Classification *Severe* indicates stage 2 for hepatocellular ballooning, stage 3 for steatosis, stage 2 for inflammation, and stage 2+3 for fibrosis. Asterisks denote significance compared with the corresponding first (left) bar for each classification. Each classification is indicated by the light-grey, the dark-grey, and the colored bars. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. The hashtag (#) notation represents significance compared with the corresponding second (middle bar) for each classification. # $p < 0.001$. NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis.

Plasma ALT does not correlate with the NAFLD histological pattern in children

Currently, plasma ALT is primarily used in the clinic as a non-invasive marker to detect NASH. In order to make a comparison between cathepsin D and ALT, plasma ALT levels were also measured. Opposite to cathepsin D, a trend towards increasing ALT levels in parallel with increasing NAFLD severity was found (Figure 5.2). However, this difference was not significant, neither for Brunt's or Kleiner's criteria. Unlike cathepsin D, ALT levels remained similar upon severe degrees of hepatocellular ballooning and steatosis. ALT levels rose with increasing severity of inflammation, but did not reach a statistically significant level. During fibrosis, a trend towards an increase in ALT levels was found between patients who displayed no fibrosis and those who demonstrate mild or severe fibrosis. Altogether, ALT is not able to differentiate between the different stages of pediatric NAFLD and is more associated with late-stage fibrosis.

Figure 5.2 Plasma alanine aminotransferase (ALT) levels in children with biopsy-proven non-alcoholic fatty liver disease (NAFLD). ALT levels in children with NAFLD divided by biopsy-proven steatosis, borderline non-alcoholic steatohepatitis (NASH), and NASH (light-grey bars) or divided upon the NAS score (dark-grey bars). The colored bars show plasma ALT for the individual stages of NAFLD. NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis.

CK-18 can differentiate between steatosis and NASH and correlates with some histological features of pediatric NAFLD

As previous research has shown that plasma levels of CK-18 are accurate in predicting NASH vs. no NASH in adults⁵ and children⁶, we point to confirm these data in our cohort. In the current study, CK-18 was increased in pediatric subjects with borderline NASH and definite NASH compared to steatosis. However, CK-18 levels remained

identical between children with borderline and definite NASH (Figure 5.3). CK-18 was significantly increased in subjects with a high NAS score (3-4 and 5-7) compared to children with an NAS score of 1-2. To determine whether there is an association between CK-18 and severity of NAFLD, we categorized CK-18 according to the histological stages of hepatocyte ballooning, steatosis, inflammation and fibrosis. In this cohort CK-18 was significantly increased upon mild hepatocyte ballooning and according to steatosis and inflammation severity. No difference in CK-18 levels between the different stages of fibrosis was observed. Altogether, we confirm that CK-18 is able to distinguish NASH from steatosis in children with NAFLD.

Figure 5.3 Cytokeratin-18 (CK-18) levels in plasma of children with non-alcoholic fatty liver disease (NAFLD). CK-18 measurements were plotted against the several stages of NAFLD. Asterisks denote significance compared with the corresponding first (left) bar for each classification. Each classification is indicated by the light-grey, the dark-grey, and the colored bars. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. The hashtag (#) notation represents significance compared with the corresponding second (middle bar) for each classification. # $P < 0.001$. NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis.

Plasma cathepsin D holds a better predictive value for the differentiation between steatosis and NASH in children than CK-18 and ALT

We next investigated the clinical potential of plasma cathepsin D for diagnosing pediatric NASH and hereby made use of plotted ROC curves. For the comparison between steatosis and NASH, the ROC curve of cathepsin D demonstrated a significant higher area under the curve (AUC) of 0.94, compared to the AUC values of ALT (0.59; $p = 0.0004$) and CK-18 (0.72; $p = 0.0225$) (Figure 5.4A and Table 5.3). Regarding the differentiation between borderline NASH and definite NASH, an AUC value of 0.85 was

reached by using cathepsin D, which was significantly higher than the AUC values of ALT (0.57; $p=0.0011$) and CK-18 (0.57; $p=0.0003$). An AUC value of 0.88 for cathepsin D was given for the differentiation between steatosis + borderline NASH vs. NASH, compared to an AUC of 0.52 and 0.53 for ALT and CK-18 respectively (Figure 5.4C). For this differentiation, further statistical analysis revealed that the AUC value of cathepsin D is higher than the AUC values of ALT ($p<0.0001$) and CK-18 ($p<0.0001$) (Table 5.3). No significant differences were observed between the AUCs of cathepsin D (AUC: 0.81), ALT (AUC: 0.66) and CK-18 (AUC: 0.74) to distinguish steatosis vs. borderline NASH + NASH (Figure 5.4D and Table 5.3).

Figure 5.4 The diagnostic value of cathepsin D, CK-18 and ALT in pediatric NAFLD. ROC curve analysis was performed to assess the area under the curve (AUC) of using plasma cathepsin D, CK-18 or ALT to predict hepatic inflammation for the following comparisons; steatosis vs. NASH (A); borderline NASH vs. NASH (B); steatosis combined with borderline NASH vs. NASH (C) and steatosis vs. borderline NASH + NASH (D).

The diagnostic performances of cathepsin D, ALT and CK-18 in the prediction of NASH are depicted in Supplementary Table S5.1. The best cut-off point for cathepsin D was <18445 pg/ml, which demonstrated the highest sensitivity (100%) and specificity

(89.5%). Positive and negative predictive values (PPV and NPV) were calculated and demonstrated 92.9% and 100% respectively. In contrast to cathepsin D, ALT and CK-18 displayed lower sensitivity and specificity rates and less accurate PPV and NPV percentages to predict pediatric NASH.

In short, compared to ALT and CK-18, cathepsin D holds better diagnostic value to predict pediatric NASH and is accurate in making the differentiation between pediatric NASH subjects from those who have steatosis. Similar to steatosis vs. NASH subjects, cathepsin D also improves the prediction of NASH compared to the combination of steatosis + borderline NASH, and also compared to borderline NASH separately.

Table 5.3 Overview of the calculated p-values between the different AUCs.

	Cathepsin D vs. ALT	Cathepsin D vs. CK-18	CK-18 vs. ALT
Steatosis vs. NASH	p=0.0004	p=0.0225	p=0.2840
Borderline NASH vs. NASH	p=0.0011	p=0.0003	p=0.9629
Steatosis + Borderline NASH vs. NASH	p<0.0001	p<0.0001	p=0.9204
Steatosis vs. Borderline NASH + NASH	p=0.1030	p=0.4299	p=0.4189

P-values in bold indicate statistically significant differences.

Adding CK-18 to cathepsin D improves the diagnostic accuracy to predict pediatric NASH

To explore whether adding a liver-specific marker, CK-18, to cathepsin D would lead to a better prediction of pediatric NASH, we plotted ROC curves of cathepsin D combined with CK-18. Whereas cathepsin D alone demonstrated an AUC of 0.94 for the comparison of steatotic subjects vs. patients with NASH (Figure 5.4A), the combination CK-18 with cathepsins D resulted in an AUC of 0.998 (Figure 5.5A).

Compared to using cathepsin D alone, combining CK-18 with cathepsin D did not increase the AUC values for the differentiations borderline NASH vs. NASH, and steatosis + borderline NASH vs. NASH (Figure 5.4B-C; 5.5B-C). When distinguishing steatosis from borderline NASH + NASH patients by using cathepsin D individually, an AUC value of 0.81 was reached (Figure 3.4D). Upon the addition of CK-18, an AUC value of 0.85 was obtained (Figure 5.5D). These data illustrate that adding CK-18 to cathepsin D contributes to reaching an optimal ROC curve for the diagnostic prediction of pediatric steatosis vs. NASH.

Figure 3.5 The diagnostic value of adding CK-18 to cathepsin D. ROC curve analysis was performed to assess the area under the curve (AUC) of using the combination of plasma CK-18 with cathepsin D to predict hepatic inflammation for the following differentiations; steatosis vs. NASH (A); borderline NASH vs. NASH (B); steatosis combined with borderline NASH vs. NASH (C) and steatosis vs. borderline NASH + NASH (D).

Discussion

Diagnosing NASH during childhood is of critical importance in order to prevent further progression into NAFLD-related cirrhosis and end-stage liver disease during adolescence. Therefore, understanding the mechanisms that cause progression to NASH at an early age is crucial.

With the help of lysosomal enzymes, lysosomes are best known for its primary role in protein degradation. However, lysosomal function is not merely restricted to degradation of proteins, but increasing evidence now demonstrates that the lysosomal

compartment plays also a role in the immune system and can be seen as secretory vesicles.^{23,24} Numerous studies have shown that lysosomal cholesterol accumulation inside macrophages is an event that occurs during inflammation and has been detected in NASH as well as atherosclerosis.^{7,25} In line, cathepsins have been shown to be significantly involved in mediating the inflammatory response and cholesterol trafficking.^{13,26,27}

Furthermore, it has been reported that cathepsin expression was impaired in the liver of NAFLD patients, suggesting a pivotal role of these proteins in the setting of liver inflammation that characterize the disease.¹⁴ In addition, these findings would indicate that cathepsins are more likely to be involved during early stages of NAFLD, rather than a reflection of a late consequence of inflammation. Indeed, in the current study we showed that levels of plasma cathepsin D are decreased at early stages of NASH, while these remain similar upon the different stages of fibrosis. This was in contrast to plasma ALT, which showed an increase during late-stage fibrosis and was more representative for liver damage. Likewise, a correlation exists between circulating cathepsin D and carotid intima-media thickness, an indicator for atherosclerosis.²⁸ In line with these results, abnormal cathepsin D fractions were associated with a lysosomal storage disease²⁹, a disease predominantly characterized by chronic systemic inflammation. Thus, it seems that lysosomal cholesterol accumulation in KCs, and its subsequent effect on lysosomal enzyme homeostasis, plays a central role in the development of childhood NASH. While many evidences point to the link between lysosomal enzymes and NASH, the exact mechanisms that lead to the secretion of the lysosomal content in plasma are not yet known.

Lysosomes are able to secrete its content, including lysosomal enzymes, via fusion with the plasma membrane. Such fusion is a calcium-dependent process and has been shown to be sensitive for cholesterol levels. As a result, inducing lysosomal cholesterol accumulation completely inhibited lysosomal exocytosis, suggesting that storage of lysosomal cholesterol plays a crucial role in pediatric NASH.³⁰ These data are in line with previous results showing high cholesterol levels in KCs, as was indicated by numerous cholesterol-filled droplets in KCs of livers of NASH patients, while livers of steatotic patients did not show this typical foam cell-like phenotype.¹⁰ However, foam-cell like structures were so far only detected in NASH livers of adults, and still needs to be confirmed in NASH livers of children.

Excessive cholesterol inside lysosomes can also damage the lysosomal membrane and cause lysosomal membrane permeabilization. In turn, there is leakage of cathepsins into the cytosol, whereby cathepsins can mediate the apoptotic signaling pathway.³¹ Thus, instead of extracellular secretion, less viable cells remain left to secrete cathepsin D due to increased apoptosis.

The gold standard to diagnose pediatric NASH is still an invasive liver biopsy procedure. However, due to its invasiveness, biopsies are not performed as a first step. Therefore,

clinicians rely on several non-invasive methods to detect NASH in children such as, liver markers (ALT/AST) and imaging techniques (ultrasound, CT scan and MRI). Currently, ALT is the primary clinical parameter to evaluate NAFLD in children. However, by using a biopsy-confirmed NAFLD cohort it was found that AST and ALT have limited predictive power to discriminate between low and high NAS scores in children with AUC values varying from 0.59 to 0.76.³² In line, normal ALT levels were observed despite the presence of NAFLD, confirming a low correlation between ALT and NAFLD.³³ These data are confirmed in our study by demonstrating low AUC (0.53-0.59) and predictive values (PPV: 72.7% and NPV: 56.5%) for ALT in the discrimination between NASH and no NASH.

A promising serum marker in adults is CK-18, which is representative for hepatocyte apoptosis. Recently, CK-18 was tested in a biopsy-proven children cohort, which confirmed that CK-18 is also an accurate way of predicting NASH vs. steatosis already during early life.³⁴ Similarly, a second pediatric study confirmed the high diagnostic accuracy for CK-18 in predicting NASH (AUC value: 0.93) and a PPV and NPV of 94% and 72%, respectively.⁶ Comparable to these studies, we now found a high AUC value (0.72) and similar PPV and NPV values of CK-18 (72 and 100% respectively) for the detection of NASH vs. steatosis, confirming that CK-18 is accurate at diagnosing NASH in children. However, in our cohort, plasma cathepsin D correlated better with pediatric NAFLD disease progression than CK-18. Altogether, in contrast to other plasma parameters tested for pediatric NASH, our data suggest that plasma cathepsin D is a highly accurate and early non-invasive marker to distinguish steatosis vs. NASH in children. In contrast to CK-18, cathepsin D correlated significantly with NAFLD severity.

Despite our significant findings, the cohort that was used is relatively small in patient size and is of cross-sectional nature. Additional cohorts and future longitudinal studies are essential to validate plasma cathepsin D as a useful tool to detect pediatric hepatic inflammation. Moreover, in view of statistics, a larger sample size would also boost the statistical power. Furthermore, intervention studies should be performed to test whether plasma cathepsin D could be implicated in the clinical follow-up. As obtaining liver biopsies from children with normal aminotransferases is rarely performed, all pediatric individuals in the current study demonstrate abnormal aminotransferase levels. As such, children with normal levels of aminotransferases are not included and consequently, the level of plasma cathepsin D under healthy conditions is not known. The same restriction holds true for studying cathepsin D in relation to NAFLD in children or adults with a normal body mass index.

Altogether, in this study, we used a well-defined biopsy-proven population of children and evaluated plasma levels of cathepsin D. We have demonstrated for the first time that plasma cathepsin D is significantly decreased in children with NASH compared to children with either steatosis or borderline NASH. Plasma cathepsin D holds high

diagnostic value and could be used as a promising non-invasive clinical marker to distinguish pediatric NASH patients from subjects with steatosis, especially upon the addition of CK-18. Furthermore, this is the first human study to show that the lysosomal compartment changes under inflammatory conditions. However, future research is warranted to fully understand the novel role of lysosomes during the development of early NASH. Moreover, whether cathepsin D can be used in clinical practice as a non-invasive marker for NASH, should be validated in additional larger well-defined NAFLD cohorts in children and adults.

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Supplementary table

Table S3.1 Different cut-off values of cathepsin D, CK-18 and ALT for distinguishing pediatric NASH from steatosis.

Cathepsin D (pg/ml)	Sensitivity %	Specificity %	PPV %	NPV %
<19811	100.0	79.0	86.7	100.0
<19070	100.0	84.2	89.6	100.0
<18445	100.0	89.5	92.9	100.0
<16471	96.2	89.5	92.6	94.4
<14612	92.3	89.5	92.3	89.5
<13172	88.5	89.5	92.0	85.0
CK-18 (U/L)	Sensitivity %	Specificity %	PPV %	NPV %
>257.0	100.0	47.4	72.2	100.0
>318.0	84.0	57.9	73.7	72.6
>327.5	72.0	63.2	72.8	62.2
>345.5	60.0	73.7	75.7	57.4
>364.5	48.0	84.2	80.6	54.2
>396.5	8.0	89.5	51.0	42.2
>411.5	4.0	100.0	100.0	43.2
ALT (U/L)	Sensitivity %	Specificity %	PPV %	NPV %
>34.0	84.6	21.1	59.5	50.0
>42.0	76.9	36.8	62.5	53.8
>54.5	69.2	52.6	66.7	55.6
>64.5	61.5	68.4	72.7	56.5
>76.5	34.6	79.0	69.2	46.9
>87.0	26.9	84.2	70.0	45.7
>169.0	19.2	100.0	100.0	47.5

PPV: positive predictive value, NPV: negative predictive value.

Chapter 6

Plasma cathepsin D correlates with histological classifications of fatty liver disease in adults and responds to intervention

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Abstract

Non-alcoholic steatohepatitis (NASH) is characterized by liver lipid accumulation and inflammation. The mechanisms that trigger hepatic inflammation are poorly understood and subsequently, no specific non-invasive markers exist. We previously demonstrated a reduction in the plasma lysosomal enzyme, cathepsin D (CatD), in children with NASH compared to children without NASH. Recent studies have raised the concept that non-alcoholic fatty liver disease (NAFLD) in adults is distinct from children due to a different histological pattern in the liver. Yet, the link between plasma CatD to adult NASH was not examined. In the current manuscript, we investigated whether plasma CatD in adults correlates with NASH development and regression. Biopsies were histologically evaluated for inflammation and NAFLD in three complementary cohorts of adults (total n=248). CatD and alanine aminotransferase (ALT) were measured in plasma. Opposite to our previous observations with childhood NASH, we observed increased levels of plasma CatD in patients with NASH compared to adults without hepatic inflammation. Furthermore, after surgical intervention, we found a reduction of plasma CatD compared to baseline. Our observations highlight a distinct pathophysiology between NASH in children and adults. The observation that plasma CatD correlated with NASH development and regression is promising for NASH diagnosis.

Introduction

The current obesity epidemic is paralleled by an increasing prevalence of non-alcoholic steatohepatitis (NASH). NASH is characterized by hepatic lipid accumulation (steatosis) and inflammation. While steatosis itself is generally considered benign and reversible, the presence of inflammation will eventually set the stage for further liver damage, including fibrosis, cirrhosis and liver cancer.^{1,2} Currently, elevations in plasma alanine transaminase (ALT), a liver enzyme, are the primary clinical abnormality detected in NASH patients. When hepatocellular injury is present, this liver enzyme is released into the circulation. Although ALT is used as a tool for the routine diagnosis of NASH, it lacks specificity and sensitivity to distinguish between NASH and steatosis.^{3,4} In order to improve non-invasive diagnosis of NASH, it is of clinical relevance to investigate novel underlying mechanisms during NASH pathophysiology. Although several processes have been identified that participate in the development of hepatic inflammation, the actual mechanisms for the inflammatory response remain uncertain.

We previously demonstrated a clear and direct association between hepatic inflammation and lysosomal cholesterol accumulation inside Kupffer cells (KCs) of low-density lipoprotein receptor knockout (*Ldlr*^{-/-}) mice fed a high-fat, high-cholesterol diet.⁵⁻⁷ In accordance with our observation in mice, cholesterol-containing KCs were also demonstrated recently in livers of NASH patients.⁸ The occurrence of lysosomal cholesterol accumulation has been shown to induce disturbances in the lysosomal (enzyme) trafficking pathway.^{9,10} In line, we previously detected modified levels of the lysosomal enzyme cathepsin D (CatD) in the plasma of children with NASH compared to children without hepatic inflammation.¹¹

Recent studies have raised the concept that non-alcoholic fatty liver disease (NAFLD) in children and adults is distinct due to differences in the histological pattern in the liver as well as pathological characteristics of the disease.¹²⁻¹⁵ Thus far, the relationship between plasma CatD and adult NASH has not been explored. Therefore, as a follow-up study from our data in children, we investigated in the current paper whether plasma CatD in adults correlates with the development and regression of NASH.

Here, we observed in three complementary cohorts of adults (average age >40 years), with an average obese body mass index (BMI) (BMI >30 kg/m²), that plasma CatD is significantly increased along the development of NASH. As the plasma CatD levels in adults with NASH were the opposite of those previously found in children with NASH,¹¹ we point toward the existence of a potential different pathophysiology between NASH in children and adults. Our data further show that CatD responds to surgical intervention, which underlines the potential clinical relevance and diagnostic value of plasma CatD in the context of NASH.

Methods

Human cohorts; Cleveland cohort, Maastricht cohort and the Kuopio cohort

The design of this study includes three adult cohorts. All three cohorts demonstrated a similar adult age group (average age >40 years) and a similar average obese BMI (average BMI >30 kg/m²). Across the three cohorts, all liver biopsies were histologically classified for NAFLD/NASH according to the criteria of Brunt and/or Kleiner, which includes steatosis, ballooning, lobular inflammation and portal inflammation.^{16,17}

The Cleveland cohort consists of NASH patients after the detection of abnormal plasma ALT values. The second and third population (the Maastricht and Kuopio cohort, respectively) include obese patients that are unselected for ALT, *i.e.*, biopsies were taken during bariatric surgery regardless of their ALT levels. Patients from these cohorts are considered to have a wide spectrum of fatty liver diseases, including early stages.

Definition Cleveland cohort

A total of 127 adults (average age >40 years) with an average obese BMI >30 kg/m² were included, of which 43 had biopsy-proven NASH. Subjects with NAFLD were recruited from the metabolic clinics of the Cleveland Clinic and MetroHealth Medical Center in Cleveland, OH. Excluding criteria, the procedure of obtaining and scoring of liver biopsies as well as blood sampling of this NASH Cleveland cohort have been described previously¹⁸⁻²⁰. See Supplementary Table S6.1 for the patient characteristics of the Cleveland cohort.

Definition Maastricht cohort

47 obese adult patients (average BMI >30 kg/m², average age >40 years), undergoing bariatric surgery at the Maastricht University Medical Center or at the Atrium Medical Center Parkstad, Heerlen, the Netherlands, were included in the study. Excluding criteria, the procedure of obtaining liver biopsies and blood sampling of this Maastricht obese cohort has been described previously.^{21,22} See Supplementary Table S6.2 (online available) for the population characteristics of the Maastricht cohort. Liver biopsies were evaluated by a trained pathologist, according to the criteria of Brunt¹⁷ and Kleiner²⁰ and graded for severity from NASH grade 1 (early-stage), to NASH grade 3 (late-stage) (see Supplementary Table S6.3).

Definition Kuopio cohort

All patients undergoing obesity surgery in Kuopio University Hospital are recruited into our ongoing study investigating metabolic consequences of obesity surgery (Kuopio Obesity Surgery Study).^{23,24} The study group included 74 obese adult subjects (average BMI >30 kg/m², average age >40 years), who were accepted for Roux-en-Y gastric bypass (RYGB) operation over the years 2005-2010. See Supplementary Table S6.4 (online available) for the general population characteristics at the start of intervention. Excluding criteria, the procedure of obtaining and scoring of the liver biopsies, and blood sampling of the Kuopio obese cohort has been described previously.²³⁻²⁵

Use of human subjects

With regard to the Cleveland cohort, the methods in this study were carried out in accordance with approved guidelines by the Institutional Review Boards at the Cleveland Clinic and MetroHealth Medical Center and was conducted according to the relevant guidelines of the protocol. For the Maastricht cohort, the study was carried out in accordance with the approved guidelines by the Medical Ethical Committees of both the Maastricht University Medical Centre and the Atrium Medical Centre Parkstad and conducted in accordance with the revised version of the Declaration of Helsinki (October 2008, Seoul). For the Kuopio cohort, the study protocol was carried out in accordance with approved guidelines by the Ethics Committee of the Northern Savo Hospital District (54/2005, 104/2008 and 27/2010), and it was performed according to the Helsinki Declaration. Written informed consent was obtained from all the subjects.

