Small molecules, big consequences

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Small molecules, big consequences:
**Novel therapeutic approaches for treating chronic
inflammatory disorders**

Tulasi Yadati

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**Small molecules, big consequences:
Novel therapeutic approaches for treating chronic inflammatory
disorders**

DISSERTATION

to obtain the degree of Doctor at Maastricht University,
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Chapter 1

General introduction

Inflammatory responses and inflammation-associated diseases

Inflammation is a normal physiological reaction of the body to harmful stimuli, be they physical, chemical, or biological. These stimuli include infectious agents, damaged cells, toxic substances, or irradiation. Inflammation is the process involved in removing such injurious stimuli by initiating the healing process [1]. The primary function of the inflammatory response is to identify and destroy the danger stimuli, repair or replace the injured tissue and reestablish tissue homeostasis. Inflammation can affect all organs of the body. The four cardinal signs of inflammation are redness (rubor), heat (calor), pain (dolor), swelling (tumor) and loss of function [2]. The inflammatory response includes a complex network of several cell types and is explained in the following steps:

1. At the site of injury, the epithelial and endothelial cells of the damaged tissue release inflammatory factors such as cytokines and chemokines, which are chemical substances that are rapidly synthesized and released in response to infection.
2. These inflammatory factors then induce vasodilation (widening of blood vessels leading to redness and heat), subsequently helping in extravasation of neutrophils (professional killer cells) and leakage of plasma (swelling) into the damaged tissue.
3. Followed by neutrophils, monocytes, leukocytes (white blood cells) and mast cells are deployed to damaged tissues via chemotaxis, a process that involves migration of cells to site of inflammation. Monocytes can further differentiate into macrophages.
4. Neutrophils and macrophages kill the damaging stimuli by a process called phagocytosis (cell eating), which involves internalizing and degrading of the infectious agent and subsequently clearing the debris.
5. The infectious agent is thus eliminated and is followed by tissue repair. This step majorly involves the synthesis of a fibrous extracellular matrix (ECM) to replace the injured tissue. ECM is the non-cellular compartment that lines the tissues to provide structural support to the tissues. Cells such as fibroblasts, macrophages and keratinocytes participate in this healing process. Inflammatory response thus is normally rapid, and it should be noted that prolonged inflammation can lead to tissue damage [3-5]. The main steps involved in the inflammatory response are illustrated in **Figure 1**.

Figure 1 Major events in the local inflammatory response. Upon an inflammatory stimulus, cells release chemokines and cytokines that help in the vasodilation of blood vessels. This leads to recruitment/ infiltration of leucocytes (neutrophils and macrophages) which engulf the pathogen and initiate tissue repair. Figure is created with permission from Servier Medical Art Image bank.

As mentioned above, macrophages due to their phagocytic ability represent an integral part of the inflammatory processes and are critical regulators of both normal homeostasis as well as pathology. Based on the site of the origin, macrophages are classified either as blood monocyte-derived or tissue-resident macrophages. Macrophages are seeded throughout the body in various organs including liver, bone, lungs and intestine. During inflammation, macrophages mediate three major functions: antigen (foreign body) presentation, phagocytosis, and production of various cytokines and growth factors [6]. Further, macrophages themselves are activated by cytokines including interferon gamma, tumor necrosis factor alpha (TNF α), extracellular matrix proteins and other chemical mediators. Macrophages also respond to metabolic cues such as glucose and lipids [7]. Thus, macrophages play an important role in metabolic and immune signaling in the body.

Based on the duration of the inflammatory response, inflammation can be either acute or chronic. Acute inflammation is the early response of the body to an inflammatory stimulus and is terminated after resolution of the inflammatory process. Therefore, acute inflammation typically lasts only for minutes to days and is mainly carried out by neutrophils. Failure to resolve inflammation or prolonged exposure to the triggering agent makes acute inflammation to become chronic. Chronic inflammation has a longer time course that can last from days up to years and is mediated by lymphocytes and macrophages rather than neutrophils. Moreover, acute inflammation is critical to maintain tissue homeostasis while chronic inflammation results in tissue damage leading to pathologies [8-10]. Major differences between acute and chronic inflammation are mentioned in **Table 1**.

Table 1: Differences between acute and chronic inflammation

Feature	Acute inflammation	Chronic inflammation
Trigger	Injured tissue, pathogens	Persistent pathogens, metabolic disturbances
Duration	Short (days to weeks)	Long (months to years)
Cell types	Neutrophils, eosinophils, mast cells	Macrophages, lymphocytes
Location	Local	Systemic
Outcome	Abscess formation and resolution of inflammation	Tissue damage and fibrosis

Chronic low-grade inflammation is a mild sustained inflammation with low- levels of inflammatory markers such as C-reactive protein, interleukin-6 (IL-6) and tumor necrosis factor (TNF α). Low- grade chronic inflammation observed in metabolic disorders is termed as ‘metabolic inflammation’ or ‘meta- inflammation. While pathogens such as bacteria or viruses trigger classical or infectious inflammation, metabolic disturbances induce meta-inflammation. Pathologies associated with excess diet such as obesity, type 2 diabetes and non- alcoholic fatty liver disease (NAFLD) show meta-inflammation [11-13]. The chronic state of inflammation in these diseases is mediated by macrophages (among other immune cells) located within the liver, muscle, adipose tissue and colon. Another chronic inflammatory disorder, inflammatory bowel disease (IBD), is also partly governed by metabolic disturbances where the intake of a diet is believed to elicit certain inflammatory responses. On the other hand, the gut lining is populated with a multitude of bacteria, viruses and fungi, which also have been suggested to contribute to the inflammatory response observed during IBD. Therefore, the type of inflammation in IBD is likely a combination of metabolic and infectious inflammation. For example, the intake of a high fat diet alters the intestinal microbiota composition and disturbs the intestinal barrier leading to inflammation. Further, because the liver exists in proximity to the gastrointestinal tract, also the liver is strongly affected by the alterations in the intestinal microbiota. In fact, NAFLD and atherosclerosis are associated with changes in gut microbiota profile. Thus, gut microbiota forms a common ground for underlying pathologies including IBD and NAFLD. Even though these diseases have different triggers and affect different organs and display distinctive symptoms, they are known to share some common immunological features in their pathology. While inflammation seems to be more stable in metabolic diseases, IBD patients experience severe flares of acute inflammation [14]. Within the cluster of inflammatory diseases, the contents of this thesis will particularly focus on NAFLD and IBD.

Inflammation at the center of non-alcoholic fatty liver disease

Nonalcoholic fatty liver disease (NAFLD) is characterized by hepatic steatosis (which is $>/$ 5% fat accumulation in the liver) with the lack of secondary causes such as significant alcohol consumption and other chronic liver diseases such as viral hepatitis [15]. NAFLD encompasses a whole spectrum of liver diseases ranging from hepatic steatosis, referred to as non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH) which is NAFL along with inflammation with or without liver fibrosis (damage of liver tissue). NASH can lead to liver cirrhosis (scarring of liver tissue) including the development of a hepatocellular carcinoma (HCC or liver cancer) [16,17], **emphasizing the driving role of inflammation in the progression of NAFLD**. Although NAFL is a largely benign and reversible condition, an estimated 20–33% of individuals with NAFL show indication of NASH on histopathology.

Further, it is known that people with NASH progress to fibrosis and cirrhosis at a yearly rate of 7–10%. Liver-related death in patients with NASH-related cirrhosis is around 1.4–3% [18].

In the United States alone, the annual economic burden of NAFLD has been estimated at \$103 billion [19], while in European countries (i.e., the United Kingdom, Germany, France and Italy) these costs account for €35 billion and thus, have a large economic burden on health care systems [20]. Globally, it is currently estimated that NAFLD affects about one-quarter of the general population, while NASH has been calculated at 2–5% of the general population [21]. Estes *et al.* predicted a 63% increase in the prevalence of NASH by 2030 accounting to 27 million patients [22]. The primary risk factors for NAFLD development are obesity, type 2 diabetes, and the metabolic syndrome including dyslipidemia [23]. Additionally, genetic, environmental and demographic factors are likely to have some influence on the advancement of fatty liver disease [24]. Metabolic inflammation in the liver is a key driver of NAFLD disease progression. For example, excess lipid accumulation inside the liver activates intrahepatic inflammatory pathways leading to activated immune cells and generation of cytokines that further contribute to liver inflammation. The relationship between lipids and inflammation in NASH will be further elaborated in the following sections.

The majority of the NAFL patients are asymptomatic except for general complaints like fatigue and irritation in the upper right quadrant of the abdomen, often hindering a faster diagnosis [25]. Currently, liver biopsy continues to be the gold standard for diagnosing NAFLD/NASH; however, because of the invasiveness and cost of the biopsy, its use is limited [26]. On the other hand, reliable noninvasive methods to detect NASH are few, if any. Commonly, plasma levels of liver enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are often used in clinical practice as a surrogate for inflammation but have poor predictive value [27,28]. In general, aminotransferases cannot distinguish patients from steatosis to NASH. Next to the use of plasma markers, many imaging techniques such as ultrasound [29], computed tomography (CT) and magnetic resonance imaging (MRI) and FibroScan are increasingly used to detect different stages of NAFLD [30], but with limited assessment of inflammatory levels [31]. Therefore, future advances in the field of NASH diagnostics focusing on developing sensitive and specific non-invasive markers of inflammation are required.

Inflammation as driving factor in Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal (GI) tract. The major types of IBD are Crohn's disease (CD) and ulcerative colitis (UC) which are associated with chronic intestinal inflammation and alterations in gut microbiota composition [32]. While UC is a condition in which the inflammatory responses are confined to colon, CD can involve any part of the GI tract. The gut microbiome is defined as the totality of microorganisms including bacteria, viruses, protozoa, and fungi present in the gastrointestinal tract that help in executing several physiological functions of the body [33]. Individuals between the age group of 15-35 are more often diagnosed with IBD. The signs of IBD include stomach pain, blood and mucus in the stools, diarrhea and a poor quality of life [34]. The prevalence of IBD is highest in the Western countries affecting up to 0.5% of the general population. Overall, Europe has a higher prevalence of UC than CD while the contrary is seen in Australia; UC and CD are equally distributed in North America [35].

The etiology of IBD is largely not known. However, genetic, environmental and individual's factors including immune status and intestinal microbiota composition are shown to be implicated in the pathogenesis of IBD. Risk factors for IBD include smoking, hygiene, drugs, diet and stress. Moreover, it is also known that obesity might contribute to the development of IBD [36]. Importantly, the combination of genetic factors and environmental factors is known to impair intestinal barrier function leading to translocation of beneficial gut microorganisms into the bowel wall. Damage to the intestinal barrier is critical as it may allow increased access and assimilation of toxic and immunogenic factors. As a result of dysbiosis and intestinal barrier breach, the microbiota and its components (that might act as endotoxins) are leaked into the circulation and subsequently trigger inflammation. The inflammation cascade further triggers the inflow of neutrophils and macrophages that increase the pro-inflammatory cytokine production, subsequently leading to perpetuation of epithelial cell damage, gut inflammation and ulceration [37].

Similar to NAFLD, the pathogenesis of IBD is also at least partly driven by the contribution macrophages. Majority of the tissue-resident macrophages are formed before birth from embryonic macrophages or fetal liver cells and sustain themselves through limited self-renewal. In contrast, gut-resident macrophages are the most abundant and are derived from lymphocyte antigen (Ly6C+) blood monocytes, cells which are associated with the inflammatory phenotype and are constantly renewed from hematopoietic stem cells (HSCs) [38]. The failure of Ly6C monocytes to differentiate into mature intestinal macrophages leads to their accumulation which causes inflammation in the intestine. This step subsequently results in excessive secretion of IL-23 and TNF α . CD is characterized by enhanced production of interferon- γ and TNF α . In ulcerative colitis, the local immune response is characterized by increased Interleukin-13 levels [39].

Pathophysiology of inflammation

Hub of lipid metabolism: The liver

The liver is one of the largest organs in the body driving many important metabolic functions. The adult human liver weighs about 1.4 kilograms and is found in the right upper abdomen, below the diaphragm. Beneath the liver, the gallbladder is located. The gallbladder stores bile, which facilitates the digestion and absorption of fats (among other functions). The liver is a unique organ due to its dual blood supply: nutrient-rich blood from the portal vein (approximately 75%) that drains the stomach, intestines, pancreas and spleen and the oxygen-rich arterial blood from the hepatic artery (approximately 25%). The portal vein carries the blood from the digestive organs to the liver, carrying toxic substances, drugs and nutrients. The liver processes these substances with the help of its enzymes such as cytochromes. Once degraded, modified or detoxified they are sent back into the blood or excreted. Major cell types found in the liver are hepatocytes, endothelial cells, Kupffer cells (liver resident macrophages) and stellate cells (hepatic development and regeneration). Hepatocytes majorly participate in metabolic functions; endothelial cells play a role in immunity by scavenging substances, while Kupffer cells mediate inflammatory responses [40].

Liver is the main organ involved in body's energy metabolism. This energy is primarily derived from carbohydrates, sugars and fats from the food we consume. After a meal, dietary sugars and fats are digested in different organs of the body to release energy. Lipids are fat-like substances that are

absorbed from food or synthesized in the liver and are critical for cell structure, function, and energy, as well as for insulation and protection. Important type of lipids are triglycerides and cholesterol [41]. Due to their hydrophobic nature (insoluble in water), lipids are packaged and transported through the bloodstream in the form of lipoproteins. Based on their content (lipid and protein combination), density and size, lipoproteins are classified as chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). Lipid metabolism and transport is organized into three transport systems: (a) exogenous transport that is associated with dietary lipids (b) endogenous transport which is associated with internally produced lipids and (c) reverse transport which transports the lipids from the periphery (e.g., skeletal muscle and adipose tissue) to the liver.

The exogenous pathway begins with the intestinal absorption of triglycerides and cholesterol from dietary sources. After absorption, these dietary lipids are coupled with various proteins to form chylomicron particles. Chylomicrons are hydrolyzed by lipoprotein lipase (LPL) expressed by endothelial cells into free fatty acids and glycerol that are ultimately taken up by the cell via cell surface receptors like cluster of differentiation 36 (CD36). The muscle, white and brown adipose tissue are major recipients of such triglyceride cargo. The remnants of chylomicrons are endocytosed by hepatocytes by receptors on the liver such as the low-density lipoprotein (LDL) receptor (LDLr), LDL receptor-related protein (LRP). An overview on lipoprotein metabolism is depicted in **Figure 2**.

Figure 2 **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; SRB1: scavenger receptor class B type I. Figure is created with permission from Servier Medical Art Image bank.

When the supply of dietary lipids is low, chylomicron remnants are combined with newly synthesized lipids in the liver to form VLDL particles which initiate the endogenous pathway. Similar to chylomicrons, VLDL are hydrolyzed by LPL by detachment of the remaining triglycerides and its substitution by cholesterol esters to form intermediate-density lipoproteins (IDLs) and LDLs. Majority of these LDL particles are cleared by liver. After the uptake of LDL particles by hepatic macrophages, they are directed to lysosomes for subsequent degradation. Lysosomes are 'recycling centers' of the cell and are characterized by an acidic interior, which enables the activity of many hydrolytic enzymes including proteases, lipases, and nucleases [42,43]. Under normal conditions, cholesterol is hydrolyzed by the lysosomal acid lipase (LAL) in the lysosomes and the free unesterified cholesterol that is formed is transferred to the cytoplasm with the help of Niemann-pick disease type C1 (NPC1) and NPC2 proteins. While NPC2 plays an obligate role in shuttling cholesterol through the lysosomal lumen, NPC1, which is located on the lysosomal membrane is known to efflux the free cholesterol into the cytoplasm [44]. Some of the LDL particles absorbed by the extrahepatic peripheral tissue such as skeletal muscle.

In reverse cholesterol transport, cholesterol is transferred from peripheral tissues back to the liver for ultimate excretion with the help of HDLs. This is important for homeostasis as most cells in peripheral organs cannot catabolize cholesterol. HDL particles are synthesized as cholesterol-free particles by intestinal enterocytes and liver. Acquiring cholesterol generates mature HDL particles which then return to the liver via scavenger receptor class B type 1 (SR-B1) receptors on macrophages. Cholesterol is hydrolyzed and excreted into bile acid. Finally, the cholesterol depleted HDLs are either enter the circulation to be reused or excreted [45].

Excess lipid accumulation in the macrophages induces lysosomal dysfunction and inflammation

Lipid homeostasis in the cells is maintained by balancing lipid synthesis, transport and metabolism. Any imbalance to the normal process including lipid excess that is commonly observed in obesity, disrupts lipid homeostasis [46]. Especially, cholesterol accumulation leads to a shift in the lipoprotein profile towards increased LDL levels and reduced HDL levels, a condition referred to as dyslipidemia. Also, metabolic disorders such as NAFLD, atherosclerosis and insulin resistance are usually associated with dyslipidemia [47,48].

Due to elevated LDL levels during dyslipidemia, cholesterol metabolism in macrophages is overwhelmed leading to excessive accumulation of LDL. Moreover, cholesterol overload is associated with abnormalities such as oxidative stress [49]. Oxidative stress is defined as a process caused by an imbalance between production and detoxification of reactive oxygen species (ROS) in cells and tissues. Due to increased presence of oxidative stress in dyslipidemic conditions, LDL particles are highly prone to oxidation, which increases the levels of oxidized LDL (oxLDL) particles [50,51]. Unlike LDL, which is endocytosed by LDL receptors, oxLDL is taken up by scavenger receptors present on the macrophages. The two main scavenger receptors responsible for oxLDL uptake are scavenger receptor A (SR-A) and CD36 [52]. Since the structure of oxLDL particles mimics the epitopes of pathogens, oxLDL is taken up by scavenger receptors as a part of protective response. However, this might cause oxLDL overload in the macrophages [53]. Moreover, normal LDL is efficiently cleared from lysosomes as described earlier, but oxLDL is resistant to normal degradation and efflux and therefore accumulates in the lysosomal compartments [54]. This chain of events is known to trigger

inflammatory pathways during atherosclerosis and NASH [55-58]. Thus, inflammation and metabolism are capable of regulating one another.

One of the potential mechanisms that explains the link between oxLDL and hepatic inflammation is via lysosomal membrane permeabilization. Excessive oxLDL inside the lysosomes can damage and disturb lysosomal membrane leading to the release of the lysosomal components including its enzymes into cytoplasm and subsequently into extracellular space, a process called as lysosomal exocytosis [59,60]. The intracellular cholesterol metabolism in normal and dyslipidemic conditions is illustrated in **Figure 3**.

Figure 3 Intracellular cholesterol metabolism. LDL is taken up by the cells via LDLR. Cholesteryl esters (CEs) from the endosome are directed towards the lysosome, where they are hydrolyzed by lysosomal acid lipase (LAL), generating free unesterified cholesterol (FC). FC is transported to the lysosomal lumen by NPC2 and to cytoplasm via NPC1 proteins. During dyslipidemia, LDL gets modified to oxLDL and is taken up by the cells via CD36 receptor. Rather than being transported to cytoplasm, oxLDL is resistant to efflux and accumulates in the lysosomes. This leads to rupture of lysosomes by lysosomal membrane permeabilization (LMP) causing leakage of lysosomal enzymes into cytoplasm and into the extracellular space. These changes of events trigger inflammatory responses. LDL, low-density lipoprotein; CE, cholesteryl ester; FC, free unesterified cholesterol; LAL, lysosomal acid lipase; NPC, Niemann-Pick type C; LMP- lysosomal membrane permeabilization. Figure is created with permission from Servier Medical Art Image bank.

Intralysosomal oxLDL can additionally contribute to activation of inflammation by lysosomal enzymes. Lysosomes harbor several kinds of enzymes including lipases, nucleases, proteases which work together in the degradation of various substances. One of the most abundant lysosomal enzymes are cathepsins, which are proteases that help in intracellular protein degradation in the lysosomes [61]. Cathepsins are normally located in the acidic environment of lysosomes, however in some physiological and pathological conditions they can be secreted out the lysosomes into the cytosol or outside of the cell. Cytosolic cathepsins are known to be involved in mitochondrial dysfunction, apoptosis (cell death) [62] and inflammasome activation [63]. Inflammasomes are multi-protein complexes that are activated upon bacterial infections or cellular damage. Cathepsins activate/degrade components of inflammasome and thereby trigger the release of the pro-inflammatory cytokines leading to inflammation. Moreover, lysosomal dysfunction impairs

autophagy, a self-destructive process that mediates intracellular degradation of substances. Impaired autophagy enhances ROS production, inflammasome activation, and IL-1 β production. Disturbed autophagy is reported in NASH and IBD [64]. Alternatively, cathepsins in the extracellular space are known to participate extensively in the degradation of matrix substances leading to inflammation [65]. All these findings show an association between cholesterol-loaded macrophages, lysosomal exocytosis, cathepsins and inflammation during dyslipidemia. Interestingly, we have previously shown that plasma cathepsin D is implicated in NASH patients [66].

Role of cathepsin D in inflammation

Cathepsin D is an aspartic protease that is constitutively expressed in nearly all cells and is located typically in the lysosomes where it participates in protein degradation. Loss of this proteolytic activity results in accumulation of substances in different cell types leading to neurodegeneration, developmental and vision related problems. Active and zymogen (inactive) forms of cathepsin D are known to be released into cytosol and into circulation by macrophages, mammary epithelial cells, cancer cells and keratinocytes. Cathepsin D in its active form was found in biological fluids such sweat, urine, seminal fluid and also in macrophage- conditioned media [67]. The lysosomal fraction of cathepsin D has more 'house-keeping' functions while the extracellular fraction is most likely associated with pathological conditions. For example, increased levels and activity of cathepsin D in extracellular space are reported in several metabolic inflammatory disorders including atherosclerosis [68], diabetes [69], cancer [70] and IBD [71]. Moreover, it can regulate inflammation by many ways. For example, proteolytic alterations of apolipoprotein B-100 (apoB) part of LDL by cathepsin D causes LDL accumulation in arterial intima, a main feature of atherosclerosis. In IBD, cathepsin D contributes to inflammation by the destruction of extracellular matrix and basement membrane proteins that can lead to impairment of the intestinal barrier and epithelial cell damage. Furthermore, we have previously observed that plasma cathepsin D correlated with hepatic inflammation in NAFLD/ NASH patients [66] and demonstrated the functional link of cathepsin D in NASH-related hepatic inflammation [72].

Role of stress in inflammation

One of the potential risk factors for the development of inflammatory disorders is stress [73]. Stress is defined as the outcome of any internal or external stimuli that disrupts the body's homeostasis. The concept of stress has two components: 1) stressors (the triggers or factors that cause stress) and 2) stress (the bodily reaction to stressors). Stress can be physical (trauma, illness and dietary) or psychological (fear, anxiety, depression etc.). Humans are constantly subjected to variety of stressors from pre-natal life to adulthood that can largely influence the health status of the individual. Evidence suggests that exposure to stressors such as smoke, diet, medication and psychosocial stress during early life and childhood is known to have adverse effects in adult life. Socio-economic status, work conditions and trauma majorly trigger stress in adult life [74].

Stress is linked to many diseases including diabetes, cardiovascular diseases, obesity and IBD. Stressful conditions are known to trigger unhealthy eating habits [75]. Moreover, stress is known to disrupt lipid metabolism and increase postprandial levels of triglycerides [76]. Not just disrupting metabolic activities, stress is often accompanied by elevated inflammatory responses. Severe stress

leads to hyperactive immune system leading to disturbed inflammatory responses. In fact, chronic low-grade inflammation is considered as a link between stress and the underlying pathophysiology of metabolic diseases like diabetes. Evidence from preclinical and clinical studies demonstrated stress stimuli to the brain activates autonomic nervous system and hypothalamic-pituitary-adrenal axis (HPA axis) which increase pro-inflammatory cytokine production by modulating several pathways [77]. For example, in IBD, stress induced changes in the brain are known to affect the gut microbiota composition, intestinal permeability and cytokine secretion, thereby increasing the severity of the disease. However, the complex mechanisms responsible for the stress-induced increase of plasma inflammatory markers in inflammatory diseases are beyond the scope of this thesis.

Resolving inflammation

Lifestyle modifications

Lifestyle modifications are the first-line approach to manage chronic inflammatory diseases [78]. Healthy lifestyle which includes calorie restriction, healthy food choices, physical activity and stress management are proven to reduce levels of proinflammatory markers such as CRP [79], delay or prevent the onset of type 2 diabetes [80] and NASH [81].

Diet: Obesity is usually associated with adipose tissue hypoxia that results in triggering of the NF- κ B-inducible signaling leading to production of proinflammatory cytokines. Although the mechanism is unclear, foods rich in fibers, whole grains, omega-3 fatty acids and antioxidant vitamins are known to reduce blood concentration of inflammatory markers such as CRP and TNF α [82]. Though the first-line therapy for NASH is calorie restriction, majority of NASH patients are unable to attain and retain the required amount of weight loss [83]. For morbidly obese individuals who are unable to reduce weight, bariatric surgery is considered. Bariatric surgery introduces changes in the digestive system restricting the amount of food that enters the stomach. Though bariatric surgery is known to improve physical health and ameliorate NASH histology, it is known to have complications and relapse of NASH after surgery [84].

Physical activity: Physical exercise is one of the important intervention strategies that is widely prescribed for lowering the risk of chronic diseases and is known to have modulatory effect on inflammation. Literature suggests that moderate physical activity in young and elderly population as well as in metabolic syndrome patients led to beneficial anti-inflammatory effects [85]. Although the underlying mechanism is not completely known, exercise is known to increase adipose tissue blood flow and thus blunting the hypoxia-associated inflammation. Besides the reduction of inflammatory markers, exercise has been documented to restore liver enzyme levels and improve the histological alterations associated with metabolic syndrome [86].

Relaxation: Similar to diet and physical activity, various relaxation methods to reduce stress are known to have beneficial effects in patients. Effective stress management training is known to reduce inflammatory markers in metabolic diseases and IBD [87]. Stress management includes self-relaxation techniques such as yoga, meditation, mind relaxation and guided therapy (amongst others) which mainly help in reducing heart rate, anxiety and improving quality of life in general [88]. The role of stress-management strategies in tackling inflammation are discussed in this thesis.

Despite strong evidence for the benefits of lifestyle modification, achieving and maintaining these changes remains a challenge. Major concerns include the compliance of the patients to lifestyle modifications due to economic problems, physical disabilities, or the difficulty to present a personalized plan for patients. Moreover, each patient might require different dietary recommendations, duration and/ frequency of food intake. These factors will influence participant's adherence and their success in achieving the best results. Unfortunately, most health professionals currently lack training and means to manage lifestyle interventions for their patients [89]. Lack of data on prolonged effects of lifestyle medicine and heterogeneity in the observed effects between patient groups also makes it difficult to formulate lifestyle plans. Additionally, stress interventions didn't prove to be consistently effective in producing effects in work settings [90]. Failure to adhere to stress-management techniques and constantly emerging stress situations in the workplaces signal that stress will increase or at least continue to be a problem in the near future. For all of these reasons, lifestyle interventions are often supported with pharmacological tools.

Pharmacological targets in inflammatory disorders

To support the dietary and lifestyle interventions, pharmacological agents to reduce inflammation are widely used in treating metabolic diseases. Pharmacological therapies for NASH are being extensively researched, although none of them can convincingly reverse NASH yet. The NASH drug market is set to increase from \$618 million in 2016 to approximately \$25.3 billion by 2026, pointing towards the huge demand for NASH treatments [91]. Guidelines from the American Association for the Study of Liver Diseases advise pioglitazone as a treatment for NASH, but this drug is shown to have increased risk of prostate or pancreatic cancer, body weight gain and fluid retention [92]. Four drug candidates for NASH are currently in phase 3 clinical trials namely, obeticholic acid, elafibranor, cenicriviroc, and resmetirom. These candidates are yet to receive approval from the food and drug administration (FDA) to be used as prescription medications [91]. Therefore, currently the only curative therapy for NASH is liver transplantation, which involves replacing the patient's liver with a healthy donor liver accompanied by persistent lifestyle changes. Scarcity of the donor organs and post-surgery complications urges the need to develop alternate therapeutic tools [93]. Therefore, research dedicated at identifying the complex molecular pathways triggering inflammation is highly desirable to formulate effective treatment options for NASH.

Management of IBD relies on a range of pharmacological and surgical interventions based on age [94]. Aminosalicylates are often the first option in the treatment of IBD to reduce inflammation but are limited by their efficacy [95]. Corticosteroids are used as the second option for the management of moderate-to-severe IBD. Though they offer rapid relief of symptoms and reduce inflammation, side effects including bone damage and risk of infection are observed in patients [96]. Immunosuppressants that control the hyperactive immune system are although extremely useful for IBD patients, they cannot offer complete treatment. Normally used immunosuppressant drugs include azathioprine, methotrexate, monoclonal antibodies against inflammatory cytokines, TNF, IL-12 or IL-23, and $\alpha 4\beta 7$ integrin [97]. In elderly, surgery to remove the colon is considered curative for UC, but it is not a treatment of choice for most patients [98].