Human cathepsin D enzyme-linked immunosorbent assay

Plasma samples were diluted and CatD levels were determined by the CatD enzyme-linked immunosorbent assay according to the manufacturers' protocol (Uscn Life Science Inc, Wuhan, China). The detection limit ranges approximately from 46.88 to 3,000 pg/ml. Coefficients of variation (CV%) for intra- and inter assays are <10 and <12% respectively. CatD measurements were performed blinded to the histology findings of the study participants.

Immunohistochemistry

Bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDMs) were isolated as described previously.²⁶ Cells were grown in 12-well plates (Greiner Bio-One) on coverslips (Ø 20 mm; Thermo Scientific). BMDMs were washed twice with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Fixed BMDMs were stored at 4°C in PBS-NaN3

(0.03%) or washed three times with PBS-/- (Gibco) and directly used for immunocytochemistry. Subsequently, cells were permeabilized (0.1% Triton X-100 and 0.2% BSA in PBS) for 15 min and blocked (2% BSA-PBS) for 30 min at room temperature. Primary and secondary antibodies (2% BSA-PBS) were incubated for 1h at room temperature. Coverslips were washed and mounted onto glass slides using DABCO-glycerol medium (Sigma-Aldrich) containing DAPI (1:10,000; Sigma-Aldrich) in order to counterstain nuclei. The following antibodies were used: goat anti-mouse CatD antibody (1:100, AF1029, R&D systems), rabbit anti-mouse lysosome-associated membrane protein-1 (LAMP-1) antibody (1:200, Ab24170, Abcam), donkey anti-goat IgG NorthernLights (NL)557-conjugated antibody (1:200; NL001, R&D systems), goat anti-rabbit Alexa488 (1:200; Invitrogen). Cells were imaged using a Leica TCS SPE confocal laser scanning microscope (Leica Microsystems GmbH) equipped with an air-cooled argon-krypton mixed gas laser, using oil immersion objectives. ImageJ software was used to process and analyze the images.

Liver biopsy specimens

Immunohistochemistry for CatD was performed on paraffin-embedded liver sections originating from the Maastricht cohort. Sections were deparaffinized in xylene and ethanol and rehydrated in water. Antigen retrieval was achieved by incubating sections in 10 mM sodium citrate buffer in 0.05% Tween20, pH 6.0, at 95°C. After cooling down, sections were quenched in hydrogen peroxide (3%) to suppress endogenous peroxidase activity. Prior to incubation with the first antibody, slides were first incubated with 1×PBS plus an amplification step: 1:5 Avidin D Block solution followed by an incubation step with Biotin Block solution 1:5 in 1×PBS (Avidin/Biotin Blocking kit, SP-2001, Vector Laboratories, Burlingame, CA, USA). Sections were then incubated overnight at 4°C with the primary CatD antibody (Cell Signaling Technology, no. 2284, Danvers, MA, USA; [1:50]) dissolved in 1× PBS containing 0.05% Tween20. As negative controls sections were incubated without primary antibody. Incubation with the second antibody for biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; [1:50]) was carried out in 1×PBS containing 0.05% Tween20 for 30 min. Afterwards the slides were washed and incubated for an additional amplification step in 1×PBS+1:50 Avidin D solution+1:50 Biotin solution (Vectastain Elite ABC kit, PK-6100, Vector Laboratories, Burlingame, CA, USA). Then, 13-amino-9-ethylcarbazole (AEC) was applied using an AEC Peroxidase Substrate kit (Vector Laboratories, SK-4200, Burlingame, CA, USA) and haematoxylin for nuclear counterstaining. Sections were enclosed with Faramount aqueous mounting medium. Pictures were taken with a Nikon DMX1200 digital camera and ACT-1 version 2.63 software (Nikon Instruments Europe, Amstelveen, the Netherlands).

Statistical analysis

The data were analysed by performing two-tailed non-paired *t*-tests using GraphPad Prism for comparing the different groups. The data were expressed as mean $\pm$ SEM and considered significant at $p < 0.05$. The diagnostic accuracy of the combination ALT with CatD was calculated as described previously.¹¹

Results

General population characteristics of the Cleveland cohort

ALT was significantly higher in patients with hepatic inflammation compared to their age- and gender-matched control subjects ($p < 0.001$) (see Supplementary Table S6.1). BMI, low-density lipoprotein (LDL), triglycerides and aspartate aminotransferase (AST) were all increased in subjects scored for hepatic inflammation compared to their matched controls. In line, plasma high-density lipoprotein (HDL) and the AST/ALT ratio were reduced in the group with hepatic inflammation.

6

Elevated CatD levels in plasma of patients with hepatic inflammation

First, CatD and ALT were measured in plasma of the Cleveland cohort. We found a significant increase of CatD in relation to liver biopsies of patients that demonstrate inflammation, compared to those without hepatic inflammation (No inflammation vs. inflammation: $p < 0.001$) (Figure 64.1A). Next, we investigated the correlation between plasma ALT and CatD. Plasma CatD demonstrated a strong positive correlation with ALT (Pearson's $r = 0.57$, $p < 0.001$) (Figure 6.1B). These results suggest that plasma CatD levels are linked to the pathogenesis of NASH.

Population characteristics of the Maastricht cohort

To test whether plasma CatD is elevated already during early-stage of NASH, we used the Maastricht cohort consisting of patients with a wide range of fatty liver disease. Clinical characteristics of the patient population from this cohort are presented in Supplementary Table S6.2. The BMI from the cohort tested, ranged from 30.7 to 73.6 kg/m^2 with an overall average of 46.7 kg/m^2 . The average plasma levels of ALT and AST were significantly higher in NASH patients as compared to subjects with a healthy liver ($p = 0.018$ and $p = 0.019$ respectively). Patients in the NASH group were significantly older compared to patients with a healthy liver ($p = 0.021$) and to those who had steatosis ($p = 0.049$). Importantly, there was no difference with respect to all other parameters (*i.e.*, BMI, plasma total cholesterol, HDL, LDL, triglycerides and the AST/ALT ratio). Liver specimens of 27 NASH patients were histologically classified according to the scoring

systems of Brunt and Kleiner. Most NASH subjects (14 out of 27) included displayed mild NASH (grade 1), as summarized in Supplementary Table S6.3. The majority of NASH patients showed steatosis score 2, ballooning score 1, lobular inflammation score 1, and no or little fibrosis.

Figure 6.1 CatD associated with ALT and hepatic inflammation. (A) CatD in plasma of subjects with or without hepatic inflammation (n = 127). (B) The Pearson correlation test was used to calculate the correlation coefficient (r) between plasma CatD and ALT. *** indicates $p < 0.001$.

Plasma CatD distinguishes NASH patients from subjects without hepatic inflammation and correlates with histological classifications of NAFLD

The level of plasma CatD was significantly higher in subjects with NASH compared to individuals with either simple steatosis or a healthy liver (NASH vs. normal: $p = 0.031$; NASH vs. steatosis: $p = 0.0065$) (Figure 6.2A). More importantly, patients with a mild score for NASH grade, *i.e.*, grade 1, showed an increase in plasma CatD compared to patients without NASH ($p = 0.0018$) (Figure 6.2B). The same significant trend holds true with respect to lobular inflammation, where CatD levels were already increased with mild disease as observed for NASH grade 1 ($p = 0.0295$) (Figure 6.2C). In addition, CatD

was strongly elevated upon mild steatosis, *i.e.*, score 1, compared to patients without steatosis ($p=0.019$), and decreased upon severity of steatosis (Figure 6.2D). Similar to NASH grade, lobular inflammation and steatosis, CatD was primarily elevated upon mild fibrosis (score 1 and 2) compared to patients that did not show fibrosis ($p=0.002$ and $p=0.019$, respectively) (Figure 6.2E). Moreover, total cholesterol levels correlated with plasma CatD (Figure 6.2F). In summary, the plasma levels of the lysosomal enzyme CatD are increased in plasma of patients with NASH compared to the patients with a normal liver phenotype or steatosis alone. These data also suggest that plasma CatD correlates specifically with NAFLD severity, already from the early phase, and to cholesterol levels.

Figure 6.2 Lysosomal CatD in human plasma. (A) CatD in plasma of obese patients with a normal liver phenotype, steatosis and NASH ($n = 47$). According to Brunt's criteria, patients were divided for NASH grade (B). Lobular inflammation (C), steatosis (D) and fibrosis (E) were categorized according to Kleiner's scoring system. (F) The Pearson correlation test was used to calculate the correlation coefficient (r) between plasma CatD and total cholesterol levels. *Significantly different from normal. * and ** indicate $p<0.05$, and 0.01 , respectively.

In contrast to ALT, CatD correlates with early stages of NASH

Currently, plasma ALT is the most common diagnostic tool in the clinic to detect hepatocellular injury during NASH. Significantly increased ALT levels were observed in patients with NASH and steatosis (NASH vs. normal: $p=0.018$; steatosis vs. normal: $p=0.019$) (Figure 6.3A). While plasma CatD could differentiate NASH from steatosis, there was no difference in ALT concentrations between livers with NASH and steatosis,

indicating that CatD correlates better with the spectrum of NAFLD than ALT. Moreover, ALT was only increased upon more advanced degrees of hepatic inflammation (NASH grade 2 vs. grade 0: $p=0.003$; NASH grade 3 vs. grade 0: $p<0.001$) (Figure 6.3B), whereas CatD demonstrated to be elevated already during mild NASH (NASH grade 1). Plasma CatD was significantly higher in the early degrees of steatosis, lobular inflammation and fibrosis, whereas this was not true for ALT (Figure 6.3C-E). A correlation was found between CatD and total cholesterol, whereas no similar correlation could be detected for ALT (Figure 6.3F). Thus, in contrast to ALT, CatD was elevated upon early NAFLD severity and could distinguish NASH from steatosis in adult subjects with obesity.

Figure 6.3 Human plasma ALT levels. (A) Plasma ALT in obese patients with either a normal, steatotic or NASH liver (n = 47). Patients were classified for NASH grade (Brunt's score) (B), lobular inflammation (C), steatosis (D) and fibrosis (E) (Kleiner's score). (F) The Pearson correlation test was used to calculate the correlation coefficient (r) between plasma ALT and total cholesterol levels. *Significantly different from normal. *, ** and *** indicate $p<0.05$, 0.01, and 0.001 respectively.

Potential clinical significance of using plasma CatD as an additional non-invasive screening method for NASH

Since our results suggest a strong association between increased plasma CatD and early-stage NASH, we next investigated the potential significance of plasma CatD for the diagnosis of NASH by using the area under the curve (AUC) of plotted receiver-

operating characteristic (ROC) curves in the Maastricht cohort. Such a statistical analysis in the Maastricht cohort, with a relative small sample size, gives an indication whether plasma CatD could be potentially clinical relevant for the non-invasive detection of NASH.

Normal & Steatosis vs. NASH

First, we compared plasma ALT and CatD levels in normal + steatotic subjects vs. patients with NASH. The AUC of ALT alone was estimated to be 65%, whereas CatD displayed an AUC of 77% (Figure 6.4A). Notably, combining both enzymes led to an increase in the AUC from 65% to 77% compared to ALT alone (see Supplementary Figure S6.1). Thus, the addition of CatD to ALT improved the diagnostic value for distinguishing NASH subjects from those who are healthy or have steatosis.

Steatosis vs. NASH

Whereas ALT alone demonstrated low predictive power to discriminate steatosis from NASH (AUC: 51%), CatD demonstrated improved the predictive value with an AUC of 84% (Figure 6.4B). In comparison to CatD alone, similar diagnostic values were obtained when ALT and CatD were combined with an AUC value of 84% (see Supplementary Figure S6.1 online). In short, taking plasma CatD into account, the diagnostic prediction for NASH became considerably more accurate than by using ALT alone.

Figure 6.4 Diagnostic accuracy of ALT and CatD for predicting NASH. ROC curve analysis was used to assess the area under the curve (AUC), the sensitivity and specificity of ALT and CatD in predicting NASH for the following comparisons: healthy & steatotic subjects vs. subjects with NASH (A); subjects with steatosis vs. NASH (B); and normal vs. NASH subjects (C).

Normal vs. NASH

Almost similar AUC values were obtained for ALT and CatD (74% and 73% respectively) for the differentiation between NASH from healthy individuals (Figure 6.4C). Combining the two enzymes resulted in a minor increase of the AUC value to 77% (see Supplementary Figure S6.1). The relatively high AUC value of ALT alone for the differentiation between NASH and healthy individuals remained roughly equal upon addition of CatD.

Plasma CatD responds to intervention

To test the effect of an intervention on plasma CatD levels, we included an additional liver biopsy-proven cohort (*i.e.* the Kuopio cohort), which was monitored following a one-year surgical intervention. Before the start of the surgical intervention, CatD levels were increased in adults with NASH compared to adults who did not show signs of hepatic inflammation (normal vs. NASH: $p=0.039$) (Figure 6.5A), herewith confirming our previous data in this manuscript (Figure 6.1A and Figure 6.2A). Additionally, when merging all three individual cohorts (Cleveland, Maastricht and Kuopio cohort) into one single cohort, plasma CatD levels remained increased in patients with hepatic inflammation compared to those without hepatic inflammation ($p=0.0003$) (see Supplementary Figure S6.2). Similar to the Maastricht cohort, CatD correlated with total cholesterol levels in plasma (Figure 6.5B). The absolute difference of plasma CatD was calculated before and after surgical intervention and demonstrated a significant reduction upon intervention of NASH patients compared to intervention of their healthy individual controls ($p=0.007$) (Figure 6.5C). These data suggest for the first time that plasma CatD can be used in the clinical follow-up of adult NASH patients.

Figure 6.5 Plasma CatD levels in the Kuopio cohort. A) CatD in plasma of obese patients with a normal liver phenotype, steatosis and NASH ($n = 74$). B) Correlation between CatD and total cholesterol. C) The absolute difference of plasma CatD was calculated before and after one-year of surgical intervention of normal, steatotic and NASH subjects.

CatD localization

Additional to plasma CatD measurements, a CatD staining on normal, steatotic and NASH livers was performed and revealed that CatD expression in liver is similar between the three groups and is mainly located around the pericentral vein (see Supplementary Figure S6.3). Next to this, we examined intracellular CatD levels and CatD location in more detail. Bone marrow-derived macrophages (BMDMs) were loaded with oxidized low-density lipoproteins (oxLDL), *i.e.* to mimick hyperlipidemia, or with control medium for 24h. Our data pointed towards an increase of intracellular CatD upon oxLDL loading, compared to control. Additionally, CatD was located closer to the plasma membrane in oxLDL-treated BMDMs, compared to control treatment (see Supplementary Figure S6.4). Additionally, we observed yellow-orange dots in the merged images, which indicates that CatD co-localized with lysosomes. Thus, intracellular CatD expression is increased particularly in lysosomes during high cholesterol conditions, and is located closer to the plasma membrane.

6

Discussion

The exact mechanisms leading to NASH remain largely unknown. Diagnosing NASH is of critical importance in order to prevent further progression into NAFLD-related cirrhosis and end-stage liver disease. Therefore, better understanding of the mechanisms that cause progression to NASH is important.

We have recently established a strong association between CatD in pediatric NASH and children without hepatic inflammation.¹¹ In the current paper, we observed an association between plasma CatD and NASH in three biopsy-proven cohorts of adults. Strikingly, whereas we found a decrease of plasma CatD in NASH children, we here show an increase of CatD in the plasma of adults with NASH. These findings suggest a difference in disease pathology between adult and pediatric NASH. Furthermore, our observations point to plasma CatD as a useful non-invasive tool to improve NASH diagnosis in adults.

Under normal conditions, lipoprotein-derived cholesterol is internalized by macrophages and directed to the lysosome for further processing. After hydrolysis, free cholesterol is transferred from the lysosome to the cytoplasm where the free cholesterol is degraded into bile acid precursors or secreted via cholesterol efflux. The difference in pathophysiology between NASH in children and adults could be due to age-dependent changes of the lysosomal compartment. Indeed, lysosomal function has previously been shown to be an age-dependent process, whereby the amount of lysosomes increase upon ageing.²⁷⁻²⁹ These data suggest that at a young age, the

amount of lysosomes is low and that, consequently, the cells have difficulties to cope with excessive amounts of cholesterol. As such, upon high-cholesterol circumstances, the stability of the lysosomal membrane is likely to decrease,³⁰ which can eventually lead to lysosomal rupture and subsequent lysosomal enzyme release into the cytosol. Lysosomal enzyme secretion into the plasma is hereby prevented and instead, the cytosolic lysosomal enzymes activate the apoptotic signaling pathway inducing rapid cell death.³¹ This mechanism could explain the reduction of CatD in the plasma of pediatric subjects with NASH compared to subjects without hepatic inflammation.¹¹ In contrast to young cells, older cells possess a higher number of lysosomes,²⁷⁻²⁹ suggesting increased cholesterol storage capabilities inside the cells. It is therefore likely that adult lysosomes can cope better with the cholesterol accumulation and would be less likely to rupture. Nevertheless, cholesterol-filled lysosomes have been shown to induce disturbances in the lysosomal enzyme trafficking pathway^{9,10} that can potentially lead to increased levels of lysosomal enzymes in plasma.^{32,33} Likewise, we observed that CatD is increased intracellularly during high cholesterol conditions and is located closer to the plasma membrane. Since the plasma membrane is the primary site where secretion takes place into plasma,³⁴ these data suggest that cholesterol-rich lipoproteins, including oxLDL, increases the contact of lysosomes with the plasma and as such leads to elevated levels of plasma CatD. This potential mechanism has been illustrated in a schematic figure (see Figure 6.6).

Figure 6.6 Potential mechanism for enhanced plasma CatD levels during adult NASH. In adults, under normal conditions, circulating cholesterol-rich lipoproteins are endocytosed and directly distributed to the lysosomes. There, lipoprotein-derived cholesteryl esters are further processed following release into the cytoplasm. Under conditions with high cholesterol-rich lipoprotein levels, which is commonly associated with NASH, Kupffer cells internalize high amounts of lipoproteins, leading to enlarged lysosomes. These large cholesterol-filled lysosomes can potentially disturb the trafficking of lysosomes, which can lead to migration of the lysosome towards the plasma membrane. Hence, enhanced extracellular secretion of CatD into the plasma can occur.

Plasma LDL oxidation can be viewed as a representative parameter of oxidative stress that has been shown to be induced in healthy elderly compared to young controls, and therefore is strongly associated with the course of ageing.^{35,36} In vitro, particularly the intracellular accumulation of the oxidized cholesterol fraction has been shown to enhance extracellular secretion of pro-CatD.^{37,38} Immunohistochemical stainings of CatD on human liver underlined these findings, as CatD was mainly present in the pericentral area of the liver. Compared to the periportal and the midzonal zones, which are relatively nearer to the entering blood supply, the pericentral zone receives blood that is lower in oxygen and high in oxidative stress.³⁹ As oxidative stress is increased in NASH patients,⁴⁰ these data are in line with our observations in plasma, in which increased levels of CatD are associated with NASH in adults. Altogether, the observed differences between NASH in children and adults could be explained by the fact that secretion of CatD into the plasma is dependent on age-related changes of the lysosomes and therefore is dependent on accumulation of specific lipid species. In agreement, several studies indeed describe that the histological and pathological characteristics of NASH are age-dependent.¹²⁻¹⁵ Further studies are warranted to precisely determine the contribution of lysosomes to NASH disease pathology in children and adults.

With the help of lysosomal enzymes, lysosomes are best known for its primary role in protein degradation. However, protein degradation during NASH, for example of cholesterol, seems to be disturbed, as we have recently established a clear association between hepatic inflammation and increased cholesterol accumulation in lysosomes of KCs.⁵ This is underlined by the fact that KCs compromise 15-20% of the total number of liver cells⁴¹ and account for the main hepatic uptake of modified cholesterol-rich lipoproteins from the circulation.⁴² Additionally, numerous studies have shown that lysosomal cholesterol accumulation inside macrophages is an event that occurs during inflammation and has been detected in NASH as well as in atherosclerosis.^{5,6} These data are in line with previous results showing high cholesterol levels in KCs, as was indicated by numerous cholesterol-filled droplets in KCs of livers of NASH adults, while livers of steatotic patients did not show this typical foam cell-like phenotype.⁴³

Lysosomal function is not merely restricted to degradation of proteins, such as cholesterol, but increasing evidence now demonstrates that the lysosomal compartment can also be seen as vesicles that can secrete its content, for example lysosomal enzymes.^{44,45} Cathepsins, a specific class of lysosomal enzymes, have been shown to be significantly involved in mediating the inflammatory response and cholesterol trafficking.⁴⁶⁻⁴⁸ Furthermore, we have recently reported that plasma CatD was strongly associated with pediatric NASH.¹¹ Likewise, a correlation exists between CatD and carotid intima-media thickness, an indicator for atherosclerosis,⁴⁹ as well as with a lysosomal storage disease,⁵⁰ diseases which both are predominantly

characterized by chronic systemic inflammation and excessive cholesterol storage inside macrophages. In line, in the current paper, we show that CatD is correlated with histological classifications for NASH as well as with cholesterol. These evidences point to a strong link between CatD and cholesterol-mediated inflammation. Therefore, it seems reasonable that lysosomal cholesterol accumulation in KCs, and its subsequent effect on lysosomal enzyme homeostasis, is a central mechanism in the development of NASH.

Several plasma markers that have been tested as a potential screening method for NASH are in relation to apoptosis (cytokeratin-18 (CK-18) fragments),⁵¹ inflammation (cytokines and adipokines)⁵² and advanced glycation end products.⁵³ Numerous studies screening NASH patients for individual CK-18 fragments described a high diagnostic accuracy with an AUC between 71 and 93%.⁵⁴ Our observations for analyzing CatD individually to distinguish NASH from steatotic patients are within the same high accuracy range as CK-18 with an AUC of 84%. However, whereas CatD correlated with early signs of inflammation, CK-18 represents liver damage and fibrosis.⁵¹ Combining two biomarkers such as total CK-18 with interleukin-6 yielded an AUC of 83% and 84% for the combination adiponectin and total CK-18.⁵⁵ In line with this approach, we presented the combination of ALT and CatD with a similar high AUC of 84% for separating NASH from steatotic patients. These data suggest that including one liver-specific parameter (*i.e.*, ALT) could increase the diagnostic accuracy for detecting a liver disease such as NASH. In contrast to ALT, increased CatD levels in the plasma are representative for early-stage hepatic inflammation.