Alternative therapies include agents such as statins because of their anti-inflammatory as well as cholesterol modulating properties. Statins are known to reduce LDL concentrations up to ~30%. Statins reduce cholesterol by inhibiting HMG-coA (3-hydroxy-3-methyl-glutaryl-CoA) reductase, the

main enzyme involved in cholesterol synthesis in liver. Statins are known to modulate inflammation by reduction of pro-inflammatory cytokine production and by inhibition of T-cell activation and proliferation at the site of infection. Side effects of statin treatment include joint and muscle pains and potential interaction of statins with other medications [99]. One of the available statin alternatives is 2-hydroxypropyl- β -cyclodextrin (CD). CD is an FDA-approved compound that is normally used for delivering therapeutic drugs because of its ability to solubilize lipophilic substances. Due to its cholesterol solubilizing properties, CD is a well-established treatment option for Niemann-Pick disease type C1 disease (NPC1), a lysosomal storage disorder. Moreover, in mouse models of atherosclerosis, cyclodextrin showed beneficial effects as it converted the cholesterol crystals into water-soluble 27-hydroxycholesterol, promoted cholesterol efflux and exerted anti-inflammatory effects [100]. However, CDs are shown to have toxic effects on different organs including ear (hearing loss) and kidney. Moreover, long term administration of CD is known to cause bone loss [101]. Hence novel therapeutic targets that modulate inflammation with minimal or zero side effects are essential. To this end, targeted drug approaches that involves selective inhibition of targets are considered safer and novel [102]. In this regard, small molecules are being investigated as stand-alone drugs because of their ability to reach precise target areas. Besides, they are well tolerated and easy to synthesize and optimize [103]. This thesis focuses on understanding the underlying mechanism of inflammation in metabolic diseases and optimization of pharmacological targets in pre-clinical rodent models.

Pre-clinical models for validation of putative targets against NAFLD

To study the mechanistic details of inflammatory processes, we made use of rodent models in this thesis. Preclinical animal models are critical for not only studying the disease progression, but also to test the potential of therapeutic drugs. Since excess dietary intake is one of the main causal factors for NAFLD in humans, dietary animal models are usually preferred to perform NAFLD/NASH research. Many dietary rodent models have tried to mirror the metabolic disturbances as well as histological features observed in human NAFLD and NASH [104]. In rodents, NASH is usually induced by feeding high-carbohydrate or high-fat diets (HFDs), methionine and choline-deficient (MCD) diet, choline-deficient L-amino acid-defined (CDAA) diet and others.

Hyperlipidemic *Ldlr*^{-/-} as a mouse model for NASH

One of the existing mouse models that mimics most features of human NASH is the low-density lipoprotein receptor *Ldlr*^{-/-} mouse model. This is the combinatorial model based on the genetic deletion of the low-density lipoprotein receptor (Ldlr) with HFD for a period of 3-12 weeks. Due to the absence of Ldlr, these mice lack the ability to uptake LDL into the cells and thus display high levels of plasma LDL and low levels of HDL, hence resembling human-like lipoprotein profile. Moreover, *Ldlr*^{-/-} mice show sustained hepatic inflammation. While these mice do not develop severe fibrosis, they show mild to moderate fibrosis compared to normal C57BL/6 mice on the same diet. Therefore, *Ldlr*^{-/-} model serves as an excellent pre-clinical model for early inflammation studies associated with human NASH [105,106].

Sprague-Dawley rats as model for hepatic steatosis

Similar to mice, rat models differing in nutritional and genetic factors are frequently used as preclinical models for NAFLD/NASH. In fact, compared to mouse models, rat models are more susceptible to HFD, and are shown to display severe early histological features of NAFLD [107]. When given a HFD (71% fat/11% carbohydrates/18% proteins) for a period of 3 weeks, Sprague-Dawley rats were reported to develop steatosis, insulin resistance and mild to marked inflammation, features also observed in human NASH [108].

Thesis aim and outline

Low-grade systemic inflammation plays a crucial role in the development of NAFLD and IBD. Current treatment of chronic inflammatory diseases relies mainly on lifestyle changes and lowering lipid or inflammatory mediators. However, there are no existing approved FDA treatments. Good understanding of the pathophysiological processes involved in these diseases can help to formulate safe and effective therapies. In the current thesis, we aimed to characterize the mechanisms linking lysosomal lipid overload and inflammation investigated the effects of different intervention strategies to treat metabolic inflammation.

Chapter 2 provides extensive information about the role of cathepsins in pathophysiological conditions. Further, we specifically explain the mechanisms of cathepsin translocation and address the site-specific functions of cathepsins with emphasis on the importance of selective cathepsin targeting in chronic inflammatory diseases. **Chapter 3** explores the pharmacological value of a selectively targeting extracellular cathepsin D in NAFLD using a small-molecule inhibitor. This chapter demonstrates the causal role of extracellular cathepsin D in lipid metabolism using Sprague-Dawley rats on high fat diet. In addition to steatosis, we found improved insulin sensitivity in these rats. Since steatotic rats display different hyperlipidemic profile than humans, we further expanded this research in **chapter 4**, where we investigated the individual roles of intracellular and extracellular cathepsin fractions in hepatic inflammation. We made use of *Ldlr*^{-/-} mice which were treated with respective cathepsin inhibitors and fed a high- fat, high-cholesterol diet for a period of 10 weeks. Furthermore, we used proteomic approaches to understand the mechanisms of intra and extracellular cathepsin D action during NASH. In **chapter 5**, we explain the complex role of the gut/microbiota/brain axis with regard to the gastrointestinal inflammation associated with IBD. Further this chapter focuses on the benefits of non-pharmacological stress management techniques in modulating inflammation in IBD. In **chapter 6**, the effects of 2-hydroxypropyl- β -cyclodextrin (CD), a cholesterol mobilizing drug were examined in various models of metabolic inflammation Using *in vivo* and a series of *in vitro* experiments, we demonstrated that besides its beneficial cholesterol depleting properties, CD can cause time-dependent pro-inflammatory effects. Thus, care should be taken when using CD in clinic. Finally, **chapter 7** discusses the important conclusions of this thesis with respect to the current knowledge in the field and highlights the clinical implications of the findings.

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Chapter 2

The Ins and Outs of Cathepsins: Physiological function and role in disease management

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Abstract

Cathepsins are the most abundant lysosomal proteases that are mainly found in acidic endo/lysosomal compartments where they play a vital role in intracellular protein degradation, energy metabolism, and immune responses among a host of other functions. The discovery that cathepsins are secreted and remain functionally active outside of the lysosome has caused a paradigm shift. Contemporary research has unraveled many versatile functions of cathepsins in extra lysosomal locations including cytosol and extracellular space. Nevertheless, extracellular cathepsins are majorly upregulated in pathological states and are implicated in a wide range of diseases including cancer and cardiovascular diseases. Taking advantage of the differential expression of the cathepsins during pathological conditions, much research is focused on using cathepsins as diagnostic markers and therapeutic targets. A tailored therapeutic approach using selective cathepsin inhibitors is constantly emerging to be safe and efficient. Moreover, recent development of proteomic-based approaches for the identification of novel physiological substrates offers a major opportunity to understand the mechanism of cathepsin action. In this review, we summarize the available evidence regarding the role of cathepsins in health and disease, discuss their potential as biomarkers of disease progression, and shed light on the potential of extracellular cathepsin inhibitors as safe therapeutic tools.

1. Introduction

Lysosomes are intracellular membrane-bound organelles characterized by an acidic interior and harbor a variety of hydrolytic enzymes including lipases, proteases and glycosidases that participate in cellular catabolism [1,2]. The functions of most of these enzymes require an acidic lumen, which is maintained by the vacuolar H⁺ ATPase (V-ATPase), an ATP-driven proton pump located on the lysosomal transmembrane. Lysosomes can fuse with endosomes, phagosomes, autophagosomes and break down both endogenous and exogenous cargo consisting of various biomolecules such as lipids, proteins, polysaccharides, and certain pathogens. Additionally, lysosomes play critical roles in some of the most vital processes such as metabolic signaling, repair of the plasma membrane and nutrient sensing [3–5]. Lysosomes perform this multitude of coordinated events with the help of their enzymes.

Among the variety of enzymes that lysosomes harbor, cathepsins are a family of lysosomal proteases with an astonishingly broad spectrum of functions. Cathepsins help in intracellular housekeeping where they for example, participate in the antigen processing during immune responses and degrade several proteases and chemokines to maintain cellular homeostasis (reviewed in [6,7]). Mammalian proteases have been classified into 5 different families namely metallo, serine, threonine, aspartic, and cysteine proteases based on the type of amino acid at the active site. All cathepsins fall into three different protease families viz; serine proteases (cathepsins A and G), aspartic proteases (cathepsin D and E) and eleven cysteine cathepsins (cathepsins B, C, F, H, K, L, O, S, V, X, and W) [6,8,9]. Serine proteases constitute up to 31% of total proteases expressed in human body, while cysteine and aspartic proteases make up for 25% and 4% of the total protease population respectively [6,10]. Cathepsins show highest activity in the low pH environment of lysosomes. However, certain cathepsins are also found to be active outside of their optimal pH range of 5 [6,11]. The pH optimum of cathepsin S was found to be 6.5 [12,13]. While cathepsin D shows optimal activity at pH 4, its activity was detected even at pH of 7.4 (although at reduced kinetic rates) [14]. Moreover, cathepsin K and H displayed stable activity at pH 7 [15] indicating their wide range of proteolytic activity. Due to the retained activity far outside the optimal pH range cathepsins have been identified with specific proteolytic functions even outside of the endo/lysosomal system [16,17].

Although cathepsins exhibit some level of similarity in their proteolytic functions in physiological processes, consequences of cathepsin dysfunction are very diverse in terms of clinical symptoms. Deregulated cathepsin synthesis and activity has been associated with several diseases including the metabolic syndrome, cancer and inflammatory neurological diseases (reviewed in [18]). Cathepsins are known to activate and/or degrade several important neuronal proteins, and thus have important roles in neurodegenerative disorders (reviewed in [19,20]). For example, cathepsin D plays an important role in neuronal cell homeostasis whose dysfunction leads to impaired proteolysis of target proteins such as huntingtin, α -synuclein, tau, lipofuscin, apoE resulting in Huntington's and Parkinson's amongst other neurological disorders [20]. In addition to cathepsin D, several cathepsins are associated with inflammatory neurological diseases including Niemann–Pick type C (NPC) disease, neuronal ceroid lipofuscinosis (NCL) and Alzheimer's (reviewed in [21–23]). Cardiovascular disorders such as cardiomyopathy, hypertension, myocardial infarction, atherosclerosis and aortic aneurysms are characterized by extensive extracellular matrix (ECM) degradation and remodeling, one of prime processes mediated by cathepsins (reviewed in [24]). Similarly, in cancer, tumors

metastasize by ECM degradation and cathepsins are known to breakdown the constituents of ECM, epithelial membrane and cell–cell junctions facilitating cancer cell migration. Further, cathepsins are involved in growth, invasion, angiogenesis, and therapeutic resistance associated with tumors [25]. Obesity and diabetes are most common metabolic disorders and cathepsins L, S, and K are found to have potential roles in these pathologies (reviewed in [26]). Thus, the range and complexity of biological activities reliant on cathepsins make them a common point of interest in diverse diseases. Importantly, owing to their differential function during physiological and pathological conditions, cathepsins are considered as highly relevant targets for therapeutic intervention in this range of diseases.

This review is divided into four parts. In the first part, we provide a basic overview of cathepsin expression and function within the endo/lysosomal compartments. In the next two parts, we emphasize on the mechanisms leading to the translocation of cathepsins into the cytosol and extracellular milieu, respectively. Finally, this review further highlights the prospect of using highly specific, targeted cathepsin inhibitors for clinical implementation in various diseases.

2. Cathepsins in lysosomes

Cathepsins exhibit similarities in their cellular localization and biosynthesis with some differences in their expression pattern. Of all the lysosomal proteases, cathepsins L, B, and D are the most abundant with their lysosomal concentrations equivalent to 1 mM [27]. Cathepsins B, H, L, C, X, V, and O are ubiquitously expressed while cathepsins K, S, E, and W show cell or tissue-specific expression. Cathepsin K is expressed in the osteoclasts (multinucleated cells of bone) and in epithelial cells. Cathepsins S, E, and W are mainly expressed in immune cells.

2.1. Regulation

Cathepsins undergo transcriptional, translational, post-translational and epigenetic regulation. At transcriptional level, except for cathepsin D all other cathepsins show TATA-independent transcription initiation which defines the transcription initiation site. Cathepsin L, K, C, and B are known to require various transcription factors such as nuclear factor (NF- κ B), specificity proteins, Sp1, Sp3 and erythroblast transformation-specific (Ets) family factors for transcription initiation and regulation [28,29]. Cathepsin D is known to be transcriptionally regulated by Peroxisome Proliferator-Activated Receptor γ in dendritic cells [30] and by estrogen in breast cancer cells [31]. Transcript variants for cathepsin B and L have been reported as a result of alternative splicing. These transcripts differ in their mRNA stability and thus are known to be accumulated during tumors [32]. Cathepsin L is found to be translationally regulated by internal ribosomal entry site (IRES) [33]. Cysteine cathepsins are known to also contain CpG islands in their promoter region and thus are epigenetically regulated by methylation [25]. Additionally, cathepsin enzyme activity is regulated by pH and endogenous protein inhibitors such as stefins, cystatins, and kiniogens [8].

2.2. Synthesis

The primary structure of all cathepsins consists of a signal peptide, a propeptide, and a catalytically active mature functional enzyme. Cathepsins are synthesized as preprocathepsins in the endoplasmic reticulum (ER). A N- terminal signal peptide of 20-25 amino acids directs preprocathepsins into the ER lumen where they are cleaved co-translationally by a signal peptidase generating procathepsins, the less active zymogen forms. Simultaneously, N-linked glycosylation occurs within the ER, producing high levels of mannose in the procathepsins [34]. Procathepsins later travel through the Golgi stacks where the mannose residues are modified to mannose-6-phosphate moieties (M6P). The M6P-tagged procathepsins are recognized and bound by M6P receptors in the trans-Golgi network (TGN) and are either directly sorted to the endo/lysosomes or first pass the plasma membrane from where they enter the endo/lysosomes in an indirect manner. Both direct and indirect routes rely on clathrin-coated vesicles to carry the cathepsins from the TGN or plasma membrane to the endo/lysosomes. Once the cathepsins are trafficked to endo/lysosomes, free M6P receptor is transported back to the TGN [35]. Inside lysosomes cleavage of propeptide converts procathepsins to mature active cathepsins. This activation occurs via different modes such as auto-activation or trans-activation or both. During the auto activation the pH inside the lysosomes enables the cleavage of propeptide by the catalytic site of the same enzyme, while trans-activation requires help of other proteases. For instance, cathepsin B, H, L, S, and K are activated by auto-activation, while cathepsins C and X need cathepsins L and S for their activation [36,37]. Cathepsin D is found to be processed by partial auto-activation and requires cathepsin B and L for further maturation [38]. Further, procathepsin D is known to undergo a two-step maturation process in the endo/lysosomal compartments: in the first step, propeptide is partially cleaved to generate an active single-chain intermediate which upon reaching lysosome undergoes second processing in the region of T155–K173 to yield a double-chain mature cathepsin D [39].

2.2.1 M6P-independent sorting

While most of the lysosomal enzymes are dependent on M6P for their transport, several studies have reported the existence of M6P-independent transport routes of cathepsins to reach endo/lysosomes with the aid of alternative receptors. One such alternative receptor protein is sortilin, a trans-membrane Golgi protein, which was shown to be involved in the sorting of cathepsin H and cathepsin D in COS-7 cells [40]. In mouse embryonic fibroblasts cathepsin B and D are known to be captured by the membrane proteins LRP1 (low-density lipoprotein receptor-related protein 1) and LDLR (low-density lipoprotein receptor) [41,42]. While procathepsin D is known to complex with sphingolipid activator precursor protein prosaposin, procathepsin B is known to rely on its membrane association to enter lysosomes independent of M6P route [43–45]. Recently, type 1 transmembrane protein SEZ6L2 was linked with sorting of cathepsin D in neurons [46]. Thus, cell-type, post-translational processing, and modifications play an important role in the sorting of cathepsins towards lysosomes.

2.3. Physiological functions of cathepsins in the endo/lysosomes

Cathepsins carry out many proteolytic events in the compartments of the endocytic pathway thus contributing to the protein turn-over and normal metabolism of the cell. The pH inside the

endo/lysosomal compartment favors cathepsin activity while it induces conformational changes in the substrates leading to their cleavage by cathepsins, and thereby helping cathepsins to successfully degrade the cargo transported to the endo/lysosomes [9,47].

2.3.1. Immune responses

Cathepsins display significant roles predominantly in the endosomes of immune cells. They are known to participate both in the innate and adaptive immune responses. During the innate immune responses, lysosomal cathepsins have been shown to cleave the ectodomains of Toll-like receptors (TLRs) 7 and 9 that are expressed on endo/lysosomal membranes, where TLRs recognize nucleic acids of phagocytosed microbes. The processed forms of TLR 7 and 9 then recruit the adaptor protein MyD88 leading to activation of TLR signaling pathways [48]. During adaptive immune responses, T lymphocytes recognize processed antigens on the surface of antigen-presenting cells (APCs) that are bound to major histocompatibility complex (MHC) molecules. While MHC class I molecules present processed antigenic peptides that are derived from the cytosol, MHC class II molecules present peptides derived from the endo/lysosomal compartment. Further, MHC class II molecules require an invariant chain (Ii), a glycoprotein necessary for their folding and assembly. Various cathepsins are found to be involved in the (I) proteolytic processing of antigens into short peptides and (II) degradation of the invariant chain thus facilitating adaptive immune responses [49,50]. However, *Deussing et al.* [51] demonstrated that cathepsins B and D are non-essential in MHC II mediated antigen presentation.

2.3.2. Autophagy

Cathepsins participate in autophagy, an essential catabolic process which delivers cytosolic constituents to lysosomes for their subsequent degradation in order to maintain cell homeostasis [52]. As demonstrated by *Dennemarker et al.* [53], cathepsin L deficient primary mouse embryonic fibroblasts showed normal initiation of the autophagy process, autophagosome formation as well as autophagosome–lysosome fusion but impaired degradation of autolysosomal content. Despite this impairment of autolysosomal turnover due to the lack of cathepsin L, the viability of the cells was not affected. This implies that autolysosomal degradation is not solely dependent on cathepsin L and is likely compensated by cathepsin D as evidenced by the increased levels of cathepsin D in cathepsin L knock out cells. In addition, cathepsins also regulate lysosome and autophagosome populations. Cathepsin B is known to degrade the calcium channel MCOLN1/TRPML1 in the lysosomes, leading to the suppression of transcription factor TFEB and thus inhibiting the expression of autophagy-related proteins. This response is known to keep in check the lysosomal biogenesis and population of cellular autophagosomes [54]. Cathepsin S is required for the fusion processes of autophagosomes and lysosomes and its deficiency results in increased number of autophagosomes. Moreover, cathepsin S-mediated autophagic flux is known to induce M2-type polarization of tumor associated macrophages, which would then contribute to tumor [55].

2.3.3. Growth and development related functions

Cathepsins regulate growth and development related processes by processing various hormones and growth factors. For instance, cathepsin B degrades a variety of substrates including insulin-like growth factor-1 (IGF-I), glucagon, pituitary hormone, thyroglobulin [56,57]. Additionally, cathepsin B in the adipocytes is known to cleave perilipin 1 (PLIN1), a lipid droplet-associated protein leading to increased lipolysis in obese adipose tissue [58]. Further, the proteolytic events regulated by cathepsins are critical in the control of biological processes including ovulation, neuronal development and fertilization. For instance, cathepsin L is one of the key proteases upregulated in granulosa cells of ovulatory follicles mediating follicular rupture during ovulation [59]. Cathepsin D is known to process vitellogenin and together with B and L, is involved in oogenesis in lower vertebrates. Cathepsins are extensively involved in embryo development. Relevantly, knock down of cathepsin D led to tissue defects including eye, skin and swim bladder in zebra fish [60] and caused intestinal mucosa damage, atrophy of myelin sheath and early death in mice [61]. Similarly, mice deficient in cathepsins B and L developed atrophy in the cerebral and cerebellar regions of the brain, suggesting their necessity for neuronal development [62].

2.4. Pathological role of cathepsins in the endo/lysosomes

Inactivation or loss-of-function of cathepsins results in inappropriate degradation and abnormal accumulation of substrates in lysosomes leading to various diseases including lysosomal storage disorders (LSD). Further, defective proteolytic events by cathepsins can lead to several LSDs, namely, galactosialidosis [63] neuronal ceroid lipofuscinosis (CLN) type 10 and 13 [63,64], Papillon–Lefèvre syndrome [65], pycnodysostosis [66], and Alzheimer’s disease [21]. Further, given the important role of cathepsins in autophagy, impaired proteolysis by cathepsins can lead to massive accumulation of autophagosomes which eventually can cause pathologies including cellular senescence, inflammasome activation [67] and Saposin (Sap) C deficiency, a rare variant form of Gaucher disease [68]. Impaired activity of Cathepsin D and L would result in accumulation of α -synuclein amyloid fibrils in the brain tissue leading to synucleinopathies [69]. Alterations or loss of functions in the cathepsin activity are known to have adverse effects on reproduction and fertility [70]. Thus, consequences of cathepsin inactivation translate to a wide variety of clinical complications. Detailed functions of individual cathepsins and the associated pathologies are mentioned in **Table 1**.

Table 1: Cathepsins in the endo/lysosomal compartment

Cathepsin	Enzyme Commission Number	Catalytic type	Function	Pathology	OMIM ID	Reference
Cathepsin A	3.4.16.5	serine	dual function: a. protective: β -galactosidase and neuraminidase b. degradative: bioactive peptides like bradykinin, angiotensin, oxytocin, endothelin 1	hypertension Galactosialidosis	256540	[63]
Cathepsin B	3.4.22.1	cysteine	degrades amyloid- β ; activation of pro-hormones and pro-enzymes; trypsin activation; promotes viral entry into cells	Alzheimer’s; gaucher disease acute pancreatitis		[21,68]
Cathepsin C	3.4.14.1	cysteine	inflammatory responses and activation of serine proteases	Papillon–Lefèvre syndrome Periodontitis	245000	[65]

			including neutrophil elastase and cathepsin G			
Cathepsin D	3.4.23.5	aspartic	embryo and neuronal development brain antigen processing of α -Synuclein; tau, amyloid β , apoE; degradation of hormones, proenzymes and growth factors	Alzheimer's disease; CLN 10 Parkinson's; Huntington's	610127	[20,61,64]
Cathepsin E	3.4.23.34	aspartic	carboxypeptidase A and IgE processing	atopic dermatitis		[71]
Cathepsin F	3.4.22.41	cysteine	li chain processing and MHC-II class responses	CLN 13	615362	[63]
Cathepsin G	3.4.21.20	serine	auto antigen processing	auto-immune diseases		[72]
Cathepsin H	3.4.22.16	cysteine	prohormone processing	type 1 diabetes		[9]
Cathepsin K	3.4.22.38	cysteine	TLR signaling; processing of β -endorphin in brain	periodontitis; pseudosyndactylism	265800	[73,74]
Cathepsin L	3.4.22.15	cysteine	antigen and li chain processing; prohormone processing; degradation of α -Synuclein, tau; promotes viral entry into cells	Parkinson's disease; Frontotemporal dementia		[19,49]
Cathepsin S	3.4.22.27	cysteine	antigen processing and presentation; li chain processing	auto-immune diseases		[50]
Cathepsin X	3.4.18.1	cysteine	T-cell migration and invasion	-		[75]
Cathepsin O	3.4.22.42	cysteine	-	-		
Cathepsin V	3.4.22.43	cysteine	natural killer cell and CD8+ cytotoxic cell production	thymic pathology		[76]
Cathepsin W	3.4.22.-	cysteine	component of endoplasmic reticulum proteolytic machinery	-		[77]
Cathepsin Z	3.4.18.1	cysteine	intracellular protein turnover	-		[78]

- implies that no function or pathology is yet discovered for the respective cathepsins. CLN stands for ceroid lipofuscinosis, neuronal; OMIM stands for online mendelian inheritance in man.

3. Cathepsins in cytosol

As described in the previous section, proteolytic functions of cathepsins mainly occur in the endo/lysosomal compartments. However, most of the cathepsins are released into the extralysosomal locations such as cytosol, nucleus, and mitochondria where they perform crucial tasks. In the current section, we describe the mechanism of cathepsin translocation into different regions of the cell and their respective functions.

3.1. Mechanism of translocation

Perhaps one of the most simplistic mechanisms through which cathepsins are translocated to cellular compartments other than lysosomes is by leaking outside of lysosomes. Lysosomes are surrounded by a limiting membrane, which is a phospholipid bilayer that is characterized by variety of integral membrane proteins, which together protect the organelle from the degradative enzymes [79]. Damage to the lysosomal membrane components or disturbances in membrane fluidity and structure can influence lysosomal stability and subsequently lead to rupture of lysosomes, a process called lysosomal membrane permeabilization (LMP). LMP is induced by a plethora of stimuli including oxidative stress, lysosomotropic agents, as well as some endogenous cell death effectors. While partial permeabilization of the lysosomal membrane induces apoptosis (programmed cell death), complete lysosomal rupture leads to necrosis (reviewed in [80]).

3.1.1. Oxidative stress induced LMP

Lysosomal destabilization is recognized feature during oxidative stress-mediated cell damage. The initial burst of reactive oxygen species (ROS) may be generated by different triggers inside or outside of the lysosomes. For example, ceramide accumulation or ingestion of heavy metals such as silica or asbestos is known to increase NADPH oxidase in the lysosomes to generate ROS. The generated free radicals interact with free intralysosomal iron forming highly reactive hydroxyl radicals in a Fenton-type reaction [80]. Such hydroxyl radicals induce LMP by causing lipid peroxidation of lysosomal membranes thereby forming lipofuscins and further damaging lysosomal membrane proteins (reviewed in [81]). An oxidative burst elicited by interferon- γ was reported to induce LMP and cathepsin release [82]. Further, ROS is known to modify lipids and lysosomal trapping of oxLDL has the potential to damage and disrupt the lysosomal membrane leading to the secretion of cathepsins [83,84]. Additionally, ROS is found to be generated outside of the lysosomes by destabilized mitochondria, inhalation of exogenous pollutants and ionizing radiation leading to LMP (reviewed in [85,86]).

3.1.2. Lysosomotropic agents

Lysosome-targeting agents, referred to as lysosomotropic agents and lipid detergents cause LMP either by direct membrane lysis or by osmotic lysis of lysosomal membrane. Examples of agents that cause direct membrane lysis include O-methyl-serine dodecylamide hydrochloride (MSDH), N-dodecylimidazole, n- β -naphthylamide, and endogenous compounds such as lipofuscin. Due to their amphiphilic nature, these agents partition between the water phase and the phospholipid bilayer of lysosomal membrane. Further, accumulation of the lysosomotropic agents and molecules leads to thinning of the lysosomal bilayer and subsequent solubilization of lysosomal membrane (LM) (reviewed in [87]). Lysosomotropic amines, such as chloroquine or ammonium chloride and peptides such as (LeuLeu)nOMe are known to change the permeability of LMs and thus leading to increased influx of solutes into lysosomes and eventually leading to osmotic lysis [88]. Loss of membrane cholesterol or modification of membrane lipids by lipases such as sphingomyelinase and ceramidase are known to induce LMP by increasing the lysosomal permeability for potassium ions and protons and subsequent osmotic lysis [89,90]. Thus, various molecules can cause LMP, leading to leakage of cathepsins into the cytosol.

3.2. Transport of cathepsins to different regions of cytosol

Besides LMP, other mechanisms such as alternative translation or exon skipping can lead to extra lysosomal translocation of cathepsins. Translation initiation at a different start site produces cathepsins which are devoid of signal peptide, while exon skipping generates truncated cathepsins with modified signal sequences thus misrouting cathepsins from their biosynthetic pathway to locations such as cytosol, nucleus, and mitochondria. For instance, translational initiation at downstream AUG site is known to localize cathepsin L in nucleus [91] and alternative splicing in cathepsin B mRNA with missing exons at 2 and 3 is known to direct cathepsin B to mitochondria [92]. Another report by Bestvater *et al.* [93] proposed an alternative targeting signal besides the usual N-

terminal signal peptide for cathepsin B namely, a signal patch within its heavy chain domain that facilitates its nuclear import.