Furthermore, we have shown for the first time that plasma CatD responded to a 1-year follow-up period after a gastric bypass intervention of NASH patients. Although no follow-up liver biopsies were obtained in the current study, gastric bypass intervention has previously been shown to resolve histopathological features of NASH.⁵⁶ While plasma CatD responded to the intervention, changes in plasma CK-18 levels following intervention of NASH patients have not been defined so far.⁵⁷ Contradictive results have been found concerning plasma transaminases after surgical intervention, while others demonstrated a correlation between histological improvement of NASH and lower ALT and AST after follow-up,⁵⁸ some did not find a difference postoperatively.⁵⁶ Thus, besides NASH diagnosis, plasma CatD can potentially be used in the clinical follow-up of NASH. Further trials are necessary to validate the response of plasma CatD upon a surgical intervention.

Thus, in the current study, we have found a strong association between plasma CatD and NASH in adults. In contrast to childhood NASH, we now demonstrated elevated CatD levels in plasma of NASH adults. These data suggest the existence of a distinct disease pathology between NASH in children and adults. Plasma CatD holds potential

clinical utility as it correlated with histological characteristics of NASH and regression of this disease. Mechanistically, these findings point to the important role of lysosomes in the development of NASH and should be further investigated.

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Supplemental figures and tables

Figure S6.1 Diagnostic accuracy of ALT + CatD combined together to predict NASH.

Cleveland cohort + Maastricht cohort + Kuopio cohort

Figure S6.2 Plasma CatD levels in three merged cohorts (Cleveland, Maastricht and Kuopio).

Figure S6.3 Representative pictures of the immunohistochemical staining of CatD on liver biopsy specimens (total n=5). CatD, which is depicted in red, occurs mainly in the pericentral area of the liver. Magnification 200x.

Bone marrow-derived macrophages C57/Bl6

Figure S6.4 CatD expression in bone marrow-derived macrophages (BMDMs) loaded with and without oxidized low-density lipoproteins (oxLDL). Cells were fixed and stained with an anti-cathepsin D antibody and anti-lysosomal-associated membrane protein 1 (LAMP-1) antibody that was detected by a NothernLights557-labeled (in red) and Alexia488-labeled secondary antibody (in green), respectively. DAPI was used to visualize the nuclei (in blue). Magnification 630x.

Table S6.1 Population characteristics of the Cleveland cohort

	Cleveland cohort	
	No inflammation	Inflammation
n	84	43
Sex, male/female	35/49	17/26
Age, y	41.8±1.3	45.8±1.8
BMI, kg/m ²	28.2±0.7	34.8±0.8***
Total cholesterol, mmol/L	4.7±0.1	5.0±0.2
HDL, mmol/L	1.3±0.1	1.1±0.04*
LDL, mmol/L	2.8±0.1	3.2±0.1*
Triglycerides, mmol/L	1.3±0.1	1.8±0.1*
ALT, IU/L	24.1±2.1	77.0±8.5***
AST, IU/L	24.2±1.0	60.2±6.3***
AST/ALT ratio	1.2±0.1	0.9±0.1***

Data are represented as mean ± SEM. Significantly different from *No inflammation*, * and *** indicates p<0.05 and p<0.001, respectively.

Table S6.2 Population characteristics of the Maastricht cohort

	Maastricht cohort		
	Normal	Steatosis	NASH
n	12	8	27
Sex, male/female	5/12	0/8	8/19
Age, y	40.5±2.3	40.0±3.9	48.5±2.0* [#]
BMI, kg/m ²	43.5±2.0	44.6±2.5	48.8±1.8
Total cholesterol, mmol/L	4.9±0.3	5.4±0.3	5.5±0.3
HDL, mmol/L	0.9±0.1	1.0±0.1	1.0±0.1
LDL, mmol/L	3.1±0.2	3.4±0.4	3.5±0.2
Triglycerides, mmol/L	1.8±0.2	2.2±0.4	2.8±0.6
ALT, IU/L	18.2±1.1	28.7±5.1*	29.0±2.9*
AST, IU/L	18.3±2.2	22.4±2.8	29.5±2.6*
AST/ALT ratio	1.1±0.2	0.8±0.1	1.1±0.1

Data are represented as mean ± SEM. * and # indicate p<0.05. Asteriks (*) indicate significant changes compared with Normal. Hashtag (#) indicates significant changes compared with Steatosis.

Table S6.3 Histological scoring of liver biopsies from NASH subjects of the Maastricht cohort

Brunt score	Definition	NASH subjects (n = 27)
Grade 1	Mild	14
Grade 2	Moderate	7
Grade 3	Severe	6
Kleiner score		
Steatosis	<5% (score 0)	0
	5-33% (score 1)	7
	33-66% (score 2)	12
	>66% (score 3)	8
Ballooning	None (score 0)	6
	Few balloon cells (score 1)	18
	Prominent ballooning (score 2)	3
Lobular inflammation	None (score 0)	2
	<2 foci per 200x field (score 1)	14
	2-4 foci per 200x field (score 2)	7
	>4 foci per 200x field (score 3)	4
Fibrosis	None (score 0) / Nondefined	10 / 2
	Perisinusoidal or periportal (score 1)	6
	Perisinusoidal and portal/periportal (score 2)	5
	Bridging fibrosis (score 3)	2
	Extensive bridging fibrosis, cirrhosis (score 4)	2

Table S4.4 Population characteristics of the Kuopio cohort

	Kuopio cohort		
	Normal	Steatosis	NASH
n	29	23	22
Sex, male/female	8/21	3/20	10/12
Age, y	50±1.7	46±2.0	47±2.0
BMI, kg/m ²	42.8±1.2	44.6±1.0	44.7±1.3
Total cholesterol, mmol/L	4.1±0.1	4.0±0.2	5.0±0.3**, ##
HDL, mmol/L	1.1±0.05	1.0±0.04	1.1±0.09
LDL, mmol/L	2.3±0.1	2.3±0.2	3.0±0.2**, ##
Triglycerides, mmol/L	1.4±0.1	1.6±0.1	1.9±0.2*
ALT, IU/L	36.9±5.0	42.7±3.8	64.1±10.6*
AST, IU/L	28.6±2.8	27.1±1.7	46.8±8.4 *, #
AST/ALT ratio	0.8±0.07	0.7±0.03	0.8±0.1

Data are represented as mean ± SEM. Similar to * and **, # and ## indicate p<0.05 and p<0.01, respectively. Asteriks (*) indicate significant changes compared with Normal. Hashtag (#) indicates significant changes compared with Steatosis.

Chapter 7

Cathepsin D regulates lipid metabolism in murine steatohepatitis

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Abstract

Due to the obesity epidemic, non-alcoholic steatohepatitis (NASH) is a prevalent liver disease, characterized by fat accumulation and inflammation of the liver. However, due to a lack of mechanistic insight, diagnostic and therapeutic options for NASH are poor. Recent evidence has indicated cathepsin D (CTSD), a lysosomal enzyme, as a marker for NASH. Here, we investigated the function of CTSD in NASH by using an *in vivo* and *in vitro* model. In addition to diminished hepatic inflammation, inhibition of CTSD activity dramatically improved lipid metabolism, as demonstrated by decreased plasma and liver levels of both cholesterol and triglycerides. Mechanistically, CTSD inhibition resulted in an increased conversion of cholesterol into bile acids and an elevated excretion of bile acids via the feces, indicating that CTSD influences lipid metabolism. Consistent with these findings, treating Wt BMDMs with PepA *in vitro* showed a similar decrease in inflammation and an analogous effect on cholesterol metabolism.

Conclusion

CTSD is a key player in the development of hepatic inflammation and dyslipidemia. Therefore, aiming at the inhibition of the activity of CTSD may lead to novel treatments to combat NASH.

Introduction

Due to the increasing obesity epidemic, non-alcoholic fatty liver disease (NAFLD) has emerged as the most important liver disease in the world.¹ NAFLD comprises a spectrum of liver diseases ranging from benign hepatic steatosis to more advanced liver diseases such as cirrhosis and fibrosis, which eventually might result in liver failure and death. The combination of steatosis and inflammation is referred to as non-alcoholic steatohepatitis (NASH). NASH is a disease stage that can further progress into such advanced liver diseases, underscoring the value of preventing/treating the inflammatory aspect of NAFLD. Currently, the etiology and mechanisms leading to obesity-induced liver inflammation are not clear, resulting in the lack of well-defined effective therapies.^{2,3}

Using mice lacking the low-density lipoprotein receptor (*Ldlr*^{-/-}), we previously demonstrated a positive correlation between hepatic inflammation and cholesterol accumulation in lysosomes of Kupffer cells (KCs).^{4,5} Moreover, lysosomal enzymes such as cathepsin D (CTSD) are increasingly recognized for their involvement in inflammatory responses.^{6,7} Whereas it was initially believed that proteases such as CTSD were only involved in non-specific protein degradation inside acidic lysosomes, research of the past decade has indicated that CTSD has a wide spectrum of functions, both of physiological and pathological nature.^{8,9} Under pathological inflammatory conditions, CTSD has been associated with distinct disorders such as rheumatoid arthritis, Alzheimer's disease, atherosclerosis and inflammatory bowel disease (IBD).¹⁰⁻¹³ Furthermore, recent data also revealed a correlation between hepatic inflammation and the activity and expression of CTSD in the liver.¹⁴⁻¹⁶ Relevantly, plasma CTSD levels correlated with different stages of NAFLD.¹⁷ These data suggest the involvement of lysosomes, and more specifically the lysosomal enzyme CTSD, in mediating the inflammatory response in NASH. However, despite the established relationship between lysosomes and inflammation as well as the awareness of an association between the lysosomal enzyme CTSD and hepatic inflammation, the exact function of CTSD in the context of NASH has not yet been investigated.

In the current study, we hypothesized that proteolytic inhibition of CTSD leads to reduced steatohepatitis. To induce NASH, *Ldlr*^{-/-} mice were put on a high-fat, high-cholesterol (HFC) diet for three weeks. This model was chosen due to its close resemblance with a human-like lipoprotein profile combined with the presence of hepatic inflammation, thereby serving as a physiological model to study NASH.¹⁸ To examine whether inhibition of CTSD decreases hepatic inflammation, *Ldlr*^{-/-} mice were injected with pepstatin A (PepA), an inhibitor of aspartyl proteases, for three weeks or only in the final week of the experiment. In agreement with our hypothesis, inhibition of the proteolytic activity of CTSD reduced hepatic inflammation. Remarkably,

proteolytic inhibition of CTSD dramatically improved lipid and lipoprotein metabolism, which is demonstrated by decreased plasma and liver levels of both cholesterol and triglycerides. Mechanistically, CTSD inhibition resulted in an increased conversion of cholesterol into bile acids as well as an elevated excretion of bile acids via the feces, indicating that CTSD influences lipid metabolism. Consistent with these findings, treating wildtype (Wt) bone marrow-derived macrophages (BMDMs) with PepA showed a similar decrease in inflammation and an analogous effect on cholesterol metabolism. These data demonstrate for the first time a key regulatory role for CTSD in lipid metabolism in the development of NASH. Hence, aiming at the modulation of CTSD activity in NASH holds therapeutic promise and should be further investigated in the future.

Methods

Mice, diet and intervention

Experiments were performed according to the Dutch regulation and approved by the Committee for Animal Welfare of Maastricht University. *Ldlr*^{-/-} mice on a C57BL/6 background were housed under standard conditions and given free access to food and water. Female 12 week-old *Ldlr*^{-/-} mice were fed either regular chow or a high-fat, high-cholesterol (HFC) diet (SAFE, Augy, France) for 3 weeks and were divided in three groups (n=11 for each group). The HFC diet contained 21% milk butter, 0.2% cholesterol, 46% carbohydrates and 17% casein. To examine whether proteolytic inhibition of CTSD would decrease hepatic inflammation, *Ldlr*^{-/-} mice were injected intraperitoneally with pepstatin A (PepA; 50 µg/g body weight; P5318, Sigma, Zwijndrecht, the Netherlands), a proteolytic inhibitor of aspartyl proteases. Mice were injected with DMSO (8%) or PepA two times every week. An overview of all experimental groups is depicted in Supplementary Figure S7.1. Collection of blood and tissue specimens, fluorescence-activated cell sorting (FACS), liver histology, plasma alanine transaminase levels (ALT), RNA isolation, cDNA synthesis and qPCR were determined as described previously^{4,15,19,20}.

Bone marrow-derived macrophages (BMDMs)

BMDMs were isolated from the tibiae and femurs of C57BL/6 mice. Cells were cultured in RPMI-1640 (GIBCO Invitrogen, Brede, the Netherlands) with 10% heat-inactivated fetal calf serum (Bondinco B.V. Alkmaar, the Netherlands), penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine 2 mM (GIBCO Invitrogen, Breda, the Netherlands), supplemented with 20% L929-conditioned medium (LCM) for 8-9 days to generate BMDMs. After attachment, macrophages were seeded at 350,000 cells per well in 24-well plates and incubated for 24h with oxLDL (25 µg/ml) or medium

(control). Afterwards, cells were washed and exposed to PepA (10µg/ml; dissolved in DMSO) or DMSO (0.06%) for 4h. Next, cells were washed and stimulated with or without LPS (100 ng/ml) for 4h. Finally, conditioned medium was used for enzyme-linked immunosorbent assays and cells were lysed for mRNA expression analysis. All *in vitro* data are the result of at least three independent experiments.

Statistical analysis

Data were statistically analyzed by performing two-tailed unpaired *t* test and Area Under the Curve (AUC) analysis using GraphPad Prism version 6 for Windows. Data were expressed as the mean and standard error of the mean (SEM) and were considered significantly different compared to control-treated mice on chow diet (* $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$) and compared to control-treated mice on HFC diet (# $p \leq 0.05$; ## $p < 0.01$; ### $p < 0.001$). Pearson correlation coefficients (*r*) and respective *p* values were calculated to assess the statistical significance of the correlation.

Results

Decreased systemic and hepatic inflammation in *Ldlr*^{-/-} mice after inhibition of CTSD

To elucidate the systemic immune effects of proteolytic inhibition of CTSD, blood leukocyte levels were determined via fluorescence-activated cell sorting after two weeks and at the end of the experiment. Administration of PepA for one week to *Ldlr*^{-/-} mice on HFC diet resulted in a significant decrease of blood leukocyte, B and CD4⁺ T cells after 3 weeks (Figure 7.1A and Supplementary Figure S7.2). Though not significant, similar trends were also observed for T cells, monocytes, NK cells, Ly6C^{high} monocytes, Ly6C^{int} monocytes, Ly6C^{low} monocytes and CD8⁺ T cells, confirming the reduced systemic inflammatory phenotype after proteolytic inhibition of CTSD (Figure 7.1A and Supplementary Figure S7.2). In contrast, no statistical differences were observed in blood granulocyte levels between the groups (Supplementary Figure S7.2).

To determine the effect of the proteolytic inhibition of CTSD on hepatic inflammation, immunohistochemical stainings were performed on liver sections for the inflammatory cell markers Mac-1, CD3, CD68 and F4/80. Quantification of all immunohistochemical stainings showed no effect of one week PepA treatment on hepatic inflammation in *Ldlr*^{-/-} mice on HFC diet at histological level (Figure 7.1B-D and Supplementary Table S7.1). This observation was also confirmed by H&E-staining (see Supplementary Figure S7.3A-B). Additionally, plasma alanine transaminase levels, indicative for liver damage, did not change upon one week PepA treatment (data not shown).

Figure 7.1 Parameters of systemic and hepatic inflammation in control- and PepA-injected mice. (A) Absolute levels of leukocyte, T cell, monocyte and B cell populations at week 2 and 3 of the experiment. (B) Liver sections were stained for infiltrating macrophages and neutrophils (Mac-1), T cells (CD3) and resident monocytes/macrophages (CD68). Positive cells were counted (infiltrated macrophages/neutrophils and T cells) or scored (CD68). (C, D) Representative pictures of the Mac-1 (C) and CD68 staining (D) (original magnification, 200x). (E) Gene expression analysis of *Tnfa*, *Ccl2*, *Caspase1* and *Cd68*. Data are shown relative to control mice on chow diet. Error bars represent $\pm$ SEM; * Indicates $p \leq 0.05$ and *** $p < 0.001$ compared to mice on chow diet; # indicates $p \leq 0.05$ and ### $p < 0.001$ compared to control-injected mice on HFC diet by use of two-tailed unpaired t test; for FACS $n=4$ animals per group and for immunohistochemistry and gene expression $n=8-11$ animals per group.

As inflammatory changes at histological level usually require administration of the therapeutic agent for more than one week before these effects can be observed¹⁹⁻²¹, we also performed hepatic gene expression analysis on the inflammatory markers tumor necrosis factor alpha (*Tnfα*), chemokine (C-X-C motif) ligand-2 (*Ccl2*), *Caspase1*, cluster of differentiation 68 (*Cd68*), interleukin-12 (*Il12*) and vascular cell adhesion protein (*Vcam*). As shown in Figure 7.1E, Supplementary Figure S7.3D and Supplementary Table S7.1, when *Ldlr*^{-/-} mice on HFC diet were treated with PepA for one week, hepatic gene expression levels of *Tnfα*, *Ccl2*, *Caspase1*, *Cd68* and VCAM were significantly reduced compared to control-treated *Ldlr*^{-/-} mice on HFC diet (Figure 7.1E). A similar trend was also observed for hepatic gene expression of *Il12* and for the M2 markers cluster of differentiation 206 (*Cd206*), Early growth response protein 2 (*Egr2*) and ratio of inducible nitric oxide (*iNos*) and arginase 1 (*Arg1*) (see Supplementary Figure 7.3C and Supplementary Table S7.1). Accordingly, hepatic protein levels of the inflammatory cytokines TNFα and IL12 were also reduced upon PepA administration (see Supplementary Table S7.1), indicating CTSD as a contributing compound in evoking the hepatic inflammatory response. Moreover, when injected over the total three weeks, *Ldlr*^{-/-} mice showed a nearly significant decrease of hepatic T cell levels, which was combined with an even stronger reduction of hepatic inflammatory markers at gene and protein expression level (see Supplementary Table S7.1). Altogether, these data demonstrate that inhibition of the proteolytic site of CTSD in *Ldlr*^{-/-} mice reduces the development of systemic inflammation and hepatic inflammatory gene expression, thereby pointing to a pro-inflammatory role of CTSD.

Improved hepatic lysosomal function after inhibition of intracellular and circulating CTSD is linked to hepatic inflammation in *Ldlr*^{-/-} mice

To investigate the effect of PepA on hepatic lysosomal function in diet-induced hepatic inflammation, plasma and hepatic CTSD activity as well as the activity the lysosomal enzyme acid phosphatase (AP) were measured in total liver. Treating *Ldlr*^{-/-} mice on HFC diet with PepA for one week resulted in a striking decrease of plasma CTSD activity compared to control-treated mice (Figure 7.2A). Furthermore, PepA-treated *Ldlr*^{-/-} mice on HFC diet demonstrated a reduction in hepatic CTSD activity (Figure 7.2B). In contrast, one week PepA-treatment did neither affect plasma nor hepatic cathepsin E (CTSE) activity (Supplementary Figure S7.4A-B). Collectively, these results indicate that PepA is a proteolytic inhibitor of intracellular and circulating CTSD. Of note, while *Ldlr*^{-/-} mice on a HFC diet showed increased plasma and hepatic CTSD activity compared to *Ldlr*^{-/-} mice on chow diet (Figure 7.2A-B), when compared to *Wt* mice on HFC diet, plasma CTSD (and not hepatic CTSD) activity increased in HFC-fed *Ldlr*^{-/-} mice (data not shown). These data suggest that circulating CTSD likely plays the most important role in developing hepatic inflammation.

Figure 7.2 Parameters of lysosomal function in control- and PepA-injected mice. (A, B) Plasma (A) and hepatic (B) activity of CTSD. (C) Hepatic gene expression analysis of *Ctsd*. Data are shown relative to control mice on chow diet by use of two-tailed unpaired t test. (D) Plasma levels of CTSD. (E-G) Correlation between plasma levels of CTSD and hepatic gene expression of *Tnfa*, *Ccl2* and *Caspase1* by Pearson correlation. Error bars represent $\pm$ SEM. ** Indicates $p < 0.01$ and *** $p < 0.001$ compared to mice on chow diet; # indicates $p \leq 0.05$, ## $p < 0.01$ and ### $p < 0.001$ compared to control-injected mice on HFC diet by use of two-tailed unpaired t test. $n = 9-11$ animals in each group. For correlation analysis, $n = 4$ animals on chow diet and $n = 10$ for mice on HFC diet.

Subsequently, the activity of acid phosphatase (AP) was measured in the liver. Although total hepatic AP activity was similar among all groups, control-treated mice on a HFC diet for 3 weeks showed reduced levels of lysosomal AP activity compared to mice on chow diet, confirming lysosomal dysfunction upon HFC diet (see Supplementary Figure S7.4C-D). In contrast, PepA-treated *Ldlr*^{-/-} mice on HFC diet demonstrated elevated levels of hepatic lysosomal AP activity compared to control mice on HFC diet (see Supplementary Figure S7.4D). To confirm these effects on lysosomal function, hepatic gene expression was measured for the lysosomal enzymes cathepsin D (*Ctsd*), cathepsin S (*Ctss*) and *Ap*. As shown in Figure 7.2C and Supplementary Figure S7.4E, hepatic gene expression of *Ctsd*, *Ctss* and *Ap* returned to chow levels after inhibition of CTSD, confirming the rebalancing of lysosomal function after CTSD inhibition. Additionally, to validate whether plasma CTSD levels correlate with the level of hepatic inflammation, plasma CTSD levels were measured. *Ldlr*^{-/-} mice on an HFC diet showed increased levels of plasma CTSD levels, whereas mice that received the PepA treatment, showed reduced plasma CTSD levels (Figure 7.2D). Moreover, hepatic gene expression levels of the inflammatory markers *Tnfa*, *Ccl2* and *Caspase1* correlated significantly with plasma levels of CTSD (Figure 7.2E), underlining the link between

hepatic inflammation and plasma CTSD levels. Together, these data reveal that proteolytic inhibition of intracellular and circulating CTSD improves diet-induced hepatic lysosomal dysfunction, which is connected to hepatic inflammation in *Ldlr*^{-/-} mice.

Improved lipid and lipoprotein metabolism in PepA-treated *Ldlr*^{-/-} mice on HFC diet

To determine whether inhibition of CTSD affects lipid metabolism, plasma and hepatic lipid levels were measured. Whereas plasma cholesterol and triglycerides were elevated upon feeding an HFC diet, administration of PepA significantly decreased both cholesterol and triglycerides in the plasma (Figure 7.3A-B). As shown in the fast protein liquid chromatography (FPLC) profiles, the reductions of both lipids were mainly attributed to a decrease in the VLDL fraction (see Supplementary Figure S7.5A-D). Similar to plasma cholesterol and triglyceride levels, hepatic cholesterol and triglyceride levels were also elevated in control-treated *Ldlr*^{-/-} mice on a HFC diet and decreased upon treatment with PepA (Figure 7.3C-D). These changes in hepatic lipid levels were confirmed by Oil red O staining (Figure 7.3E and Supplementary Figure S7.5E) and are in line with the changes observed in the relative liver weights of these mice (Supplementary Figure S7.5F).

To further define the influences of PepA-mediated CTSD inhibition on hepatic lipid metabolism, hepatic gene expression analysis was performed on markers involved in lipid homeostasis. Compared to control-injected *Ldlr*^{-/-} mice on HFC diet, PepA administration resulted in reduced gene expression levels of Cluster of differentiation 36 (*Cd36*), Niemann-Pick C2 (*Npc2*) and Liver X receptor alpha (*Lxra*), suggesting a reduced uptake, intralysosomal presence and efflux of cholesterol in these mice respectively (Figure 7.3F). In addition, *peroxisome proliferator-activated receptor gamma* (*Ppar-γ*) expression levels, a lipid-activated transcription factor of genes controlling lipid metabolism, were also decreased upon PepA administration, indicating a dramatic improvement in lipid homeostasis (Figure 7.3F).

Figure 7.3 Plasma and hepatic lipids in hyperlipidemic mice with or without PepA-treatment. (A, B) Plasma total cholesterol and triglyceride measurements. (C, D) Total hepatic cholesterol and hepatic triglyceride measurements. (E) Representative pictures (original magnification, 200x) of the Oil red O staining. (F) Gene expression analysis of *Cd36*, *Npc2*, *Lxra* and *Ppar-γ*. Gene expression data are shown relative to control mice on chow diet. Error bars represent $\pm$ SEM; * Indicates $p \leq 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to mice on chow diet; # indicates $p \leq 0.05$, ## $p < 0.01$ and ### $p < 0.001$ compared to control-injected mice on HFC diet by use of two-tailed unpaired t test. $n=9-11$ animals for each group analysis.

As the proteolytic inhibition of CTSD also appeared to influence hepatic cholesterol metabolism, hepatic synthesis and degradation of cholesterol were investigated in more detail. Inhibition of CTSD by PepA decreased hepatic cholesterol synthesis, as shown by measurements of hepatic desmosterol levels (Figure 7.4A). This finding was confirmed by gene expression levels of the rate-limiting enzyme for cholesterol synthesis, *3-hydroxy-3-methyl-glutaryl-Coenzyme A reductase (HmG-CoAR)* (Figure 7.4B).

Besides cholesterol synthesis, also cholesterol degradation was affected upon inhibition of CTSD. Specifically, whereas also 27-hydroxycholesterol (27HC; see Supplementary Figure S7.6A) levels were nearly significantly elevated, the conversion of cholesterol into 7 α -hydroxycholesterol (7 α HC) was significantly increased in PepA-injected *Ldlr*^{-/-} mice on a HFC diet compared to control-injected mice on HFD diet (Figure 7.4C). In line, hepatic gene expression levels of the cytochrome P450 7A1 (*Cyp7a1*) enzyme, responsible for this conversion, were elevated in PepA-treated *Ldlr*^{-/-} mice on a HFC diet, corroborating increased cholesterol degradation after inhibition of CTSD (Figure 7.4D). Furthermore, whereas plasma and hepatic bile acid levels remained unaffected (see Supplementary Figure S7.6B and S7.6C), PepA-treatment increased fecal bile acid (significant) (Figure 7.4E) and cholesterol (not significant) levels (Figure 7.4F). Overall, these data indicate that inhibition of CTSD activity results in increased bile acid synthesis eventually leading to excretion via the feces, thereby indicating an effect of CTSD on lipid metabolism.

Inhibition of the proteolytic function of CTSD reduces inflammation in oxLDL-loaded BMDMs

In order to explore the specific role of macrophages on the reduced inflammatory response upon CTSD inhibition, bone marrow cells of *Wt* mice were isolated, differentiated to macrophages and incubated with oxLDL for 24 hr. Subsequently, cells were treated with PepA for 4hr, followed by 4 hr stimulation with lipopolysaccharide (LPS). Upon incubation with PepA, cytokine levels of the pro-inflammatory marker TNF α , measured in the supernatant of the BMDMs, were significantly reduced compared to carrier (dimethyl sulfoxide (DMSO))-treated cells (Figure 7.5A, *left panel*). Additionally, cytokine levels of the anti-inflammatory marker IL10 increased after PepA treatment (Figure 7.5A, *right panel*), confirming the reduction in inflammation upon PepA treatment and the pro-inflammatory properties of CTSD in lipid-induced inflammation (as CTSE activity was also here not affected by PepA (see Supplementary Figure S7.7)). In line, *Tnfa* gene expression measured in BMDMs showed a decrease after PepA treatment. A similar trend was also observed for the expression of *Ccl2*, but this did not reach statistical significance (Figure 7.5B, *right panel*). The aggregated *in vitro* findings are in line with a reduction in hepatic inflammation upon PepA treatment, and by extension a pro-inflammatory action of CTSD.

Figure 7.4 Hepatic de novo synthesis and degradation of cholesterol. (A, C) Hepatic concentrations of desmosterol (A) and 7 α -hydroxycholesterol (7 α HC; C). Both values are shown relative to the hepatic cholesterol concentrations. (B, D) Gene expression analysis of HmG-CoAR and Cyp7a1. (E, F) Fecal bile acid (E) and cholesterol levels (F). Both measurements are relative to mg dry weight of the fecal sample. Gene expression data are shown relative to control mice on chow diet. Error bars represent $\pm$ SEM; * Indicates $p \leq 0.05$ and *** $p < 0.001$ compared to mice on chow diet; # indicates $p \leq 0.05$, ## $p < 0.01$ and ### $p < 0.001$ compared to control-injected mice on HFC diet by use of two-tailed unpaired t test; $n = 11$ for each group. DCA, deoxycholic acid; CA, cholic acid; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid; α MCA, α -muricholic acid; β MCA, β -muricholic acid.

To study whether the PepA-dependent reduction in inflammation was accompanied by an improvement in cholesterol metabolism, gene expression analysis was performed for *Cd36*, *Cyp27a1* and *HmG-CoAR*. In line with the hepatic *in vivo* gene expression data, cholesterol uptake (*Cd36*) and synthesis (*HmG-CoAR*) were reduced, though not significant, after PepA treatment in BMDMs. Next, *Cyp27a1*, the main enzyme responsible for the degradation of cholesterol in macrophages, appeared to be

upregulated, indicating higher levels of cholesterol degradation in BMDMs (Figure 7.5C). Thus, oxLDL-loaded BMDMs demonstrate a reduced inflammatory response upon proteolytic inhibition of CTSD, likely caused by an improved cholesterol metabolism.

Figure 7.5 Gene expression and cytokine levels of oxLDL-loaded bone marrow-derived macrophages (BMDM) treated with PepA. (A) TNF α and IL10 cytokine secretion in supernatant of *Wt* mouse BMDMs, exposed to oxLDL for 24hr and, subsequently, treated with or without PepA (4hr) and LPS (4hr). (B-C) Gene expression analysis of *Tnf α* , *Ccl2*, *Cd36*, *HmG-CoAR* and *Cyp27a1* measured in *Wt* mouse BMDMs. Gene expression data are shown relative to DMSO-treated BMDMs. All data represent the mean value of three or four independent experiments (with n=13-16 per group). Error bars represent $\pm$ SEM; * Indicates $p \leq 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to DMSO-treated bone marrow-derived macrophages by use of two-tailed unpaired *t* test.

Discussion

A major impediment to the development of well-defined, effective therapies for NASH has been the lack of understanding the mechanisms leading to the inflammatory component of this disorder. In the current study, we show for the first time that the lysosomal enzyme CTSD is a key player in the pathogenesis of NASH. Mechanistically, our data suggest that the pro-inflammatory function of CTSD is tied to a regulation of general lipid metabolism. In addition, we provide evidence for the central role of macrophages in exerting the lipid-induced effects of CTSD in the liver. These findings unravel CTSD as a promising, novel target for the treatment of NASH.

While CTSD was shown to have anti-inflammatory properties in the context of neutrophils²⁴, ample evidence points towards the pro-inflammatory effects of CTSD. In human intestinal epithelial cells and a mouse model for IBD, it was shown that activation of the lysosomal enzyme CTSD contributes to intestinal inflammation.^{12,25,26} Furthermore, inhibition of CTSD and CTSS reduced cardiovascular inflammation and attenuated atherosclerotic lesion progression, reiterating the contribution of cathepsins to pathological inflammatory responses.^{7,11,27,28} Notably, in the aforementioned IBD and atherosclerotic studies, the pro-inflammatory effects of CTSD were suggested to be derived from macrophages.^{7,12,25,26} Indeed, lysosomal enzymes in macrophages appear to play a critical role in withstanding the lipid challenge in obesity-induced inflammatory processes.^{29,30} In line, cytokine release was reduced in oxLDL-loaded BMDMs treated with PepA, substantiating the contribution of macrophages to CTSD-mediated hepatic inflammation. Surprisingly, hepatic macrophage levels (indicated by CD68 quantification) were not reduced after CTSD inhibition. Potential explanations for this finding might be a delayed egress of macrophages from the liver upon resolution of inflammation²³ or due to a prolonged half-life of macrophages.^{22,31} Relevantly, while we demonstrate a positive correlation between hepatic CTSD expression and hepatic inflammation, Fukuo *et al.* showed reduced levels of hepatic CTSD in NAFLD patients.¹⁴ However, these patients were not evaluated for the presence of inflammation and, thereby, cannot be used to link hepatic inflammation to reduced CTSD expression. Together, our current observations are in line with a causal relation between lipid-induced lysosomal dysfunction in hepatic macrophages and the level of inflammation in the liver.¹⁵ Thus, our data point towards a pivotal role for macrophage-derived CTSD in the development of hepatic inflammation.

Relevantly, proteolytic inhibition of CTSD resulted in reduced cholesterol and triglyceride levels in plasma and liver. This finding suggests that the improved NASH-phenotype in PepA-treated *Ldlr*^{-/-} mice is due to an effect of CTSD on lipid metabolism. In agreement with this finding, prevention of the proteolytic activation of the lysosomal

enzyme acid sphingomyelinase was shown to reduce cholesterol and triglyceride levels in the liver.³² Therefore, the influence of CTSD on lipid metabolism, as observed in this study, might not be unique to CTSD, but could rather be a shared mechanism of several lysosomal enzymes to control lipid homeostasis. In support of this concept, a deficiency in the gene encoding for CTSD results in a specific type of Batten disease, a disorder which is characterized by the cellular accumulation of lipid-containing residues.^{33,34}

There is considerable evidence contributing to the concept that a disturbance in bile acid metabolism interferes with cholesterol and, by extension, triglyceride metabolism. For example, a deficiency in the enzyme CYP7A1 was shown to lead to a hypercholesterolemic phenotype,³⁵ which is in line with our results that showed the same inverse relation between *Cyp7a1* and plasma cholesterol levels. Of note, patients with CYP7A1 deficiency were also reported to be hypertriglyceridemic. In agreement, triglyceride homeostasis and bile acid metabolism were revealed to be closely intertwined by regulation of farnesoid X receptor (FXR) and small heterodimer partner (SHP) on sterol regulatory element-binding protein (SREBP-1c), which is a known transcription factor of fatty acid and cholesterol synthesis.^{36,37} Similar to cholesterol levels, plasma and liver triglyceride levels in our study were inversely correlated with 7 α HC levels, the first precursor of the classical pathway of bile acid formation. In addition, hypertriglyceridemia has been associated with increased systemic inflammation.³⁸ Therefore, the overall observed effects on lipid metabolism in our study might be explained by a direct link between CTSD and bile acid metabolism. In agreement with this concept, though bile acids levels in plasma and liver were similar, fecal levels were dramatically increased after CTSD inhibition. Therefore, our data indicate that modulation of CTSD has the potential to increase bile acid excretion, resulting in reduced lipid levels in hyperlipidemic conditions (Figure 7.6).

The observation that both plasma (circulating) and hepatic (intracellular) CTSD activity were decreased after PepA inhibition suggests that the results in this study are derived from intra- and extracellular effects of CTSD. Indeed, intracellular CTSD-induced apoptosis was demonstrated to induce inflammation in the liver.³⁹ Therefore, though the strongest decrease in activity was observed in the circulating fraction of CTSD, the observed effects are likely a combination of the modulation of intra- and extracellular CTSD. Furthermore, besides CTSD, PepA can also inhibit the aspartyl protease CTSE, renin and pepsin, arguing the specificity for CTSD. Renin- and pepsin are proteases found in the kidney (renin) and digestive system (pepsin). As the reductions in inflammation were also observed *in vitro*, it is unlikely that either of them are involved in the observed effects in this study. Additionally, CTSE activity was unaffected in plasma, liver and BMDMs after PepA treatment, suggesting that the observed effects are mainly related to the inhibition of CTSD. Yet, it cannot be completely excluded that,

PepA also influenced other processes that contributed to the observed inflammatory effects in this study.

Figure 7.6 **Schematic overview illustrating the proposed by which CTSD participates in the development of hepatic inflammation.** Based on findings of the current study, increased levels of CTSD lead to a disturbance in lipid metabolism, which is known to result in increased plasma cholesterol (hypercholesterolemia) and triglyceride (hypertriglyceridemia) levels. Consequently, hypercholesterolemia and hypertriglyceridemia can lead to hepatic inflammation.

Enhancing lysosomal exocytosis in several lysosomal storage diseases strongly reduced substrate accumulation, suggesting a pivotal role for this mechanism in the setting of cellular homeostasis.⁴⁰ In the context of lipid storage, exocytosis of lysosomes may be a compensatory mechanism to ‘dump’ undigested lipids into the extracellular space as well as an elegant way to conduct signaling to protect the cell from lipid overload. In line with this concept, elevated plasma CTSD levels in mice on HFC diet were diminished after reduction of liver lipid levels. As such, the excretion of lysosomal enzymes may be a secondary consequence of a survival mechanism to protect the cell

from lipid overload. Furthermore, changes in lysosomal pH have been shown to alter processing and secretion of CTSD.⁴¹ Notably, intracellular accumulation of poorly degradable, oxidized lipid-protein cross-links was shown to alter the turnover of CTSD, leading to mistargeting of CTSD into the extracellular space.⁴² These data suggest that, by targeting specifically the circulating fraction of CTSD, lipid levels may be adjusted.

In summary, our study elucidates a key role for CTSD in the development of NASH. Our data suggest a novel mechanism by which CTSD affects lipid metabolism. Future studies are necessary to determine the exact underlying mechanisms explaining the observed effect in this study and whether other lysosomal enzymes possess similar effects as CTSD. Taken together, targeting CTSD has the potential to become an auspicious, therapeutic strategy for NASH.

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Supplementary information

Figure S7.1 Overview of all experimental groups of the *in vivo* experiment. Arrows indicate time of i.p. injection.

Figure S7.2 Blood leukocyte profile of granulocytes, NK cells, the monocyte subpopulations (Ly6C^{high}, Ly6C^{int} and Ly6C^{low}) and T cell subpopulations (T-helper (CD4) and cytotoxic T cell (CD8)) by use of two-tailed unpaired *t* test. Error bars represent $\pm$ SEM. * Indicates $p < 0.05$ compared to mice on chow diet and # $p < 0.05$ compared to control-injected mice on HFC diet. *n* = 4 animals per group. ns, not significant.

Figure S7.3 (A) General histology of the liver by a hematoxylin and eosin staining of control- and PepA-injected mice on a chow or HFC diet (original magnification, 200x). (B) Scoring for inflammation of H&E staining. (C) Hepatic gene expression analysis of *IL12* and *Vcam*. Data are shown relative to control mice on chow diet by use of two-tailed unpaired *t*. Error bars represent $\pm$ SEM. * Indicates $p \leq 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to mice on chow diet; # indicates $p < 0.05$ compared to control-injected mice on HFC diet. $n = 8-11$ animals per group. ns, not significant.

Figure S7.4 (A, B) Hepatic (A) and plasma (B) activity of CTSE. (C, D) Total and lysosomal activity of the lysosomal enzyme acid phosphatase (AP) in the liver of control- and PepA-injected mice. (E) Hepatic gene expression analysis of *Ctss* and *Ap*. Data are shown relative to control mice on chow diet by use of two-tailed unpaired *t* test. Error bars represent $\pm$ SEM. *** Indicates $p < 0.001$ compared to mice on chow diet; # indicates $p \leq 0.05$ compared to control-injected mice on HFC diet by use of two-tailed unpaired *t* test. $n = 9-11$ animals in each group.

Figure S7.5 (A, B) Using FPLC, plasma lipid and lipoprotein profiles were analyzed in all experimental groups. Sera of mice were pooled, creating $n=4$ for each experimental condition. On the chromatogram, the X-axis represents the fractions present in the mixture as a peak, thereby identifying the different components of the mixture. On the Y-axis, the amount of the different fractions can be read (mM). (C, D) Quantification of plasma cholesterol (C) and triglycerides (D) fractions. (E) Quantification of the Oil Red O staining. (F) Relative liver weight of control- and PepA-injected mice on a chow or HFC diet. * Indicates $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to mice on chow diet; # indicates $p<0.05$ and ## indicates $p<0.01$ compared to control-injected mice on HFC diet by use of the two-tailed unpaired t test. Error bars represent $\pm$ SEM.

Figure S7.6 (A) Hepatic concentration of 27-hydroxycholesterol (27HC) by use of two-tailed unpaired *t*. (B) Plasma bile acid levels. (C) Total hepatic bile acid levels. Error bars represent $\pm$ SEM. *** Indicates $p < 0.001$ compared to mice on chow diet. Plasma bile acid levels data are pooled from 11 mice, thereby creating $n=4$ for each experimental group; $n=9-11$ animals in each group. DCA, deoxycholic acid; CA, cholic acid; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid; αMCA, α-muricholic acid; βMCA, β-muricholic acid.

Figure S7.7 CTSE activity in BMDMs. Error bars represent $\pm$ SEM ($n=3$).

Table S7.1 Hepatic inflammation after 1 or 3 weeks of pepstatin A treatment.

	Chow		HFC	
	Control	Control	PepA (1w)	PepA (3w)
Histology				
<i>Infiltrating macrophages and neutrophils (Mac1)</i> (# positive cells/mm ²)	32.58 ± 5.191	122.7 ± 11.71***	124.6 ± 13.33	124.8 ± 6.16
<i>T cells (CD3)</i> (# positive cells/mm ²)	71.88 ± 9.59	130.6 ± 22.61*	132.8 ± 13.85	74.77 ± 7.77 a
<i>Monocytes/macrophages (CD68)</i> (A.U.)	1.4 ± 0.22	3.5 ± 0.31***	3.00 ± 0.33	3.00 ± 0.26
<i>Macrophage (F4/80)</i> (% positive area)	1.1 ± 0.21	1.3 ± 0.3	1.3 ± 0.2	0.9 ± 0.13
Gene expression (Rel. Exp.)				
<i>Tnfa</i>	1.00 ± 0.12	9.52 ± 1.27 ***	5.89 ± 0.91 #	5.25 ± 0.86 #
<i>Caspase1</i>	1.00 ± 0.10	1.98 ± 0.14 ***	1.45 ± 0.14 #	1.36 ± 0.11 ##
<i>Ccl2</i>	1.00 ± 0.14	3.724 ± 0.29 ***	2.00 ± 0.33 ###	1.87 ± 0.47 ##
<i>Cd68</i>	1.00 ± 0.08	3.98 ± 0.22 ***	2.77 ± 0.20 ###	2.52 ± 0.34 ##
<i>Il12</i>	1.00 ± 0.18	4.57 ± 0.63 ***	3.60 ± 0.54	2.55 ± 0.35 #
<i>Vcam</i>	1.00 ± 0.24	1.50 ± 0.14	1.05 ± 0.11 #	1.01 ± 0.15 #
<i>Cd206</i>	1.00 ± 0.09	1.27 ± 0.12	1.03 ± 0.15	0.96 ± 0.12 b
<i>Egr2</i>	1.00 ± 0.18	7.06 ± 0.77***	4.48 ± 0.53#	3.87 ± 0.77##
<i>iNos/Arg1</i>	1.00 ± 0.3	7.37 ± 1.1***	6.13 ± 1.23	5.44 ± 0.82
Protein levels (pg/mg protein)				
TNFα	38.94 ± 4.19	58.37 ± 11.20	24.62 ± 3.28 ##	18.42 ± 2.27 ##
IL12	0.03 ± 0.01	0.12 ± 0.04 *	0.02 ± 0.01 #	0.02 ± 0.01 #

*Indicates $p < 0.05$ and *** $p < 0.001$ compared to mice on chow diet; # indicates $p \leq 0.05$, ## $p < 0.01$ and ### $p < 0.001$ compared to control-injected mice on HFC diet; a indicates $p = 0.06$ and b indicates $p = 0.09$ compared to control-injected mice on HFC diet by use of two-tailed unpaired t test. Standards of error represent $\pm$ SEM.

Supplementary Methods

Acid phosphatase activity assay

Hepatic acid phosphatase (AP) activity was determined by the acid phosphatase assay kit (10008051, Cayman Chemical Company, USA). Liver homogenates were diluted 20 times in assay buffer, and transferred to a 96-well plate containing 20 μ L assay buffer. Next, 20 μ L AP substrate solution was added to each well to initiate the reaction. After 20 minutes incubation at 37°C, the reaction was stopped by adding 100 μ L of stop solution to all wells. The absorbance was measured using a Bio-Rad Benchmark 550 Micro-plate reader at 405-414 nm (170-6750XTU, Bio-Rad Laboratories, Veenendaal, the Netherlands).

Cathepsin D activity assay

Cathepsin D activity was measured using the cathepsin D activity assay kit (MBL International, Woburn, MA) according to the manufacturer's protocol. In summary, 50 μ g of liver homogenate or 5 μ L plasma was lysed in cathepsin D lysis buffer on ice for 10 minutes. Following centrifugation for 5 minutes at top speed, 5 μ L of clear cell lysate was transferred to a well of a 96-well plate, and the total volume was made up to 50 μ L with cathepsin D cell lysis buffer. To each assay, 52 μ L of mastermix (50 μ L if CTSD Reaction Buffer and 2 μ L of CTSD Substrate) was added, and the plate was incubated at 37°C for 1 hour. Samples were then measured using a fluorescence plate reader with a 328-nm excitation filter and 460-nm filter. Cathepsin D activity is expressed by the relative fluorescence units.

Cathepsin E activity assay

Cathepsin E activity was measured using the cathepsin E activity assay kit (BioVision, Milpitas, CA) according to the manufacturer's protocol. In summary, 50 μ g of liver homogenate, 5 μ L plasma or 50 μ g bone marrow-derived macrophages was lysed in cathepsin E lysis buffer on ice for 5 minutes. Following centrifugation for 10 minutes at top speed, 5 μ L of clear cell lysate was transferred to a well of a 96-well plate, and the total volume was made up to 50 μ L with cathepsin E cell lysis buffer. To each assay, 50 μ L of Substrate mix (50 μ L if CTSE Assay Buffer and 2 μ L of CTSE Substrate) was added, and the plate was incubated at 37°C for 1 hour. Samples were then measured using a fluorescence plate reader with a 320-nm excitation filter and 420-nm filter. Cathepsin E activity is expressed by the relative fluorescence units.