3.3. Physiological functions of cathepsins in the cytosol

Although cathepsins do not retain optimal activity at the neutral pH of the cytosol, their proteolytic activity is known to be preserved by substrate binding and acidification of the cytosol observed under certain conditions like apoptosis and cathepsins in the cytosol are known to mediate key physiological processes as described below.

3.3.1. Apoptosis and necroptosis

Apoptosis is a highly regulated, fundamental physiological process of cell death responsible for removal of damaged/aging cells by activation of caspase family of proteins. Apoptosis involves two different pathways; intrinsic pathway, also known as the mitochondria pathway, or the extrinsic pathway involving death ligands (reviewed in [94]). The mitochondria pathway is regulated by B-cell lymphoma-2 (Bcl-2) family proteins. Bcl-2 family consists of both anti-apoptotic members such as Bcl-2 and Bcl-xL and pro-apoptotic proteins including Bax, Bak, and Bid [95]. By genetic modification or use of pharmacological inhibitors, several studies have established that cathepsins in the cytosol play an important role in apoptosis, specifically by activating other apoptotic proteases [96]. Cytosolic cathepsins B, D, and L are implicated in the degradation of Bid, resulting in its activation and translocation to mitochondria. This translocation leads to cytochrome C release from mitochondria followed by caspase activation, and thus initiating apoptotic cell death. Simultaneously, cathepsins are involved in the degradation of anti-apoptotic proteins Bcl-2, Bcl-xL, Mcl-1, and XIAP (X-linked inhibitor of apoptosis), promoting apoptosis [97]. Additionally, in T cells, cathepsin-D-mediated apoptosis involves the activation of Bax, and the release of apoptosis-inducing factor (AIF) and cystatin c. This process has been shown to be independent of Bid cleavage and initiates apoptosis by directly activating the initiator caspase-8 [98–100].

Recent studies have shown that cathepsins regulate programmed necrosis termed necroptosis. Necroptosis is initiated by various stimuli and requires the kinase activity of receptor-interacting serine/ threonine kinase1 (Rip1). In macrophages, it has been reported that cathepsins B and S are known to cleave receptor-interacting protein 1 (Rip1) kinase and thus limit macrophage necroptosis [101].

3.3.2. Inflammation

Cytosolic cathepsins are shown to be involved in mediating inflammatory responses via activation of inflammasomes. The NLRP3 inflammasome is a multi-protein complex which is activated upon bacterial infections, LMP or cellular damage. Upon activation, pro-caspase1 is converted to active caspase1 by autocatalysis. Active caspase1 then proceeds to cleave the cytokine precursors pro IL-1 β and IL-18 into their mature secreted forms (reviewed in [102]). Gene knockout and siRNA knockdown of cathepsins B, C, S, L, and Z resulted in a suppression of IL-1 β secretion [103–105]. However, cathepsins are not shown to directly cleave either caspase-1 or IL-1 β suggesting that they

operate upstream of inflammasome activation [103]. Additionally, Cathepsins Z and S are known to compensate for the activity of cathepsins B, C, and L in LMP-mediated inflammasome activation by unknown mechanisms [106].

3.3.3. Functions of nuclear cathepsins

Cathepsins in the nucleus are known to process transcription factors that control cell cycle progression thus facilitating cell proliferation and differentiation (reviewed in [107]). The CDP/Cux/Cut transcription factors are a group of highly conserved proteins in higher eukaryotes that are involved in cell cycle proliferation, particularly in the transition from G1 to S phase. Cathepsin L in the nucleus is known to cleave the CDP/Cux, which accelerates progression into S phase of the cell cycle [108]. Nuclear cathepsin D promotes cell proliferation by acting as co-factor for Tricho-rhino-phalangeal syndrome Type 1 (TRPS1) transcription factor and is known to enhance mammary gland differentiation by cleaving histone H3 [109]. Nuclear cathepsins are known to regulate transforming growth factor- β (TGF- β) signaling, an important pathway normal growth and tissue development whose mis regulation can lead to carcinogenesis. The downstream effectors of TGF- β signaling, the Smad proteins, are phosphorylated and activated by receptors such as importin β which mediate pSmad nuclear translocation, where they regulate transcription. Cathepsins B, K, L, and S are known to localize to nuclear membrane where they exert differential effects in the translocation of pSMAD2 and pSMAD3 proteins by modulating importin β expression and thus regulate TGF- β signaling [110]. Physiological functions of cytosolic cathepsins are listed in **Table 2**.

Table 2: Function of cathepsins in the cytosol

Cathepsin	Extra lysosomal location	Function	Reference
Cathepsin B, D and L	cytosol	proteolytic processing of Bid during apoptosis	[97,111]
Cathepsin B, C, L, S and Z	cytosol	NLRP3 inflammasome activation	[103–106]
Cathepsin B	cytosol	regulation of hepatic lipid metabolism by degrading liver fatty acid binding protein	[112]
Cathepsin L and H	nucleus	cell cycle regulation	[91]
Cathepsin B, K, L and S	nucleus	TGF- β signaling	[110]
Cathepsin B	nucleus	bile-salt induced apoptosis	[113]
Cathepsin A, E, G, S, X, O, V, W, Z *	-	-	-

* Roles for these remaining cathepsins outside of the lysosome have not yet been reported.

3.4. Pathological functions of cathepsins in the cytosol

Lysosomal disruption and the subsequent release of cathepsins in the cytosol can have detrimental effects. For instance, though apoptosis is a highly regulated fundamental physiological process, there are many pathological conditions including neurodegeneration and ischemia that involve excessive apoptosis in which cytosolic cathepsins are known to play an active role [96,114]. Accordingly, substantial evidence supports the contribution of cathepsins from ruptured lysosomes in the pathology of many neurodegenerative diseases [115]. In contrast to healthy brain, in Alzheimer's brain, cathepsin D is found in the cytosol and such cytosolic cathepsin D is known to cleave tau protein generating truncated form of tau which in turn forms paired helical filaments leading to

neurofibrillary degradation [115]. Cytosolic cathepsin D is also implicated in glaucoma by promoting apoptosis in trabecular mesh work cells, which maintain intraocular pressure of the eye [116]. Furthermore, cytosolic cathepsin D has been proposed as a biomarker of age-related neurodegenerative disorders [117]. In line, it has been found that cathepsin D translocation into the cytosol led to pronounced age-related changes in rats, by increasing the degeneration of neurons [117,118]. NLRP3 inflammasome is essential for defending against bacterial infections and misregulated NLRP3 inflammasome has been implicated in metabolic inflammatory disorders including type 2 diabetes, atherosclerosis, heart reperfusion injuries, and chronic kidney diseases (reviewed in [119]). Given the known role of cathepsins in modulating inflammasome activation it then also indirectly suggests a pathological relevance of cathepsins in these disorders.

Another cytosolic cathepsin that is associated with disease conditions is cathepsin L. In pathological conditions such as proteinuria and glomerular kidney disease, translocation of cathepsin L to cytosol has been documented [120]. Here, in contrast to its lysosomal counterpart, cytosolic cathepsin L in podocytes (cells in the Bowman's capsule of the kidneys), is known to degrade cytoskeleton proteins, namely, CD2-associated protein, synaptopodin and dynamin, thus leading to the reorganization of the actin cytoskeleton, proteinuria and subsequent renal failure [121]. Nuclear cathepsin L activity is associated with polycystic kidney disease [121] and alterations in its activity significantly influenced colorectal cancer disease progression [108]. Nuclear cathepsin F activity is found to be correlated with markers of transcriptional regulation in hepatic stellate cells [122]. Mitochondrial procathepsin B is known to induce morphological changes in mitochondrial integrity and thus can lead to cell death [123]. Taken together, cathepsins perform dynamic functions outside of the lysosomes based on their cytosolic location.

4. Cathepsins in the extracellular space

Numerous lines of evidence demonstrated the presence of cathepsins in the extracellular space. Although the extracellular localization of cathepsins is more commonly observed during pathological conditions, cathepsins are mostly involved in the bone remodeling and plasma membrane repair during physiological conditions.

4.1. Mechanism of translocation

Cathepsins are normally secreted via lysosomal exocytosis or by alternative sorting from Golgi. Usually, secretion of cathepsins is often accompanied by their over expression which is commonly observed in cancer and inflammatory conditions [16]. Immune cells are known to secrete high levels of cathepsins. Additionally, osteoclasts, keratinocytes, thyroid cells, and smooth muscle cells also release cathepsins into the extracellular space [124].

4.1.1. Lysosomal exocytosis of cathepsins

The secretory pathway of lysosomes, known as lysosomal exocytosis, has been reported in many types of cells and is induced by various stimuli such as wound, cellular stress, cancer, or by signals from cytokines [125]. The induction of exocytosis occurs by the recruitment of lysosomes to the

periphery of the cells. This movement of lysosomes is mediated along microtubule track with the help of various kinesins and a multi subunit complex named BLOC-one-related complex (BORC) that promotes kinesin-mediated lysosome movement toward the cell periphery [126]. The movement of lysosomes to the periphery is followed by docking, where the lysosome and plasma membrane are brought into closer contact with the help of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes, namely the interaction of v-SNARE (vamp7) on lysosomal membrane with t-SNARE (syntaxin 4 and SNAP-23) on the cytoplasmic side of the plasma membrane (PM) [127]. Both lysosomes and PM contain negatively charged lipids in their outer and inner layers, respectively. Hence, cells rely on calcium ions (Ca^{2+}) to bridge the opposing charges on the membranes. Further, lysosomal docking and fusion are regulated by transcription factor EB (TFEB). EB modulates lysosomal exocytosis by triggering intracellular Ca^{2+} elevation through the endo/lysosomal cation-channel mucolipin 1 (MCOLN1) [128]. Finally, the fusion of lysosomes with the PM results in the secretion of lysosomal components into the extracellular space. Castro-Gomes *et al.* [129] elegantly demonstrated that cathepsin B and L are released extracellularly by lysosomal exocytosis and participate in PM repair using in vitro systems.

4.1.2. Alternative sorting of cathepsins into extracellular space

As described in the section 2.1 of this review, cathepsins are trafficked to endo/lysosomal compartments of the cell with the help of M6P receptors. Changes in the pH are known to disrupt the recycling of M6P to the Golgi, where its absence leads to the potential re-routing of procathepsins into the extracellular space directly or packaged into the secretory vesicles [37,130]. In contrast, during conditions such as bone resorption, secretion of active form of cathepsins is observed suggesting that the secretion is context dependent. Once secreted, cathepsins can be found attached to the caveolae on the PM, as seen in case of cathepsin B [131] or released in the extracellular space directly or packed inside the secretory vesicles.

4.2. Physiological function of extracellular cathepsins

Cathepsins require acidic pH for their optimum activity in contrast to the neutral pH found in extracellular space. However, cathepsins are often secreted as less active procathepsins which are normally stable at neutral pH [132]. For cathepsins secreted in the mature active form, vacuolar H^{+} -ATPases (V-ATPase) are known to provide a local acidic hub in the pericellular to facilitate their prolonged activity in the extracellular space [133]. For instance, in bone lacunae, V-ATPase and the chloride channel create a low pH extracellular environment with the help of H^{+} and Cl^{-} ion flow [134]. Cathepsins in the extracellular matrix are known to degrade many components of the ECM and thus participate in physiological processes including wound healing [135], bone remodeling [136,137] and processing of prohormones [8].

4.2.1. ECM degradation

ECM is composed of fibrous proteins such as elastin, collagen, proteoglycans, and fibronectins that form the intricate meshwork for holding the cells embedded within the tissues. Further, ECM undergoes constant remodeling and ECM components have several binding sites for growth factors

that ultimately control cell adhesion, proliferation, migration, and polarity [138]. ECM conducts mechanical signals that further activate cytoskeletal and intracellular signaling pathways. In healthy tissue, ECM homeostasis is mainly regulated by cathepsins and matrix metalloproteases (reviewed in [139]).

Bone remodeling is a continuous process that involves bone resorption and remodeling performed by specialized cells called osteoclasts. Further, bone and cartilage predominantly contain type 1 collagen. During bone resorption, osteoclasts attach to the surface of bone leading to the creation of an extracellular compartment which is isolated from general extracellular fluid (reviewed in [140]). Numerous studies showed that the active cathepsin K released from osteoclasts into the resorption lacunae is known to degrade type 1 collagen and elastin and thus plays a pivotal role in bone resorption [17,137,139,141]. Moreover, cathepsins are known to degrade two bone ECM proteins, osteocalcin, and osteonectin. While osteocalcin is involved in bone formation and insulin metabolism, osteonectin helps in cell matrix interactions [139,142]. Further, proteoglycans are major constituents of ECM are composed of core protein with covalently attached glycosaminoglycan molecules (GAGs). Various cathepsins are known to cleave the protein core of proteoglycan. For example, cathepsins B and L cleave perlecan, a heparan sulfate proteoglycan, generating LG3 peptide, which is known to have neuroprotective role in brain ischemia [143,144].

4.2.2. Functions of cathepsins on the plasma membrane

Cathepsins on the cell surface are known to be involved in plasma membrane repair. Cathepsin B released from keratinocytes attaches to cells surface where it is known to be involved in keratinocyte migration by degrading components of ECM during wound healing [145]. Another cathepsin that translocate to the plasma membrane is cathepsin X which helps with processes of cell adhesion and signaling [75]. β integrins are cell surface proteins that help in cell adhesion and invasion. Cathepsin X on the plasma membrane is known to cleave regulatory motifs in two different types of $\beta 2$ integrin receptors. By activating $\beta 2$ integrin receptor Mac-1, cathepsin X enhances the adhesion of immature dendritic cells to ECM, leading to their activation. Additionally, cathepsin X activates another $\beta 2$ integrin receptor, LFA-1 enhancing the proliferation of T lymphocytes thus accelerating immune responses [75,146].

4.2.3. Functions of cathepsins in the secretory vesicles

Neurons and endocrine cells carry out cell–cell communication with the help of peptide neurotransmitters. Secretory vesicles in these cells provide regulated secretion of neurotransmitters, which are first synthesized as inactive prohormones. Proteolytic processing of the proproteins or prohormones occurs in the secretory vesicles by cathepsins [147]. Numerous studies have identified cathepsin L and V as key processing enzymes for production of numerous peptide neurotransmitters including neuropeptide Y, enkephalin, cholecystokinin, and dynorphins [25,148]. The environment inside the vesicles is known to have acidic pH which promotes the function of cathepsins to generate active peptides [149,150].

4.3. Pathological functions of extracellular cathepsins

While ECM remodeling is an important physiological process, aberrant ECM dynamics can lead to uncontrolled cell proliferation, invasion, and differentiation leading to fatal pathologies including atherosclerosis, cancer, and tissue fibrosis [138]. Extracellular cathepsin activity have been implicated in many of these diseases as elaborated below.

4.3.1. Cancer

Cancer results from abnormal cell proliferation in the body. Increased or abnormal proteolytic activity of cathepsins is known to degrade ECM components facilitating migration and invasion of tumors leading to malignancy (reviewed in [124]). Cathepsins can be secreted from cancer cells and infiltrating immune cells called tumor-associated macrophages (TAMs). Over secretion of cathepsins is associated with their abnormal expression. Several molecular factors that regulate expression of cathepsins in tumor microenvironment are defined. Signal transducer and activator of transcription 3 and 6 (STAT3, STAT6) are known to promote the secretion of procathepsins B, C, S, and Z mainly from macrophages [151]. Collagen 1 is known to induce secretion of procathepsin B by regulating Ets1 transcription factor [152]. Additionally, it has been postulated that cancer microenvironment downregulates M6P receptor mRNA due to overexpression of cathepsins. Due to its weak affinity towards M6P receptor, cathepsin L is known to be directly secreted from TGN before binding to M6P receptor in fibrosarcoma cells [153]. Metastatic tumor cells are known to have defective lysosomal sorting of procathepsin D resulting in its secretion. Further, the acidic tumor microenvironment not only favors the maturation of procathepsins but also promotes their activity [154].

Elevated levels of extracellular cathepsins have been identified in various cancers such as breast, lung, colon, pancreas, skin, prostate, bladder, ovary, and head and neck [25]. In addition to increased levels, increased activity of cathepsins is often associated with activation of tumor-associated cytokines, shedding and cleaving cell–cell adhesion molecules, thereby destroying cell contact and contributing to metastasis. For example, E-cadherin is an important cell adhesion molecule and epithelial tumor suppressor. Extracellular cathepsins B, L and S are known to cleave E-cadherin promoting tumor invasion into surrounding tissues [155]. In contrast, secreted cathepsin D is known to cleave 23 kDa prolactin, a lactogenic hormone produced by pituitary gland to a 16 kDa fragment which has antiangiogenic properties in rat mammary epithelial cells [156] and in bovine corpus luteum but not in humans [157]. Another mechanism through which extracellular cathepsins contribute to cancer progression is by shedding, a process by which proteases release the extracellular domains of cell surface proteins from the cells [158]. Shedding converts membrane-associated proteins into soluble ones and thus reduces their cell surface expression that in turn is a means of regulation for subsequent physiological processes. Apart from several cell adhesion molecules (CAMs) identified as substrates for shedding, cathepsins also target the Ras signaling pathway, a major intracellular signaling pathway in cancer progression. Extracellular cathepsins S and L are known to shed plexins and epidermal growth factor receptor (EGFR), both of which are substrates for Ras pathway activation [16,158]. Similarly, extracellular cathepsin D is known to degrade the ECM proteins thus freeing the embedded growth factors such as fibroblast growth factor. Growth factors are complex polypeptides that have critical roles in basement membrane disruption, cell migration, and tumor metastasis in auto and paracrine manner [154].

4.3.2. Metabolic disorders

Lipoprotein accumulation and metabolism are important contributors to various diseases including cardiovascular diseases and obesity -associated disorders such as non-alcoholic steatohepatitis (NASH). Extracellular cathepsins are found to be involved in transport, efflux, and processing of lipoprotein molecules or their receptors (reviewed in [159]). For instance, extracellular cathepsins F, K, and S are known to degrade cholesterol acceptors on the cell surface, thereby reducing cholesterol efflux and initiating foam cell formation, a key feature of atherosclerosis [24]. Increased presence of low-density lipoprotein (LDL) is a characteristic of both atherosclerosis and NASH. Extracellular cathepsin D is known to proteolytically modify apolipoprotein B-100 (apoB), component of LDL and subsequently leading to LDL accumulation in arterial intima [160,161]. In agreement, extracellular cathepsin D inhibition is known to reduce hepatic steatosis [162]. Further, LDL accumulation in the arterial intima can lead to its oxidation which enhances inflammatory responses, that in a positive feedback loop promotes further secretion of cathepsins leading to exacerbation of lipid accumulation and inflammation (reviewed in [163]). Similar to extracellular cathepsin D, extracellular cathepsin S is known to promote inflammation by cleaving chemokines such as fractalkine (CX3CL1) that help in leukocyte migration and neuropathic pain [164]. Finally, extracellular cathepsin activity is involved in lung fibrosis, osteoarthritis, osteoporosis and rheumatoid arthritis which are summarized in **Table 3**.

Table 3: Cathepsins in the extracellular space

Cathepsin	Substrate	Pathological State	Reference
Cathepsin B, K, and L	proteoglycan	osteoarthritis	[137,165]
Cathepsin B, L, G, and S	fibronectin	cancer and adipogenesis	[166–169]
Cathepsin B, L, and S	laminin	cancer neovascularization, intestinal trauma	[166–168]
Cathepsin K	collagen type I	osteoporosis, rheumatized arthritis, osteoarthritis	[137]
Cathepsin B, K, L, and S	collagen type 2	lung fibrosis, cardiovascular diseases and cancer	[138,139]
Cathepsin B	tenascin	cancer	[170]
Cathepsin B, K, L, and S	aggrecan	osteoarthritis	[136,171]
Cathepsin L, S, and B	plexin	tumorigenesis	[158]
Cathepsin S	fractalkine	neuropathic pain	[164]
Cathepsin D	fibroblast growth factor	breast cancer	[172]
Cathepsin V	elastin	cancer	[173]
Cathepsin X	CXCL-12	-	[174]
Cathepsin W	-	cell-mediated cytotoxicity	[175]
Cathepsin A, C, E, F, O, and Z*	-	-	

* Role of these extracellular cathepsins in pathologies is not known.

The mechanisms involved in the translocation of cathepsins and their respective site-specific functions are illustrated in **Figure 1**.

Figure 1 **Site-specific functions of cathepsins.** (A) cathepsins in the lysosomes (represented by orange arrows): Cathepsins are synthesized as preprocathepsins in the endoplasmic reticulum and transported to endo/lysosomes via Trans-Golgi network where the acidic pH enables their maturation. Cathepsins in the lysosomes are mostly involved in protein degradation besides participating in autophagy. (B) cathepsins in the cytosol (represented by purple arrows): Lysosomotropic agents, ROS or accumulation of modified lipids (oxLDL) leads to lysosomal membrane permeabilization (LMP), releasing cathepsins into the cytosol. Cytosolic cathepsins participate in various activities. For example, cathepsins trigger the inflammasome and promote apoptosis and necroptosis by cleaving various proteins. (C) Cathepsins in the extracellular space (represented by blue arrows): Lysosomal exocytosis involves the secretion of lysosomal contents into the extracellular space with the help of several protein-receptor interactions and Ca^{+2} ion gradient. Cathepsins are released in the form of procathepsins or enclosed in the secretory vesicles or as active cathepsins. Secreted cathepsins remain attached to the plasma membrane or are released into the extracellular space. Cathepsins on the plasma membrane cleave proteins like integrins. Secreted cathepsins mainly participate in extracellular matrix degradation and thus help in wound healing. However, excessive ECM cleavage facilitates tumor invasion and promotes cancer. While in the extracellular space cathepsins also shed the ectodomains of transmembrane receptors, leading to either activation or inhibition of cell signaling. ROS: reactive oxygen species; LMP: lysosomal membrane permeabilization; NPC1: Niemann–Pick disease type C1; CD36: cluster of differentiation 36; Figure is created with permission from Servier Medical Art image bank.

5. Targeting cathepsins in disease management

Localization of cathepsins (endo/lysosomal/cytosolic/extracellular space) governs several aspects of cathepsin function. A wealth of knowledge has been published on the differential expression and functional profiles of cathepsins in various pathologies making them potential diagnostic biomarkers and most desirable therapeutic targets.

Cathepsin levels and activity have been found to be upregulated in sera and tumors of many cancer patients [176,177]. For instance, expression and activity levels of cathepsins B and L correlated with breast cancer progression [178]. Furthermore, B and L cathepsins also correlated with relapse rate after treatment in primary breast cancers [178]. In cancers concerning colon, lung, brain and head and neck, concentration of cathepsins B and L within tumors correlated with survival probability [179]. Moreover, cathepsin L levels were increased in sera of patients with pancreatic [180] and liver cancers [181]. Serum cathepsin H levels were found to be increased in patients with lung [182], melanoma [183], and colorectal cancers [184]. The findings of these recent studies have alluded to a potential of utilizing cathepsins as biomarkers of cancer though few studies show some discrepancies in the outcomes of cathepsin expression and activity during cancer (reviewed in [185]). In addition to cancer, plasma levels of cathepsin S, K, and L have been proposed as biomarkers in coronary artery disease, aneurysm, adiposity, and peripheral arterial disease [186]. Similarly, plasma cathepsin D levels associated with metabolic alterations in liver during NAFLD [187]. Recent studies suggested that plasma cathepsin D levels correlated with type 2 diabetic patients [188] and moreover plasma cathepsin D activity is suggested as biomarker for hepatic insulin sensitivity [189]. Cathepsins Z and K are proposed diagnostic markers for osteoporosis [190,191]. Another promising development is the use of cathepsins as fluorescent probes in diagnostic non-invasive imaging which has had success in preclinical mouse models [192].

In diseases caused by inactivation or loss-of-function of cathepsins, supplying functional cathepsins could be a valuable means to restore cellular function and ameliorate the disease. For instance, enzyme replacement therapy (ERT) replacing defective lysosomal cathepsin D by recombinant procathepsin D has been proven beneficial for CLN10 [193]. However, due to known associations of excessive extracellular cathepsin D with other pathological conditions highlighted previously in this review caution must be exercised when utilizing this therapeutic approach to avoid overaccumulation of cathepsin D in extra lysosomal locations which could possibly lead to activation of extracellular cathepsin D mediated pathological processes. When implementing the ERT approach for cathepsins, the recombinant cathepsins' mannose-6-phosphate content must be maximized to ensure their optimal uptake by tissues and to avoid their extra lysosomal accumulation. In addition, conjugating recombinant cathepsins with chaperones might help to diffuse them across cell membranes and reach target tissues including central nervous system (CNS) [194]. Additionally, close monitoring of injected levels of recombinant enzyme in patients might help in preventing any negative effects.

Increased understanding of the structure, differential expression and localization of cathepsins in various pathologies has opened a new avenue for the design of small molecule inhibitors of cathepsins with the hope of producing highly specific, targeted drugs for many diseases. Several small molecule inhibitors of cathepsin S and K are being tested in clinical trials [192]. Additionally, combinatorial therapies involving cathepsin inhibition are gaining more attention. For example,

cathepsin inhibitors conjugated with radio/chemotherapy would be a potential anti-cancer treatment [195]. Further, cathepsin K clinical inhibitors for treatment of osteoporosis might have potential to attenuate cancer [196]. Unfortunately, one of the bone-specific cathepsin K inhibitors to complete phase III clinical trials, odanacatib (Merck) had to be discontinued due to risk of stroke [197]. The failure was likely due to the fact that the active-site inhibitor of K also blocked the other essential protease functions of cathepsin K [17]. One of the strategies to alleviate this problem was to identify inhibitors specific to the exosites or allosteric sites, that essentially inhibit the pathological collagenolytic activity only and thus limiting the cytotoxicity [198]. One of such exosite inhibitors of cathepsin K was successfully demonstrated in a mouse model of osteoporosis [199]. However, since not all cathepsin activities are modulated by exosite interactions it presents a limitation for the use of exosite inhibitors in certain cathepsin related pathologies. Other novel ways such as site-specific inhibition of cathepsins would also reduce their off-target limits. For instance, small-molecule inhibitors targeting only the secreted extracellular fraction of cathepsin D and not the lysosomal fraction had beneficial results in a rodent model of NAFLD [162]. Moreover, antibodies against extracellular fraction of cathepsin S efficiently inhibited tumor growth and neovascularization in xenograft tumors [200] and improved chemotherapy efficacy in colorectal carcinomas [201]. There are still a few inhibitors targeting either extracellular cathepsins or their substrates that require further validation in clinical setting [192] as described in **Table 4**. Taken together, while evidence that shows a direct link between extracellular cathepsins and various pathologies are few, current studies to date show promising therapeutic result of targeting specifically the extracellular fraction of cathepsins in their respective pathologies. Therefore, further research looking into the specific role of extracellular cathepsins and the therapeutic value of targeting them is recommended.

Table 4: List of available extracellular cathepsin inhibitors

Inhibitor	Target	Reference
Fsn0503	antibody against extracellular cathepsin S	[200]
Nitroxoline	extracellular cathepsin B	[202]
LNC-NS-629	extracellular cathepsin B	[203]
CTD-002	extracellular cathepsin D	[162]

6. Conclusion

In conclusion, cathepsins are a group of enzymes with distinct functions at different locations inside and outside of the cells. While many recent findings helped us to further understand these roles and establish the potential for targeting, many mechanistic aspects of cathepsin action are still to be explored. Currently, the extracellular role of cathepsins have gained enormous attention in the biomedical field, establishing them as non-invasive diagnostic markers and pharmacological targets in immune disorders, cancer, osteoarthritis, and metabolic diseases. Many selective cathepsin inhibitors with limited side effects are being developed and have shown success in the preclinical animal models and are awaiting validation in the clinical setting. With the advent of new mass spectrometry technologies and systems biology approaches, the future research should focus to better understand the specific substrates of cathepsins in the physiological and pathological environment. Finally, the future of cathepsins in targeted drug delivery looks more promising than ever.