Plasma lipid analysis

Both plasma and liver lipid levels were measured with enzymatic color tests (cholesterol CHOD-PAP; 1489232; Roche, Basel, Switzerland; serum triglyceride (TG) determination kit, TR0100; Sigma-Aldrich; NEFAC, ACS-ACOD, 999-75406; Wako Chemicals, Neuss, Germany) according to the manufacturer's protocols on a Benchmark 550 Micro-plate Reader (170-6750XTU; Bio-Rad, Hercules, CA).

Liver lipid analysis

Approximately 50 mg of frozen liver tissue was homogenized for 30 seconds at 5000 rpm in a closed tube with 1.0-mm glass beads and 1.0 mL SET buffer (sucrose 250 mmol/L, EDTA 2 mmol/L, and Tris 10 mmol/L). Complete cell destruction was done by 2 freeze-thaw cycles and 3 times passing through a 27-gauge syringe needle and a final freeze-thaw cycle. Protein content was measured with the bicinchoninic acid (BCA) method (23225; Pierce, Rockford, IL). Cholesterol and triglycerides (TG) were measured as described above. Protocols were followed according to the manufacturers' instructions.

Fluorescence-activated cell sorting

FACS analysis was performed as previously described.¹ Graphs shown show the absolute values after 3 weeks (T3) and 2 weeks (T2).

Lipoprotein profiles

Lipoprotein profiles were determined on plasma samples from 4 mice (pooled from 11 to 4 mice of the same experimental group) and determined as described previously.²

Enzyme-linked immunosorbent assay (ELISA)

TNF α , IL10 and IL12 ELISA assays (resp. 88-7324-88, 88-7105-88 and 88-7121-88, eBioscience, Frankfurt, Germany) were performed on supernatant or liver homogenates according to manufacturer's instructions. Analysis was performed on a Bio-Rad Benchmark 550 Micro-plate reader 450 nm.

Measuring cholesterol precursors, oxysterols and bile acids

Cholesterol precursors, oxysterols and bile acids were measured via highly specific and sensitive gas chromatography-mass spectrometry as described previously.³⁻⁵ Fecal measurement was performed on feces isolated at sacrifice. Total hepatic bile acid levels were specifically determined as described previously.⁶

Primer Sequences for Quantitative PCR

Gene	Primer forward	Primer reverse
TNF α	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
CCL2	GCTGGAGAGCTACAAGAGGATCA	TCTCTCTTGAGCTTGGTGACAAAA
Caspase1	GGGACCCTCAAGTTTTGCC	GACGTGTACGAGTGGTTGTATT
CD68	TGACCTGCTCTCTAAGGCTACA	TCACGTTGCAAGAGAAACATG
CD36	GCCAAGCTATTGCGACATGA	AAAAGAATCTCAATGTCCGAGACTTT
NPC2	CGGAGCCCTGCACTTC	ACAGGGATCGGTGGGACAT
LXR α	CAACAGTGTAAACAGGCGCT	TGCAATGGGCCAAGGC
ABCA1	CCCAGAGCAAAAAGCGACTC	GGTCATCATCACTTTGGTCCTTG
PPAR γ	TCGCTGATGCACTGCCTATG	GAGAGGTCCACAGAGCTGATT
HmG-CoAR	ACCATGCCATCGATAGAGATAGGA	CGTGCGTTTTCTCCAGGATT
Cyp7a1	CATTACAGAGTGCTGGCCAAGA	CGCAGAGCCTCCTTGATGAT
Cyp27	CTGCACTTCTGCTGACCAAT	AGGGCCCATGTCAGTGTGTT
IL12	GGAAGTACACAAGAAGAGAG	AAGTCCTCATAGATGCTACCA
VCAM	GTGTTGAGCTCTGTGGGTTTTG	TTAATTACTGGATCTTCAGGGAATGAG
CTSD	CCTCCATTCAATGCAAGATACTTG	CACATAGGTGCTGGACTTGCTACT
CTSS	AAAGATTACTGGCTTGTAACAAACAG	GCAATTCCGCAGTGATTTTTATT
AP	GACCCTAATGGCAACTACCTCTCA	GGGAGCTTGCTTCCCATTTG
CD206	TGCAAAGGACTGAAAGGAAACC	CCAGTCCAGGCATTGAAAGTG
EGR2	CTACCCGGTGGAAGACCTC	AATGTTGATCATGCCATCTCC
iNOS	GCAAACCAAGGTCTACGTTCA	CCTCATTGGCCAGCTGCTT
Arg1	CATGGGCAACCTGTGTCCTT	CGATGTCTTTGGCAGATATGCA

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Chapter 8

Hematopoietic *Npc1* mutation shifts gut microbiota composition in *Ldlr*^{-/-} mice

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Submitted

Abstract

Background

While the link between diet-induced changes in gut microbiota and lipid metabolism in metabolic syndrome (MetS) has been established, the exact contribution of non-dietary factors explaining this relation is unclear. As several findings suggested a role for the lysosomal lipid transporter Niemann-Pick type C1 (NPC1) in macrophages for disturbing lipid homeostasis in MetS, we here explored whether a hematopoietic *Npc1* mutation also influences gut microbiota composition.

Methods

To mimic a human plasma lipoprotein profile, low-density lipoprotein receptor knockout (*Ldlr*^{-/-}) mice were fed a high-fat, high-cholesterol (HFC) diet for 12 weeks. Subsequently, they were transplanted with bone marrow from Niemann-Pick type C1 mutant (*Npc1*^{mut}) mice, which are known to develop lysosomal lipid accumulation. As a control, *Ldlr*^{-/-} mice were transplanted with wildtype (*Npc1*^{wt}) bone marrow. Fecal samples were used to profile the microbial composition by 16s ribosomal RNA gene sequencing.

Results

The hematopoietic *Npc1* mutation shifted the gut microbiota composition and increased microbial richness and diversity. Variations in plasma lipid levels correlated with microbial diversity and richness as well as with several bacterial genera.

Conclusion

This study shows that non-dietary disturbances in lipid metabolism such as a hematopoietic *Npc1* mutation affect the gut microbiome. Future research investigating the role of non-dietary factors (such as host genetics) on gut microbiota can therefore lead to identification of novel diagnostic and therapeutic targets for MetS.

Introduction

Metabolic syndrome (MetS) is a complex disorder that identifies centrally obese patients at increased risk for developing cardiovascular disease (CVD) and diabetes mellitus type 2.^{1,2} Due to the continuous increased prevalence of obesity, MetS is considered a global epidemic, putting an enormous pressure on health care services.³ Next to insulin resistance and hypertension, another central abnormality observed within MetS are disturbances in lipid metabolism, resulting in dyslipidemia.^{1,4} Dyslipidemia is characterized by increased plasma triglyceride-rich lipoproteins, decreased plasma high-density lipoprotein (HDL) and increased plasma low-density lipoprotein (LDL), leading to MetS-associated pathologies.⁴

Next to disturbances in plasma lipid levels, alterations in intracellular lipid metabolism have also been observed in MetS. While mutations in the gene encoding for the lysosomal membrane protein Niemann-Pick type C1 protein (NPC1) are well-known to induce a rare lysosomal storage disease, recent findings have also linked a dysfunctional NPC1 protein to the development of obesity and MetS.^{5,6} Mechanistically, a dysfunctional NPC1 protein leads to lysosomal lipid accumulation, thereby disturbing lipid metabolism.⁷ Likewise, the phenomenon of lipid accumulation within the endo-lysosomal compartment of cells has also been observed in pathologies associated with MetS.⁸ While lysosomal dysfunction in proximal tubular cells contributed to obesity-related kidney diseases,⁹ lysosomal lipid storage within macrophages is a well-described mechanism disturbing general lipid metabolism and results in the induction of a chronic low-grade inflammatory response in non-alcoholic steatohepatitis^{10,11} and atherosclerosis.^{8,12-14} These findings imply a role for macrophage NPC1 in disturbing lipid homeostasis in MetS.

Relevantly, several studies have shown a link between the composition of the gut microbiota and lipid parameters such as LDL, triglyceride and HDL levels.¹⁵⁻¹⁹ Moreover, modulating the gut microbiota by transferring the microbiota of lean donors has been shown to improve MetS in patients,²⁰ indicating the gut microbiota as a promising target to tackle metabolic disorders. However, the underlying mechanisms through which the gut microbiota modulates host's metabolism remain largely elusive.²¹ Gaining knowledge into the exact interactions between dietary and non-dietary factors (such as host genetics) on gut microbiota and associated pathophysiological processes is therefore essential to improve our diagnostic and therapeutic approaches to tackle metabolic pathologies.

The current study investigated whether a hematopoietic *Npc1* mutation influences the gut microbiota. To mimic a human plasma lipoprotein profile, we used *Ldlr*^{-/-} mice, which were fed a high fat, high cholesterol (HFC) diet for 12 weeks.²² *Ldlr*^{-/-} mice received bone marrow from Niemann-Pick type C1 mutant (*Npc1*^{mut}) mice. This study

demonstrates that non-dietary disturbances in lipid metabolism such as a hematopoietic *Npc1* mutation shifts the gut microbiota composition and increases microbial richness and diversity. Variations in plasma lipid levels correlated with microbial diversity and richness as well as with several bacterial genera. Future research investigating the role of non-dietary factors (such as host genetics) on gut microbiota can therefore lead to identification of novel diagnostic and therapeutic targets for MetS.

Materials and methods

Experimental design

Niemann-Pick type C1^{nh} mutant (*Npc1*^{mut}) mice (a kind gift from Prof. Dr. Lieberman from University of Michigan Medical School) were backcrossed into a C57BL/6 background for more than 10 generations. *Npc1*^{mut} and *Ldlr*^{-/-} mice were housed under standard conditions and had access to food and water ad libitum. Experiments were performed according to Dutch regulations and approved by the Committee for Animal Welfare of Maastricht University.

To generate myeloid *Npc1*^{mut} deficient *Ldlr*^{-/-} mice, a bone marrow transplantation was performed. Twenty-two week-old female *Ldlr*^{-/-} mice received one week before and four weeks after irradiation antibiotic water containing 100 mg/l neomycin (Gibco, Breda, the Netherlands) and 6*10⁴ U/l polymyxin B Sulphate (Gibco, Breda, the Netherlands). One day before and on the day of the transplantation, *Ldlr*^{-/-} mice were lethally irradiated with 6 Gray of γ -radiation, thus receiving 12 Gray in total. Lethally irradiated *Ldlr*^{-/-} mice were then injected with 1*10⁷ bone marrow cells donated from either *Npc1*^{mut} mice or wildtype littermate controls (*Npc1*^{wt}). In order to fully ensure bone marrow replacement, mice had a nine week recovery period. After nine weeks of recovery, transplanted (-tp) mice received an HFC diet, containing 21% butter and 0.2% cholesterol (diet 1635; Scientific Animal Food and Engineering, Villemoissons-sur-Orge, France) for 12 weeks.

Sample collection and processing

Mice were housed in groups of 4 in separate cages, but in the same room throughout the study. Fecal samples were snap-frozen with liquid nitrogen and stored at -80°C. DNA isolation was done using the FavorPrep Stool DNA Isolation Mini Kit (FASTI 001-1) according to manufacturer's protocol.

Sequencing

Amplicon libraries and sequencing was performed according to previously published protocols.^{15,23} Briefly, the hypervariable region V4 of the 16S rRNA gene was PCR amplified from each DNA sample using forward primer 515F and reverse primer 806R as described previously.¹⁵ Illumina MiSeq paired end sequencing of equimolarly pooled amplicons was subsequently used to determine the bacterial composition of fecal samples. Custom scripts were used to remove primer sequences, align paired end reads and quality-filter the sequencing reads. Details can be found in Fu *et al.*¹⁵ and Bonder *et al.*²³

Data analysis and statistics

The V4 16S rDNA bacterial sequences that were used in this paper have been submitted to the Qiita databases under study ID 10939.

Sequences were clustered into Operational Taxonomic Units (OTUs) using UCLUST (version 1.2.22q) at 97% similarity. For taxonomic assignments of OTUs, a filtered and truncated version of the full Greengenes reference database version 13.5 was used. OTUs that did not cluster against any of the sequences in the reference database were suppressed.

A total of 1,503,804 paired-end sequences, with a sequencing depth ranging from 15,637-117,585 reads/sample, were clustered in a total of 13,312 OTUs. To further reduce spurious OTUs and normalize for sequencing depth, data were rarefied to 10,000 reads/sample and OTUs containing less than 5 reads or occurring in only a single sample were discarded, resulting in a final number of 2,695 OTUs. Downstream analysis were conducted in QIIME version 1.9²⁴ and R version 3.1.3.

Microbial data-analyses and statistics

Taxonomic composition

Differences in the relative abundance of bacterial phyla and genera between *Npc1*^{wt}-tp and *Npc1*^{mut}-tp *Ldlr*^{-/-} mice were compared using Metastats (q=0.15; 750 permutations). Relations between bacterial phyla/genera and other continuous variables were analyzed via Spearman correlation using GraphPad Prism, version 6.0 for Windows. Though correlations were calculated on pooled data to increase the power, it was verified whether both groups showed the same effect.

Microbial richness & diversity and microbial community structure

The following metrics of species richness and diversity within communities (alpha-diversity) were determined: observed OTUs (observed richness), Chao1 index (estimated richness), and Shannon diversity index. Alpha diversity metrics between the

wild-type and mutant animals were compared by Mann Whitney tests using GraphPad Prism, version 6.0 for Windows. Beta-diversity, or diversity shared across samples was determined by the Bray-Curtis dissimilarity (BC) at a rarefaction depth of 10,000seq/sample. Clustering of samples was visualized using Principal Coordinate analysis (PCoA) followed by distance-based redundancy analysis (db-RDA), a constrained extension of PCoA. Db-RDA was performed in R²⁵ package *vegan* using the *capscale* function. To determine to what extent the hematopoietic *Npc1* mutation contributed in explaining the microbial community structure, we used variance partitioning with distance-based redundancy analysis (db-RDA) of Bray-Curtis distances. In a subsequent db-RDA, the following data were additionally included as explanatory variables to examine whether they had a significant impact on the microbial community structure: liver cholesterol, liver triglycerides, plasma cholesterol, plasma triglycerides, plasma free fatty acids, plasma copper oxLDL, plasma EO6, cage number and hepatic gene expression of *Abcg1*, *Npc2* and *Abca1*.

Enterotyping analyses

Enterotype analyses were performed as described previously by Armougom and colleagues.²⁶ Bray-Curtis (BC) distances were calculated for the genus-level relative abundance profiles. We used the R²⁵ package “*vegan: Community ecology*”, version 2.2-1 by Oksanen et al. from 2011 for calculating the Bray-Curtis distances.²⁷

To cluster the samples based on these distance metrics, we used the Partitioning Around Medoids (PAM) clustering algorithm in the R package “*Cluster analysis basics and extensions*”, version 2.0.1 ed. by Maechler et al. from 2012.²⁸ The optimal number of clusters was chosen based on Calinski-Harabasz (CH) index and validated by the silhouette index²⁹ using the R package ‘*clusterSim*’.

An optimal number of 2 clusters was identified based on the CH index, which was confirmed by the silhouette index although clustering was weak (SI 0.28).

Subsequent visualization and identification of relevant taxa was conducted for analyses based on BC distance. Between-class analysis (BCA) was performed to plot the samples using the R package “*Analysis of Ecological Data: Exploratory and Euclidean Methods in Environmental Sciences*” version 1.7.2. by Dray et al. from 2015. The similarity percentage analysis (SIMPER)³⁰ was used to identify taxa contributing to similarity within- and dissimilarity between groups.

Results

Hematopoietic *Npc1* mutation disturbs intracellular and circulating lipid levels

To confirm successfulness of the bone marrow transplantation, intracellular and circulating lipid levels were measured in *Npc1^{mut}-tp Ldlr^{-/-}* mice. Subjecting *Npc1^{mut}-tp Ldlr^{-/-}* mice to a HFC diet for 12 weeks resulted in a strong increase in hepatic cholesterol levels, while a reduction was observed for hepatic triglyceride levels (Table 8.1). Moreover, due to the hematopoietic *Npc1* mutation, hepatic lipids accumulated in the lysosomal fraction of hepatic macrophages (Figure 8.1A–B).

Figure 8.1 Hepatic phenotype of HFC-fed *Ldlr^{-/-}* mice carrying a hematopoietic *Npc1* mutation. (A) Representative images of hepatic staining for hematoxylin and eosin (H&E). (B) Representative electron microscopy image of hepatic macrophages carrying a hematopoietic *Npc1* mutation in HFC-fed *Ldlr^{-/-}* mice.

Additionally, plasma cholesterol, triglyceride and free fatty acid levels decreased significantly, indicating a decrease of circulating lipids in *Npc1^{mut}-tp Ldlr^{-/-}* mice (Table 8.1). In line with these data, hepatic gene expression levels of ATP-binding cassette

sub-family G member 1 (*Abcg1*), Niemann-Pick type C2 (*Npc2*) and ATP-binding cassette transporter A1 (*Abca1*) increased in *Npc1^{mut}-tp Ldlr^{-/-}* mice, confirming the disturbances in lipid metabolism. Overall, these data show that a hematopoietic *Npc1* mutation induces lysosomal lipid storage in hepatic macrophages and disturbs general lipid metabolism in *Ldlr^{-/-}* mice on a HFC diet.

Table 8.1

	<i>Npc1wt-tp Ldlr^{-/-}</i>	<i>Npc1mut-tp Ldlr^{-/-}</i>
Liver total cholesterol (µg/mg dry weight)	47.03 ± 4.47	75.47 ± 2.51 ***
Liver total triglycerides (mg/mg protein)	0.51 ± 0.06	0.26 ± 0.04 **
Plasma total cholesterol (mM)	38.53 ± 1.44	22.1 ± 1.09 ***
Plasma total triglycerides (mM)	3.82 ± 0.31	1.42 ± 0.15 ***
Plasma free fatty acids (mM)	1.33 ± 0.04	0.96 ± 0.05 ***
<i>Abcg1</i> (Rel. Expr.)	1.00 ± 0.09	2.65 ± 0.13 ***
<i>Npc2</i> (Rel. Expr.)	1.00 ± 0.1	3.06 ± 0.12 ***
<i>Abca1</i> (Rel. Expr.)	1.00 ± 0.12	1.13 ± 0.06

Microbial richness and diversity are increased and linked to lipid metabolism

To investigate the influence on microbial richness and diversity, α -diversity metrics were calculated. Both the observed microbial richness (observed number of OTUs) as well as the estimated richness as indicated by the Chao 1 index, was significantly higher in *Ldlr^{-/-}* mice carrying the hematopoietic *Npc1* mutation (Figure 8.2A and 8.2B). Likewise, microbial biodiversity, measured via the Shannon index (Figure 8.2C) was significantly increased in *Npc1^{mut}-tp* mice. To further define which variables are linked to microbial richness and diversity in this mouse model, Spearman correlation analysis was performed on all variables and the Chao 1 (richness) or Shannon (biodiversity) index. Plasma free fatty acids levels were negatively correlated with microbial richness ($r=-0.47$; $p=0.03$) (Figure 8.2D). Concomitantly, plasma total cholesterol levels showed the same inverse correlation with microbial richness ($r=-0.41$; $p=0.07$) (Figure 8.2E), though this correlation did not reach statistical significance. Furthermore, biodiversity correlated positively with hepatic gene expression levels of the intracellular cholesterol transporter Niemann-Pick type C2 (*Npc2*) ($r=0.47$; $p=0.04$) (Figure 8.2F) and negatively with plasma total cholesterol levels ($r=-0.56$; $p=0.007$) (Figure 8.2G). Together, these data demonstrate that a hematopoietic *Npc1* mutation in *Ldlr^{-/-}* mice increases microbial richness and biodiversity, which is correlated to changes in lipid metabolism.

Figure 8.2 Alpha diversity metrics in *Ldlr*^{-/-} mice on HFC diet carrying a hematopoietic *Npc1* mutation compared to wildtype. (A-C) The number of OTUs (A), Chao 1 index (B) and Shannon index (C) for both groups are shown. (D-G) Spearman correlation between alpha diversity metrics and lipid parameters. * Indicates $p < 0.05$. All errors are SEM.

The microbial community structure is related to the hematopoietic *Npc1* mutation but the latter is not linked to enterotypes

The dissimilarity in the microbial community composition (beta-diversity) of stool samples was assessed using the Bray-Curtis (BC) dissimilarities. Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarities showed that samples could be partly separated based upon the hematopoietic *Npc1* mutation (Figure 8.3A). Moreover, the within-group distance in microbial community structure (i.e. average Bray-Curtis dissimilarity between samples from the same treatment group) was statistically significantly smaller than the between-group distance, indicating that the microbiota composition of animals within the same treatment group is more similar compared to animals from different treatment groups (Figure 8.3B). Enterotype analyses revealed two enterotypes driven by *Allobaculum*, *Lactobacillus* and S24-7 (Figure 8.3C). However, the clustering was not associated with the bone marrow-specific *Npc1* mutation.

Figure 8.3 Principal Coordinate Analyses (PCoAs) based on Bray-Curtis distance and assortment of gut microbial communities into enterotypes for HFC-fed *Ldlr*^{-/-} mice carrying a hematopoietic *Npc1*^{wt} or *Npc1*^{mut} mutation. (A) PCoA-plot based on unweighted UniFrac metrics for all samples. (B) Bray-Curtis distances were calculated within *Npc1*^{wt}-transplanted and *Npc1*^{mut}-transplanted HFC-fed *Ldlr*^{-/-} mice (average pairwise distance in microbiota composition between samples of the same experimental group) and between *Npc1*^{wt}-transplanted and *Npc1*^{mut}-transplanted HFC-fed *Ldlr*^{-/-} mice (average pairwise distance between samples of different experimental groups). (C) Shown are between-class analysis visualizations of enterotypes, as identified by PAM clustering, with closed dots and squares representing individuals mice and numbered white rectangles marking the center of each enterotype. * Indicates $p < 0.05$.