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Chapter 2

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Chapter 3

Inhibiting extracellular cathepsin D reduces hepatic steatosis in Sprague–Dawley rats

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Abstract

Dietary and lifestyle changes are leading to an increased occurrence of non-alcoholic fatty liver disease (NAFLD). Using a hyperlipidemic murine model for non-alcoholic steatohepatitis (NASH), we have previously demonstrated that the lysosomal protease cathepsin D (CTSD) is involved with lipid dysregulation and inflammation. However, despite identifying CTSD as a major player in NAFLD pathogenesis, the specific role of extracellular CTSD in NAFLD has not yet been investigated. Given that inhibition of intracellular CTSD is highly unfavorable due to its fundamental physiological function, we here investigated the impact of a highly specific and potent small-molecule inhibitor of extracellular CTSD (CTD-002) in the context of NAFLD. Treatment of bone marrow-derived macrophages with CTD-002, and incubation of hepatic HepG2 cells with a conditioned medium derived from CTD-002-treated macrophages, resulted in reduced levels of inflammation and improved cholesterol metabolism. Treatment with CTD-002 improved hepatic steatosis in high fat diet-fed rats. Additionally, plasma levels of insulin and hepatic transaminases were significantly reduced upon CTD-002 administration. Collectively, our findings demonstrate for the first time that modulation of extracellular CTSD can serve as a novel therapeutic modality for NAFLD.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver diseases in the world with an estimated prevalence of 25% in the adult population [1]. Histologically, NAFLD ranges from steatosis, which is the benign accumulation of macro-vesicular and micro-vesicular lipid droplets in hepatocytes, to the more severe form of NAFLD referred to as non-alcoholic steatohepatitis (NASH). NASH is a combination of hepatic steatosis and inflammation with or without fibrosis [2], which can progress further to severe conditions such as liver cirrhosis and liver failure [3,4]. Due to the lack of mechanistic insights into the pathogenesis of NAFLD [5], clinically approved treatment options do not yet exist.

NAFLD is characterized by an impaired hepatic lipid profile, which is in turn characterized by dysregulation of cholesterol and triglyceride metabolism [6]. It has been well-established that lysosomes, in particular their lysosomal proteases such as cathepsins, play a crucial role in maintaining metabolic processes such as lipid homeostasis [7–10]. Specifically, it has been shown that changes in the expression of the lysosomal enzyme cathepsin D (CTSD) are associated with differences in cholesterol metabolism [11–13]. Relevantly, though CTSD is mainly functional within the intracellular, acidic milieu of lysosomes, in response to certain physiological and pathological conditions, CTSD is secreted extracellularly [11]. Strikingly, and similar to its intracellular fraction, it has been shown that CTSD is also proteolytically active upon secretion into the extracellular space [14,15]. Indeed, given that CTSD retains its catalytic activity at neutral pH [16], these data indicate a potential role for extracellular CTSD in lipid-related disorders such as NAFLD [17,18].

Recently, we have demonstrated that targeting the proteolytic activity of CTSD using pepstatin-A (Pep-A), a known inhibitor of CTSD, remarkably reduced steatohepatitis in a hyperlipidemic mouse model, thereby pointing towards a role for CTSD activity in NAFLD [19]. However, the observed effects in this study were the result of a targeted inhibition of intracellular and extracellular CTSD. Moreover, Pep-A is a potent but relatively unspecific inhibitor of aspartic proteases [20]. Hence, in the current study, we developed a highly specific and potent small-molecule inhibitor of extracellular CTSD, referred to as CTD-002 (IC₅₀ = 28 nM) in order to investigate its efficacy in treating NAFLD.

We hypothesized that specific inhibition of extracellular CTSD activity has a therapeutic benefit for NAFLD. In order to test our hypothesis, Sprague–Dawley (SD) rats fed a high-fat diet (HFD), known as the Lieber–Decarli diet, were used as an *in vivo* NAFLD model [21] and were injected with the CTD-002. In addition, wild-type (Wt) bone marrow-derived macrophages (BMDMs) treated with CTD-002 were used as an *in vitro* model to assess the ability of CTD-002 to reduce inflammation. Moreover, to investigate whether the CTD-002 inhibitor influences the inflammatory crosstalk between hepatic immune and parenchymal cells, a conditioned medium derived from BMDMs was transferred to HepG2 liver cells. Treating BMDMs with CTD-002 resulted in a decrease in inflammation and improvement in lipid metabolism. In addition, incubating hepatic HepG2 cells with the conditioned medium derived from CTD-002-treated BMDMs showed a decreasing trend in inflammation compared to control cells. Sprague–Dawley rats fed an HFD showed decreased hepatic steatosis and inflammation upon treatment with CTD-002. Altogether, these data suggest that targeting extracellular CTSD potentially represents a novel and effective therapeutic strategy for NAFLD.

2. Materials and methods

2.1. Design and development of CTD-002

CTD-002 was designed using the Schrodinger Small Molecule Drug Design Suite and medicinal chemistry efforts. In this process, a library of ~500,000 commercially available compounds were screened *in silico* using the pharmacophore modelling [22,23] in PHASE module and docking the top 10,000 molecules via the GLIDE XP [24,25] module of the Schrodinger suite (v7.2, Schrödinger, LLC). This resulted in the identification of a set of 100 molecules with diverse chemical structures. These molecules were then further screened against CTSD in a cell-free assay to identify those compounds that had micromolar or higher potency. The compounds with the lowest permeability and efflux were selected as exerting most of its effects extracellularly. Finally, these compounds were further refined and modified to create a library of compounds with nanomolar efficacy against CTSD, with CTD-002 being the most potent small-molecule inhibitor, showing a dose-dependent inhibition of CTSD activity (Figure S1). While the compound may have minor effects of intracellular CTSD, toxicity studies in Sprague–Dawley rats and BALB/c mice, a widely used mouse strain, showed no toxic reactions at the therapeutic dose of 50 mg/kg, which was injected twice a week for six weeks.

2.2. Isolation and culturing of bone marrow-derived macrophages

Tibiae and femurs of wild type mice belonging to C75Bl/6 strain were broken and used for harvesting bone marrow-derived macrophages (BMDMs). The culture medium in which the BMDMs were cultured was an RPMI medium (GIBCO Invitrogen, Breda, the Netherlands) supplemented with 10% heat-inactivated fetal calf serum (Bodinco B.V. Alkmaar, the Netherlands), penicillin (100 U/mL), streptomycin (100 µg/mL) and L-glutamine 2 mM (all derived from GIBCO Invitrogen, Breda, the Netherlands). To differentiate between monocytes and macrophages, the culture medium was also supplemented with 20% L929-conditioned medium (LCM) for 9 days. At day 10, BMDMs were cultured in a 24-well plate at 350,000 cells per well. Next, the BMDMs' culture medium was enriched with oxidized low-density lipoprotein (oxLDL) for 24 h (25 µg/mL; Alfa Aesar: J65591, Wardhill, MA, USA). Then, the macrophages were treated with CTD-002 (Aten Porus Lifesciences Pvt Ltd., India) or with carrier-control dimethyl sulphoxide (DMSO) for 4 h. In order to increase the effects on inflammation, cells were washed and further stimulated with lipopolysaccharide (LPS; 100 ng/mL) for 4 h.

To explore the possible effects of secreted CTSD on neighboring cells, hepatic HepG2 (ATCC HB-8065) cells were incubated with the conditioned medium from CTD-002-treated BMDMs, or control-treated BMDMs, for 4 h. Subsequently, cells were washed and stimulated with LPS (100 ng/mL) for 4 h, after which the supernatant was collected for protein measurements and cells were lysed for mRNA expression analyses.

2.3. Rats, diet and intervention

Animal experiments were conducted at TheraIndx Lifesciences Private Limited, following all ethical practices as formulated in the guidelines for animal care and approved by the Institutional Animals

Ethics Committee (IAEC; protocol no. IAEC/05/2017/064), India. SD rats were given free access to food and water and were housed under standard conditions. Following an acclimatization period of seven days, rats were fed with either low-fat (LFD) or HFD for three weeks. The composition of the diets is listed in Table S1. To test its therapeutic efficacy, CTD-002 was administered twice a week intraperitoneally (dose 50 mg/kg) for three weeks. The experimental conditions are also shown in Figure S2. After three weeks, the animals were sacrificed using CO₂ anesthesia. Following blood collection, the rats were dissected, liver tissues were excised, weighed and cut into small pieces weighing ~100 to 200 mg. These liver tissues were frozen immediately in liquid nitrogen and stored at –80 °C for further analyses. In addition, liver samples were also fixed in 4% formaldehyde for histologic examination. Blood was collected by cardiac puncture followed by termination, centrifuged and plasma was harvested for biochemical analyses.

2.4. Histological analyses

Formalin-fixed liver samples were stained with Hematoxylin-Eosin (H&E) in order to score fat vesicles as minimal, mild, moderate and severe steatosis. Lobular inflammatory activity was scored as follows: 1) focal collections of mononuclear inflammatory cells; 2) diffuse infiltrates of mononuclear inflammatory cells and 3) focal collections of polymorphonuclear cells in addition to mononuclear cell infiltrates. In addition to H&E, formalin-fixed liver samples stained with Chromotrope Aniline Blue and Sirius Red were quantified for total hepatic fat percentage by means of digital image analysis in ImageJ.

2.5. Plasma measurements

Plasma concentrations of alanine aminotransferase (ALT) [S.G.P.T ERBA kit, 120903; Baddi, India] and aspartate aminotransferase (AST) [S.G.O.T ERBA kit, B081717; Baddi, India] were measured with an enzymatic color test according to the manufacturer's protocols and were measured using an ERBA semi-automated biochemistry analyzer. Plasma insulin was measured using a rat enzyme-linked immunosorbent assay kit [ab100578, Abcam; Cambridge, MA, USA].

2.6. Liver tumor necrosis factor-alpha levels

The rat tumor necrosis factor-alpha (TNF- α) ELISA assay was performed on liver homogenates according to the manufacturer's instructions [ab100785, Abcam; Cambridge, MA, USA] using a microplate reader.

2.7. Statistical analyses

Data were statistically analyzed by performing two-tailed unpaired *t*-tests using GraphPad Prism, version 6.0 for Windows. Data were expressed as mean $\pm$ SEM and considered significant at $p < 0.05$. *, ** and *** indicate $p < 0.05$, 0.01 and 0.001, respectively.

3. Results

3.1. Decreased inflammation after inhibition of extracellular CTSD in oxLDL-loaded primary mouse macrophages

With the aim of exploring the influence of extracellular CTSD on inflammation in macrophages and to test the efficacy of the small-molecule inhibitor CTD-002, Wt BMDMs were isolated and incubated with oxLDL for 24 h to stimulate the secretion of CTSD. Subsequently, cells were treated with either carrier control (DMSO) or CTD-002 (100 μ M) for 4 h, followed by 4 h stimulation with LPS. Tumor necrosis factor α (TNF α) protein levels, a pro-inflammatory cytokine, measured from the medium of CTD-002-treated BMDMs, were significantly reduced compared to control cells (Figure 1A). Consistent with these results, gene expression levels of the pro-inflammatory cytokines *Tnf α* and chemokine (C-X-C motif) ligand-2 (*Ccl2*) significantly decreased in CTD-002-treated macrophages compared to control (DMSO)-treated cells (Figure 1B), confirming the reduction of inflammation upon treatment with CTD-002. Additionally, extracellular CTSD inhibition also improved cholesterol metabolism, as evidenced by the upregulation of cytochrome P450 27a1 (*Cyp27a1*), the main enzyme responsible for the conversion of cholesterol into bile acids (Figure 1C). Similar data were also observed in control conditions without oxLDL and LPS stimulation, showing reduced *Tnf α* and *Ccl2* levels and improved lipid and energy metabolism as evidenced by *Cyp27a1*, acetyl-CoA acetyltransferase 2 (*Acat2*), carnitine palmitoyltransferase1 (*Cpt1*) and scavenger receptor A (Sr-a) expression after CTD-002 treatment (Figure S3). Collectively, these data indicate that modulating the activity of extracellular CTSD has beneficial effects on inflammation and cholesterol metabolism, at least *in vitro*.

Figure 1 Effects of CTD-002 on inflammation and cholesterol metabolism in bone marrow-derived macrophages. (A) TNF α protein levels measured from supernatant of oxidized low-density lipoprotein (oxLDL)-loaded bone marrow-derived macrophages (BMDMs). (B) Gene expression of inflammation-related genes *Tnf α* and *Ccl2* and (C) the cholesterol breakdown enzyme *Cyp27a1* in oxLDL-loaded bone marrow-derived macrophages. Each bar represents a technical triplicate $\pm$ SEM; * means $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to dimethyl sulfoxide (DMSO)-treated BMDMs by means of two-tailed unpaired *t*-test.

3.2. Inhibition of macrophage derived extracellular CTSD reduces inflammation in HepG2 cells

In order to confirm whether macrophage-derived extracellular CTSD influences neighboring parenchymal cells, hepatic HepG2 cells were incubated with a conditioned medium derived from macrophages that were treated either with carrier-control or with CTD-002 (100 μ M) for 4 h. To boost the effects on inflammation, HepG2 cells received LPS for 4 h. HepG2 cells that received the conditioned medium from macrophages that were treated with CTD-002 showed a trend towards reduced TNF α secretion (Figure 2A). Additionally, HepG2 gene expression levels of *Ccl2* showed a decreasing trend compared to HepG2 cells that were incubated in the control-treated conditioned medium, while *Tnfa* did not show any effect (Figure 2B). Additionally, we also observed a reduced expression of the cluster of differentiation 36 (*Cd36*) in hepatocytes receiving the conditioned medium that was derived from BMDMs treated with CTD-002, suggesting an effect on hepatocyte lipid metabolism (Figure S4). These *in vitro* findings suggest that macrophage derived extracellular CTSD affects the inflammatory status of neighboring parenchymal cells and that inhibiting extracellular CTSD activity reduces this inflammatory response.

Figure 2 Effect of medium derived from CTD-002-treated BMDMs on HepG2 cells. (A) TNF α cytokine secretion of HepG2 cells cultured in a macrophage-conditioned medium that was treated with or without CTD-002. (B) Gene expression levels of *Tnfa* and *Ccl2* measured in HepG2 cells. Error bars represent mean $\pm$ SEM.

3.3. Improved metabolic features after inhibition of extracellular CTSD in HFD-fed Sprague–Dawley rats

To elucidate the metabolic effects of extracellular CTSD inhibition *in vivo*, metabolic parameters were tested in HFD-fed SD rats that were injected with or without the CTD-002 inhibitor. Though food consumption was statistically similar among all experimental groups, a trend towards reduced food intake was apparent (Figure S5). By performing an H&E staining, hepatic steatosis was assessed based on the scoring of fat vesicles in the liver (Figure S6). The impact of HFD CTD-002-treated HFD-fed SD rats showed a significant reduction in the amount of fat vesicles relative to control HFD-fed rats, demonstrating the therapeutic benefit of extracellular CTSD inhibition in the context of hepatic

steatosis, a main feature of NAFLD (Figure 3A). The impact of the HFD on hepatic fat deposition was further confirmed by quantification of H&E, Chromotrope Aniline Blue and Sirius Red staining, showing increased fat deposition with the HFD (Figure S7).

To further assess whether inhibition of extracellular CTSD influences pathological parameters related to NAFLD, plasma insulin levels were measured as an indicator of insulin sensitivity. Plasma insulin levels were significantly increased in HFD-fed SD rats compared to LFD-fed rats (Figure 3B). Further, CTD-002-treated HFD-fed rats had significantly lower plasma insulin levels compared to HFD-fed rats (Figure 3B). This finding suggests an improvement in insulin sensitivity, further emphasizing the metabolic benefit induced by inhibition of extracellular CTSD. Altogether, these data indicate that modulating extracellular CTSD activity improves metabolic features associated with NAFLD.

Figure 3 Metabolic parameters of high-fat diet (HFD)-fed Sprague–Dawley (SD) rats treated with or without the extracellular inhibitor CTD-002. (A) Scoring of hepatic steatosis by means of Hematoxylin-Eosin (H&E) staining. (B) Plasma levels of insulin. Error bars represent mean $\pm$ SEM; $n = 6$ for each group; * represents $p < 0.05$ and *** $p < 0.001$ compared to rats on HFD; ### represents $p < 0.001$ compared to the rats on low-fat diet (LFD) by means of two-tailed unpaired t -test.

3.4. Reduced liver damage after inhibition of extracellular CTSD in HFD-fed Sprague–Dawley rats

To determine the effects of extracellular CTSD inhibition on hepatic inflammation, the protein levels of hepatic TNF α were measured in HFD-fed SD rats that were treated with or without the CTD-002 inhibitor of extracellular CTSD. CTD-002-treated HFD-fed SD rats showed a trend towards reduced hepatic TNF α levels ($p = 0.06$) compared to the HFD-fed group (Figure 4A), thereby suggesting an improvement in hepatic inflammation. To further assess the effects of extracellular CTSD inhibition on liver damage, plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured. Upon treatment with CTD-002, HFD-fed SD rats showed a significant reduction in the aspartate transaminase/alanine transaminase (AST/ALT) ratio (Figure 4B), pointing towards an improvement in liver damage.

Figure 4 Hepatic TNF α levels and aspartate transaminase/alanine transaminase (AST/ALT) ratio in HFD-fed SD rats treated with or without the extracellular inhibitor of cathepsin D (CTSD). (A) Hepatic TNF α levels. (B) Plasma transaminase levels. Error bars represent mean $\pm$ SEM; $n = 6$ for each group. * indicates $p < 0.05$ compared to rats on the HFD.

4. Discussion

Treating NAFLD is of critical importance to prevent further progression to liver cirrhosis and end-stage liver disease. The present study provides the first evidence that modulating extracellular CTSD activity can significantly improve metabolic parameters associated with NAFLD. These findings suggest the potential therapeutic benefits of targeting extracellular CTSD within the context of metabolic diseases and opens new perspectives for therapeutic interventions in NAFLD.

To elucidate the effects of extracellular CTSD inhibition in NAFLD, we have used SD rats that were fed a high-fat diet for three weeks. Sprague–Dawley rats are known to develop micro-vesicular steatosis with [21] or without inflammation [26], depending on the composition and duration of the HFD. Here, SD rats developed hepatic steatosis. Similar to the findings of Lieber and Ahmed et al. [21,26], no changes in relative liver weights were observed between different groups of rats (Table S2). Comparable to human NAFLD patients, we observed that rats on an HFD developed insulin resistance (IR) [27], as shown by elevated plasma insulin levels with the HFD. Indeed, it has been shown that insulin resistance is commonly associated with obesity-induced hepatic steatosis [28,29]. Furthermore, hepatic lipid accumulation is known to activate intrahepatic inflammatory pathways, which stimulate the pro-inflammatory cytokine production, in turn leading to both hepatic and peripheral insulin resistance [30,31]. Upon administration of the extracellular CTSD inhibitor, we observed that HFD-fed rats showed a significant reduction in plasma insulin levels and improvement of steatosis, two important characteristics of diet-induced NAFLD. Hence, these data provide initial evidence that extracellular CTSD activity is involved with the regulation of lipid metabolism and thereby controls insulin sensitivity. However, the trend towards reduced food intake suggests additional potential effects on energy expenditure, which should be further investigated in the future. Additionally, though hepatic damage and inflammation were not increased in SD rats fed an HFD, treatment with the extracellular CTSD inhibitor showed reduced hepatic TNF α levels and AST/ALT ratio, indicating an improvement in hepatic damage and inflammation. Moreover, we validated this finding in primary macrophages and in an *in vitro* setup in which hepatic cells were incubated with a macrophage-derived conditioned medium, an approach that has been previously used to investigate crosstalk between hepatic immune and parenchymal cells [32,33]. As such, our findings suggest that macrophage derived extracellular CTSD activity influences hepatic lipid metabolism and inflammation. However, despite the promising first results, the potential minor

effects of intracellular CTSD and of the trend towards reduced food intake in the inhibitor-treated group suggest additional non-beneficial effects related to intracellular CTSD inhibition. To determine whether these non-beneficial metabolic effects are related directly to intracellular CTSD, a comparison with a compound with high permeability and low efflux (indicating that the compound mainly exerts its effects intracellularly) is necessary. Likewise, lower concentrations of the compound should be investigated in several NAFLD models before transition to the clinic is made.

Substantial evidence points towards the role of cathepsins in the context of NAFLD. Inactivation of cathepsin B in a dietary murine model of NAFLD prevented the development of hepatic steatosis [34]. Additionally, cathepsin B was found to be involved in hepatic injury and in progression of liver fibrosis [35,36]. Similarly, we have shown that lysosomal cholesterol accumulation inside Kupffer cells of *Ldlr*^{-/-} mice leads to increased CTSD activity in the liver [37]. CTSD is a key lysosomal protease that affects many fundamental functions in the cell. A reduction in cellular CTSD expression or catalytic activity leads to devastating neurodegenerative disorders [38,39]. In contrast, an increase in extracellular CTSD expression and activity is associated with many types of cancers [40–42]. Owing to this differential expression and secretion of CTSD in many pathophysiological processes, there is increased awareness for the extracellular fraction of CTSD to play a role in health and disease. However, to date, research primarily focuses on targeting the whole fraction of CTSD [43], which is known to disturb physiological processes, resulting in serious side effects [44]. Hence, in the present study, we specifically investigated the benefits of targeting the extracellular fraction of CTSD in the context of NAFLD.

Though the mechanisms underlying the secretion of CTSD are not yet known, changes in the lysosomal pH or lysosomal accumulation of poorly degradable lipids are known to cause mistargeting of CTSD into the extracellular milieu [45,46]. Additionally, cholesterol-filled lysosomes have been shown to induce disturbances in the lysosomal enzyme trafficking pathway that can potentially lead to increased levels of lysosomal enzymes in the plasma [47,48]. In vitro, particularly, the intracellular accumulation of the oxidized fraction of cholesterol has been shown to enhance extracellular secretion of CTSD [49,50]. Building further on this knowledge, we found in this study that targeting the activity of the extracellular fraction of CTSD in oxLDL-loaded macrophages improves inflammation and lipid metabolism, suggesting that macrophage-derived extracellular CTSD has a key role in lipid metabolism and inflammation.

Our observation that extracellular CTSD activity is involved with the pathogenesis of NAFLD implies that CTSD remains active in the circulation. Indeed, modulating the vacuolar type H⁺ ATPase pump by macrophages creates a more acidic pericellular space, in which lysosomal enzymes such as CTSD can remain active [51]. Moreover, several findings have implicated that upon secretion, anchorage of CTSD to the cell surface ameliorates its ability to interact with specific extracellular substrates [52]. The combination of an acidic pericellular environment and localization proximal to the cell proposes a potential mechanism by which extracellular CTSD activity can influence physiological processes. However, the question remains of which underlying mechanism is responsible for the observed improvements in lipid metabolism and inflammation upon inhibition of extracellular CTSD activity. In the current study, extracellular inhibition of CTSD upregulated the expression of *Cyp27a1* in macrophages. *Cyp27a1* is a gene encoding for an essential enzyme responsible for regulating cholesterol, fatty acid and bile acid metabolism through modulation of the mitochondrial P450 enzyme sterol 27-hydroxylase. In the liver, sterol 27-hydroxylase catalyzes the first step of the alternative bile acid biosynthetic pathway from cholesterol [53]. Previously, we have demonstrated

the potential of Cyp27a1 to modulate intracellular cholesterol distribution in Kupffer cells [37]. Of note, Cyp27a1 has been shown to regulate ATP-binding cassette transporter A1 (ABCA1), an exporter of cellular lipids [54]. Based on this evidence, a possible explanation for our current findings is that the extracellular CTSD binds to one of the cell surface lipid transporters such as ABCA1 or scavenger receptors, which in turn blocks cholesterol transport, preventing regulation of cellular cholesterol and efflux pathways. It is therefore feasible that CTSD acts as an extracellular messenger interacting with an as yet unidentified cell surface receptor and regulates lipid homeostasis in addition to inflammation. In this regard, extracellular CTSD has recently been shown to be involved with ABCA1 regulation via influencing the low-density lipoprotein receptor-related protein 1 (LRP1) [55]. Additionally, we here observed differential expression of *Cd36* and *Sr-a* upon extracellular CTSD inhibition, suggesting an involvement of these scavenger receptors. Moreover, modulating proteases is known to influence the transcriptional regulation of cholesterol handling via *Srebp* [56] and *Nfe2l1* [57]. However, further studies are warranted to decipher the exact underlying mechanism of extracellular CTSD function.

In conclusion, we provide evidence for the first time that extracellular CTSD has a central role in the progression of NAFLD. In contrast to conventional therapeutic targeting of cathepsins, our data demonstrate that inhibiting specifically the extracellular fraction of CTSD can be a valuable therapeutic strategy for NAFLD. Further studies that investigate the downstream targets regulated by extracellular CTSD will provide deeper understanding of the mechanisms of NAFLD pathogenesis.

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Supplemental material

Figure S1 Inhibitory activity of CTD-002 on CTSD activity

Figure S2 Schematic representation of the *in-vivo* setup

Figure S3 Effect of CTD-002 in bone marrow-derived macrophages under control conditions

Figure S4 *Cd36* gene expression levels of BMDMs and HepG2

Figure S5 Food consumption of Sprague-Dawley rats

Figure S6 Representative images of fat droplets stained by haematoxylin and eosin staining

Figure S7 Impact of high-fat diet on hepatic fat deposition in Sprague-Dawley rats

Table S1: Composition of low and high fat diets

Ingredients	Low fat diet (gm)	High fat diet (gm)
Casein	200	200
L-Cystine	3	3
Corn starch	506	0
Maltodextrin 10	125	125
Sucrose	68.8	68.8
Cellulose	50	50
Soybean Oil	25	25
Lard*	20	245
DiCalcium Phosphate	13	13
Calcium Carbonate	5.5	5.5
Choline Bitartrate	2	2
Potassium Citrate	16.5	16.5
Mineral Mix	10	10
Vitamin Mix	10	10
FD&C Blue Dye	0.01	0.05
FD&C yellow Dye	0.04	0
*Typical analysis of cholesterol in lard = 0.72 mg/gram		

Table S2: Relative liver weight (per 100 g of body weight) of the experimental groups of rats

Group	Liver weight mg/100g body weight (Mean $\pm$ SD)
LFD	3.67 $\pm$ 0.61
HFD	3.73 $\pm$ 0.27
HFD + CTD-002	3.73 $\pm$ 0.47

Chapter 4

Inhibition of extracellular cathepsin D reduces hepatic lipid accumulation and leads to mild changes in inflammation in NASH mice

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Abstract

Background & Aims

The lysosomal enzyme, cathepsin D (CTSD) has been implicated in the pathogenesis of non-alcoholic steatohepatitis (NASH), a disease characterized by hepatic steatosis and inflammation. We have previously demonstrated that specific inhibition of the extracellular CTSD leads to improved metabolic features in Sprague- Dawley rats with steatosis. However, the individual roles of extracellular and intracellular CTSD in NASH are not yet known. In the current study, we evaluated the underlying mechanisms of extracellular and intracellular CTSD fractions in NASH-related metabolic inflammation using specific small-molecule inhibitors.

Methods

Low-density lipoprotein receptor knock-out (*Ldlr*^{-/-}) mice were fed a high-fat, high cholesterol (HFC) diet for ten weeks to induce NASH. Further, to investigate the effects of CTSD inhibition, mice were injected either with an intracellular (GA-12) or extracellular (CTD-002) CTSD inhibitor or vehicle control at doses of 50 mg/kg body weight subcutaneously once in two days for ten weeks.

Results

Ldlr^{-/-} mice treated with extracellular CTSD inhibitor showed reduced hepatic lipid accumulation and an associated increase in faecal bile acid levels as compared to intracellular CTSD inhibitor-treated mice. Furthermore, in contrast to intracellular CTSD inhibition, extracellular CTSD inhibition switched the systemic immune status of the mice to an anti-inflammatory profile. In line, label-free mass spectrometry-based proteomics revealed that extra- and intracellular CTSD fractions modulate proteins belonging to distinct metabolic pathways.

Conclusion

We have provided clinically translatable evidence that extracellular CTSD inhibition shows some beneficial metabolic and systemic inflammatory effects which are distinct from intracellular CTSD inhibition. Considering that intracellular CTSD inhibition is involved in essential physiological processes, specific inhibitors capable of blocking extracellular CTSD activity, can be promising and safe NASH drugs.

1. Introduction

Non-alcoholic fatty liver disease [NAFLD] is an expanding global health burden. NAFLD is an umbrella term that encompasses liver conditions ranging from steatosis to non-alcoholic steatohepatitis [NASH] and cirrhosis. While steatosis represents abnormal fat accumulation in the liver, NASH is steatosis accompanied by inflammation with or without fibrotic scarring [1]. NASH can further progress to end-stage liver diseases such as cirrhosis and hepatocellular carcinoma [2]. Though there have been considerable advances in recent years in elucidating the pathophysiological mechanisms involved in NASH, much of the underlying mechanisms remain elusive, which is hampering drug development [3, 4]. Currently, no Food and Drug Administration (FDA)-approved drugs are available for this disease [5, 6] and therefore, there is an urgent need for effective treatment options to manage the complex pathogenesis of NASH.

We have previously demonstrated the relationship between lysosomal lipid accumulation in macrophages and hepatic inflammation during NASH [7, 8]. Excessive accumulation of lipids in the lysosomes or changes in lysosomal pH leads to lysosomal dysfunction which subsequently disrupts the trafficking and sorting of lysosomal proteases causing their leakage into the circulation [9–12]. In particular, the secreted lysosomal proteases, cathepsins, are known to participate in several inflammatory responses. For example, in bone disorders and cancer, cathepsins secretion is known to be a common part of the inflammatory response [13, 14].