To determine whether the hematopoietic *Npc1* mutation or other variables had an additional effect on the gut microbiota community structure, we subsequently performed distance-based redundancy analysis (dbRDA); a constrained ordination technique. Constrained ordination techniques attempt to explain differences in microbial composition between samples by differences in explanatory variables (e.g. hematopoietic *Npc1* mutation). In db-RDA, the information from explanatory variables is combined with the eigenvalues obtained from PCoA. The hematopoietic *Npc1* mutation ($p=0.034$, explained variance =18.9%) as well as hepatic gene expression levels of the cholesterol transporter *Abcg1* ($p=0.041$, explained variance =20.8%) showed a relationship with the microbial community structure (Figure 8.4A-B), whereas *Abca1*, *Npc2*, plasma total cholesterol, plasma total triglycerides, plasma free fatty acids, plasma copper oxLDL, plasma EO6, cage number, liver total cholesterol and liver total triglycerides did not.

Figure 8.4 Distance-based Redundancy Analysis (db-RDA) plot showing the relationship of the hematopoietic deficiency for *Npc1* and *Abcg1* to the microbial community structure. The plots represent a dbRDA ordination based upon the Bray-Curtis distance including (A) *Npc1*^{wt-tp} and *Npc1*^{mut-tp}-transplanted *Ldlr*^{-/-} mice and with the hematopoietic *Npc1* mutation as explanatory variable or (B) *Npc1*^{wt-tp} and *Npc1*^{mut-tp}-transplanted *Ldlr*^{-/-} mice, colored to hepatic expression of *Abcg1*, and with plasma cholesterol, plasma triglycerides, liver triglycerides, plasma free fatty acids, cage number, liver cholesterol, plasma EO6, plasma CuOxLDL and hepatic gene expression of *Abcg1*, *Npc2*, *Npc1* and *Abca1* as explanatory variables. MDS1 represents the unconstrained axis. CAP1 and CAP2 represent, respectively, the first and second constrained axes used in the CAP (canonical analysis of principal coordinates).

Hematopoietic *Npc1* mutation shifts gut microbiota composition

Based on 16S sequencing, 4 phyla and 44 genera were analyzed (Figure 8.5A-B). Firmicutes, Bacteroidetes and, to a lesser extent, Actinobacteria and Proteobacteria were the dominant observed phyla (Figure 8.5A). The relative abundance of the bacterial phyla was not significantly different between *Npc1*^{wt-tp} and *Npc1*^{mut-tp} mice (Figure 8.5A), however, within the Firmicutes phylum significant differences were observed in the relative abundance of several bacterial genera. Specifically, whereas *Staphylococcus spp.* ($p=0.008$; $q=0.34$) and unclassified *Mogibacteriaceae spp.* ($p=0.008$; $q=0.34$) showed significantly higher levels in relative abundance in the *Npc1*^{mut-tp} mice compared to *Npc1*^{wt-tp} mice, the abundance of *Allobaculum spp.* ($p=0.01$, $q=0.34$) was significantly lower (Figure 8.5B-E). Relevantly, the unclassified *Mogibacteriaceae spp.* correlated with hepatic gene expression levels of *Abcg1* ($r=0.57$; $p=0.009$) (Figure 8.5F) and plasma total triglyceride levels ($r=-0.41$; $p=0.07$), though the latter association was not statistically significant (Figure 8.5G). Furthermore, the genus *Allobaculum spp.* showed a positive correlation with plasma free fatty acid levels ($r=0.52$; $p=0.02$) (Figure 8.5H). Thus, a hematopoietic *Npc1* mutation alters the composition of gut microbiota, which are correlated with changes in lipid metabolism.

Relative abundance

Sample	Actinobacteria	Bacteroidetes	Firmicutes	Proteobacteria
Npc1wt-tp	0.01	0.24	0.75	0.00
Npc1mut-tp	0.01	0.22	0.77	0.00

C

Staphylococcus

Relative abundance

NPC1^{wt,tp} NPC1^{mut,tp}

Genotype	Relative abundance
NPC1 ^{wt,tp}	~0.0003
NPC1 ^{mut,tp}	~0.04

D

[Mogibacteriaceae]: Other

Relative abundance

0.015

0.010

0.005

0.000

NPC1^{wt,tp} NPC1^{mut,tp}

**

E

Allobaculum

Relative abundance

NPC1^{wt-tp} NPC1^{mut-tp}

* indicates statistical significance (p < 0.05).

Group	Median	Q1	Q3	Min	Max
NPC1 ^{wt-tp}	~0.20	~0.17	~0.22	~0.13	~0.26
NPC1 ^{mut-tp}	~0.10	~0.05	~0.20	~0.02	~0.26

Figure 8.5 Taxonomy at phylum and genus level for *Npc1*^{wt}- and *Npc1*^{mut}-transplanted HFC-fed *Ldlr*^{-/-} mice. (A-B) An overview of the mean relative abundance at phylum level is given. (C) An overview of the mean relative abundance at genus level is given. Significant differences between the groups at genus level are presented in separate box-whiskers for *Staphylococcus* spp. (D), unclassified *Mogibacteriaceae* spp. (E) and *Allobaculum* spp. (F). (G-I) Spearman correlation between lipid parameters and relative abundances of unclassified *Mogibacteriaceae* spp. and *Allobaculum* spp. * Indicates $p < 0.05$ and ** $p < 0.01$.

Discussion

Whereas increasing evidence has linked diet-induced changes in gut microbiota to perturbations in lipid metabolism in MetS, the exact contribution of non-dietary factors explaining this relation is unclear. This study shows that non-dietary disturbances in lipid metabolism such as a hematopoietic *Npc1* mutation affect the gut microbiome.

Though *Npc1* was previously identified in a human GWAS for obesity,⁵ our study is the first to identify its direct impact on the gut microbiome in MetS. This observation suggests that host-derived genetic disturbances in lipid metabolism can impact the gut microbiome. Indeed, an increasing amount of reports have established the capacity of the host's genetic profile to shape the gut microbiome, thereby supporting our findings.^{31,32} Also, the observation that a hematopoietic *Npc1* mutation impacts the gut microbiota confirms the previously established link between lipid metabolism and gut microbiota composition. However, in contrast to genetically manipulating lipid metabolism, the link between lipid metabolism and gut microbiota has been mainly established based on studies wherein dietary components (i.e. high fat diet, prebiotics) altered lipid metabolism via influences on gut microbiota.^{33,34} Therefore, in addition to gut microbiota-mediated influences of dietary compounds on lipid metabolism, our study implies that also non-dietary factors that influence lipid metabolism can impact the gut microbiome. Overall, our findings suggest that the gut microbiota and lipid metabolism may be equivalent partners in a relationship where one affects the other and vice versa in order to maintain the host's homeostasis.

Our results also shed new light on the association between overall health status and the gut bacterial richness and diversity. Here, induction of the pathological hematopoietic *Npc1* mutation elevated bacterial richness and diversity. However, this is opposed to the concept that increased bacterial richness and diversity reflect ecosystem stability and resilience and is associated with overall health.³⁵⁻³⁹ Yet, our apparent discordant finding on the association between disease and richness/diversity has also been observed by Kasai *et al.* who observed increased levels of bacterial diversity in obese compared to lean subjects.⁴⁰ Moreover, Vandeputte *et al.* associated stool consistency, potentially due to long colonic transit time,^{41,42} with higher gut bacterial richness, hampering the view on bacterial richness and diversity as a marker for overall health status.

Among the shifted gut bacteria after hematopoietic *Npc1* mutation, we identified reduced relative abundances of the genus *Allobaculum spp.*. Several studies have previously shown that *Allobaculum spp.* abundance is reduced in response to dietary fat intake^{43,44} and correlates with variations in plasma HDL concentrations⁴⁵ and body weight.⁴⁶ Therefore, our finding is consistent with the proposed view that *Allobaculum spp.* can be considered beneficial for the physiology of the host.³³ Next, in a prospective cross-sectional study, *Staphylococcus spp.* abundance was positively correlated with energy intake in obese children.⁴⁷ Here, we found an increased abundance of *Staphylococcus spp.* in HFC-fed *Ldlr*^{-/-} mice carrying the hematopoietic *Npc1* mutation. As such, *Staphylococcus spp.* might be involved in regulating the host's lipid metabolism. Finally, increased relative abundances were observed of unclassified *Mogibacteriaceae spp.* in this study. Relevantly, a recent study by Harach *et al.* correlated levels of unclassified *Mogibacteriaceae spp.* with amyloid beta 42 levels in the brain of mice suffering from Alzheimer's disease.⁴⁸ As multiple reports have previously linked a dysfunctional NPC1 protein to Alzheimer's disease,⁴⁹⁻⁵¹ relative abundance changes in unclassified *Mogibacteriaceae spp.* might be directly related to mutations in *Npc1* and deserves further investigation.

Overall, this study shows the significant impact of non-dietary disturbances in lipid metabolism such as a hematopoietic *Npc1* mutation on the gut microbiome in the context of MetS, confirming the link between lipid metabolism and gut microbiota. In addition, in contrast to previous studies, our findings suggest that increased microbial richness and diversity do not necessarily associate with improved health. Future research investigating the role of non-dietary factors (such as host genetics) on gut microbiota can therefore lead to identification of novel diagnostic and therapeutic targets for MetS.

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Chapter 9

General discussion

A lack of mechanistic insight into the processes driving hepatic inflammation in NASH hampers the development of novel diagnostic and therapeutic tools. In the current thesis, we aimed to provide translational insight into the underlying mechanisms leading to NASH and metabolic syndrome. The most essential findings of the studies presented in the previous chapters will be discussed in the context of the current knowledge and suggestions for future research will be given.

Lysosomal lipid accumulation in blood-derived hepatic macrophages triggers hepatic inflammation

Upon receptor-mediated endocytosis in macrophages, cholesterol-rich low-density lipoprotein (LDL) particles enter the endocytic pathway, which involves breakdown of their internal components by the endo-/lysosomal compartment of the macrophage.¹ After a series of events, the lysosomal enzyme lysosomal acid lipase (LAL) hydrolyzes cholesteryl esters and triglycerides into free fatty acids and free cholesterol. Subsequently, free cholesterol is transported to the cytoplasm by the Niemann-Pick type C1 (NPC1) protein, a lysosomal membrane transporter. Following lysosomal efflux, cholesterol is stored in cytoplasmic lipid droplets as cholesteryl esters or is excreted by the cell.² When plasma LDL concentrations increase, due to obesity for example, the time that these particles reside in the circulation is prolonged, increasing the likelihood of LDL becoming modified (or oxidized). While oxLDL levels only makes up about 0.001% of native LDL in healthy individuals, this percentage can increase almost 2000-fold (i.e. up to 1.8%) in patients with metabolic syndrome.³ Also, hyperlipidemic mice demonstrated increased levels of atherosclerosis⁴ and hepatic inflammation⁵ which was related to increased circulating oxLDL levels. Therefore, under hyperlipidemic conditions, the tightly regulated process of lipoprotein uptake/efflux by macrophages is disturbed, leading to the formation of lipid-filled macrophages, or foam cells.⁶

While the development of foam cells is best described in the context of atherosclerosis, previous studies also demonstrated the presence of foamy hepatic macrophages both in hyperlipidemic mice⁷ as well as in NASH patients,⁸⁻¹⁰ suggesting a central role for hepatic macrophages in NASH pathogenesis. This hypothesis was reinforced by findings showing that inactivation or depletion of Kupffer cells (KCs), the resident hepatic macrophages, prevented both steatosis and inflammation.¹¹⁻¹³ Though we previously confirmed the essential role of hepatic macrophages, our data demonstrated that the level of hepatic inflammation was not correlated with the size of hepatic macrophage nor was the presence of foamy macrophages *per se* associated with an inflammatory response.¹⁴ Instead, we observed an association between hepatic inflammation and the amount of lipids that specifically accumulated in the lysosomes of hepatic

macrophages, suggesting that the subcellular localization of lipids rather than lipid accumulation alone determines the level of inflammation.^{5,15} A causal link, however, between lysosomal lipid accumulation in macrophages and hepatic inflammation was not yet found. To prove such a link, we transferred the bone marrow of Niemann-Pick type C1 mutant (*Npc1*^{mut}) mice to *Ldlr*^{-/-} mice that were then put on a high-fat, high-cholesterol diet (Chapter 4). By limiting the *Npc1*^{mut} gene to the hematopoietic cell line, we were able to assess the effects of increased lipid storage specifically in the lysosomes of macrophages. As such, we used these mice as a proof-of-concept model to causally link lysosomal lipid accumulation in macrophages to hepatic inflammation. As expected, mice exhibiting lysosomal lipid accumulation in macrophages developed severe hepatic inflammation and even showed early stages of fibrosis. Our data therefore suggest that lysosomal lipid accumulation in macrophages triggers hepatic inflammation.

Increasing anti-oxLDL IgM antibodies in plasma reduced lysosomal lipid accumulation, hepatic inflammation and fibrosis development, suggesting that a considerable fraction of the lysosomal lipids is derived from the oxLDL particle (Chapter 4). This is supported by the finding that the content of oxLDL particles is essential in mediating foam cell-induced inflammation.⁶ However, which of the components carried by the oxLDL particle accumulate in lysosomes of macrophages is not yet clear. While the cholesterol oxidation products 7 β -hydroxycholesterol and 7-ketocholesterol have been shown to correlate with inflammatory reactions^{16,17} and to accumulate in lysosomes,¹⁸ it remains to be proven whether their lysosomal accumulation indeed leads to inflammation. Other components of oxLDL particles that might cause lysosomal accumulation-induced inflammation include oxidized phospholipids, free fatty acids and other related oxidized lipid derivatives.¹⁹ All of these components are worthwhile candidates for further investigation in the context of hepatic inflammation.

The liver macrophage population consists of both resident KCs of embryonic origin and infiltrating monocytes derived from the bone marrow which differentiate into functional macrophages in the target organ^{20,21}. Under inflammatory conditions, however, resident KCs have been shown to attract monocytes from the circulation into the liver, eventually resulting in a dominance of blood-derived tissue-macrophages.²² Indeed, the functional role of resident KCs is maintenance of tissue homeostasis and resolution of inflammation,²³ while the blood-derived macrophages promote inflammation in response to a signal transmitted by the resident KCs.²⁴ Therefore, a functional repertoire of blood-derived hepatic macrophages is essential for an adequate response to inflammatory stimuli. These observations support our finding that functional KCs alone could not resolve diet-induced inflammation when blood-derived macrophages were dysfunctional due to the *Npc1* mutation. Therefore, while both blood-derived and resident macrophages play a role in coping with inflammatory

stimuli, functional blood-derived macrophages are essential in order to overcome such an insult.

OxLDL-induced inflammation is mediated via the release of lysosomal enzymes

In the current thesis, we have shown that plasma levels of the lysosomal enzyme cathepsin D (CTSD) have a significant diagnostic value for the detection of NASH in children and adults (Chapters 5 & 6). We also demonstrated that reducing CTSD activity under hyperlipidemic conditions reduced hepatic inflammation and improved lipid metabolism (Chapter 7). Together, these observations suggest a central role for lysosomes, and lysosomal enzymes, in regulating lipid metabolism and inflammation.

Initially, lysosomes were perceived as acidic, enzyme-containing intracellular compartments for the digestion of endocytosed cargo, but more recently lysosomes and their enzymes have also been associated with a variety of other processes involved in cell physiology.²⁵⁻²⁷ In Chapters 2 & 4, we highlighted oxLDL as a toxic compound leading to disturbances in lysosomal function and subsequently to inflammation. The effects of oxLDL on lysosomes (and their enzymes) and inflammation can be propagated by several underlying mechanisms.

Firstly, lysosomal accumulation of oxLDL-derived lipids has been shown to result in cholesterol crystal formation,²⁸ subsequently inducing lysosomal membrane permeabilization.²⁹ OxLDL-induced lysosomal membrane permeabilization in foam cells leads to the release of lysosomal enzymes into the cytoplasm. Once relocated to cytoplasm, these lysosomal enzymes can activate the inflammasome³⁰ and disturb autophagy.³¹ The release of CTSD into the cytoplasm can also activate the mitochondrial apoptotic pathway,^{32,33} which was shown to lead to inflammation.³⁴ Therefore, the release of lysosomal enzymes to the cytoplasm due to oxLDL-induced lysosomal membrane permeabilization can lead to an inflammatory response.^{29,35,36}

Inhibition of CTSD resulted in reduced hepatic inflammation and improved lipid metabolism (Chapter 7). It is therefore highly likely that CTSD has a functional role in mediating NASH pathogenesis. We observed increased levels of bile acid and cholesterol excretion, which likely resulted in reduced hepatic inflammatory levels. Though CTSD inhibition was achieved both extra- and intracellularly, the largest inhibition was observed in the circulating fraction. This observation suggests that the circulating (or plasma) fraction of CTSD likely contributed most to the observed effects.

Similar to the concept of cytokines and chemokines, the regulated exocytosis of endo-/lysosomal content (referred to as extracellular vesicles (EVs)) to the plasma also influences the presence of lysosomal enzymes in non-lysosomal compartments. EVs have been shown to operate as a signal transduction platform inducing the stimulation or inhibition of certain targets.³⁷⁻³⁹ Compounds found in EVs range from lipids (cholesterol, sphingomyelin and fatty acids)⁴⁰ to signaling molecules,⁴¹ microRNAs,⁴² cytokines^{37,43} and lysosomal enzymes. Exosomal (particles derived from endo- or lysosomes)-derived CTSD and lysosome-associated protein 1 (LAMP-1) were higher in the plasma of preclinical Alzheimer disease patients⁴⁴ and the cysteine cathepsins L and S have been identified in the exosomes of tumor fibroblasts.⁴⁵ In metabolic inflammatory diseases, EV-derived plasma cathepsin S was increased in atherosclerotic and diabetic patients and was proposed as a marker for adiposity.⁴⁶⁻⁴⁸ In patients suffering from the lysosomal lipid storage disorder Niemann-Pick type C1, EV-derived plasma cathepsin B, D and S concentrations were increased compared to healthy controls.⁴⁹⁻⁵¹ Together, these findings indicate that regulated exocytosis of EVs is an important mechanism by which lysosomal enzymes can enter the circulation.

Moreover, Truman *et al.* showed that oxLDL activates lysosomal acid sphingomyelinase (ASMase), the enzyme responsible for the conversion of sphingomyelin to ceramide.⁵² Together with others,^{53,54} they also showed that the activity of ASMase was required for the release of exosomes. Ceramides, which are essential for budding off exosomes to the extracellular space,⁵⁵ are also activators of CTSD, suggesting that CTSD is a downstream effector of the acid sphingomyelin pathway.^{56,57} Relevantly, immune cells such as macrophages show the highest degree of EV secretion.^{38,58,59} Therefore, changes in plasma CTSD levels in NASH patients (Chapters 5 & 6) might be triggered by oxLDL-induced lysosomal dysfunction in macrophages (Chapter 2 & 4), which activates the ASMase-ceramide pathway. The regulated exocytosis of endo-/lysosomal content should be considered an essential function of lysosomes and might therefore explain the presence of CTSD in the circulation.

Besides its role in intercellular communication, 'defecation' of lysosomal content into the extracellular environment has also been proposed as a mechanism to prevent intracellular accumulation of undigested cargo (such as lipids).^{60,61} Therefore, whether the observed plasma CTSD levels in NASH patients (Chapter 5 & 6) are the result of changes in the regulated exocytosis of EVs or rather are the consequence of a different mechanism (or a combination of both) is not clear and should be investigated in the future.

Alterations in gut microbiota are an additional component of metabolic syndrome

The human intestinal tract is estimated to harbor up to 10^{14} microbes, thereby outnumbering the amount of human cells in the body by ten-fold.⁶² Recent improvements in sequencing technologies and analytical methods have resulted in the recognition of the gut microbiota as an endocrine organ contributing to a variety of physiological processes.^{63,64} Owing to their essential role in maintaining host physiology (described in more detail in Chapter 3), alterations in the gut ecosystem trigger a wide range of perturbations leading to low-grade inflammation, excess lipid accumulation and loss of insulin sensitivity.^{65,66}

The tight interplay between gut microbiota, metabolism and inflammation makes it extremely difficult to unravel cause-consequence relationships that can explain disorders such as metabolic syndrome. Germ-free rodent models^{67,68} and cohousing experiments⁶⁹ have clearly indicated the impact of the gut microbiome on metabolic pathologies, but which parameters influence the gut microbiota (or vice versa) is not completely clear. In Chapter 8, we show that a hematopoietic *Npc1* mutation shifts the gut microbiota composition, providing evidence for a role for the host's genetic profile in determining gut microbiota composition. Likewise, other reports have supported the contribution of the host's genetic profile in shaping the gut microbiome.^{70,71} Besides genetics, another obvious component that influences gut microbiota is the composition of an individual's diet. High fat diet-induced changes in gut microbiota have been associated with metabolic syndrome-associated pathologies such as atherosclerosis,^{72,73} NAFLD/NASH,⁶⁵ insulin resistance⁷⁴ and adipose tissue dysfunction.^{65,75} However, malondialdehyde (which is a lipid peroxidation product contained inside oxLDL particles)-induced hepatic inflammation was independent of gut microbiota composition,⁷⁶ questioning the mechanistic role of gut microbiota to hepatic inflammation in this particular model. Other studies found associations between oxidized lipids and NASH development,^{77,78} but none of these studies could mechanistically prove causality for the gut microbiome leading to hepatic inflammation.

Other components influencing gut microbiota composition range from antibiotic treatments⁷⁹ and smoking⁸⁰ to air pollution⁸¹ and even the change of seasons.⁸² Therefore, it is clear that the gut microbiota is extremely sensitive to a whole range of changes. Nevertheless, how microbiota are exactly related to the aforementioned pathologies still remains a black box.

Overall, it is clear that the composition of the gut microbiome associates with physiological processes such as metabolism and inflammation. The major challenge for future researchers, however, encompasses the unravelling of the mechanisms by which the gut microbiota potentially contributes to diseases. Such knowledge can provide novel diagnostic and therapeutic tools to a wide range of metabolic and inflammation-related disorders.

Are pediatric and adult NASH two different diseases?

While plasma CTSD levels were reduced in pediatric NASH patients (Chapter 5), they increased in adult NASH patients relative to steatotic controls (Chapter 6). These remarkable differences between adult and pediatric NASH patients suggest that we are dealing with two different diseases.

Differences between adult and pediatric NASH have been described previously. Histological examination of 100 livers of pediatric NASH patients revealed two types of NASH.⁸³ Type 1 NASH resembled the histological pattern of adult NASH, featuring steatosis in acinar zone 3 with hepatocyte ballooning degeneration and lobular inflammation, but without portal inflammation (Table 9.1). In contrast, type 2 NASH (which was found in 51% of the children) was characterized by steatosis in acinar zone 1 with portal inflammation, but without hepatocyte ballooning (Table 9.1). Though follow-up studies showed the presence of a mixed histological NASH pattern within some adult patients (type 1 + type 2), type 2 NASH was mostly found in children.^{84,85}

Table 9.1 Histological characteristics of pediatric and adult NASH

Adults/Children (type1)	Histological characteristic	Children (type 2)
Yes	<i>Ballooning</i>	Generally not
Acinar zone 3	<i>Steatosis</i>	Acinar zone 1
Lobular	<i>Inflammation</i>	Portal

Based on these histological differences, several experts in the field have postulated that pediatric NASH has a distinct pathophysiology compared to adult NASH.⁸⁶ In line with this view, the observed discrepancies in plasma CTSD levels between children (Chapter 5) and adult (Chapter 6) NASH patients may be related to differences in disease pathogenesis.

A distinct pathophysiology between pediatric and adult NASH can also be related to differences in lysosomal physiology. As previously mentioned in this thesis, the presence of endo-/lysosomal content in the extracellular environment can be achieved

by regulated exocytosis of EVs.^{38,39} A central component in guiding these EVs to the plasma membrane is the cellular cytoskeleton. Age-related changes in components of the cytoskeleton, such as F-actin and vimentin, have been observed before.^{87,88} Therefore, age-induced changes in the cytoskeleton might have resulted in different regulation of exocytosis of EVs, resulting in different plasma CTSD levels in children and adult NASH.

Alternatively, age positively correlates with the amount of lysosomes.⁸⁹ Since accordingly the number of lysosomes is lower in children, it is expected that their overall capacity for a high influx of lipids (such as oxLDL) is also lower. Excess influx of lipids may then result in rupture of the lysosomal membrane.^{90,91} As a consequence, lysosomal enzymes will enter the cytosol where they will initiate apoptosis, thereby preventing the exocytosis of CTSD-containing EVs.³³ In contrast, adults have more lysosomes⁸⁹ and, therefore, a higher capacity for oxLDL handling. As the uptake of oxLDL increased exocytosis of EVs⁵² and influenced the trafficking of lysosomal enzymes,⁹² an increased uptake of oxLDL by lysosomes of macrophages in adult NASH patients could directly result in increased plasma levels of CTSD. Together, it is possible that the observed differences in plasma CTSD between pediatric and adult NASH patients is related to differences in the lipid storage capacity of lysosomes in children and adults.

Differences in pediatric and adult NASH may also be related to the gut microbiota, since pediatric and adult gut microbiota composition differ.^{93,94} The liver is considered to be a biological filter between the contaminated bowel and the sterile systemic circulation.^{95,96} It is therefore directly exposed to gut microbiota-derived toxins, which can impact liver physiology and pathology significantly.⁹⁷ Intriguingly, gut microbiota composition has been shown to affect EV release,⁹⁸ suggesting a possible mechanism by which gut microbiota can influence to the release of CTSD to the plasma.

However, in contrast to the hypothesis of a different pathology between pediatric and adult NASH, others believe that the heterogeneity of the histological pattern in pediatric NASH can be explained as an ongoing pathology (that starts in childhood and normally progresses in adulthood).⁸⁶ Yet, even if pediatric NASH is merely preceding adult NASH, the histological differences between NASH patients in general highlight a high variability in the disease and suggest, to the least, that the disease course is multifactorial. For instance, the pediatric and adult NASH patients that were included in our cohorts did not have the same country of residence (pediatric patients are Italian, while adults patients are Dutch, American and Finish). It is therefore possible that the differences in plasma CTSD levels between pediatric and adult NASH patients are due to a different place of residence or genetic/epigenetic background. To exclude the potential confounding effect of geographical location on plasma CTSD levels, we aim to

measure plasma CTSD levels in an Italian adult NASH cohort or in a pediatric Dutch, American or Finish cohort.

Overall, the findings described in this thesis highlight the differences between adult and pediatric NASH. Whether pediatric and adult NASH are indeed two different pathologies or whether one merely precedes the other needs to be meticulously investigated as the answers to this question might dramatically change our perspective on the disease course of NASH.

Clinical implications of the findings

Diagnosis

Currently, the clinical standard for evaluating the progression of hepatic inflammation is the histological examination of a liver biopsy.⁹⁹ However, acquiring a liver biopsy is an invasive procedure associated with procedural discomfort, hospitalization, sampling error and potentially severe complications.^{99,100} The development of non-invasive techniques for the diagnosis of hepatic inflammation in the context of NASH is therefore highly desirable.

Available non-invasive markers for assessing the presence of steatosis include a combination of blood tests such as aminotransferases (ALT, AST) and gamma-glutamyltransferase (GGT). Although these markers effectively diagnose steatosis, they do not allow to make the distinction between NASH and steatosis,¹⁰¹ suggesting that they are not suitable for the detection of hepatic inflammation. In the present thesis, we showed both in children and adults that plasma CTSD levels are a promising, novel tool to diagnose NASH (Chapter 5 & 6). Moreover, plasma CTSD significantly outperformed the currently used biomarker ALT in differentiating NASH from steatotic patients (Chapter 5 & 6). In line, by developing a computational model for identifying novel non-invasive markers for NASH, Yoshimura *et al.* identified CTSD as a marker to diagnose NASH, thereby confirming our initial findings.¹⁰²

The choice of investigating plasma CTSD as a diagnostic marker for NASH was based on the mechanistic finding of oxLDL-induced lysosomal dysfunction in macrophages (Chapter 4). Therefore, plasma markers that provide information about oxLDL levels might provide novel non-invasive tools to monitor NASH. Indeed, NASH patients carried increased levels of plasma lipid peroxidation products. In addition, naturally occurring IgM anti-oxLDL antibodies negatively correlated with hepatic inflammation.¹⁰³ Besides oxLDL, disturbances in lysosomal function might also be reflected in the plasma, for example by the modulated exocytosis of EVs. Therefore, next to CTSD, also plasma

levels of other lysosomal enzymes should be investigated in the future as a potential marker of NASH.

In conclusion, the results of this thesis suggest that plasma CTSD (and potentially other lysosomal enzymes) and plasma markers related to oxLDL should be further investigated as non-invasive markers for NASH.

Therapy

Due to a lack of knowledge in the underlying mechanisms leading to hepatic inflammation, no effective therapy for NASH is currently available. Given that recent estimations predict NASH to become the leading cause for liver transplantation worldwide in 2019,¹⁰⁴ the development of alternative therapeutic tools to combat NASH is warranted.

Currently, the most prescribed drugs for NASH patients are aimed at reducing lipid levels in general.^{105,106} While these drugs often demonstrated a successful reduction in steatosis, a concomitant decrease in hepatic inflammation could not be observed.^{105,107} Therefore, therapeutic interventions aimed at a specific molecular aspect of the pathogenesis might be more successful in reducing hepatic inflammation.

In the current thesis, we demonstrated that 1) lysosomal storage of oxLDL-derived lipids (Chapter 4) and 2) plasma CTSD increases hepatic inflammation (Chapter 7) *in vivo*. Therapies aimed at targeting these components might therefore lead to novel therapeutical tools for NASH. As described in Chapter 2, multiple approaches (e.g., vitamin E, betaine, pentoxifylline) have been taken to investigate the potential of antioxidants in reducing hepatic inflammation in NASH. Yet, an effective therapeutic drug that improves hepatic inflammation by modulating the oxidation process is not yet found. However, based on data from us (Chapter 4) and others,⁴ increasing anti-oxLDL antibodies by pneumococcal immunization reduces inflammation in the liver⁵ and arteries.⁴ Relevantly, as infections with *S. pneumoniae* regularly occur, it is generally advised that children are vaccinated against this bacterium at a young age.¹⁰⁸ For this reason, it will be useful to investigate whether these vaccinations have an influence on NASH development in humans.

We demonstrated that inhibiting CTSD activity leads to hepatic inflammation (Chapter 7). To our knowledge, no data has so far been generated on the effect of targeting plasma levels of CTSD to reduce inflammation. Additional research is therefore necessary to demonstrate whether plasma CTSD can indeed be considered as a therapeutic target for NASH.

Finally, we also show that a hematopoietic *Npc1* mutation (which leads to lysosomal lipid accumulation) can shift the gut microbiota composition (Chapter 8), suggesting that the gut microbiota might also contribute to phenotypes that are characterized by lysosomal lipid accumulation. Targeting the gut microbiota therefore has the potential to alleviate symptoms in certain diseases. For instance, dietary approaches such as prebiotics, which are known to modulate the gut microbiota composition, improved metabolism in obese and insulin resistant mice¹⁰⁹. Alternatively, dietary administration of living organisms, referred to as probiotics, could attenuate atherosclerosis¹¹⁰ and improve oxidative damage¹¹¹. As the gut microbiota appears to be a central endocrine organ, modulating its composition in metabolic diseases should be investigated in the future.

Overall, in the current thesis, we demonstrated two novel underlying mechanisms, i.e. lysosomal accumulation of oxLDL-derived lipids in macrophages and plasma CTSD, for developing NASH. Approaches that are aimed at modulating targets involved in these mechanisms might therefore provide novel therapeutic tools for NASH. In contrast to the increasing amount of drugs that are tested in clinical trials¹¹², the drugs we proposed emanate from a hypothesis-driven approach, thereby providing a strong translational platform which can be used to develop new drugs for NASH.

Novel findings of this thesis

In the present thesis, we aimed to provide translational insight into the underlying mechanisms leading to NASH and metabolic syndrome in general. The findings of the studies described in this thesis are summarized below.

- OxLDL is an essential inflammatory component in the pathogenesis of NASH. (Chapter 2 & 4)
- The gut microbiota is located at a crucial position in both metabolism and inflammation. (Chapter 3 & 8)
- Accumulation of oxLDL-derived lipids in lysosomes of hepatic macrophages triggers hepatic inflammation. (Chapter 4)
- Plasma cathepsin D is able to differentiate NASH from steatosis in children. (Chapter 5)
- Plasma cathepsin D might be used as a tool to monitor disease progression and regression in adult NASH patients. (Chapter 6)
- Plasma cathepsin D is involved in lipid metabolism and might be a novel target to reduce lipid levels and hepatic inflammation in NASH. (Chapter 7)
- A hematopoietic *Npc1* mutation influences gut microbiota composition (Chapter 8)
- Pediatric and adult NASH might be two different diseases. (Chapter 5 & 6)

Possible non-invasive markers for the diagnosis of NASH

- Plasma Cathepsin D
- Plasma oxLDL and related products
- Levels and activity of other lysosomal enzymes in the plasma

Possible therapeutic approaches/options for NASH

- Improving lysosomal function of macrophages
- Blocking activity of plasma cathepsin D
- Approaches that increase IgM anti-oxLDL antibodies
- Modulating gut microbiota composition

Summary of the main findings of this thesis. (A) Under normal conditions, lipids derived from low-density lipoproteins (LDL) are internalized by macrophages, where they enter the endo-lysosomal compartment. After degradation, they are exported from the lysosome to the cytoplasm via the Niemann-Pick type C1 (NPC1) transporter. Afterwards, lipids are stored in the cytoplasm or directed out of the cell. (B) In metabolic syndrome, patients have increased levels of plasma oxLDL. Though the uptake of oxLDL by macrophages can be blocked by binding of anti-oxLDL IgM antibodies, when oxLDL is internalized, it cannot be degraded by the endo-lysosomal compartment. Lysosomal accumulation of oxLDL-derived content in macrophages induces hepatic inflammation (Chapter 4) and shifts the composition of gut microbiota (Chapter 8). Additionally, plasma cathepsin D, likely as a result of lysosomal lipid storage, can be considered a marker (Chapter 6) and potential target (Chapter 7) for NASH. (Several elements of this figure were taken from Servier Medical Art).

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Summary

Summary

In **Chapter 1**, the central role of the liver in metabolic syndrome is introduced. After highlighting the clinical challenges of NASH (which is the hepatic manifestation of metabolic syndrome), lysosomal lipid accumulation in macrophages was proposed as a novel mechanism leading to NASH. Furthermore, we explain how this mechanism can be used as a translational platform to tackle the aforementioned clinical challenges. Finally, the content of this thesis is summarized.

Chapter 2 provides information on the central role of oxidized LDL in NASH. There, the biochemical generation of oxidized lipids are explained, and the mechanisms by which these oxidized lipids affect hepatic inflammation are summarized. Finally, the implications of these findings for patients are emphasized.

Chapter 3 describes the interplay between the gut microbiota and the intestinal immune system in the context of metabolic syndrome. An overview of the molecular aspects of the gut microbiota-immune system axis is given, and the influence of the diet on the regulation of this axis is highlighted. Finally, future perspectives for gut microbiota research are suggested.

In **Chapter 4**, we investigate whether lysosomal lipid accumulation in blood-derived hepatic macrophages is a causal trigger for the development of hepatic inflammation. We hypothesize that oxLDL-derived lipids are essential in mediating lysosomal lipid-induced hepatic inflammation. For this purpose, irradiated *Ldlr*^{-/-} mice were transplanted with bone marrow from either Niemann-Pick Type C1 mutant (*Npc1*^{mut}) or wildtype (*Npc1*^{wt}) mice and fed a high-fat, high-cholesterol (HFC) diet for 12 weeks. Due to the hematopoietic deficiency of the gene encoding the NPC1 protein, which is a lysosomal lipid transporter, lipids accumulate in lysosomes of blood-derived hepatic macrophages. As expected, hepatic inflammation and fibrosis were increased in the *Npc1*^{mut} transplanted mice. Accordingly, preventing the uptake of oxLDL by macrophages by increasing circulating anti-oxLDL IgM antibodies reduced hepatic inflammation and hepatic fibrosis. These findings were also confirmed *in vitro*. We conclude that blood-derived macrophages prone to accumulate lysosomal lipids trigger oxLDL-dependent murine steatohepatitis.

In **Chapter 5**, we hypothesize that plasma levels of the lysosomal enzyme cathepsin D correlate with the level of hepatic inflammation in children suffering from fatty liver disease. Liver biopsies of ninety-six children were evaluated and classified as having steatosis, borderline NASH or definite NASH, respectively. We demonstrated that plasma cathepsin D levels were significantly lower in children with definite NASH compared to children with borderline NASH or steatosis. Moreover, a reduction of

plasma cathepsin D levels corresponded with increasing hepatic inflammation, steatosis, hepatocellular ballooning and a higher NAFLD activity score. We conclude that plasma cathepsin D is a novel non-invasive tool to predict pediatric hepatic inflammation.

In **Chapter 6**, we hypothesize that the progression and regression of adult NASH correlates with plasma cathepsin D levels. We observed increased levels of plasma cathepsin D in adults with NASH compared to adults without NASH. Furthermore, after surgical intervention, plasma cathepsin D levels were reduced compared the levels measured before the intervention. We conclude that plasma cathepsin D levels correlate with adult NASH progression and regression, and propose implementation of plasma cathepsin D determination to monitor adult NASH patients.

In **Chapter 7**, we hypothesize that cathepsin D plays an active role in the development of hepatic inflammation during NASH. By injecting *Ldlr*^{-/-} mice with pepstatin A, an inhibitor of aspartyl proteases, we demonstrated that inhibition of cathepsin D reduced hepatic inflammation. Mechanistically, our data suggested that increased fecal excretion of bile acids improved lipid metabolism, resulting in decreased hepatic inflammation. The largest reduction in cathepsin D activity was observed in the circulating fraction, suggesting that circulating cathepsin D contributed most to the observed effects. Collectively, this chapter shows for the first time the role of cathepsin D in the pathogenesis of NASH.

In **Chapter 8**, we hypothesize that a hematopoietic *Npc1* mutation would shift the gut microbiota composition in *Ldlr*^{-/-} mice. Therefore, irradiated *Ldlr*^{-/-} mice were transplanted with bone marrow from either Niemann-Pick Type C1 mutant (*Npc1*^{mut}) or wildtype (*Npc1*^{wt}) mice and fed a high-fat, high-cholesterol (HFC) diet for 12 weeks. Due to the hematopoietic deficiency of the gene encoding the NPC1 protein, which is a lysosomal lipid transporter, lipids accumulate in lysosomes of blood-derived hepatic macrophages. The hematopoietic *Npc1* mutation shifted gut microbiota composition and increased microbial richness and diversity. We conclude that non-dietary changes in lipid metabolism such as a hematopoietic *Npc1* mutation can shift the gut microbiota composition.

Finally, **Chapter 9** discusses the major findings of the thesis, places them in perspective relative to their research field(s) and provides information regarding their value for clinicians.

Samenvatting

Samenvatting

In **hoofdstuk 1** wordt de centrale rol van de lever in het metabool syndroom geïntroduceerd en worden de klinische uitdagingen van NASH, de hepatische manifestatie is van het metabool syndroom, benadrukt. Wij stellen lysosomale lipide stapeling in macrofagen voor als een nieuw, onderliggend mechanisme dat leidt tot NASH. Vervolgens leggen we uit hoe dit mechanisme kan gebruikt worden als translationeel platform om de hiervoor aangehaalde klinische uitdagingen aan te pakken. Ten slotte wordt de inhoud van de thesis samengevat.

Hoofdstuk 2 verstrekt informatie betreffende de centrale rol van geoxideerd LDL in NASH. De biochemische reacties die aan de basis liggen van de vorming van geoxideerde lipiden worden uitgelegd. Daarnaast vat dit hoofdstuk ook samen via welke mechanismen deze geoxideerde lipiden leverontsteking beïnvloeden. Uiteindelijk worden de implicaties van deze bevindingen voor patiënten nog benadrukt.

Hoofdstuk 3 beschrijft de wisselwerking tussen darmbacteriën en het darm-geassocieerde immuun systeem in de context van het metabool syndroom. Naast het geven van een overzicht betreffende de moleculaire aspecten van deze as, wordt de invloed van het dieet op het reguleren van deze as benadrukt. Uiteindelijk worden de toekomstperspectieven van darmbacterie-onderzoek samengevat.

In **hoofdstuk 4** onderzochten we of er een oorzakelijk verband bestaat tussen leverontsteking en lysosomale stapeling van lipiden in levermacrofagen afkomstig van het bloed. We stelden de hypothese dat lipiden afkomstig van oxLDL partikels essentieel zijn voor het mediëren van lysosomale lipiden-geïnduceerde leverontsteking. Hiervoor verwijderden we eerst het aanwezige hematopoietische systeem van *Ldlr*^{-/-} muizen door ze bloot te stellen aan radioactieve straling. Vervolgens ontvingen deze muizen beenmerg afkomstig van Niemann-Pick type C1 mutant of wildtype muizen en kregen ze een hoog-vet, hoog-cholesterol dieet van 12 weken. Door de hematopoietisch-specifieke uitschakeling van het gen dat codeert voor het NPC1 eiwit, stapelen de lipiden zich op in de lysosomen van levermacrofagen afkomstig van het bloed. Zoals we verwachtten, waren leverontsteking alsook leverfibrose verhoogd in *Npc1*^{mut} getransplanteerde muizen. Verder stelden we vast dat het voorkomen van oxLDL-opname in macrofagen door anti-oxLDL IgM antilichamen te verhogen, resulteerde in een daling van leverontsteking en leverfibrose. Deze bevindingen konden wij ook *in vitro* bevestigen. We concluderen dat bloed-afgeleide macrofagen die vatbaar zijn voor de stapeling van lysosomale lipiden een oorzaak zijn voor oxLDL-afhankelijke steatohepatitis in muizen.

In **hoofdstuk 5** stelden we de hypothese dat plasma waarden van het lysosomale enzyme cathepsine D correleren met de hoeveelheid leverontsteking in kinderen, die gediagnosticeerd zijn met vette leverziekte. Leverbiopten van 96 kinderen werden geëvalueerd en geclassificeerd als steatose, borderline NASH of definitief NASH. We toonden aan dat plasma cathepsine D waarden significant lager waren in kinderen met definitieve NASH vergeleken met kinderen met borderline NASH of steatose. Verder kwam de daling van plasma cathepsine D waarden overeen met verhoogde leverontsteking, steatose, hepatocellulair ballooning en de NAFLD activiteitscore. We concludeerden dat plasma cathepsine D een nieuwe niet-invasieve methode is om leverontsteking te voorspellen in kinderen.

In **hoofdstuk 6** stelden we de hypothese dat er een verband bestaat tussen plasma cathepsine D waarden en de ontwikkeling en regressie van NASH in volwassenen. In volwassen NASH patiënten observeerden we verhoogde waarden van plasma cathepsine D vergeleken met volwassenen zonder NASH. Verder stelden we vast dat na een chirurgische ingreep, de plasma cathepsine D waarden daalden t.o.v. de meting uitgevoerd voor de ingreep. We concluderen dat plasma cathepsine D waarden correleren met de ontwikkeling en regressie van NASH in volwassenen. Wij stellen voor om plasma cathepsine D metingen te gebruiken om NASH op te volgen in volwassenen.

In **hoofdstuk 7** stelden we de hypothese dat cathepsine D een actieve rol speelt in de ontwikkeling van NASH. Door *Ldlr*^{-/-} muizen te injecteren met pepstatine A, een inhibitor van aspartyl proteasen, toonden we aan dat het blokkeren van cathepsine D leidde tot een daling van leverontsteking. Mechanistisch gezien, suggereerden onze bevindingen dat door een verhoogde fecale excretie van galzuren, het lipide metabolisme verbeterde waardoor leverontsteking daalde. Omdat het cathepsine D in de circulatie het sterkst werd geblokkeerd, is het waarschijnlijk dat het plasma cathepsine D het meest bijdraagt aan de door ons beschreven effecten. Dit hoofdstuk beschrijft voor de eerste keer de rol van cathepsine D in de ontwikkeling van NASH.

In **hoofdstuk 8** stelden we de hypothese dat een hematopoietische *Npc1* mutatie een verschuiving zou veroorzaken in de samenstelling van de darmbacteriën van *Ldlr*^{-/-} muizen. Hiervoor verwijderden we eerst het aanwezige hematopoietische systeem van *Ldlr*^{-/-} muizen door ze bloot te stellen aan radioactieve straling. Vervolgens ontvingen deze muizen beenmerg afkomstig van Niemann-Pick type C1 mutant of wildtype muizen en kregen ze een hoog-vet, hoog-cholesterol dieet van 12 weken. Door de hematopoietisch-specifieke uitschakeling van het gen dat codeert voor het NPC1 eiwit, stapelen de lipiden zich op in de lysosomen van levermacrofagen afkomstig van het bloed. De hematopoietische *Npc1* mutatie veranderde de samenstelling van de darmbacteriën en verhoogde de microbiële rijkheid en diversiteit. We concluderen dat

niet-dieet gerelateerde veranderingen in het lipide metabolisme, zoals een hematopoietische *Npc1* mutatie, de samenstelling van darmbacteriën kan veranderen.

Tot slot bediscussieert **hoofdstuk 9** de belangrijkste bevindingen van de thesis in het licht van de huidige stand van zaken in de respectievelijke onderzoeksgebieden. Verder wordt de klinische waarde van deze bevindingen besproken.