CTSD is an aspartyl lysosomal protease synthesized in the rough endoplasmic reticulum from where it is sorted to lysosomes [15]. As a result of excess lipids, extracellular CTSD secretion is elevated, where its increased activity in the plasma is known to be associated with metabolic inflammatory disorders such as NAFLD [16], atherosclerosis [17] and type 2 diabetes [18]. We have established that inhibition of CTSD proteolytic activity using the generic aspartyl protease inhibitor pepstatin A ameliorated NASH [19]. However, given the essential role of intracellular CTSD fraction in important cellular processes such as protein degradation and autophagy, its inhibition can have harmful effects if applied in humans [20, 21]. Taking this into consideration, we have recently designed and tested a novel non-toxic inhibitor, CTD-002, that specifically targets the extracellular CTSD fraction. Our promising results showed that CTD-002 reduces hepatic steatosis and improves insulin sensitivity in Sprague-Dawley rats [22]. While we successfully demonstrated the importance of extracellular CTSD activity in steatosis, the underlying mechanism of extracellular CTSD action compared to intracellular CTSD inhibition in NASH-associated hepatic inflammation was never investigated. Such evaluation is important in order to establish extracellular CTSD as an effective and less-toxic treatment for NASH.

By using specific small-molecule inhibitors of CTSD, we demonstrated that while intracellular CTSD is involved in essential processes such as mitochondrial oxidative phosphorylation and electron transport function, extracellular CTSD is mainly involved in pathways related to lipids and inflammation. In line, extracellular CTSD inhibition led to increased beneficial effects in NASH compared to intracellular CTSD inhibition, including reduced hepatic triglyceride levels, increased faecal bile acids and reduced systemic inflammation.

2. Materials and methods

2.1 Primary culture of bone marrow-derived macrophages (BMDMs)

BMDMs were isolated from the tibiae and femurs of wildtype C57BL/6 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 mg/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, macrophages were seeded at 350,000 cells per well in 24-well plates and incubated for 24 hours with oxidized low-density lipoprotein (oxLDL) (25 mg/ml; Alfa Aesar, Wardhill, MA, USA). Subsequently, macrophages were treated with 100 μ M CTD-002 or GA-12 (Aten Porus Lifesciences Pvt Ltd., India) or with vehicle control [0.1% dimethyl sulphoxide (DMSO)] for 4 hours. CTD-002 is a potent small-molecule active site inhibitor of CTSD designed based on the binding interactions of pepstatin A. Docking studies showed that CTD-002 interacts with polar side chain residues of ASP33 and GLY133 of ligand binding site of the human CTSD (PDB ID: 4od9).

2.2 Mice, diet and intervention

Ldlr^{-/-} mice, on a C57BL/6 background, were housed under standard conditions and given free access to food and water. Experiments were performed according to the Dutch regulation and approved by the Committee for Animal Welfare of Maastricht University (Project license number: AVD107002016743; working protocol: 2016-003-003). Female *Ldlr*^{-/-} mice (8–14 weeks of age) were fed high-fat, high-cholesterol (HFC) diet (containing 21% milk butter, 0.2% cholesterol, 46% carbohydrates and 17% casein; SAFE, Augy, France) for ten weeks and were subsequently divided into three groups (n=17–20 per group; schematic overview in Figure S1). To evaluate the potential side effects of intracellular CTSD inhibition, a specific small-compound inhibitor, GA-12, was designed and used in this study. HFC-fed mice were subcutaneously injected with extracellular CTSD inhibitor (CTD-002; 50 mg/kg body weight) or intracellular CTSD inhibitor, GA-12 (50 mg/kg body weight) once every two days. Vehicle treatment (5% DMSO, 40% PEG400, 10% Ethanol, 45% Saline) served as control. *Ldlr*^{-/-} mice fed a regular chow diet (9% fat, 67% carbohydrates and 24% protein) for 10 weeks were included as a control group for NASH disease phenotype (n=20). At the end of treatment, animals were euthanized by CO₂ inhalation. Collection of blood and tissue specimens, RNA isolation and cDNA synthesis were performed as described previously [7, 19].

2.3 Liver lipid analysis

Liver tissue was isolated and snap-frozen in liquid nitrogen and stored at -80°C. The biochemical determination of liver cholesterol and triglyceride levels were carried out as described previously [7]. Briefly, 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 using the BCA method (Pierce, Rockford, IL). Liver cholesterol and triglyceride levels were quantified using

cholesterol liquicolor kit [CHOD-PAP, Roche, Basel, Switzerland; triglycerides: GPOtrinder, TRO100, Sigma Aldrich, St. Louis, MO, USA]] following the manufacturer's protocol on a Benchmark 550 microplate reader (Bio-Rad, Hercules, CA, USA).

2.4 Liver Histology

Immunostainings were carried out on frozen liver sections (7µm) as described previously [23]. Briefly, cryosections were fixated in dry acetone followed by blocking with H₂O₂ solution. Next, tissue sections were treated with avidin/biotin solution (Vector; SP2001) for 30 minutes followed by 1 hour incubation with primary antibody [infiltrated macrophages marker, Mac-1; MAB1124; clone M1/70; 1:500], [F4/80;101201; Biolegio; (1:50)]. Sections later were incubated in secondary antibody (Rabbit anti- Rat IgG Biotin (6180-08), Southern Biotech, Birmingham, USA) which was detected using the Peroxidase Substrate kit AEC (Vector Laboratories, SK-4200, Peterborough, United Kingdom). Haematoxylin was used for nuclear counterstaining. Lipid and collagen content was assessed by using neutral lipid marker Oil Red O (ORO; O0625; Sigma-Aldrich) and Sirius red, (Direct red 80; 43664; Sigma-Aldrich) respectively according to standard protocol. Images were captured with a Nikon digital camera DMX1200 and ACT-1 v2.63 software (Nikon Instruments Europe, Amstelveen, The Netherlands).

2.5 CTSD activity measurements

CTSD activity was measured using the CTSD activity assay kit (MBL International, Woburn, MA) as described previously [16]. Briefly, cell medium was incubated with CTSD substrate and reaction buffer for 1 hour at 37°C. Samples were measured using a fluorescence plate reader with a 328-nm excitation filter and 460-nm emission filter and CTSD activity is expressed as relative fluorescence units.

2.6 FACS analysis

Fluorescence activated cell sorting (FACS) analysis was performed as described previously [19]. Tail vein blood was collected from mice before the start of the treatment (T0) as well at the end of the treatment (T1). Using Trucount tubes (BD Biosciences, Breda, The Netherlands), staining was performed according to the manufacturer's instructions, to detect the monocyte population (NK1.1-Ly6G-CD11b⁺; Ly6C). Briefly, heparinized blood samples were mixed and incubated for 10 minutes in the dark at room temperature (RT) with CD16/32 antibody (eBioscience, Halle-Zoersel, Belgium) to block Fc receptor. Samples were then gently vortexed with the appropriate antibodies (PE Mouse Anti-Mouse NK-1.1 (1:100); APC-Cy[™]7 Rat Anti-Mouse Ly-6G (1:100); Anti-Mouse Ly-6C-APC (1:10) (Miltenyi, Bergisch Gladbach, Germany) and incubated in the dark at RT for 20 minutes [24]. All antibodies were diluted in FACS buffer (PBS, 0.1% BSA, 0.01% sodium azide). Finally, samples were mixed and incubated in the dark at RT for 15 minutes with an RBClysis solution (8.4g NH₄Cl + 0.84g NAHCO₃ in 1-liter H₂O, pH 7.2-7.4). Sample stainings were quantified using BD FACSCanto II flow cytometer (BD Biosciences).

2.7 Faecal bile acid measurements

Faecal measurement was performed on faeces collected after 24 hours from individually caged mice. Total faecal bile acid levels were specifically determined as described previously [25].

2.8 Label-free LC-MS (Liquid Chromatography Mass Spectrometry) based proteomics

For proteomics experiments, liver homogenates were used (n=3 for each group of mice). The samples were prepared as described previously [26]. Briefly, proteins were extracted, reduced with 20 mM dithiothreitol for 45 minutes and alkylated with 40 mM iodoacetamide for 45 minutes in the dark. Alkylation was terminated using 20 mM DDT which is followed by digestion with a mixture of LysC and trypsin at a ratio of 1:25 (enzyme:protein) at 37 °C overnight. The digestion was stopped by formic acid. Peptide separation was performed on a Thermo Scientific (Dionex) Ultimate 3000 Rapid Separation UHPLC system equipped with a PepSep C18 analytical column (15 cm, ID 75 µm, 1.9 µm Reprosil, 120Å). Peptide samples were first desalted on an online installed C18 trapping column. After desalting peptides were separated on the analytical column with a 90-minute linear gradient from 5% to 35% Acetonitrile (ACN) with 0.1% FA at 300 nL/min flow rate. The UHPLC system was coupled to a Q Exactive HF mass spectrometer (Thermo Scientific). DDA settings were as follows. Full MS scan between 250 – 1,250 m/z at resolution of 120,000 followed by MS/MS scans of the top 15 most intense ions at a resolution of 15,000.

For protein identification and quantification, DDA spectra were analyzed with Proteome Discoverer version 2.2. The search engine Sequest was used with the Swiss-Prot mouse database (Mus musculus, TaxID 10090). The settings for the database search were similar as before [25]. Proteins with a false-discovery rate < 1% were considered for the analysis and were normalized to the total peptide amount. Statistical significance of changes observed in protein abundance was performed using ANOVA. The Benjamini–Hochberg method was used to correct P-values for multiple testing. Proteins were considered differently regulated if the fold change was ≥ 1.5 ($\log_2 \geq 0.58$) and a p-value of ≤ 0.05 . The differentially regulated proteins were then imported to the EnrichR tool and KEGG database (Version 2019, mouse) was used to demonstrate the top 10 pathways of up or down-regulated proteins ranked by the combined score which is calculated by multiplying the unadjusted, rather than the adjusted, p-values with the z-scores [26].

2.9 Statistical analyses

Except for proteomics, rest of the data were analysed using GraphPad Prism software (GraphPad, San Diego, California, USA), version 6.0 for Windows. Experimental groups were compared using two-tailed student's t-test or two-way ANOVA where appropriate or by one-way ANOVA followed by a Tukey post-hoc test. Data are expressed as mean $\pm$ SEM and were considered significant at $p < 0.05$, in which * $p < 0.05$; ** $p < 0.01$; or *** $p < 0.001$ respectively.

3. Results

3.1 Cathepsin D activity is reduced using low-dose extracellular CTSD inhibitor

To evaluate their potency and specificity, both the intracellular and extracellular CTSD small-compound inhibitors were tested using in vitro methods. CTSD activity was measured from the medium of bone marrow-derived macrophages (BMDMs) that were treated with either of the inhibitors. CTD-002 (i.e., the extracellular CTSD inhibitor) at a concentration of 100 μ M significantly blocked CTSD activity when compared to DMSO (vehicle) as measured from the supernatant of BMDMs. In contrast, the intracellular CTSD inhibitor, GA-12, had no effect on CTSD activity in the supernatant, thus confirming the selective targeting of respective inhibitors (Figure 1).

Figure 1 Efficacy of the cathepsin inhibitors. CTSD activity measurements from the supernatant of the Wt mouse BMDMs, exposed to 24 hours and subsequently treated with either vehicle control (0.1% DMSO) or 100 μ M CTSD inhibitors for 4 hours. Error bars represent $\pm$ SEM; $n=5$ from two independent experiments. * represents $p<0.05$ compared to control mice. ## represents $p<0.01$ compared to intracellular CTSD inhibitor- treated mice by means of one-way ANOVA.

3.2 Extracellular CTSD inhibition led to reduced hepatic lipid levels compared to intracellular CTSD inhibition

To test the effects of CTSD inhibition in NASH-associated dyslipidemia, *Ldlr*^{-/-} mice on HFC diet were injected with either control or intra- or extracellular CTSD inhibitors for a period of 10 weeks. The small-compound CTSD inhibitors showed no visible signs of toxicity in mice. Food intake, body morphology and growth of all mice stayed normal throughout the 10-week experimental period. No abnormalities were detected in any of the animals at autopsy. To investigate the effects of CTSD inhibition on lipid metabolism, hepatic and plasma lipid levels were measured. After ten weeks of HFC diet, both plasma and liver cholesterol and triglycerides were significantly elevated in HFC-fed mice compared to mice on a chow diet, confirming the effect of HFC on hepatic and plasma lipid levels (Figures S2A–D). Further, treating HFC-fed mice with either the intracellular or the extracellular CTSD inhibitor decreased hepatic cholesterol levels compared to control mice (Figure 2A). However, only upon administration of the extracellular CTSD inhibitor, a reduction in hepatic triglyceride was observed (Figure 2B). To confirm the hepatic changes in lipid content at the histological level, we

performed an oil red O staining which demonstrated elevated fat deposition in HFC mice compared to chow mice (Figure S2C). Moreover, in line with hepatic lipid measurements, extracellular CTSD-inhibitor treated mice showed significant reduction of intracellular and near significant reduction of sinusoidal lipid deposition compared to HFC mice (Figures 2C, D) suggesting a clear reduction in hepatic lipid content upon extracellular CTSD inhibition. To further define the impact of extracellular CTSD inhibition on hepatic lipid metabolism, hepatic gene expression analysis using qPCR was performed. Compared to control and intracellular CTSD inhibitor-treated mice, inhibition of extracellular CTSD led to increased hepatic expression of cytochrome P450 7A1 (Cyp7a1), a rate-limiting enzyme in the breakdown of cholesterol to bile acids, suggesting increased cholesterol conversion only upon extracellular CTSD inhibition (Figure 2E). No pronounced effects in other lipid metabolism-related genes were observed (Figure S4A). However, plasma lipid levels remained similar among all experimental groups (Figure 2F). Altogether, these findings demonstrate that extracellular, rather than intracellular, CTSD inhibition decreases hepatic lipid accumulation in a mouse model for NASH.

Figure 2 Hepatic and plasma lipids in hyperlipidaemic *Ldlr*^{-/-} mice with or without CTSD inhibition: (A, B) Total hepatic cholesterol and triglyceride levels. (C) Representative pictures of oil red o staining on frozen liver sections (original magnification, 200x). (D) Scoring of hepatic lipid content of oil red o staining using arbitrary units (A.U.) (E) Hepatic gene expression analysis of *Cyp7a1*. (F) Plasma triglyceride and cholesterol measurements. Error bars represent $\pm$ SEM; n = 16-20 per each group; * represents p < 0.05 and ** represents p < 0.01 compared to control mice. # represents p < 0.05 compared to intracellular CTSD inhibitor- treated mice by means of one-way ANOVA.

3.3 Extracellular CTSD inhibition led to increased faecal bile acid levels compared to intracellular CTSD inhibition

To confirm the increased conversion of cholesterol into bile acid precursors, we opted to further investigate bile acid metabolism by assessing faecal bile acid levels. Faecal levels of primary bile acids, chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA) were significantly enhanced in the extracellular inhibitor-treated group as compared to mice that received the intracellular inhibitor, indicating increased cholesterol breakdown in the former mice (Figure 3A). In line, secondary bile acids including α -muricholic acid and β -muricholic acid showed increased faecal levels in extracellular inhibitor-treated mice as compared to intracellular inhibitor-treated mice (Figure 3B). No differences in the faecal bile acid levels were observed between control and extracellular inhibitor-treated mice. Collectively, our data suggest that inhibition of extracellular CTSD activity in NASH mice results in increased disposal of cholesterol via increased conversion into bile acids leading to their subsequent excretion.

Figure 3 Increased faecal bile acid levels upon extracellular CTSD inhibition in *Ldlr*^{-/-} mice. (A) Faecal measurements of primary bile acids (B) Secondary bile acids in the faeces. Error bars represent $\pm$ SEM. $n=4$ per group. # represents $p<0.05$ compared to intracellular CTSD inhibitor- treated mice as determined by one-way ANOVA.

3.4 Extracellular CTSD inhibition led to reduced systemic inflammation compared to intracellular CTSD inhibition

Next, we examined the role of extracellular CTSD on inflammation at both hepatic and systemic levels. Firstly, inflammatory status was significantly elevated in HFC-fed mice compared to chow mice both at systemic and hepatic levels (Figures S2E–G). To explore the status of systemic inflammation

upon extracellular CTSD inhibition, we analysed the profile of circulating monocytes using fluorescence-activated cell sorting (FACS). However, no statistical differences were observed in the number of total and pro-inflammatory monocytes (DT1-T0) between three different groups of HFC mice (Figures 4A, B). Further, indicating the decrease of systemic inflammation upon extracellular CTSD inhibition, extracellular inhibitor-treated mice showed a significant increase in the total number of anti-inflammatory monocytes (Ly6c-) compared to the control mice and intracellular CTSD inhibitor-treated mice (near significant; $p=0.07$) (Figure 4C). However, no significant changes in T-cell population were observed upon extracellular CTSD inhibition (Figures 4D–F). Together, these data demonstrate minor reduction in systemic inflammation upon extracellular CTSD inhibition. Next to systemic inflammation, the effects of extracellular CTSD inhibition on hepatic inflammation were assessed by hepatic immunostainings for the inflammatory cell markers Mac-1 and F4/80. The levels of infiltrated macrophages and F4/80 positive macrophage remained unaffected upon extracellular CTSD inhibition (Figures 4G–J). Lastly, as expected, the extent of hepatic fibrosis, an advanced NASH feature was no different in chow and experimental groups of HFC as demonstrated by Sirius red staining (Figure S3).

Figure 4 Reduction in inflammatory status upon extracellular CTSD inhibition in *Ldlr*^{-/-} mice: (A–F) Total number of monocyte and T-cell population as measured by FACS. (G) Liver sections were stained for infiltrating macrophages and neutrophil cells (Mac-1). Positive cells were counted in six microscopical views. (H) Immunostaining of livers for F4/80. Positive cells were scored and given a score in arbitrary units (A.U.). (I) Representative pictures of Mac-1 staining (original magnification, 200x) and (J) F4/80 (original magnification, 400x). Error bars represent SEM; n = 5 per each group for FACS and n = 3 per group for proteomics; * represents p < 0.05 compared to control mice as determined by one-way ANOVA.

3.5 Label-free quantitative proteomics and pathway enrichment analysis

To obtain a comprehensive understanding of the proteomic changes upon extracellular and intracellular CTSD inhibition in the liver, label-free quantitative proteomics was performed. After data processing, a total of 1905 proteins were identified. The number of significantly differentially regulated proteins were plotted using volcano plots (Figure S5). The complete list of significantly different regulated proteins ($\log_2 \geq 0.58$) are mentioned in Tables S1–S6.

To further understand the functions of these differentially regulated proteins, enrichment analysis was performed to identify specific pathways. The top 10 KEGG (Kyoto encyclopaedia of genes and genomes) pathways that are most significantly enriched are displayed in Table 1. Compared to control and intracellular inhibitor-treated mice, extracellular inhibitor-treated mice showed enrichment of pathways related to lipid metabolism. Linoleic acid metabolism, steroid hormone biosynthesis, fatty acid biosynthesis and elongation were significantly enriched upon extracellular CTSD inhibition, suggesting improved lipid metabolism in these mice. Moreover, retinol metabolism, PPAR signalling, taurine/ hypotaurine metabolism and caffeine metabolism were upregulated in extracellular inhibitor- treated mice (Table 1A, B). In addition to lipid-related pathways, ferroptosis, mineral absorption, quinone biosynthesis, ascorbate and aldarate metabolism, NAFLD and chemical carcinogenesis pathways were also enriched upon extracellular CTSD inhibition. Among the downregulated pathways in extracellular inhibitor-treated group,

IL-17 pathway (proteins S100A8/A9), African trypanosomiasis, malaria (haemoglobin subunit beta 1), arginine and proline metabolism (creatinine kinase M-type) and glycosaminoglycan biosynthesis pathways (Xylosyltransferase 2) were in top 5 pathways compared to control (Table 1C). Interestingly, protein S100A8/A9, which is a ligand complex for Toll-like receptor 4 (TLR-4), was the most significantly downregulated protein suggesting that extracellular CTSD inhibition potentially reduces inflammation in a TLR-4 dependent manner. Moreover, pathways related to cardiac function (hypertrophic and dilated cardiomyopathy, cardiac muscle function), glycolysis, metabolic pathways (glycine, serine, threonine, thiamine, pyruvate, propanoate metabolism) and glucagon signalling were decreased in extracellular inhibitor-treated mice compared to intracellular CTSD inhibitor-treated mice (Table 1D).

Most importantly, intracellular inhibitor-treated mice showed downregulation of proteins involved in mitochondrial oxidative phosphorylation and electron transport chain compared to control and extracellular inhibitor-treated mice (Tables S1 and S4). According to KEGG pathways, intracellular inhibitor-treated mice showed enrichment of pathways belonging to muscle contraction and glycolysis compared to control (Table S7). Furthermore, proteins that are involved in hepatic stellate cell activation such as tropomyosin alpha-1 and beta chain, myosin regulatory light chain 2 and myosin 7 were upregulated. Taken together, these findings demonstrate that extracellular CTSD fraction regulates distinct pathways compared to intracellular CTSD in the context of NASH.

Table 1: Differentially regulated proteins were imported to EnrichR tool and KEGG database was used for pathway enrichment analysis. Top 10 enriched pathways ranked by combined score are listed below.

(A) KEGG database (Mouse) – extracellular inhibitor-treated mice/ control

Name	P-value (p)	Combined Score
Caffeine metabolism	0.005388	967.31
Linoleic acid metabolism	0.00001168	757.16
Biosynthesis of unsaturated fatty acids	0.0003734	548.11
Taurine and hypotaurine metabolism	0.009858	466.61
Ferroptosis	0.0005847	413.57
Mineral absorption	0.0007077	366.34
PPAR signaling pathway	0.002613	155.48
Steroid hormone biosynthesis	0.002860	146.24
Retinol metabolism	0.002988	141.96
Fatty acid elongation	0.02579	140.14

(B) KEGG database (Mouse) –extracellular inhibitor-treated mice/ intracellular inhibitor-treated mice

Name	P-value (p)	Combined Score
Steroid hormone biosynthesis	4,59E-12	824.33
Retinol metabolism	5,43E-09	535.98
Linoleic acid metabolism	1,59E-05	429.79
Chemical carcinogenesis	1,90E-07	407.05
Fatty acid biosynthesis	0.00006776	351.63
Peroxisome	6,11E-04	203.82
Ubiquinone and other terpenoid-quinone biosynthesis	0.001803	196.37
Non-alcoholic fatty liver disease (NAFLD)	2,59E-04	154.53
Ascorbate and aldarate metabolism	0.0005151	143.80
PPAR signaling pathway	0.00001057	138.25

(C) KEGG database (Mouse) –control/ extracellular inhibitor-treated mice

Name	P-value (p)	Combined Score
IL-17 signaling pathway	0.002357	166.22
African trypanosomiasis	0.03076	111.59
Malaria	0.03850	83.09
Arginine and proline metabolism	0.03927	80.93
Glycosaminoglycan biosynthesis	0.04158	75.00
Glutathione metabolism	0.05001	58.51
Metabolism of xenobiotics by cytochrome P450	0.05153	56.17
PPAR signaling pathway	0.06590	39.99
Protein digestion and absorption	0.06965	37.00
Chemical carcinogenesis	0.07263	34.87

(D) KEGG database (Mouse) –intracellular inhibitor-treated mice/ extracellular inhibitor-treated mice

Name	P-value (p)	Combined Score
Glycolysis / Gluconeogenesis	0.00004577	426.09
Hypertrophic cardiomyopathy (HCM)	0.00009653	307.16
Dilated cardiomyopathy (DCM)	0.0001105	289.22
Thiamine metabolism	0.01564	263.99
Cardiac muscle contraction	0.003005	141.82
Propanoate metabolism	0.03207	105.68
Glucagon signaling pathway	0.005077	98.65
Pyruvate metabolism	0.03917	81.20
Glycine, serine and threonine metabolism	0.04119	75.94
Adrenergic signaling in cardiomyocytes	0.01042	58.74

3.6 Anti-Inflammatory Effects of Extracellular CTSD Inhibitor Are LPS-Dependent

To better understand the link between extracellular CTSD and TLR-4 mediated inflammation, we investigated whether lipopolysaccharide (LPS), a TLR4 agonist, influences the inflammatory effects of extracellular CTSD inhibitor. OxLDL- stimulated mouse bone-marrow derived macrophages were treated with extracellular CTSD inhibitor in the presence or absence of LPS. Only in the presence of LPS, extracellular CTSD inhibitor reduced gene expression levels of inflammatory cytokines, *Tnfa* and *Ccl2* (Figures 5A, B) suggesting that the anti-inflammatory effects of extracellular CTSD inhibitor are TLR-4 dependent.

Figure 5 Anti-inflammatory effects of extracellular CTSD inhibitor are LPS- dependent: (A, B) Gene expression levels of *Tnfa*, and *Ccl2* from mouse *Wt* mouse BMDMs. Gene expression data are shown relative to DMSO-treated BMDMs with no LPS. Error bars represent ± SEM; n = 3 from an independent experiment. * represents p < 0.05 and **** represents p < 0.001 compared to DMSO-treated BMDMs as determined by two-way ANOVA.

4. Discussion

Except for lifestyle interventions such as caloric restriction and exercise, there are currently no well-defined approved therapeutic strategies for NASH. As lifestyle changes are often insufficient, novel and safer pharmacologic treatments for NASH are urgently needed. While inhibition of CTSD activity was proven beneficial for NASH, the specific mechanisms by which extracellular and intracellular fractions of CTSD affect NASH were not explored. In the current study, we have identified that

extracellular CTSD inhibition has more beneficial and less toxic effects in reducing metabolic inflammation over intracellular CTSD. These findings demonstrate the potential of extracellular CTSD to serve as a promising and safe target for the treatment of NASH.

Intracellular CTSD activity is accountable for cellular processes such as breakdown of intracellular proteins [28], cell signalling [29] and apoptosis [30]. Therefore, CTSD knockout mice develop intestinal necrosis, destruction of lymphoid cells and myelin sheath atrophy leading to early death, suggesting CTSD as a key protein in physiology [21]. In line, our proteomics data showed that inhibition of intracellular CTSD led to predominant downregulation of proteins specifically involved in mitochondrial oxidative phosphorylation and electron transport function namely Cox4i1, Cox6c, NADH dehydrogenase as well as CYP class of enzymes (Cyp2c29, Cyp3a41a, Cyp3a11, Cyp2c40, Cyp2c37, Cyp1a2, Cyp2e1) as found in 4/10 KEGG enriched pathways. It must be noted that downregulation of hepatic CYP enzymes might result in elevated plasma drug levels, adverse drug effects or drug-induced toxicity and inflammation [31] indicating unfavourable effects of intracellular CTSD inhibition. Besides executing its classical homeostatic activity inside the cells, CTSD also functions in the extracellular space both in physiological and pathological conditions [32]. While controlled proteolytic activity of extracellular cathepsin D is evidently important for vital processes such as extracellular matrix (ECM) degradation during tissue repair, uncontrolled CTSD expression and activity is more commonly observed under pathological conditions [33, 34]. Specifically, excessive extracellular CTSD activity has been implicated in metabolic inflammatory conditions [18, 22, 35, 36]. In line with these compelling findings, we have demonstrated that extracellular CTSD activity is associated with NASH pathogenesis. Given evidence for the site-specific role of CTSD in pathophysiological processes, targeted blocking of its activity is critical to prevent any off-target side effects [37]. To this end, selective effects of respective small-compound CTSD inhibitors obtained in our in vitro experiment strengthens the possibility that these inhibitors may have therapeutic value in treating NASH without causing disturbances of generalized lysosomal CTSD operations.