Valorisation

Socio-economical and clinical relevance

Metabolic syndrome is one of the largest health challenges of the 21st century with currently more than **600 million** adult individuals suffering from this condition.¹ While the term was initially used to exclusively identify obese patients at risk for cardiovascular diseases and type 2 diabetes, an increasing amount of evidence demonstrates the influence of metabolic syndrome on other physiological and pathological processes.² Moreover, in the last 5 years, a staggering increase of **pediatric and adolescent metabolic syndrome**³⁻⁵ has been diagnosed, emphasizing the gigantic health challenge metabolic syndrome presents for society.

In this thesis, the main research goal was to provide translational insight into the mechanisms leading to NASH. NASH is a disorder characterized by hepatic inflammation and is mostly present in obese subjects⁶. Though exact numbers remain obscure, latest findings estimate 25% of the global population suffering from a form of fatty liver disease, of which about one third suffers from NASH.⁶ The recent evidence that NASH is also becoming more prevalent in children has supported the expert prediction that **NASH will become the leading liver disorder** by the year 2019.⁷ This is worrying since NASH patients progress to more advanced liver disorders such as fibrosis, cirrhosis and liver failure. Currently, the only effective treatment option for these advanced liver disorders is liver transplantation.⁷ In addition, diagnosis of NASH can only be made by histological assessment of a liver biopsy, in itself a risky procedure which can lead to severe complications.⁸ Taking all this into account, Younossi *et al.* recently estimated the economic and clinical burden of fatty liver disease in the United States and in 4 European countries (Germany, France, Italy and United Kingdom).⁹ In the **United States**, over 64 million people were projected to suffer from NAFLD, leading to an **annual cost of about \$103 billion**. In the **4 European countries**, 52 million NAFLD patients are expected to run up **an annual bill of €35 billion**.⁹ Therefore, there is an enormous demand for better diagnostic and therapeutic tools for NASH and metabolic syndrome in general. This is also reflected by the increasing amount of projects dedicated to the identification of better therapeutic compounds and biomarkers.¹⁰

We propose plasma **cathepsin D** as a novel **non-invasive** tool to diagnose NASH in an **early stage** of the disease. This finding is exceptionally **promising from an economic point of view** as it has the potential to strongly **reduce healthcare costs** for patients and society in general. Furthermore, early diagnosis of NASH can be a huge **support for clinicians** in preventing the development of NASH but also its progression into more advanced liver diseases. From a therapeutical point of view, reducing oxLDL levels, blocking plasma cathepsin D activity and/or changing the composition of gut microbiota are suggested as novel ways to tackle NASH and metabolic syndrome and can be alternatives to treat patients in the clinic.

The findings of this thesis should also be considered of interest with respect to **other related diseases**. Firstly, excessive lysosomal lipid storage in macrophages has also been shown in the context of atherosclerosis,¹¹ suggesting that our findings can be extrapolated to **cardiovascular diseases**. Secondly, lipid disturbances induced by lysosomal dysfunction in microglia, the macrophages of the brain, were previously linked to **Alzheimer's, Parkinson's and Huntington's disease**, strongly suggesting that the observations of this thesis are also relevant for these disorders.^{12,13} Finally, **lysosomal storage disorder's** central feature is the accumulation of digested or undigested material in lysosomes.¹⁴ Our findings might therefore also be of interest for these disorders.

Knowledge dissemination among different target groups

The observations described in this thesis have enormous clinical potential and are, therefore, of great interest for clinicians, industrial partners and particularly, patients. In order to reach these target groups, we have published our observations in **peer-reviewed scientific and clinical journals** such as *The American Journal of Gastroenterology* and *Scientific Reports*. To discuss our findings with the scientific community, we also presented at multiple national and international conferences by means of **oral or poster presentations**. Furthermore, by **organizing the 1st European Fatty Liver Conference**, we facilitated fruitful meetings of scientists with clinicians and industrial representatives. To make our findings available to the general public, we have recently published an article in the Science & Technology magazine (issue of April 2017) of **Pan European Networks**. This is a media group devoted to provide the most relevant information about European advances made in the fields of politics, technology and research to the general public.

Originality and/or innovative elements

Many diagnostic and therapeutic tools for NASH that are currently used in the clinic are the result of an explorative-driven research approach. Unfortunately, this approach has resulted in non-specific diagnostic (i.e. ALT) and therapeutic (i.e. exercise) options. In contrast, we employed a **hypothesis-driven approach** that identified lysosomal lipid accumulation in macrophages at the basis of NASH development. This finding was achieved by performing experiments at *in vitro* and *in vivo* both in mouse models and in **human subjects**. This approach is **innovative** and promising as it offers a **translational** platform for the development of novel diagnostic and therapeutic tools for NASH and metabolic syndrome in general. By identifying plasma levels of the lysosomal enzyme

cathepsin D as a marker for NASH, we also prove the successfulness of our translational and hypothesis-driven approach, emphasizing the enormous clinical and industrial potential of our findings.

A main obstacle that prevents progress in the field of NASH is the wide-spread use of animal models that do not adequately mimic (patho-)physiology and metabolism of humans.¹⁵ In contrast, we here used the *Ldlr*^{-/-} mouse model, which is a **physiological model** that mimics hepatic inflammation (and other features of metabolic syndrome) in humans. The findings that are obtained from these *in vivo* experiments can therefore be **applied to the human situation**.

Also, while most diagnostic and therapeutic research of NASH focuses on alleviating and reducing advanced NASH,¹⁶ we here provide information of the **early molecular mechanisms** at the fundament of the disease. As such, our observations have the **potential to prevent**, rather than cure advanced NASH.

Products and activities

Up until now, a liver biopsy is considered the golden standard for assessing NASH. This is known as an invasive, painful and risky procedure, underlining the need for non-invasive methods to improve early diagnosis of NASH. This thesis identifies **plasma cathepsin D** as a novel, non-invasive marker to diagnose and monitor NASH. In contrast to the liver biopsy, this approach is **safer, more rapid and associated with less discomfort** for the patient. This finding has also resulted in a **filed patent application**. Our finding that oxLDL particles substantially contribute to lysosomal lipid accumulation in hepatic macrophages (Chapter 4), strongly suggests that **plasma oxLDL** levels should be investigated for their potential to predict NASH. In line, low levels of anti-oxLDL IgM antibodies were correlated with the level of hepatic inflammation in humans.¹⁷

Besides novel diagnostic tools, this thesis also provides several therapeutical alternatives for NASH and metabolic syndrome. In chapter 7, we showed that modulation of cathepsin D reduced hepatic inflammation, thereby identifying **cathepsin D as a potential therapeutic target** for NASH. In chapter 4, we demonstrated that an **immunization** protocol with **heat-inactivated pneumococci** increased anti-oxLDL IgM antibody levels, resulting in reduced hepatic inflammation. This type of immunization might be further refined for application in patients at risk of developing NASH and metabolic syndrome as oxLDL has been shown a risk factor in several metabolic syndrome-associated pathologies.¹⁸ Finally, we found that lysosomal lipid storage in

macrophages resulted in a shift in **gut microbiota composition** (chapter 8), suggesting that gut microbiota potentially contributes to lysosomal lipid storage-associated problems. Future research should point out the effect of gut microbiota on physiology and pathology, and assess whether modulating gut microbiota can be an attractive alternative for therapeutic intervention.

Future planning

While this thesis offers several novel diagnostic and therapeutic options for NASH and metabolic syndrome, more action has to be undertaken to translate our findings to patients and the general public. One of our main goals for the future is to create a novel non-invasive approach to diagnose all stages of fatty liver disease in the clinic. We recently **obtained a competitive grant from the TKI**, which will support us in translating our findings to the clinic and general public. This project involves collaborations between national (Groningen, Amsterdam, Maastricht) and international (Antwerp, BE) academic partners together with companies (Echosens, FR and Tobira, US) and patient organizations. Therefore, the first step has already been made in the direction of implementation of a novel, non-invasive approach to diagnose NASH. As mentioned, this project is also **closely connected to the Dutch patient organization of the liver**, a collaboration that we intend to intensify in the future. Furthermore, as follow-up on the 1st European Fatty Liver Conference, we are currently planning the **2nd European Fatty Liver Conference** to be held in Maastricht in March 2018. Besides close collaboration between clinicians, researchers and industrial partners, a contribution of patient organizations will also be included within the program of the conference.

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Dankwoord

Dankwoord

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Curriculum vitae

Curriculum vitae

Tom Houben was born on June 24th, 1990 in Hasselt, Belgium. After finishing primary school in his home town, he continued his education at the Sint-Martinusscholen Campus Amandina in Herk-de-Stad, where he acquired his high school diploma (Latin-Mathematics-Sciences). In September 2008, he started his Bachelor education in Biomedical Sciences at the Transnational University Limburg (tUL; campus Hasselt, Belgium). After finishing an internship at the CMK Institute of Hasselt University, he acquired his Bachelor in Biomedical Sciences in July 2011. Subsequently, he participated in the Master 'Clinical Molecular Life Sciences' at the tUL. For this degree, he performed two internships in two different departments of Maastricht University. During his first internship, which lasted 3 months, he investigated the role of glycogen synthase kinase 3 α in skeletal muscle atrophy at the department of Respiratory Medicine. In his final year, he joined the department of Molecular Genetics for an 8 month internship, during which he investigated the contribution of oxidized LDL to hepatic inflammation in the context of non-alcoholic steatohepatitis. For his work in this latter internship, he received several competitive poster awards. In July 2013, he received his diploma as Master of Science in the Clinical Molecular Sciences with distinction.

In September 2013, he started his PhD program as part of the CVON IN-CONTROL consortium at the department of Molecular Genetics within the School of Nutrition and Translational Research in Metabolism (NUTRIM). His PhD was supervised by Prof. dr. Ronit Shiri-Sverdlov, Prof. dr. Marten Hofker and dr. Eleonore Köhler. The topic of his research was to identify novel molecular pathways leading to hepatic inflammation and based on these findings, come up with novel diagnostic and therapeutic tools to combat NASH and metabolic syndrome. During this period, he mentored multiple Bachelor and Master students during their internships and he conducted various teaching activities as a tutor in Problem-Based Learning (PBL) sessions and as lecturer. He also actively attended international and national conferences, including the European Association for the Study of the Liver (EASL), the Dutch Liver Retreat (DLR), the Genetics Retreat, the Dutch Digestive Disease Days and the European Fatty Liver Conference (EFLC). During these conferences, he presented his research both orally and by poster presentation. During his period as PhD-student, he won several presentation awards (NUTRIM symposium; European Fatty Liver Conference; Dutch Liver Retreat) and was he, as co-applicant, awarded a competitive national grant by the LSH-TKI.

From September 2017 until present, he continues his work at the department of Molecular Genetics as post-doctoral researcher.

List of publications

1. **Tom Houben**, Yvonne Oligschlaeger, Albert V Bitorina, Tim Hendriks, Sofie MA Walenbergh, Marie-Hélène Lenders, Marion JJ Gijbels, Fons Verheyen, Dieter Lütjohann, Marten H Hofker, Christoph J Binder, Ronit Shiri-Sverdlov. Blood-derived macrophages prone to accumulate lysosomal lipids trigger oxLDL-dependent murine hepatic inflammation. *Sci Rep*. 2017 Oct 2;7(1):12550. doi: 10.1038/s41598-017-13058-z. (IF: 4.810)
2. Aafke WF Janssen, **Tom Houben**, Saeed Katiraei, Wieneke Dijk, Lily Boutens, Nieke van der Bolt, Zeneng Wang, J Mark Brown, Stanley L Hazen, Stéphane Mandard, Ronit Shiri-Sverdlov, Folkert Kuipers, Ko Willems van Dijk, Jacques Vervoort, Rinke Stienstra, Guido JEJ Hooiveld, Sander Kersten. Modulation of the gut microbiota impacts non-alcoholic fatty liver disease: a potential role for bile acids. *J Lipid Res*. 2017 Jul;58(7):1399-1416. doi: 10.1194/jlr.M075713. (IF: 4.810)
3. **Tom Houben**, Yvonne Oligschlaeger, Tim Hendrik, Albert V Bitorina, Sofie MA Walenbergh, Patrick J van Gorp, Marion JJ Gijbels, Silvia Friedrichs, Jogchum Plat, Frank G Schaap, Dieter Lütjohann, Marten H Hofker, Ronit Shiri-Sverdlov. Cathepsin D regulates lipid metabolism in murine steatohepatitis. *Sci Rep*. 2017 Jun 14;7(1):3494. doi: 10.1038/s41598-017-03796-5. (IF: 4.259)
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6. Jeurissen ML, Walenbergh SM, **Houben T**, Hendriks T, Li J, Oligschlaeger Y, van Gorp PJ, Gijbels MJ, Bitorina A, Nessel I, Radtke F, Vooijs M, Theys J, Shiri-Sverdlov R. Myeloid DLL4 does not contribute to the pathogenesis of non-alcoholic steatohepatitis in Ldlr^{-/-} mice. *PLoS One*. 2016 Nov 29;11(11):e0167199. doi: 10.1371/journal.pone.0167199. (IF: 2.806)

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10. Hendriks T, Walenbergh SM, Jeurissen ML, **Houben T**, van Gorp PJ, Lindsey PJ, Koek GH, Kathan S, Pihlajamäki J, Hofker MH, Shiri-Sverdlov R. Plasma IL-1 receptor antagonist levels correlate with the development of non-alcoholic steatohepatitis. *Biomark Med*. 2015;9(12):1301-9. doi: 10.2217/bmm.15.71. (IF: 2.02)
11. Walenbergh SM, **Houben T**, Hendriks T, Jeurissen ML, van Gorp PJ, Vaes N, Olde Damink SW, Verheyen F, Koek GH, Lütjohann D, Grebe A, Latz E, Shiri-Sverdlov R. Weekly treatment of 2-Hydroxypropyl- β -cyclodextrin improves intracellular cholesterol levels in LDL receptor knockout mice. *Int J Mol Sci*. 2015 Sep 2;16(9):21056-69. doi: 10.3390/ijms160921056. (IF: 3.226)
12. Hendriks T, Jeurissen ML, van Gorp PJ, Gijbels MJ, Walenbergh SM, **Houben T**, van Gorp R, Pötgens CC, Stienstra R, Netea MG, Hofker MH, Donners MM, Shiri-Sverdlov R. Bone marrow-specific caspase-1/11 deficiency inhibits atherosclerosis development in Ldlr^{-/-} mice. *FEBS J*. 2015 Jun;282(12):2327-38. doi: 10.1111/febs.13279. (IF: 3.902)
13. Walenbergh SM, **Houben T**, Hendriks T, Jeurissen ML, van Gorp PJ, Vreugdenhil AC, Adriaanse MP, Buurman WA, Hofker MH, Mosca A, Lindsey PJ, Alisi A, Liccardo D, Panera N, Koek GH, Nobili V, Shiri-Sverdlov R. Plasma cathepsin D levels: a novel tool to predict pediatric hepatic inflammation. *Am J Gastroenterol*. 2015 Mar;110(3):462-70. doi: 10.1038/ajg.2015.29. (IF: 9.566)

14. Brandsma E, **Houben T**, Fu J, Shiri-Sverdlov R, Hofker MH. The immunity-diet-microbiota axis in the development of metabolic syndrome. *Curr Opin Lipidol*. 2015 Apr;26(2):73-81. doi: 10.1097/MOL.0000000000000154. (IF: 4.096)
15. Tim Hendriks, Mike L.J. Jeurissen, Veerle Bieghs, Sofie M.A. Walenbergh, Patrick J. van Gorp, Fons Verheyen, **Tom Houben**, Yasmin Dias Guichot, Marion J.J. Gijbels, Eran Leitersdorf, Marten Hofer, Dieter Lütjohann, Ronit Shiri-Sverdlov. Hematopoietic overexpression of Cyp27a1 reduces hepatic inflammation independently of 27-hydroxycholesterol levels in Ldlr^{-/-} mice. *J Hepatol*. 2015 Feb;62(2):430-6. doi: 10.1016/j.jhep.2014.09.027. **(IF: 12.486)**

In preparation

- **Tom Houben**, John Penders, Yvonne Oligschlaeger, Marc-Jan Bonder, Debby P Koonen, Jingyuan Fu, Marten H Hofker, Ronit Shiri-Sverdlov. Hematopoietic Npc1 mutation shifts gut microbiota composition in Ldlr^{-/-} mice. *In preparation*
- Yvonne Oligschlaeger, **Tom Houben**, Mike L. Jeurissen, Albert V. Bitolina, Maurice Konings, Sabine Baumgartner, Jogchum Plat, Ronit Shiri-Sverdlov. Oxyphytosterols do not play a prominent role in macrophage-mediated inflammation. *In preparation*

Oral presentations

- Sept 2013 – Aug 2017: Progress meetings (4x a year) within the CVON IN-CONTROL consortium
- March, 2017: The Digestive Disease Days, Veldhoven, the Netherlands
Title: The lysosomal enzyme cathepsin D as a therapeutic target for NASH.
- January, 2017: Research Line 2 lunch meeting, Maastricht, the Netherlands
Title: The lysosomal enzyme cathepsin D as marker and therapeutic target for NASH.
- October, 2016: Dutch Liver Retreat, Spier, the Netherlands
Title: Cathepsin D as a novel therapeutic target to combat NASH.
(Awarded Best Oral Presentation)
- September, 2016: lecture 1st Master Biomedical Sciences, Hasselt, Belgium
Title: Mouse models to study fatty liver and steatohepatitis.

- April, 2016: Business meeting with company Echosens, Barcelona, Spain
Title: Lysosomes in motion: early non-invasive biomarkers for the detection of NASH.
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- April, 2016: Business meeting with company Tobira, Barcelona, Spain
Title: Lysosomes in motion: early non-invasive biomarkers for the detection of NASH.
- September, 2015: lecture 1st Master Biomedical Sciences, Hasselt, Belgium
Title: Mouse models to study fatty liver and steatohepatitis.
- June 23, 2015: Meeting collaborative group **Bambino Gesù** Children's Hospital, Rome, Italy
Title: Lysosomal lipid accumulation in hepatic macrophages as driver for hepatic inflammation.
- April 29, 2015: Invited presentation Centre of expertise for chronic organ failure (CIRO), Roermond, the Netherlands.
Title: Storage solutions: targeting lysosomal function to improve hepatic inflammation.
- March, 2015: Seminar Genetics and Cell biology Cluster, Maastricht, the Netherlands
Title: Storage solutions: targeting lysosomal function to improve hepatic inflammation.
- October 31, 2014: Dutch Liver Retreat, Spier, the Netherlands
Title: Storage solutions: targeting lysosomal function to improve hepatic inflammation.
- April, 2014: Seminar Genetics and Cell biology Cluster, Maastricht, the Netherlands
Title: Cholesterol in hepatic inflammation: innocent bystander or still a heavily suspect?

Poster presentations

- December, 2016: Annual NUTRIM Symposium, Maastricht, the Netherlands
Title: Cathepsin D as novel therapeutic target to combat NASH
- September, 2016: Summer Frontiers 2016 – Systems Biology of Innate Immunity, Nijmegen, the Netherlands
Title: Circulating cathepsin D regulates lipid metabolism in murine steatohepatitis

- June 2016: The Michael, Marcia, and Christa Parseghian Scientific Conference for Niemann-Pick type C Research at the Westin La Paloma Resort, Tucson, Arizona, USA
Title: Immunization against oxLDL as novel therapeutic tool for NPC1
- June 2016: The Michael, Marcia, and Christa Parseghian Scientific Conference for Niemann-Pick type C Research at the Westin La Paloma Resort, Tucson, Arizona, USA
Title: Plant stanols as a novel tool to overcome lysosomal cholesterol storage
- March, 2015: 1st European Fatty Liver Conference (EFLC), Maastricht, the Netherlands
*Title: Storage solutions: targeting disturbed lysosomes to improve hepatic inflammation (**Awarded 1st Poster prize**)*
- December, 2014: Annual NUTRIM symposium, Maastricht, the Netherlands
*Title: Storage solutions: targeting disturbed lysosomes to improve hepatic inflammation (**Selected as outstanding abstract + Awarded 1st prize for poster presentation and elevator pitch**)*
- December, 2013: Annual NUTRIM symposium, Maastricht, the Netherlands
Title: Cholesterol in hepatic inflammation: innocent bystander or still a heavily suspect?
- June, 2013: International Life Sciences Master Students Research Conference, Maastricht, the Netherlands
*Title: Oxidized cholesterol is taking the lead in non-alcoholic steatohepatitis (**Awarded 3rd Poster prize**)*
- June, 2013: Mosa Conference, International Students Research Conference on Health, Maastricht, the Netherlands
*Title: Oxidized cholesterol is taking the lead in non-alcoholic steatohepatitis (**Ranked top 4 poster presentation according to the Student Jury**)*

Other contributions

- Invited scientific chair of the session 'Immunity in NAFLD'
1st European Fatty Liver Conference (EFLC), Maastricht, the Netherlands

Awards

- November 2017: Nominated as Best tutor within the domain of Biomedical Sciences during the Education Prizes FHML 2017
- March, 2017: Twice awarded as inventor at the Brightlands Science Forum
- February, 2017: Co-applicant LSH-TKI grant: Lysosomes in motion: early non-invasive biomarkers for the detection of NASH
- October, 2016: Best Oral Presentation at Dutch Liver Retreat, 2016
- March, 2015: Poster prize European Fatty Liver Conference
- December, 2014: Poster prize Annual NUTRIM Symposium
- June, 2013: Poster prize International Life Sciences Master research conference

Filed patent applications

- Methods and compounds for the treatment of Niemann-Pick disease type C1. (Patent Number 330 EPP0; date filing: 27.03.2016)
- Method for treating nonalcoholic steatohepatitis. (Patent number 329 EPP0; date of filing: 23.02.2016)

Student supervision

Supervisor of Maria Imperatrice (Erasmus student; Mar 2017 – Jun 2017)

Supervisor of Marco Caruso (Erasmus student; Mar 2017 – Jun 2017)

Supervisor of Laura Hertz (Maastricht University College student; Sept 2017 – Dec 2017)

Supervisor of Ilona Cuijpers (Senior Master Student; Nov 2015 – Jul 2016)

Supervisor of Paula Boeira (Senior Master Student; Nov 2015 – Jul 2016)

Supervisor of Robbin de Kruijf (Senior Master Student; Nov 2014 – Jul 2015)

Supervisor of Ruby Zelissen (Junior Master Student; Jan 2015 – Jun 2015)

Supervisor of Laure Verstraeten (Bachelor Student; Mar 2015 – Jun 2015)

Supervisor of Joris Meessen (MLO Student; Jan 2015 – Jun 2015)

Supervisor of Samantha Mouse (MLO Student; Sept 2014 – Jan 2015)

Supervisor of Maria Sounidaki (Senior Master Student; Nov 2013 – Jul 2014)

Supervisor of Magda Drożdż (Bachelor Student; Mar 2014 – Jul 2014)

Supervisor of Qasem Almuhi (May 2014)

Supervisor of Angel Frijnts (MLO Student; Sept 2013 – Jan 2014)