In our current study, we found pronounced effects of CTSD inhibition in the liver such as reduced lipid levels and alterations in metabolic pathways amongst others. This observation is in line with the concept that complex interplay between lipid acquisition and lipid disposal occurs in NASH [38]. Lipids undergo constant recycling and relocation in the cell and lysosomes play an important role in this recycling as they regulate hepatic lipid catabolism and mobilization [39]. In agreement, lysosomal dysfunction accompanied by aberrant cathepsin expression and activity is shown to be involved with the onset of NASH [16, 40–42]. Similar to these observations, we have previously shown that CTSD plays a central role in lipid metabolism during NASH and that its inhibition reduces hepatic steatosis by biliary cholesterol elimination [19]. The present study confirms the findings of this earlier report and provides insight for the specific role of extracellular CTSD fraction in lipid metabolism. In this study, pathways involved in hepatic lipid metabolism such as linoleic acid, fatty acid and steroid hormone biosynthesis have been found enriched in extracellular inhibitor- treated mice. The increased lipid biosynthesis upon extracellular CTSD inhibition likely reflects a compensatory response to the reduction in hepatic lipid levels and enhancement of cholesterol clearance. Further, amino acid [glycine, serine, and threonine] and pyruvate metabolism involving L-lactate dehydrogenase, enolase, and phosphoglycerate mutase proteins were downregulated suggesting reduced hepatic gluconeogenesis to compensate for the energy excess in the hyperlipidaemic mice. In addition to lipid-related pathways, proteomics analysis revealed enrichment of taurine and hypotaurine metabolism, retinol metabolism and PPAR signalling pathways upon extracellular CTSD

inhibition. Taurine is an amino acid that is known to have diverse protective effects in liver diseases by suppressing diet- induced steatosis and inflammation among others [43]. Noteworthy, retinoic acid and PPAR are known to synergically reverse liver fibrosis [44, 45]. These findings further reinforce the potential benefits of extracellular CTSD inhibition in the context of liver diseases.

Furthermore, KEGG analysis revealed that IL-17 signalling pathway, drug and glutathione metabolism were downregulated upon extracellular CTSD inhibition. Relevantly, IL-17 signalling has been demonstrated to be involved in the pathogenesis of NASH. IL-17 pathway revealed the downregulation of S100A8/9 proteins. S100A8/9 proteins act as a ligand for Toll-like receptor 4 (TLR4) and are known to be released extracellularly, where they amplify inflammatory responses by inducing cytokine production such as IL-17 [46, 47]. Moreover, TLR-4 activation is required for IL-17-mediated inflammation [48]. Furthermore, both broad and selective cathepsin inhibitors are known to suppress IL-17 producing T helper cells during inflammatory process [49]. Intriguingly, TLR4-dependent signalling is known to drive extracellular catabolism of modified lipoproteins by lysosomal proteases [50]. These findings suggest that extracellular CTSD released upon hyperlipidaemic conditions might interact with S100A8/9 complex to activate the TLR4 signalling pathway which further exacerbates inflammation in a positive feedback mechanism.

It should however be taken into account that we could not detect profound effects of extracellular CTSD inhibition on other markers of hepatic inflammation. Moreover, while our data suggest an improvement in systemic inflammation, these data were retrieved from only a limited number of mice (n=5). More in-depth analysis including additional mice is therefore essential to confirm our findings. Further, 16 weeks of dietary intervention didn't reveal significant differences in plasma inflammatory markers in low and HFC fed *Ldlr*^{-/-} mice [51], suggesting that longer periods of diet are required to see such changes. As such the results of the current study are not sufficient to conclude that extracellular CTSD has major effects on reducing inflammation status. Therefore, it is currently unclear whether the dose of 50 mg/ kg of inhibitor and 10 weeks of HFC diet are sufficient to interfere with the inflammatory network. Future studies with higher and/or more frequent injections of CTSD inhibitor are warranted to answer these questions. Furthermore, it remains to be determined to what extent these potential mechanisms take place in humans.

Recently, we showed that extracellular CTSD inhibition led to reduced hepatic steatosis and insulin sensitivity in rats [22]. However, it is noteworthy that the translational potential of these findings to humans is limited due to substantial differences in lipoprotein metabolism between humans and rats [52]. For instance, hyperlipidaemic rats are known to display elevated plasma levels of high-density lipoprotein cholesterol (HDL-c) and reduced low-density lipoprotein cholesterol (LDL-c) levels, while humans show reversed plasma lipoprotein profiles, with higher levels of LDL-c and lower HDL-c. Relevantly, knocking out the LDL receptor in mice is known to block the uptake of LDL and results in a phenotype with excessive plasma LDL-c levels. Further, *Ldlr*^{-/-} mice have the largest amount of the cholesterol in the form of IDL/ LDL fraction [intermediate density lipoprotein/low-density lipoprotein] as seen in humans [53]. Thus, *Ldlr*^{-/-} mice possess the plasma lipoprotein profile as comparable to humans and serve as a better model to study the "human-like" profile [54]. In agreement, *Ldlr*^{-/-} mice have been used extensively as a research model to investigate pathways involved in human NASH [55–57]. The current study establishes a key role for extracellular CTSD in NASH-induced dyslipidaemia. Despite these significant findings, there are certain limitations to our study that include no advanced inflammatory data and absence of human data with CTSD inhibitors.

Therefore, future studies using human models are warranted to expand our findings on inflammation and their validation in the clinical setting.

In conclusion, in contrast to the intracellular fraction of CTSD, extracellular CTSD inhibition has substantial benefits in restoration of lipid metabolism. Although the results of the current study should be interpreted with caution given the mild changes in inflammation, our data highlight that extracellular CTSD inhibition has favourable effects compared to intracellular inhibition and thus holds potential translational value as observed in a NASH mouse model with a 'human-like' lipoprotein profile.

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Supplemental material

Figure S1 Schematic representation of the study design and the timeline of the in vivo experiment. *Ldlr*^{-/-} mice fed a regular chow diet were included as a control group for NASH disease phenotype ($n=20$). HFC mice received vehicle or CTSD inhibitors subcutaneously once in every two days for 10 weeks. Sample sizes are $n=17$ for vehicle group, $n=20$ for intracellular inhibitor (GA-12)-treated group and $n=17$ for extracellular inhibitor (CTD-002)-treated group.

Figure S2 HFC supplementation for 10 week induces NASH in *Ldlr*^{-/-} mice: (A-B) Feeding mice with HFC diet for 10 weeks lead to a significant increase in hepatic cholesterol and triglyceride levels compared to mice on chow diet. (C) Scoring of hepatic lipid content (oil red o staining) using arbitrary units (A.U.). (Original magnification, 200x) (D) Plasma lipid levels between chow and HFC mice. (E) Total number of plasma monocytes as measured by FACS. (F) Liver sections were stained for infiltrating macrophages and neutrophils (Mac-1). Positive cells were counted in six microscopical views (original magnification, 200x) (G) Hepatic immunostaining for F4/80. Postive cells were evaluated and scored according to arbitrary units (original magnification, 400x). Error bars represent $\pm$ SEM; n=17-20 animals; n=5 per each group for FACS; * represents $p < 0.05$ **** represents $p < 0.001$ compared to chow control mice as determined by two-tailed unpaired t test.

Figure S3 No changes in hepatic fibrosis between different experimental groups of mice: Representative pictures of Sirius Red staining (original magnification, 100x)

Figure S4. Hepatic gene expression of lipid-related genes: (A) Hepatic gene expression of lipid metabolism-related genes. Error bars represent $\pm$ SEM. n=16-20 per group.

Figure S5. Label-free quantitative LC-MS proteomics of different groups of mice. Volcano plots of protein changes observed in the livers of mice belonging to different groups. The $-\log_{10}$ (Benjamini–Hochberg corrected P value) is plotted against the $\log_2$ (fold change) for respective groups. (A) extracellular inhibitor-treated group vs control (B) intracellular inhibitor-treated group vs control and (C) extracellular vs intracellular inhibitor-treated groups.

Table S1: Significantly upregulated proteins found in control mice compared to intracellular inhibitor-treated mice. Proteins were selected with a ratio of at least 1.5-fold change and p-value of ≤ 0.05 . In the table, Protein accession number according to UniProt, protein names, gene ID, abundance ratio ($\log_2 \geq 0.58$) are listed.

Accession number	Upregulated proteins in control mice compared to intracellular inhibitor-treated mice	Gene ID	Control/ intracellular inhibitor Abundance ratio ($\log_2$)
	Description		
Q9D6Y9	1,4-alpha-glucan-branching enzyme	Gbe1	1,26
Q9R092	17-beta-hydroxysteroid dehydrogenase type 6	Hsd17b6	2,08
P61922	4-aminobutyrate aminotransferase, mitochondrial	Abat	1,4
A0JNU3	60 kDa lysophospholipase	Aspg	1,46
Q9DCD0	6-phosphogluconate dehydrogenase, decarboxylating	Pgd	1,78
Q80XL6	Acyl-CoA dehydrogenase family member 11	Acad11	1,35
Q99PL7	Acyl-CoA desaturase	Scd3	3,31
Q5XG73	Acyl-CoA-binding domain-containing protein 5	Acbd5	2,6
P51881	ADP/ATP translocase 2	Slc25a5	1,75
P47740	Aldehyde dehydrogenase family 3 member A2	Aldh3a2	1,79
Q8BW75	Amine oxidase [flavin-containing] B	Maob	1,49
Q91YI0	Argininosuccinate lyase	Asl	1,8
P34927	Asialoglycoprotein receptor 1	Asgr1	1,79
Q8BP47	Asparagine--tRNA ligase, cytoplasmic	NARS1	1,79
P04919	Band 3 anion transport protein	Slc4a1	2,13

P18572	Basigin	Bsg	1,61
Q61335	B-cell receptor-associated protein 31	Bcap31	1,83
Q9QXX4	Calcium-binding mitochondrial carrier protein Aralar2	Slc25a13	2,03
Q61490	CD166 antigen	Alcam	1,77
Q91WS0	CDGSH iron-sulfur domain-containing protein 1	Cisd1	2,14
P60766	Cell division control protein 42 homolog	Cdc42	2,37
Q68FD5	Clathrin heavy chain 1	Cltc	1,43
Q9QZE5	Coatomer subunit gamma-1	Copg1	1,77
Q9QZQ8	Core histone macro-H2A.1	H2afy	1,89
P50172	Corticosteroid 11-beta-dehydrogenase isozyme 1	Hsd11b1	1,88
Q99L04	Dehydrogenase/reductase SDR family member 1	Dhrs1	2,59
P50285	Dimethylaniline monooxygenase [N-oxide-forming] 1	Fmo1	2,72
P97501	Dimethylaniline monooxygenase [N-oxide-forming] 3	Fmo3	2,38
P97872	Dimethylaniline monooxygenase [N-oxide-forming] 5	Fmo5	2,57
O54734	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit	Ddost	1,67
Q91YQ5	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	Rpn1	2,31
P58252	Elongation factor 2	Eef2	1,66
Q8BHI7	Elongation of very long chain fatty acids protein 5	Elovl5	3,2
P19096	Fatty acid synthase	Fasn	2,24
O08914	Fatty acid amide hydrolase 1	Faah	1,55
P35576	Glucose-6-phosphatase	G6pc	2,12
Q64516	Glycerol kinase	Gk	1,73
Q9ET01	Glycogen phosphorylase, liver form	Pygl	1,51
P01901	H-2 class I histocompatibility antigen, K-B alpha chain	H2-K1	1,58
P10922	Histone H1.	H1-0	2,72
P15864	Histone H1.2	H1-2	2,48
P43274	Histone H1.4	H1-4	2,91
P43276	Histone H1.5	H1-5	2,61
P02301	Histone H3.3C	H3f3c	1,57
P62806	Histone H4	H4c1	1,3
Q8VCZ9	Hydroxyproline dehydrogenase	Prodh2	2,82
P01872	Immunoglobulin heavy constant mu	Ighm	1,43
P70168	Importin subunit beta-1	Kpnb1	1,97
Q9CXF0	Kynureninase	Kynu	1,67
Q9WVM8	Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial	Aadat	2,16
Q922Q8	Leucine-rich repeat-containing protein 59	Lrrc59	2,76
P58710	L-gulonolactone oxidase	Gulo	2,12
P41216	Long-chain-fatty-acid--CoA ligase 1	Acs1	1,68
Q8JZR0	Long-chain-fatty-acid--CoA ligase 5	Acs15	1,84
O35114	Lysosome membrane protein 2	Scarb2	2,1
Q99J39	Malonyl-CoA decarboxylase, mitochondrial	Mlycd	1,62
O55022	Membrane-associated progesterone receptor component 1	Pgrmc1	1,58
Q80UU9	Membrane-associated progesterone receptor component 2	Pgrmc2	2,33
Q9DD20	Methyltransferase-like protein 7B	Mettl7b	2,33
Q91VS7	Microsomal glutathione S-transferase 1	Mgst1	2,91
Q922Q1	Mitochondrial amidoxime reducing component 2	Marc2	2
Q9CW42	Mitochondrial amidoxime-reducing component 1	Marc1	1,79
Q791V5	Mitochondrial carrier homolog 2	Mtch2	2,86
Q9CZW5	Mitochondrial import receptor subunit TOM70	Tomm70	1,63
Q9D023	Mitochondrial pyruvate carrier 2 OS=Mus musculus OX=10090 GN=Mpc2 PE=1 SV=1	Mpc2	1,93
Q8VDD5	Myosin-9 OS=Mus musculus	Myh9	1,71
Q9ERS2	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13	Ndufa13	1,86
O09111	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 11, mitochondrial	Ndufb11	2,39
Q9CQC7	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 4	Ndufb4	1,73
Q9DC70	NADH dehydrogenase [ubiquinone] iron-sulfur protein 7, mitochondrial	Ndufs7	2
Q9DCN2	NADH-cytochrome b5 reductase 3	Cyb5r3	2,45
Q9QXD1	Peroxisomal acyl-coenzyme A oxidase 2	Acox2	1,84
P97372	Proteasome activator complex subunit 2	Psme2	2,13
Q9CQS8	Protein transport protein Sec61 subunit beta OS=Mus musculus OX=10090 GN=Sec61b PE=1 SV=3	Sec61b	3,18
P35293	Ras-related protein Rab-18	Rab18	1,67
P35278	Ras-related protein Rab-5C	Rab5c	2,09

P51150	Ras-related protein Rab-7a	Rab7a	2,51
Q9JIM62	Receptor expression-enhancing protein 6	Reep6	1,49
O88451	Retinol dehydrogenase 7	Rdh7	2,23
Q9D5J6	Sedoheptulokinase	Shpk	1,89
P52430	Serum paraoxonase/arylesterase 1	Pon1	1,45
Q9JIL3	Solute carrier organic anion transporter family member 1B2	Slco1b2	3,72
Q62465	Synaptic vesicle membrane protein VAT-1 homolog	Vat1	2,13
Q9D0R2	Threonine--tRNA ligase 1, cytoplasmic	Tars1	2,12
Q9QUI0	Transforming protein RhoA	Rhoa	2,27
Q62186	Translocon-associated protein subunit delta	Ssr4	1,97
Q99KF1	Transmembrane emp24 domain-containing protein 9	Tmed9	2,41
Q8JZU2	Tricarboxylate transport protein, mitochondrial	Slc25a1	2,52
P68373	Tubulin alpha-1C chain	Tuba1c	1,17
P68372	Tubulin beta-4B chain	Tubb4b	1,21
Q8BWQ1	UDP-glucuronosyltransferase 2A3	Ugt2a3	2,81
P17717	UDP-glucuronosyltransferase 2B17	Ugt2b17	2,29
Q8JZZ0	UDP-glucuronosyltransferase 3A2	Ugt3a2	2,78
O35488	Very long-chain acyl-CoA synthetase	Slc27a2	2,32
O70503	Very-long-chain 3-oxoacyl-CoA reductase	Hsd17b12	1,79
Q9QY76	Vesicle-associated membrane protein-associated protein B	Vapb	2,09
O08547	Vesicle-trafficking protein SEC22b	Sec22b	2,11
P25444	40S ribosomal protein S2	Rps2	1,32
P97461	40S ribosomal protein S5	Rps5	2,98
Q6ZWV3	60S ribosomal protein L10	Rpl10	1,24
P19253	60S ribosomal protein L13a	Rpl13a	1,5
Q9CR57	60S ribosomal protein L14	Rpl14	1,7
Q9CZM2	60S ribosomal protein L15	Rpl15	1,75
P35980	60S ribosomal protein L18	Rpl18	2,69
Q8BP67	60S ribosomal protein L24	Rpl24	1,71
P41105	60S ribosomal protein L28	Rpl28	1,74
P47915	60S ribosomal protein L29	Rpl29	1,55
Q9D1R9	60S ribosomal protein L34	Rpl34	2,36
Q6ZWV7	60S ribosomal protein L35	Rpl35	2,08
P83882	60S ribosomal protein L36a	Rpl36a	1,64
Q9D8E6	60S ribosomal protein L4	Rpl4	1,73
P47911	60S ribosomal protein L6	Rpl6	2,91
P14148	60S ribosomal protein L7	Rpl7	3,09
P12970	60S ribosomal protein L7a	Rpl7a	1,31
Q9CQX2	Cytochrome b5 type B	Cyb5b	1,84
P00405	Cytochrome c oxidase subunit 2	Mtco2	2,51
P19783	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	Cox4i1	1,96
Q9CPQ1	Cytochrome c oxidase subunit 6C	Cox6c	2,19
P20852	Cytochrome P450 2A5	Cyp2a5	2,78
Q64458	Cytochrome P450 2C29	Cyp2c29	1,35
P56654	Cytochrome P450 2C37	Cyp2c37	2,32
P56657	Cytochrome P450 2C40	Cyp2c40	1,94
P24456	Cytochrome P450 2D10	Cyp2d10	1,82
Q8CIM7	Cytochrome P450 2D26	Cyp2d26	3,06
Q05421	Cytochrome P450 2E1	Cyp2e1	2,68
Q64459	Cytochrome P450 3A11	Cyp3a11	2,87
Q9JMA7	Cytochrome P450 3A41	Cyp3a41a	1,77
Q9CQQ7	ATP synthase F(0) complex subunit B1, mitochondrial	Atp5pb	1,41
P03930	ATP synthase protein 8	Mtatp8	1,97
P56379	ATP synthase subunit ATP5MPL, mitochondrial	Atp5mpl	2,71
Q9CPQ8	ATP synthase subunit g, mitochondrial	Atp5mg	1,63
P55096	ATP-binding cassette sub-family D member 3	Abcd3	2,57

Table S2: Significantly upregulated proteins found in control mice compared to extracellular inhibitor-treated mice. Proteins were selected with a ratio of at least 1.5-fold change and p-value of ≤ 0.05 . In the table, Protein accession number according to UniProt, protein names, gene ID, abundance ratio ($\log_2 \geq 0.58$) are listed.

Accession number	Upregulated proteins in response in control mice compared to extracellular inhibitor-treated mice	Gene Id	Control/ extracellular inhibitor
	Description		Abundance ratio ($\log_2$)
Ca1	Carbonic anhydrase 1	Ca1	0,9
Col14a1	Collagen alpha-1(XIV) chain	Col14a1	0,82
Ckm	Creatine kinase M-type	Ckm	0,65
Fabp3	Fatty acid-binding protein, heart	Fabp3	0,69
Gsta1	Glutathione S-transferase A1	Gsta1	0,66
Hbb-b1	Hemoglobin subunit beta-1	Hbb-b1	0,65
Ighv3-6	Ig heavy chain V region 3-6	Ighv3-6	1,28
Nubpl	Iron-sulfur protein NUBPL	Nubpl	0,59
Mb	Myoglobin	Mb	2,35
Myl1	Myosin light chain 1/3, skeletal muscle isoform	Myl1	1,04
Prelp	Prolargin	Prelp	0,74
S100a8	Protein S100-A8	S100a8	0,83
S100a9	Protein S100-A9	S100a9	0,65
Spta1	Spectrin alpha chain, erythrocytic 1	Spta1	0,68
Tnnc2	Troponin C, skeletal muscle	Tnnc2	1,37
Xylt2	Xylosyltransferase 2	Xylt2	0,91

Table S3: Significantly upregulated proteins found in extracellular inhibitor-treated mice compared to control mice. Proteins were selected with a ratio of at least 1.5-fold change and p-value of ≤ 0.05 . In the table, Protein accession number according to UniProt, protein names, gene ID, abundance ratio ($\log_2 \geq 0.58$) are listed.

Accession number	Upregulated proteins in extracellular inhibitor-treated mice compared to control mice	Gene Id	Extracellular inhibitor/ control
	Description		Abundance ratio ($\log_2$)
Q99PL7	Acyl-CoA desaturase 3	Scd3	0,6
P07361	Alpha-1-acid glycoprotein 2	Orm2	1,12
Q9DBE0	Cysteine sulfinic acid decarboxylase	Csad	0,8
Q05816	Fatty acid-binding protein 5	Fabp5	0,71
P01864	Ig gamma-2A chain C region secreted form		0,91
P01655	Ig kappa chain V-III region PC 7132		0,8
Q8K2C9	Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 3	Hacd3	0,86
Q9R1Z8	Vinexin	Sorbs3	0,68
P18242	Cathepsin D	Ctsd	0,58
O35403	Amine sulfotransferase	Sult3a1	0,64
P00186	Cytochrome P450 1A2	Cyp1a2	0,59
O54749	Cytochrome P450 2J5	Cyp2j5	0,71
Q64481	Cytochrome P450 3A16	Cyp3a16	1,12
P04938	Major urinary protein 11	Mup11	0,66
P11589	Major urinary protein 2	Mup2	0,61
P09528	Ferritin heavy chain	Fth1	0,85
P29391	Ferritin light chain 1	Ftl1	0,9
P46978	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3A	Stt3a	0,72
Q99KR7	Peptidyl-prolyl cis-trans isomerase F, mitochondrial	Ppif	0,71
P54726	UV excision repair protein RAD23 homolog A	Rad23a	0,66

Table S4: Significantly upregulated proteins found in extracellular inhibitor-treated mice compared to intracellular inhibitor-treated mice. Proteins were selected with a ratio of at least 1.5-fold change and p-value of ≤ 0.05 . In the table, Protein accession number according to UniProt, protein names, gene ID, abundance ratio ($\log_2 \geq 0.58$) are listed.

Accession number	Upregulated proteins in extracellular inhibitor-treated mice compared to Intracellular inhibitor-treated mice	Gene ID	Extracellular inhibitor/ intracellular inhibitor
			Abundance ratio ($\log_2$)
P19096	Fatty acid synthase	Fasn	1,91
Q9ET01	Glycogen phosphorylase, liver form	Pygl	1,66
Q9DCN2	NADH-cytochrome b5 reductase 3	Cyb5r3	2,19
Q791V5	Mitochondrial carrier homolog 2	Mtch2	2,42
P35576	Glucose-6-phosphatase	G6pc	3,54
Q91YI0	Argininosuccinate lyase	Asl	1,61
P51881	ADP/ATP translocase 2	Slc25a5	1,66
P41216	Long-chain-fatty-acid--CoA ligase 1	Acs1	1,71
Q9CZM2	60S ribosomal protein L15	Rpl15	1,61
P47740	Aldehyde dehydrogenase family 3 member A2	Aldh3a2	1,73
P50172	Corticosteroid 11-beta-dehydrogenase isozyme 1	Hsd11b1	1,74
Q9DD20	Methyltransferase-like protein 7B	Mettl7b	2,42
P19783	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	Cox4i1	1,79
P01872	Immunoglobulin heavy constant mu	Ighm	1,72
P58710	L-gulonolactone oxidase	Gulo	1,73
Q922Q1	Mitochondrial amidoxime reducing component 2	Marc2	2
O35488	Very long-chain acyl-CoA synthetase	Slc27a2	1,7
Q8JZR0	Long-chain-fatty-acid--CoA ligase 5	Acs15	1,65
Q9QXX4	Calcium-binding mitochondrial carrier protein Aralar2	Slc25a13	2,03
P43274	Histone H1.4	H1-4	1,74
O88451	Retinol dehydrogenase 7	Rdh7	1,79
Q91WS0	CDGSH iron-sulfur domain-containing protein 1	Cisd1	1,83
P00405	Cytochrome c oxidase subunit 2	Mtco2	2,35
P97872	Dimethylaniline monooxygenase [N-oxide-forming] 5	Fmo5	2,29
Q9CPQ1	Cytochrome c oxidase subunit 6C	Cox6c	2,35
Q64516	Glycerol kinase OS=Mus musculus OX=10090 GN=Gk PE=1 SV=2	Gk	1,78
Q9DCD0	6-phosphogluconate dehydrogenase, decarboxylating	Pgd	1,95
Q9DCM2	Glutathione S-transferase kappa 1	Gstk1	1,7
Q91YQ5	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	Rpn1	1,86
Q9CW42	Mitochondrial amidoxime-reducing component 1	Marc1	1,82
Q9CXF0	Kynureninase	Kynu	1,73
P03930	ATP synthase protein 8	Mtatp8	1,9
Q8BP67	60S ribosomal protein L24	Rpl24	1,58
P35980	60S ribosomal protein L18	Rpl18	2,06
Q9D0R2	Threonine--tRNA ligase 1, cytoplasmic	Tars1	2,1
Q8CIM7	Cytochrome P450 2D26	Cyp2d26	2,6
Q64458	Cytochrome P450 2C29	Cyp2c29	1,85
Q99L04	Dehydrogenase/reductase SDR family member 1	Dhrs1	2,17
P14148	60S ribosomal protein L7	Rpl7	2,73
P55096	ATP-binding cassette sub-family D member 3	Abcd3	2,27
Q9QXD1	Peroxisomal acyl-coenzyme A oxidase 2	Acox2	2,03
P47911	60S ribosomal protein L6	Rpl6	1,86
Q9D5J6	Sedoheptulokinase	Shpk	1,91
P97372	Proteasome activator complex subunit 2	Psme2	1,69
Q9QUI0	Transforming protein RhoA	Rhoa	2,34
P50285	Dimethylaniline monooxygenase [N-oxide-forming] 1	Fmo1	2,07
Q91X44	Glucokinase regulatory protein	Gckr	1,67
P24456	Cytochrome P450 2D10	Cyp2d10	2,15
Q9D1R9	60S ribosomal protein L34	Rpl34	2,3
P34927	Asialoglycoprotein receptor 1	Asgr1	1,61
Q922Q8	Leucine-rich repeat-containing protein 59	Lrrc59	2,25
Q9CZW5	Mitochondrial import receptor subunit TOM70	Tomm70	1,59
P97461	40S ribosomal protein S5	Rps5	2,85
P15864	Histone H1.2	H1-2	1,69

P10922	Histone H1.0	H1-0	1,83
P17717	UDP-glucuronosyltransferase 2B17	Ugt2b17	2,28
Q9CQC7	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 4	Ndufb4	1,67
Q61335	B-cell receptor-associated protein 31	Bcap31	1,58
Q99J39	Malonyl-CoA decarboxylase, mitochondrial	Mlycd	1,58
Q9WVM8	Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial	Aadat	2,02
P60766	Cell division control protein 42 homolog	Cdc42	2,38
Q8BWQ1	UDP-glucuronosyltransferase 2A3	Ugt2a3	2,75
Q62465	Synaptic vesicle membrane protein VAT-1 homolog	Vat1	1,93
P56654	Cytochrome P450 2C37	Cyp2c37	1,78
P97501	Dimethylaniline monooxygenase [N-oxide-forming] 3	Fmo3	1,98
P56135	ATP synthase subunit f, mitochondrial	Atp5mf	1,95
Q9CQX2	Cytochrome b5 type B	Cyb5b	2,01
O35114	Lysosome membrane protein 2	Scarb2	2,12
Q8R111	Cytochrome b-c1 complex subunit 9	Uqcr10	1,77
A2AKK5	Acyl-coenzyme A amino acid N-acyltransferase 1	Acnat1	1,59
Q9DC70	NADH dehydrogenase [ubiquinone] iron-sulfur protein 7, mitochondrial	Ndufs7	1,95
O08547	Vesicle-trafficking protein SEC22b	Sec22b	1,73
P61027	Ras-related protein Rab-10	Rab10	1,56
Q62186	Translocon-associated protein subunit delta	Ssr4	1,58
P56657	Cytochrome P450 2C40	Cyp2c40	1,98
Q9QY76	Vesicle-associated membrane protein-associated protein B	Vapb	1,79
P18572	Basigin	Bsg	1,82
Q64514	Tripeptidyl-peptidase 2	Tpp2	1,71
Q8JZ20	UDP-glucuronosyltransferase 3A2	Ugt3a2	1,88
Q9D023	Mitochondrial pyruvate carrier 2	Mpc2	1,76
Q8JZU2	Tricarboxylate transport protein, mitochondrial	Slc25a1	2,32
P00186	Cytochrome P450 1A2	Cyp1a2	2,39
Q8BP47	Asparagine--tRNA ligase, cytoplasmic	NARS1	1,72
Q64459	Cytochrome P450 3A11	Cyp3a11	2
Q5XG73	Acyl-CoA-binding domain-containing protein 5	Acbd5	2,45
B1AR13	CDGSH iron-sulfur domain-containing protein 3, mitochondrial	Cisd3	1,85
O70503	Very-long-chain 3-oxoacyl-CoA reductase	Hsd17b12	1,64
Q9JMA7	Cytochrome P450 3A41	Cyp3a41a	2,87
O09111	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 11, mitochondrial	Ndufb11	2,48
Q8BHI7	Elongation of very long chain fatty acids protein 5	Elovl5	2,55
Q8VCZ9	Hydroxyproline dehydrogenase	Prodh2	2,91
P51150	Ras-related protein Rab-7a	Rab7a	2,26
P04919	Band 3 anion transport protein	Slc4a1	1,67
P35278	Ras-related protein Rab-5C	Rab5c	1,92
Q9CR61	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7	Ndufb7	1,81
Q9R092	17-beta-hydroxysteroid dehydrogenase type 6	Hsd17b6	1,82
P56379	ATP synthase subunit ATP5MPL, mitochondrial	Atp5mpl	3,36
Q92511	ATPase family AAA domain-containing protein 3	Atad3	1,63
Q80UU9	Membrane-associated progesterone receptor component 2	Pgrmc2	2,13
Q99PL7	Acyl-CoA desaturase	Scd3	2,8
Q9CQS8	Protein transport protein Sec61 subunit beta	Sec61b	3,03
Q9R1J0	Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating	Nsdhl	1,78
Q99KF1	Transmembrane emp24 domain-containing protein 9	Tmed9	2,27
Q9R1P3	Proteasome subunit beta type-2	Psmb2	1,72
P15392	Cytochrome P450 2A4	Cyp2a4	2,21
Q9CRC0	Vitamin K epoxide reductase complex subunit 1	Vkorc1	1,88
O09117	Synaptophysin-like protein 1	Sypl1	1,79
P20852	Cytochrome P450 2A5	Cyp2a5	2,92
P43276	Histone H1.5	H1-5	1,57
Q9CQW9	Interferon-induced transmembrane protein 3	Ifitm3	1,79
Q9D2R0	Acetoacetyl-CoA synthetase	Aacs	2,02
Q9CQC6	Basic leucine zipper and W2 domain-containing protein 1	Bzw1	1,68
Q9JL3	Solute carrier organic anion transporter family member 1B2	Slco1b2	3,87
Q64669	NAD(P)H dehydrogenase [quinone]	Nqo1	1,83
Q76M72	Solute carrier family 22 member 27	Slc22a27	2,31
Q61490	CD166 antigen	Alcam	1,65
Q05421	Cytochrome P450 2E1	Cyp2e1	2,07
P20852	Cytochrome P450 2A5	Cyp2a5	2,92
P15392	Cytochrome P450 2A4	Cyp2a4	2,21

Q9JMA7	Cytochrome P450 3A41	Cyp3a41a	2,87
Q64459	Cytochrome P450 3A11	Cyp3a11	2
P00186	Cytochrome P450 1A2	Cyp1a2	2,39
P56657	Cytochrome P450 2C40	Cyp2c40	1,98
Q9CQX2	Cytochrome b5 type B	Cyb5b	2,01
P56654	Cytochrome P450 2C37	Cyp2c37	1,78
Q64458	Cytochrome P450 2C29	Cyp2c29	1,85
Q8CIM7	Cytochrome P450 2D26	Cyp2d26	2,6
Q9DCN2	NADH-cytochrome b5 reductase 3	Cyb5r3	2,19

Table S5: Significantly upregulated proteins found in intracellular inhibitor-treated mice compared to control mice. Proteins were selected with a ratio of at least 1.5-fold change and p-value of ≤ 0.05 . In the table, Protein accession number according to UniProt, protein names, gene ID, abundance ratio ($\log_2 \geq 0.58$) are listed.

Accession number	Upregulated proteins in intracellular inhibitor-treated mice compared to control mice	Gene ID	Intracellular inhibitor/control
			Abundance ratio ($\log_2$)
Q9R0Y5	Adenylate kinase isoenzyme 1	Ak1	4,1
P21550	Beta-enolase	Eno3	5,97
O54724	Caveolae-associated protein 1	Cavin1	2,37
P07310	Creatine kinase M-type	Ckm	4,05
Q6P8J7	Creatine kinase S-type, mitochondrial	Ckmt2	3,2
P31001	Desmin	Des	2,42
P11404	Fatty acid-binding protein, heart	Fabp3	3,04
P97447	Four and a half LIM domains protein 1	Fhl1	3,4
P14602	Heat shock protein beta-1	Hspb1	1,98
Q5EBG6	Heat shock protein beta-6	Hspb6	3,79
Q9JKS4	LIM domain-binding protein 3	Ldb3	5,75
P16125	L-lactate dehydrogenase B chain	Ldhb	2,11
P04247	Myoglobin OS=Mus musculus	Mb	6,52
Q62234	Myomesin-1 OS=Mus musculus	Myom1	3,59
P05977	Myosin light chain 1/3, skeletal muscle isoform	Myl1	3,64
P51667	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform	Myl2	4,68
Q5SX40	Myosin-1 OS=Mus musculus	Myh1	2,31
Q5SX39	Myosin-4 OS=Mus musculus	Myh4	1,81
Q91Z83	Myosin-7 OS=Mus musculus	Myh7	1,85
Q9JK37	Myozenin-1	Myoz1	2,54
Q9JJW5	Myozenin-2	Myoz2	1,95
P00688	Pancreatic alpha-amylase	Amy2	2,89
O70250	Phosphoglycerate mutase 2	Pgam2	4,87
Q9R1C7	Pre-mRNA-processing factor 40 homolog A	Prpf40a	2,27
P58771	Tropomyosin alpha-1 chain	Tpm1	3,35
P58774	Tropomyosin beta chain	Tpm2	4,93
P20801	Troponin C, skeletal muscle	Tnnc2	4,08
P13412	Troponin I, fast skeletal muscle	Tnni2	2,25
Q9QZ47	Troponin T, fast skeletal muscle	Tnnt3	2,53
Q11136	Xaa-Pro dipeptidase	Pepd	3,81

Table S6: Significantly upregulated proteins found in intracellular inhibitor-treated mice compared to extracellular inhibitor-treated mice. Proteins were selected with a ratio of at least 1.5-fold change and p-value of ≤ 0.05 . In the table, Protein accession number according to UniProt, protein names, gene ID, abundance ratio ($\log_2 \geq 0.58$) are listed.

Accession number	Upregulated proteins in intracellular inhibitor-treated mice compared to extracellular inhibitor-treated mice	Gene ID	Intracellular inhibitor/ extracellular inhibitor
	Description		Abundance ratio ($\log_2$)
Q5SX40	Myosin-1	Myh1	2,54
P07310	Creatine kinase M-type	Ckm	5,61
P58771	Tropomyosin alpha-1 chain	Tpm1	3,31
P05977	Myosin light chain 1/3, skeletal muscle isoform	My1	3,07
P58774	Tropomyosin beta chain	Tpm2	5,16
Q9QZ47	Troponin T, fast skeletal muscle	Tnnt3	3,48
P21550	Beta-enolase	Eno3	4,56
P31001	Desmin	Des	2,71
P13412	Troponin I, fast skeletal muscle	Tnni2	2,42
P97447	Four and a half LIM domains protein 1	Fhl1	3,47
P11404	Fatty acid-binding protein, heart	Fabp3	6,52
Q9R0Y5	Adenylate kinase isoenzyme 1	Ak1	3,89
O70250	Phosphoglycerate mutase 2	Pgam2	4,04
Q62234	Myomesin-1	Myom1	3,85
Q5SX39	Myosin-4	Myh4	2,08
P16125	L-lactate dehydrogenase B chain	Ldhb	2,03
Q9JK37	Myozenin-1	Myoz1	2,45
P14602	Heat shock protein beta-1	Hspb1	2,14
O54724	Caveolae-associated protein 1	Cavin1	2,33
Q9JJW5	Myozenin-2	Myoz2	2,11
Q91WK1	SPRY domain-containing protein 4	Spryd4	1,62
P14602	Heat shock protein beta-1	Hspb1	2,14

Table S7: Top 10 enriched pathways upon intracellular CTSD inhibition

(A) KEGG database (Mouse) – Intracellular inhibitor-treated mice/ control

Name	P-value (p)	Combined Score
Hypertrophic cardiomyopathy (HCM)	1.711e-7	603.92
Dilated cardiomyopathy (DCM)	2.150e-7	568.61
Cardiac muscle contraction	0.000005430	414.48
Glycolysis / Gluconeogenesis	0.0001367	265.60
Adrenergic signaling in cardiomyocytes	0.00006793	172.92
Thiamine metabolism	0.02227	169.08
Arginine and proline metabolism	0.002548	159.27
Tight junction	0.001967	74.63
Propanoate metabolism	0.04550	66.45
Glucagon signaling pathway	0.01021	59.93

(B) KEGG database (Mouse) –control/ intracellular inhibitor-treated mice

Name	P-value (p)	Combined Score
Steroid hormone biosynthesis	6.332e-13	573.81
Ribosome	1.294e-15	519.41
Fatty acid biosynthesis	0.00009699	300.03
Retinol metabolism	8.955e-9	277.69
Chemical carcinogenesis	1.195e-8	264.64
Linoleic acid metabolism	9.232e-7	252.64
Ascorbate and aldarate metabolism	0.0007320	121.54
Peroxisome	0.00001966	117.28
PPAR signaling pathway	0.00002104	115.18
Drug metabolism	0.00001064	106.53

Chapter 5

Inflammatory Bowel Disease: A stressed "gut/feeling"

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Abstract

Inflammatory bowel disease (IBD) is a chronic and relapsing intestinal inflammatory condition, hallmarked by a disturbance in the bidirectional interaction between gut and brain. In general, the gut/brain axis involves direct and/or indirect communication via the central and enteric nervous system, host innate immune system, and particularly the gut microbiota. This complex interaction implies that IBD is a complex multifactorial disease. There is increasing evidence that stress adversely affects the gut/microbiota/brain axis by altering intestinal mucosa permeability and cytokine secretion, thereby influencing the relapse risk and disease severity of IBD. Given the recurrent nature, therapeutic strategies particularly aim at achieving and maintaining remission of the disease. Alternatively, these strategies focus on preventing permanent bowel damage and concomitant long-term complications. In this review, we discuss the gut/microbiota/brain interplay with respect to chronic inflammation of the gastrointestinal tract and particularly shed light on the role of stress. Hence, we evaluated the therapeutic impact of stress management in IBD.

1. Introduction

Inflammatory bowel disease (IBD) is a chronic and relapsing disorder [1], including Crohn's disease and ulcerative colitis. While Crohn's disease is characterized by transmural inflammation in any part of the gastrointestinal tract, ulcerative colitis is affecting the mucosal layer of the colon and rectum. Similar to other immune-mediated chronic diseases, such as rheumatoid arthritis, IBD is hallmarked by periods of remission interspersed with periods of acute flare. During disease course, symptoms like abdominal pain, cramping, loose stools or bloody diarrhea, fatigue, anemia and/or weight loss can manifest. The prevalence and incidence of IBD are increasing enormously [2]. Together with its early onset, relapsing nature, and life-threatening complications, IBD is currently a major health issue. Although the exact pathogenesis is unclear, IBD is certainly driven by disturbed crosstalk between a variety of parameters, i.e., genetic susceptibility and internal and external factors [3], which will be discussed in more detail. Although treatment options mainly focus on reducing intestinal inflammation [1], achieving/maintaining remission or improving the patient's quality-of-life, no cure for IBD is currently available. Given that IBD is a systemic disease, often associated with comorbidities such as anxiety and depression, this narrative review aims to evaluate the mutual interplay between stress and the gut/microbiota/brain axis, particularly with regard to chronic inflammation of the gastrointestinal tract. These insights set the basis for better understanding the impact of stress management on disease activity in IBD.

2. Search strategy

For this narrative review, we selected peer-reviewed preclinical and clinical articles as well as meta-analyses and important reviews from the PubMed database between January 1980 and June 2019. The following search terms were used: inflammatory bowel disease/IBD, Crohn's disease, ulcerative colitis, irritable bowel syndrome, gut/intestines, microbiota/microbiome, brain, interplay/axis, psychological/chronic/acute/cognitive stress, lifestyle factors, stress management, stress resilience, inflammation, disease activity, therapies/interventions/therapeutic strategies.

3. Gut/Microbiota/Brain interplay

In this section, we will focus on the tight association between the brain and gut, discussing the involvement of endocrine, immune, and neural pathways as well as the gut microbiota.

3.1. Brain/Gut interaction

The hypothalamic pituitary adrenal (HPA) axis is an endocrine pathway belonging to the limbic system of the brain. In response to stress [4], the activated HPA axis causes the secretion of corticotropin-releasing factor (CRF) from the hypothalamus, which stimulates the pituitary gland to release adrenocorticotrophic hormone (ACTH). In turn, ACTH triggers the immunosuppressive stress-hormone cortisol from the adrenal cortex [5], which ordinarily induces the synthesis of anti-inflammatory cytokines. However, in response to stress, sustained cortisol activity has also been associated with pro-inflammatory responses [6]. Likewise, stress-induced cortisol was shown to increase intestinal

barrier dysfunction, as recently shown by crypt analyses from rodents and humans [7]. Moreover, administration of cortisol in a porcine model caused a shift in microbiota composition, [8], pointing towards a role for cortisol in regulating intestinal inflammation and altering microbiota composition.

In addition to the HPA axis, the autonomic nervous system (ANS) coordinates the function of the gastrointestinal tract. The ANS is known to trigger efferent signals from the central nervous system (CNS; i.e., brain and spinal cord) to the intestinal wall to regulate mucosal immune responses [9] and other intestinal functions, such as nutrient absorption [10]. Vice versa, via enteric, spinal, and vagal nerves, afferent signals from the intestinal lumen are also known to regulate behavior, sleep, and stress reactivity [11,12]. Upon receiving stimuli from the diet and gut microbiota [13,14], the enteric nervous system (ENS, “second brain”), which is part of the peripheral nervous system, mainly communicates with the CNS in a bidirectional manner. However, the ENS is also capable of intrinsically innervating the gut [15] in an autonomous manner [16].

3.2. Gut microbiota

The gastrointestinal tract serves as a dynamic and local ecosystem for gut microbiota. Whereas, often being classified into two major phyla, i.e., Bacteroidetes and Firmicutes [17], the gut microbiota is composed of over 35,000 bacterial species [18]. Besides playing a role in metabolism [19], it is also essential for controlling processes related to barrier function against pathogenic microorganism colonization, such as mucosal integrity [20], immunomodulation [21], and pathogen protection [22]. Recently, preclinical [23], translational [24], and clinical [25] studies suggested that alterations in the structural composition or function of the microbiome can contribute to the development of mental illness, including depression-like behavior, and thus, is a vital component linking the gut/brain axis. In line, data have indicated strong correlations between alterations in gut microbiota and the development of multifactorial chronic inflammatory disorders, such as IBD [26,27], suggesting that dysbiosis is an important factor in both gastrointestinal and mental health.

Intestinal bacteria and their metabolites are also involved in gut-associated neuroimmune mechanisms that influence mood and behavior leading to depression. These mechanisms include tryptophan metabolism as well as neural signaling within the ENS [28]. Tryptophan is an essential amino acid, derived from the diet. While crossing the blood–brain barrier and acting as a precursor of the neurotransmitter serotonin, tryptophan can also be degraded in the gut through the kynurenine and serotonin synthesis pathways. This degradation can affect its availability to pass the blood–brain barrier. Thus, by modulating tryptophan levels, microbiota can affect the brain, resulting in behavioral changes [29].

By fermentation of dietary fibers, the gut microbiota is also responsible for producing short-chain fatty acids (SCFAs), including butyric acid, propionic acid, and acetic acid, which are typically found to be reduced in mucosa and feces of patients with IBD [30]. As extensively reviewed by Parada Venegas et al. [31], these metabolic products have shown to play an important role in promoting epithelial cell proliferation [32], barrier function [33], and cellular metabolism [34]. In addition, SCFAs have been involved in controlling intestinal inflammation through activation of G-protein coupled receptor signaling pathways [35], thereby regulating intestinal homeostasis and inhibiting pathogen colonization. Relevantly, SCFAs are also known to exert neuroprotective properties. For instance, gamma-aminobutyric acid is an inhibitory neurotransmitter involved in anxiety and depression and

can therefore modulate behavior [36]. Other mechanisms by which the intestinal microbiota affect neural responses include alterations in bacterial neurometabolites or bacterial cell wall sugars. These products can either act directly on primary afferent axons or trigger epithelial cells to release molecules that modulate neural signaling within the ENS [28].

Altogether, the multifaceted interplay between the gut, microbiota, and brain allows for intestinal and extraintestinal homeostasis, thereby coordinating gastrointestinal functions and modulating mood and higher cognitive functions, respectively.

4. Gut/Microbiota/Brain interplay in IBD development

It has become evident that factors such as genetics, environment, diet, and lifestyle are involved in dysregulation of the gut/microbiota/brain interplay, which in this section, will be discussed in the context of IBD development (**Figure 1** and **Table 1**) [37,38].

Table 1: Factors involved in the gut/microbiota/brain interplay in inflammatory bowel disease (IBD) development

Factor	Type of study	N	Intervention/Methodology	Outcome	Author(s)	Reference
Genetics	Clinical study	637	Questionnaire	First-degree relatives have 10-fold increased risk of IBD development	Orholm, M. <i>et al.</i>	37
	Meta-analysis	>75,000 cases & controls	GWAS	Identified 30 gene loci for CD and 23 for UC	Jostins, L. <i>et al.</i>	39
	Trans-ancestry association studies	238,401	GWAS	Identified 38 susceptibility loci for IBD	Liu, J.Z. <i>et al.</i>	40
	Genetic association study	6228	Association studies	Identified UPS and CYLD gene are important in IBD pathogenesis	Cleynen, I. <i>et al.</i>	41
	Genotype association study	34,819	Association studies	Insights into genetic heterogeneity between ileal and colonic CD	Cleynen, I. <i>et al.</i>	42
	Clinical study	323	Array-based transcriptome data	Identified 99 strong positional candidate genes in 63 risk loci	Momozawa, Y. <i>et al.</i>	43
	Clinical study	189 twin pairs	Questionnaire	Results highlight the importance of environmental triggers	Spehlmann, ME. <i>et al.</i>	45
	Clinical study	80 twin pairs	Questionnaire	Genetic influence is stronger in CD than in UC	Halfvarson, J. <i>et al.</i>	46
Diet	Interventional, open-label, pilot study	16	Vitamin D3 supplementation	Vitamin D3 modulates the gut microbiome	Bashir, M. <i>et al.</i>	48
	<i>In-vivo</i> mouse study	4-8 mice per group	Oral antibiotics	Increased levels of intracellular zinc led to bacterial clearance	Lahiri, A. <i>et al.</i>	49
	<i>In-vivo</i> mouse study	4-5 mice per group	High-fat diet and oral antibiotics	High-fat diet alters gut microbiome composition	Hildebrandt, MA.	55
	<i>In-vivo</i> mouse study	>100 inbred mouse strains	High-fat/high-sucrose diet	High-fat/high-sucrose diet influences gut microbiota composition	Parks, BW. <i>et al.</i>	56
	Case-control study	86	Dietary intake	Mono- and polyunsaturated fats consumption is a risk factor for IBD	Geerling, BJ. <i>et al.</i>	57

	<i>In-vivo</i> mouse study	4-5 mice per group	Diet enriched with phytosterols	Phytosterols are protective against IBD	Aldini, R. <i>et al.</i>	58
Environment	Population-based case-control study	1382	Questionnaire on 25 different topics	Altered intestinal microbiota may modulate risk of IBD	Ng, SC. <i>et al.</i>	62
	Retrospective case study	1194	Clinical history and questionnaire	Higher prevalence of CD in urban areas and UC in inland areas	Carpio, D. <i>et al.</i>	68
Smoking	Meta-analysis	245 articles	Smoking	Smoking is a risk factor for IBD	Mahid, SS. <i>et al.</i>	51
	Prospective case-control study	160	Transdermal nicotine or placebo patches	Smoking effects gut microbiota composition	Richardson, CE. <i>et al.</i>	52
Medication	Meta-analysis	11 observational studies	Antibiotic exposure	Antibiotics increases the risk of new-onset CD than UC	Ungaro, R. <i>et al.</i>	70
	Meta-analysis	20 studies	OCP	Increased risk for development of CD and UC	Ortiz, R. <i>et al.</i>	71
	Case-control study	122	NSAIDs	Provoked disease activity in IBD	Felder, JB. <i>et al.</i>	72
Exercise	<i>In-vivo</i> mouse study	4 mice per group	Exercise training	Alleviated symptoms of acute colitis	Saxena, A. <i>et al.</i>	60
	Prospective cohort study	194,711	Physical activity	Inversely associated with risk of CD	Khalili, H.	61
	Uncontrolled pilot study	12	12-week walking program	Beneficial for IBD patients	Loudon, CP. <i>et al.</i>	63
Sleep disturbances	Longitudinal, internet-based cohort data	3173	Questionnaire	Increased risk of disease flares in CD but not UC	Ananthakrishnan, AN. <i>et al.</i>	75
	Clinical study	10	Disturbances in sleep-wake cycle	Sleep disturbances led to immunologic alterations	Born, J. <i>et al.</i>	76
	Clinical study	47	Questionnaire assessing sleep quality	Impaired sleep quality is associated with pediatric IBD	Mahlmann, L. <i>et al.</i>	77
	Clinical study	32	Questionnaire assessing sleep quality	Impaired quality of life in IBD	Keefer, L. <i>et al.</i>	78
	Prospective observational cohort study	41	Pittsburgh sleep quality index (PSQI)	Strong association between poor sleep quality and IBD	Ali, T. <i>et al.</i>	79
	<i>In-vivo</i> mouse study	33	Diet and sleep disturbances	Circadian disorganization impacts intestinal microbiota	Voigt, RM. <i>et al.</i>	80

* CD = Crohn's disease; GWAS = genome-wide association studies; NSAIDs = nonsteroidal anti-inflammatory drugs; OCP = oral contraceptive pill; SNP = single nucleotide polymorphism; UC = ulcerative colitis; UPS = ubiquitin protease system

To date, genome-wide association studies revealed more than 200 susceptibility gene loci in IBD [39–43]. First, based on a model selection analysis, 163 susceptibility gene loci were identified, of which 23 and 30 loci were shown to be specific for ulcerative colitis and Crohn's disease, respectively [39]. These data were further completed with a more recent association study identifying 38 novel risk loci [40]. Based on another large genetic association study in IBD patients, it was shown that ten single nucleotide polymorphisms, in a total of four genes, were found to be significantly correlated with Crohn's disease [41]. Strongest correlations were found with CYLD, a de-ubiquitinating enzyme, pointing towards the ubiquitin proteasome system as a major contributor to IBD pathogenesis [41]. A more recent and very large study of 34,819 IBD patients investigating genotype–phenotype associations across 156,154 genetic variants also provided important insights into the genetic heterogeneity between ileal and colonic Crohn's disease, thereby rejecting the current classification of Crohn's disease versus ulcerative colitis [42].

In addition to genetics, the association between non-coding single nucleotide polymorphisms and IBD risk has gained major interest [44]. Moreover, based on several twin studies, the importance of

non-genetic environmental factors [45,46] on IBD disease manifestation has become clear. One example is the implication of micronutrients in IBD progression. Patients with IBD are commonly diagnosed with a vitamin D deficiency [47], which can be related to lowered oral intake of vitamin D or decreased sunlight exposure. A more recent study in healthy volunteers showed that, specifically, the gut microbiome of the upper gastrointestinal tract is positively influenced in response to vitamin D3 treatment [48], suggesting that vitamin D plays a protective role in IBD pathogenesis. Alternatively, intracellular levels of zinc [49] and iron [50] have been associated with bacterial clearance and consequent intestinal permeability and increased risk of IBD, respectively. Another environmental, lifestyle-related factor is smoking, which has been shown to cause a two-fold increased risk for IBD [51]. Besides affecting the nicotinic acetylcholine receptors present on gastrointestinal mucosal epithelial cells [52], smoking can modulate the human gut microbiota composition, thereby affecting the course of the disease in IBD. Although in the context of Crohn's disease, smoking has adverse effects, in ulcerative colitis patients, it may play a protective role, implying that smoking may be a disease-specific modifier [53].

As mentioned, dietary fiber intake is able to prolong IBD remission through subsequent increase in luminal production of short-chain fatty acids [31,54]. In addition, it is well-established that high intake of fat- and sugar-enriched foods are capable of regulating intestinal microbiota composition and diversity [55,56], thereby also initiating and sustaining inflammation in patients with IBD [57]. Furthermore, high intake of n-3 polyunsaturated fatty acids and plant sterols have been shown to be protective [58], suggesting that dietary changes play a role in IBD pathogenesis.

It is also well known that low-to-moderate intensity exercise positively affects immune function [59]. Indeed, preclinical studies showed that moderate intensity exercise decreased the expression of pro-inflammatory cytokines, thereby improving acute colitis [60]. Human data regarding the beneficial effects of exercise on the development of intestinal inflammation are mixed, mainly due to the variations in type and rate of exercise. Several studies suggested an inversed correlation between physical activity and the risk or onset of IBD [61]; however, these effects have shown to be disease specific [62]. Nevertheless, other studies focusing on the association between exercise and disease course or the quality of life of IBD patients found a beneficial effect on well-being, sleep, confidence, and mood [63,64].

Further evidence also suggests that geographic location and socioeconomic status are associated with the risk of IBD, thereby supporting the "hygiene hypothesis" of Bloomfield [65]. This hypothesis postulates that the recent rapid rise in IBD, especially in industrialized regions [66], may be due to the lower rate of infection during childhood. The lower infection rate may evolve from reduced exposure to enteric bacteria and improved sanitation during early life [67]. Although this reasoning might indeed explain the higher incidence of IBD in urban areas, the environmental location has been shown to differently affect the prevalence of Crohn's disease and ulcerative colitis. Whereas Crohn's disease has shown to be more frequent in urban/coastal areas, ulcerative colitis is more prevalent in inland municipalities [68].

Given that the gut microbiota is relatively diverse and unstable during early childhood, any kind of alteration is likely to affect the intestinal immune responses and predispose individuals to IBD. For instance, medications, including antibiotics, contraceptives as well as non-steroidal anti-inflammatory drugs (NSAIDs) are known to increase the risk of IBD, likely through altering the commensal flora and/or the intestinal barrier [69]. More specifically, based on a meta-analysis,

antibiotics were shown to associate with increased risk of new-onset Crohn's disease rather than ulcerative colitis [70]. In line, a multiple database search revealed that individuals exposed to oral contraceptives had a 24% and 30% increased risk for developing Crohn's disease and ulcerative colitis, respectively, compared with those not exposed to the medication [71]. Likewise, high doses and long-term treatments with NSAIDs [72] resulted in the exacerbation of IBD [73], potentially acting via non-selective inhibition of the cyclo-oxygenase [74].

Relevantly, IBD disease activity and its risk of relapse has also been associated with sleep disturbances [75]. Sleep disturbances can induce the levels of inflammatory cytokines, thereby activating an inflammatory cascade [76]. Furthermore, sleep disturbances have been shown to occur in IBD patients, including pediatric patients [77] as well as those with inactive disease [78], and can negatively impact quality of life. Indeed, optimized sleep duration (i.e., six to nine hours/day) was able to decrease the risk of ulcerative colitis. Further, based on a prospective study, a strong inversed correlation between sleep quality and the activity of IBD was demonstrated [79]. Also, disruptions of the circadian organization, a form of long-term biological stress, are known to affect health [80]. It has been suggested that the adverse effects of the host's circadian rhythm, including sleep disruption, can alter the circadian rhythm of the intestinal microbiota, thereby changing its community structure [80]. Given that the gut microbiota plays a key role in the development of IBD, it is also likely that circadian disorganization, through dysbiosis of the intestinal microbiota, negatively impacts the course of the disease.

5. Stress and intestinal microbiota: Bidirectional relationship in IBD

5.1. Influence of Stress on Gut Microbiota

Several lines of evidence suggest that stress, induced by dietary, environmental or neuroendocrine factors, can adversely affect the gut/microbiota/brain axis [81,82] (Table 2).

Table 2: Studies investigating the link between stress and gut microbiota

Factor	Type of study	N	Intervention/Methodology	Outcome	Author(s)	Reference
Prenatal/early life stress	<i>In-vivo</i> mouse study	6-20 mice per group	Maternal high-fat diet	Dysbiosis and low-grade inflammation in the intestine	Xie, R. <i>et al.</i>	83
	<i>In-vivo</i> rat study	6-10 per group	Prenatal stress	Long-lasting alterations in the intestinal microbiota composition	Golubeva, AV. <i>et al.</i>	84
	<i>In-vivo</i> mouse study	21-23 mice per group	Prenatal stress	Alterations in vaginal microbiota contributed to reprogramming of the developing brain	Jasarevic, E. <i>et al.</i>	85
	In vivo primates study	20	Maternal separation	Maternal separation-induced psychological disturbances altered intestinal microflora	Bailey, MT. <i>et al.</i>	91
	<i>In-vivo</i> rat study	22	Maternal separation	Early life stress induced alterations in gut-brain axis contributing to IBD symptoms	O'Mahony, SM. <i>et al.</i>	92
	Longitudinal clinical study	192 children	Questionnaire	Prenatal stress is associated with microbial colonization patterns in infants	Zijlmans, MA. <i>et al.</i>	104

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Chronic/social/ environmental stress	<i>In-vivo</i> mouse study	7-20 mice per group	Chronic social defeat	Stress induced complex structural changes in the gut microbiota	Bharwani, A. <i>et al.</i>	81
	<i>In-vivo</i> mouse study	10 mice per group	Lactation	Cellular transfer of bacterial translocation occurred in pregnant and lactating mice	Donnet-Hughes, A. <i>et al.</i>	87
	<i>In-vivo</i> mouse study	5 mice per group	SDR	Stress led to significant changes in intestinal microbiota colonization	Bailey, MT. <i>et al.</i>	88
	<i>In-vivo</i> mouse study	10-12 (3 independent experiments)	Unpredictable chronic mild stress	Altered intestinal microbiota composition, specifically the <i>lactobacillus</i> compartment	Marin, IA. <i>et al.</i>	89
	<i>In-vivo</i> mouse study	5 mice per group	SDR	Affected microbial populations that are closely associated with the colonic mucosa	Galley, JD. <i>et al.</i>	90
	<i>In-vivo</i> mouse study	4-6 mice per group	Chronic restraint stress	Disturbed gut microbiota and subsequent activation of immune system led to colitis	Gao, X. <i>et al.</i>	93
	<i>In-vivo</i> rat study	7-8 rats per group	WAS	Intestinal inflammation by impaired mucosal defenses against luminal bacteria	Soderholm, JD. <i>et al.</i>	94
	<i>In-vivo</i> rat study	6	Cold-restraint stress or WAS	Exacerbated intestinal inflammation due to increased uptake of immunogenic substances	Saunders, PR. <i>et al.</i>	95
	<i>In-vivo</i> rat study	not specified	Stress induction	Increased gastrointestinal permeability, allowing luminal constituents to the mucosal immune system	Meddings, JB. <i>et al.</i>	96
	<i>In-vivo</i> rat study	4 rats per group	WAS	Stress-induced epithelial mitochondrial damage and mucosal mast cell activation	Santos, J. <i>et al.</i>	97
	Field experiment in wild birds	64	Corticosterone-implant	Altered gut microbiome in free-living birds	Noguera, JC. <i>et al.</i>	101
	<i>In-vivo</i> rat study	13-14 rats per group	WAS	Altered intestinal mucus composition	Da Silva, S <i>et al.</i>	102
	<i>In-vivo</i> mouse study	18-24 mice per group	Germ-free and specific-pathogen free; acute restraint stress	Commensal microbiota can affect the postnatal development of the HPA stress response	Sudo, N. <i>et al.</i>	108
	<i>In-vivo</i> mouse study	12 mice per group	Germ-free and specific-pathogen free	Conventional intestinal microbiota influenced the development of behavior	Neufeld, KM. <i>et al.</i>	109
	<i>In-vivo</i> mouse study	7-14 mice per group	Germ-free and specific-pathogen free	Gut microbiota affected mammalian brain development and subsequent adult behavior	Diaz Heijtz, R. <i>et al.</i>	110
	Clinical study	263	Daily interview assessment	Stress is associated with digestive problems and gastrointestinal health	Walker, LS. <i>et al.</i>	103
	Clinical study	40	Depression	Increased bacterial translocation and activated immune responses against commensal bacteria	Maes, M. <i>et al.</i>	105

	Clinical study	65	Coping style instrument	IBD adolescents used more avoidant coping styles compared to healthy controls	Van der Zaag-Loonen, HJ. <i>et al.</i>	106
	<i>In-vivo</i> rat study	4-5 rats per group	WAS and probiotics	Probiotics prevented chronic stress-induced intestinal abnormalities	Zareie, M. <i>et al.</i>	99
	<i>In-vivo</i> rat study	84	Maternal separation and prebiotics/probiotics/LC-PUFA	Nutritional intervention at weaning normalized gut permeability and restored growth rate	Garcia-Rodenas, CL. <i>et al.</i>	100
	<i>In-vivo</i> mouse study	36	Probiotic formulation	Suggested the importance of probiotics in gut/brain axis in stress-related disorders	Bravo, JA. <i>et al.</i>	111
Pro/prebiotics	<i>In-vivo</i> mouse study	8 mice per group	Chronic mild stress and probiotics	Decreased pro-inflammatory cytokines and altered stress-related behaviors	Li, N. <i>et al.</i>	113
	<i>In-vivo</i> rat study	36 rats;	Probiotic formulation	Anxiolytic-like activity in rats	Messaoudi, M. <i>et al.</i>	112
	Double-blind, placebo-controlled, randomized parallel group study	66 individuals	Probiotic formulation	Beneficial psychological effects in healthy human volunteers	Messaoudi, M. <i>et al.</i>	112
	Systematic review	11 RCTs	Prebiotic supplementation	Short-term beneficial effects in intestinal microbiota composition	Rao, S. <i>et al.</i>	114

* HPA = hypothalamic pituitary adrenal axis; LC-PUFA = long-chain poly-unsaturated fatty acids; RCT = randomized controlled trials; SDR = social disruption; WAS = water-avoidance stress

5.1.1. Preclinical Studies

It was recently shown in mice that maternal nutrition can negatively affect offspring intestinal development and function. For instance, maternal high-fat diet caused a shift in microbiota composition, thereby predisposing the offspring to develop intestinal inflammation [83]. In line, early prenatal stress in rodents was shown to increase the *Oscillibacter*, *Anaerotruncus*, and *Peptococcus* genera [84] and induce a loss of Lactobacilli transmission to the neonate [84,85], pointing towards the involvement of birth canal delivery in gut microbiota colonization [86,87]. These data were further supported by other preclinical studies showing that short-term or mild chronic stress caused a reduction in *Lactobacilli* [88–90]. These data suggest that stressor-induced changes have important health implications [90]. It has also been shown, both in primates [91] and in rodents [92], that maternal separation, a form of chronic stress, was able to induce a change in fecal microbiota in newborn animals. A more recent study demonstrated that chronic stress resulted in dysbiosis of the murine gut microbiota, thereby inducing an immune system response and facilitating experimentally induced colitis [93]. Other genetically susceptible rodent models revealed that chronic psychological stress induced mucosal dysfunction, intestinal abnormalities, and subsequently intestinal inflammation [94]. Likewise, it was shown in rats that acute [95], environmental [96] as well as chronic stress [97] increased intestinal permeability, and hence, luminal molecule delivery to the mucosal immune system, thereby triggering pro-inflammatory responses. In this context, probiotics (living organisms yielding benefits on the host's microbiome [98]) were shown to revert chronic stress-induced abnormalities of the intestinal tract [99]. Also, it was shown that diets containing a combination of specific long-chain polyunsaturated fatty acids, prebiotics, and probiotics restored

the rat intestinal microbiota composition [100]. More recently, it was even demonstrated that stress hormones, through manipulation of basal corticosterone levels, were able to alter the gut microbiome of free-living birds [101]. Further, using a sophisticated rat model, water avoidance stress was shown to alter the mucus composition [102], which is known as the host's primary innate defense. Given that changes in the production of mucosal proteins have been associated with dysbiosis of the gut microbiota, it is likely that stress indirectly affects the intestinal microbiota via inflammation of the mucosal protein layer [94].

5.1.2. Clinical studies

Few clinical studies also revealed that stress is associated with digestive problems and gastrointestinal health [103]. For instance, by means of a phylogenetic microarray, one study showed that exposure to stress during pregnancy resulted in aberrant microbiota colonization patterns in pediatrics, which likely increased inflammation and gastrointestinal symptoms [104]. In line with these findings, stress-related psychiatric disorders, such as depression, were also associated with increased bacterial translocation, thereby activating immune responses against commensal bacteria [105]. Although these data imply that stress has a potent influence on intestinal microbiota, stress is a subjective experience, which makes it challenging to objectively evaluate the effects of stress. Therefore, further human studies should be performed to verify that stress results in dysbiosis of the gut microbiota.

5.2. Impact of intestinal microbiota on stress responsiveness

Appropriate physiological responses to stress and/or immunity are necessary for survival. As such, aberrant responsiveness can be detrimental to the host, leading to the development of chronic disorders, including IBD [106] and brain disorders [107].

5.2.1. Preclinical studies

Preclinical studies using germ-free animals, specific pathogen-free animals, and animals exposed to pathogens, probiotics or antibiotics have been performed to better understand how gut microbiota can regulate stress response, cognition, and behavior [28]. Previously, it has become clear that intestinal colonization with conventional microbiota at an early developmental stage is important for stress responsiveness in adult mice [108]. This study showed that the HPA stress response was exaggerated in the absence of normal gut microbiota reconstitution, whereas it could be partially corrected by reconstitution of feces from specific pathogen-free mice at an early, but not at a later stage. In line with these results, other murine studies demonstrated the influence of conventional gut microbiota on the development of behavior [109,110], and showed that this effect occurred along with neurochemical changes in the brain [109]. Another study based on a rat model of acute psychological stress demonstrated that the probiotic *Lactobacillus farciminis* reduced intestinal leakiness, thereby decreasing plasma levels of lipopolysaccharides, and consequently, diminishing the HPA axis response to stress. Moreover, by reducing stress-induced plasma corticosterone levels, Bravo et al. [111] showed that the probiotic *Lactobacillus rhamnosus* was able to reduce the stress response as well as anxiety-related behaviors and cognition in mice. Furthermore, it has been shown

that the combination of probiotics, such as *Lactobacillus helveticus* and *Bifidobacterium longum*, resulted in a reduction of anxiety-like behaviors in rodents [112,113].

5.2.2. Clinical studies

Messaoudi et al. [112] also validated their preclinical findings in healthy human volunteers. Their findings suggested that probiotic formulation attenuated psychological distress in healthy volunteers, which may be linked to decreased urinary free cortisol levels [112]. In agreement with these data, a randomized, double-blind, placebo-controlled trial suggested that the administration of probiotics helped in reducing anxiety-like behavior among patients with chronic fatigue syndrome [114]. Altogether, these data further confirm that the intestinal microbiota plays a role in controlling stress responsiveness, behavior, and cognition.

5.3. Stress and its impact on inflammation

While not having discussed in detail yet, the impact of stress on the immune system appears to be quite complex. Depending on the type of stress (short-term or chronic) and/or hormones being released, a stressor may either suppress or enhance immune function [115].

5.3.1. Preclinical studies

Several preclinical studies have shown that short-term stress induces significant changes in absolute numbers and composition of blood leukocytes [116,117]. Likewise, short-term stress was shown to increase the circulating levels of interleukin-6 (IL6) and pro-inflammatory monocyte chemotactic protein-1 (MCP1/CCL2) [118]. These findings were also further confirmed by others showing that social disruption reduced microbial diversity and richness in mice, which correlated with increased circulating levels of the pro-inflammatory cytokines MCP1 and IL6 [88]. Relevantly, based on data demonstrating that administration of antibiotics was able to abolish social stress-mediated increases in pro-inflammatory cytokines [88], it is likely that intestinal microbiota plays a role in stressor-induced pro-inflammatory responses.

5.3.2. Clinical studies

Previous studies in humans provided similar evidence that stress induces an increase of pro-inflammatory Th1 cytokines [119–121]. For instance, academic stress, referred to as the body's response to academic-related workload that goes beyond the adaptive capabilities of students [122], was shown to significantly increase the production of interferon-gamma (IFN γ) and tumor necrosis factor alpha (TNF α) [119]. Acute stress can also upregulate anti-inflammatory cytokines including IL10, while independently inhibiting pro-inflammatory cytokines such as TNF α [123]. These effects can induce a shift towards Th2-mediated humoral response [124], which may be essential to prevent hyperactivation of the stress system. Alternatively, chronic stress is known to increase the release of cortisol levels for several days, an effect that may be associated with immunosuppression, as shown by a reduction in circulating CD8 $^{+}$ lymphocytes, natural killer cells, and macrophages [125]. Nevertheless, as chronic psychological stress was also associated with increased levels of serum C-

reactive protein (CRP) [126], these data suggest that chronic stress may also exert pro-inflammatory effects. Obviously, it should be noted that the gastrointestinal tract *per se*, including its local microbiota, may serve as an essential organ mediating immune responses [127]. This is not only of relevance in the context of human IBD, but also in irritable bowel syndrome (IBS) [128] as well as major depressive disorders [129].

5.4. Stress and inflammation in IBD

Inflammatory bowel disease is a complex disease that likely does not only consist of Crohn's disease and ulcerative colitis. Extensive translational research has been conducted to better understand the role of stress and inflammation in IBD [130].

5.4.1. Preclinical studies

Previously, using rodent models of spontaneous colitis, it was shown that intestinal inflammation is associated with defects in mucosal barrier or dysfunctional regulatory T lymphocytes [131]. Other studies using mice fed a dextran–sulfate–sodium diet to induce colitis revealed the importance of intestinal adhesion molecules, such as ICAM-1, in the development of intestinal inflammation [132]. Relevantly, when dextran–sulfate–sodium-treated mice were injected with enterotoxigenic *Bacteroides fragilis*, increased colitis and colonic inflammation was observed [133]. Similarly, in cellular models, *Clostridium difficile* toxin A was shown to induce apoptosis and inflammation in enterocytes [134,135]. Moreover, using a mouse model of depression, it was shown that stress-induced release of corticosteroids can reactivate IBD [136], likely via increased production of pro-inflammatory cytokines. These data imply that stress may affect the course of the disease.

5.4.2. Clinical studies

In humans, it was shown that infections with *Bacteroides fragilis*, through secretion of its pro-inflammatory toxin, is associated with development of ulcerative colitis [137]. Likewise, infections with *Clostridium difficile* is thought to be involved in the reactivation of IBD in patients [138]. Within a similar context, it was also shown that patients with ileal Crohn's disease had a higher percentage of invasive *Escherichia coli* in the mucosa as compared to healthy controls, and these percentages even correlated with severity of the disease [139]. In line, another study demonstrated that *Escherichia coli* can replicate inside macrophages of patients with Crohn's disease and subsequently secrete large amounts of TNF α , thereby contributing to inflammation [140]. Collectively, these data point towards the strong relationship between the gut microbiota composition and intestinal inflammation.

It is also important to note that psychosocial stress, including psychological distress, anxiety, and depression, can induce low-grade chronic inflammation in the gut. For instance, in humans, it was shown that depression correlated with elevated levels of TNF α and CRP, pro-inflammatory cytokines that are known to trigger inflammation in patients with IBD [141,142]. Hence, it has been proposed that stress gradually contributes to the development or exacerbation of IBD (as reviewed elsewhere [28,143]). Vice versa, compared to the general population, young patients with IBD

displayed higher rates of psychological stress, and these results were further confirmed in gastrointestinal disorders such as IBS [144,145]. Together, these data imply that the disease itself may have a direct impact on the quality of life of patients.

Yet, it is not clear whether individuals with higher stress also experience more IBD symptoms. A large cross-sectional, population-based study of IBD patients showed that the relationship between intestinal inflammation and symptomatic disease activity differed between Crohn's disease and ulcerative colitis [146]. Whereas Crohn's disease did not associate with intestinal inflammation and disease activity symptoms, an association was found for ulcerative colitis. These findings suggest that the duration and intensity of stress factors may have differential influences on chronic inflammatory diseases. Furthermore, this study showed that perceived stress in both diseases correlated with disease activity symptoms, while not with inflammation [146]. Although the majority of conventional therapies in IBD focus on tackling intestinal inflammation, these insights open new venues for stress reduction in the management of IBD.

6. Managing stress in IBD: Does it make the gut feel better?

In recent years, significant progress has been made in the treatment of IBD, focusing either on targeted therapies [147] or on alternative and complementary strategies [148], which has recently been extensively reviewed [147,148]. Nevertheless, there is no certain cure for IBD due to the limited effectiveness of current therapies, which often even goes hand in hand with significant side effects. Quality of life as well as anxiety and depression are known predictors of negative medical outcomes in many chronic conditions, and as reviewed, stress has a profound impact on these variables in patients with IBD. Hence, a number of approaches have focused on relieving stress as a potential therapeutic option in IBD (Table 3) [148,149].

Table 3: Clinical studies investigating stress management in IBD

Factor	Type of study	N	Intervention	Outcome in IBD patients	Author(s)	Reference
Guided imagery / Relaxation training	Pilot RCT	28	Guided imagery with relaxation (GIR)	Improved QL in elderly women with osteoarthritis	Baird, CL. <i>et al.</i>	150
	Prospective RCT	39	Relaxation-training	Beneficial effects on anxiety, pain and stress in IBD patients	Mizrahi, MC. <i>et al.</i>	163
Self-directed stress management	Clinical study	45	3 types of stress management, including self-directed and conventional medical treatment	Trained CD patients showed reduced fatigue, constipation and abdominal pain, whereas no beneficial effects in conventional-treated CD patients	García-Vega, E. <i>et al.</i>	151
Lifestyle management	Clinical study	60	60-hour training program in lifestyle modification over a period of 10 weeks	Short-term benefits in the QL in UC patients, whereas no long-term effects	Langhorst, J. <i>et al.</i>	157
	Clinical study	49	8-session information about QL and stress management	No effect on anxiety levels 6 months post-intervention	Larsson, K. <i>et al.</i>	160
	Prospective, randomized waiting-control group design	30	60-hour training program on lifestyle management	Improved QL in patients with UC remission	Elsenbruch, S. <i>et al.</i>	167

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Psychotherapy	Prospective, randomized study	32	Low-intensity walking program	Improved QL of CD patients	Ng, V. <i>et al.</i>	179
	Prospective RCT	30	Moderate-intensity running	Improved QL of IBD patients	Klare, P. <i>et al.</i>	180
	Two clinical trials	36	7-session behavioral protocol	57% reduction in IBD relapse in the following 12 months	Keefer, L. <i>et al.</i>	152
	RCT	41	Primary and Secondary Control Enhancement Therapy-Physical Illness	Beneficial effects on depression in IBD adolescents	Szigethy, E. <i>et al.</i>	158
	Clinical study	178	Nurse-led counselling	Improved QL over 6 rather than 12 months in IBD patients	Smith, GD. <i>et al.</i>	161
	Prospective, uncontrolled open trial	30	Supportive-expressive group psychotherapy	No changes in QL, anxiety, or depression over the course of treatment in UC/CD	Maunder, RG. <i>et al.</i>	162
	Meta-analysis	1824 studies with 14 RCTs	Psychological therapy	Small short-term beneficial effects on QL and depression in IBD patients	Gracie, DJ. <i>et al.</i>	166
	RCT	29	Breath-Body-Mind Workshop; questionnaire	Significant long-lasting benefits for IBD symptoms, anxiety, depression and QL	Gerbarg, PL. <i>et al.</i>	155
	Control study	60	Mindfulness-based stress reduction	Improved mood and QL after six months of intervention	Neilson, K. <i>et al.</i>	156
	RCT	36	Gut-directed hypnotherapy	Gut-directed hypnotherapy may be one aspect in a disease-management program for IBD	Keefer, L. <i>et al.</i>	159
	Clinical trial	66	Multi-convergent therapy (psychotherapy)	Therapy is beneficial in the management of IBD symptoms	Berrill, JW. <i>et al.</i>	164
Medication	Retrospective observational study	30	Herbal treatment	Positive effect of cannabis on disease activity in CD	Naftali, T. <i>et al.</i>	171
	Prospective, placebo-controlled study	21	Herbal treatment	Short course of cannabis had beneficial effects in CD patients	Naftali, T. <i>et al.</i>	172
	Double-blind RCT	108	Placebo or vitamin D3	Reduced relapse risk in CD	Jorgensen, SP. <i>et al.</i>	174
	Prospective	37	Active or plain vitamin D	Active form of vitamin D has short-term beneficial effects in CD	Miheller, P. <i>et al.</i>	175
	Meta-analysis	12 studies	Serum folate and vitamin B12	Low concentration of serum folate is a risk factor for IBD and supplementation may be beneficial	Pan, Y. <i>et al.</i>	177
	Double-blind RCT	10 per group	Placebo/phyloquinone/vitamin D3	No significant beneficial effects of phyloquinone on bone health in CD patients	O'Connor EM. <i>et al.</i>	178

* CD = Crohn's disease; QL = quality of life; RCT = randomized controlled trial; UC = ulcerative colitis

Stress management is a technique used to diminish the physiological effects of stress and tension, and to help the individual to improve his/her coping skill. One variant of such therapy is relaxation, by which the individual is trained by a therapist or in a self-directed manner to create physiological and mental rest. Several studies showed the effectiveness of relaxation training in a variety of physical illnesses, including cardiovascular disease, arthritis [150], and IBD [151–153]. Previously, it was shown that stress management could significantly improve IBD symptoms such as pain and

fatigue [151]. In line, behavioral self-management therapy resulted in a 57% decrease in 1 year risk to relapse in IBD patients [152], supporting the beneficial effects of stress management in IBD. More recently, two clinical studies pointed towards the beneficial effects of mindfulness on psychological and physical symptoms, quality of life, and C-reactive protein, an established biomarker [154], in patients with IBD [155,156]. However, based on a systematic review, McCombie et al. [153] concluded that the effect of psychotherapy led to inconsistent results in IBD patients. It should be noted that the studies included in this study focused on a wide range of therapies. Thus, although meditation and relaxation may have beneficial effects on inflammatory activity and quality of life in IBD patients, the effectiveness of mindfulness-based interventions on disease activity remains to be elucidated.

Whereas several studies demonstrated that psychotherapy had a beneficial impact on anxiety [157], depression [158], and quality of life of IBD patients [159], other studies were not able to find any effect [160,161]. Yet, two other studies investigated the impact of combining a variety of techniques as a treatment option in IBD [161,162]. Indeed, when combining relaxation with guided imagery, a method focusing on mind-relaxing images to replace stressful thoughts, both anxiety status and quality of life appeared to be improved among patients with IBD [163]. Similarly, multi-convergent therapy, which combines mindfulness meditation with cognitive behavioral therapy, has been used as a therapeutic option in patients with tinnitus and IBS. Therefore, its applicability and efficacy were investigated in an IBD population that received conventional therapy [164]. This study revealed that multi-convergent therapy improved quality of life mainly in IBD patients suffering from IBS-like symptoms [164], suggesting that this strategy has beneficial effects only in a subgroup of IBD patients. Although IBS and IBD are medically distinct from each other, symptoms compatible with IBS indeed often co-exist in patients with IBD [165], and should therefore not be underestimated. Within this context, it was recently shown that 36% and 37% of CD and UC patients, respectively, met IBS diagnostic criteria [165], confirming that the presence of IBS-like symptoms in IBD is common [148]. Furthermore, patients with IBS in quiescent IBD were shown to have significantly more anxiety and depression than patients without IBS [165].

More recent data suggested that short-term cognitive behavioral therapy improved quality of life and depression scores in patients with IBD, while not affecting disease activity or other measures of psychological well-being [166]. Others particularly investigated the impact of mind-body therapy, a combination of moderate exercise, diet, stress management training, behavioral techniques and self-care strategies on patients with ulcerative colitis. Results suggested that this approach has a positive effect on IBD development by improving quality of life and mental/physical health scores [167].

As reviewed in detail elsewhere, other complementary and alternative medicines, such as herbal medicine, vitamin supplementation, and exercise have also gained attention for its anti-inflammatory properties and usefulness in the treatment of IBD [148,168]. Within this context, preclinical data from mice suggested that cannabinoid receptor activation mediates protective mechanisms in experimental colitis [169,170]. In line with these data, two clinical studies reported that cannabis was able to reduce IBD symptoms [171,172], pointing towards its ability to treat IBD. Nevertheless, whether cannabis is able to positively affect the course of disease requires further investigation. Other preclinical studies in mice showed an important role for vitamin D and its receptor in the regulation of inflammation of the gastrointestinal tract [173]. Indeed, IBD patients often lack this vitamin [47], and hence, studies investigated the role of vitamin D [174–176] in treatment of IBD

patients. Although these studies pointed towards beneficial effects on disease activity and risk of relapse, other clinical studies on the use of vitamin B [177] or K [178] in IBD treatment were inconsistent. These data imply that there is a lack of evidence to support positive effects of vitamins on IBD disease course [148]. Likewise, whereas preclinical [60] and clinical studies [179,180] suggested that low-to-moderate intensity exercise exerted beneficial effects on intestinal inflammation, overall health, and quality of life of IBD patients, further studies investigating the impact of exercise on disease activity and/or prevention of IBD are warranted [148].

Given that stress orchestrates an important influence on structural and functional aspects of the microbiome, multiple studies have also investigated the role of psychobiotics in stress-related diseases. Psychobiotics refer to probiotics or prebiotics that can manipulate commensal gut microbiota, and when ingested at adequate quantities, may indirectly have positive psychiatric effects in psychopathology [181]. As extensively reviewed, both in experimental colitis and human IBD, pre- and probiotics have shown beneficial effects in the prevention of IBD by modulating the trophic functions of the microbiota, improving the intestinal mucosal barrier and mediating anti-inflammatory responses [182]. Given that the intake of psychobiotics also seem to exert antidepressant effects, including improvements in mood and decreases in stress-related plasma and urinary free cortisol [181], it may be postulated that psychobiotics might serve as therapeutic modulators of the gut/microbiota axis and positively influence psychological functions in the context of IBD.

Altogether, the quality of life and course of IBD are regulated by psychological conditions, and as such, the implementation of stress management may play an important role in IBD disease regression (see also **Figure 1**). Nevertheless, current studies are limited in sample size and study design, and hence, may lack proper controls. Therefore, future research is needed to validate current treatment options and/or to explore novel therapeutic opportunities in order to prevent IBD onset or improve stress-affected well-being of IBD patients.

Figure 1 The gut/microbiota/brain interplay and its interactions upon exposure to stress. Under conditions of psychological stress including lack of sleep and physical inactivity, the brain (HPA axis) stimulates the production of pro-inflammatory cytokines. This can result in increased intestinal permeability and altered gut microbiota. In addition, fat- and sugar-enriched foods, long-term usage of medicines, as well as genetic predisposition can directly affect the gut microbiota composition, and subsequently, intestinal permeability. Furthermore, personal habits, such as hygiene and smoking, can also have an impact on the gut microbiome. Altogether, the multitude of stress-related factors can perturb the gut/microbiota/brain interplay, which contributes to the development of IBD. Relevantly, several stress management techniques have been proven to greatly alleviate IBD symptoms and improve the quality of life of IBD patients. Given that the exact underlying mechanisms in the context of IBD are not yet fully understood, therapeutic options aimed at improving stress management deserve further investigation.

7. Conclusion

In summary, this review summarized the tight connection between the gut, microbiota, and brain in the context of IBD and has particularly shed light on the impact of stress on this interplay. It should be noted, however, that it is rather challenging to investigate the impact of stress on IBD, as stress can arise from totally different origins and may be closely connected to potential individual confounding factors, including (mental) health status and inter-individual variability in stress responsiveness and/or vulnerability. Therefore, future research involving preclinical studies as well as large-scale, controlled clinical trials should not only focus on unravelling the exact mechanisms through which stress affects IBD. However, it is also of great interest to further investigate the exact mechanisms of how stress management can orchestrate beneficial effects in IBD and how stress-relieving therapies should be implemented in IBD care.

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Chapter 6

Pro-inflammatory implications of 2-hydroxypropyl- β -cyclodextrin treatment

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Abstract

Lifestyle- and genetically induced disorders related to disturbances in cholesterol metabolism have shown the detrimental impact of excessive cholesterol levels on a plethora of pathological processes such as inflammation. In this context, two- hydroxypropyl- β -cyclodextrin (CD) is increasingly considered as a novel pharmacological compound to decrease cellular cholesterol levels due to its ability to increase cholesterol solubility. However, recent findings have reported contra-indicating events after the use of CD questioning the clinical applicability of this compound. Given its potential as a therapeutic compound in metabolic inflammatory diseases, in this study, we evaluated the inflammatory effects of CD administration in the context of cholesterol- induced metabolic inflammation *in vivo* and *in vitro*. The inflammatory and cholesterol- depleting effects of CD were first investigated in low-density lipoprotein receptor knockout (*Ldlr*^{-/-}) mice that were transplanted with *Npc1*^{nih} or *Npc1*^{wt} bone marrow and were fed either regular chow or a high-fat, high-cholesterol (HFC) diet for 12 weeks, thereby creating an extreme model of lysosomal cholesterol-induced metabolic inflammation. In the final three weeks, these mice received daily injections of either control (saline) or CD subcutaneously. Subsequently, the inflammatory properties of CD were investigated *in vitro* in two macrophage cell lines and in murine bone marrow-derived macrophages (BMDMs). While CD administration improved cholesterol mobilization outside lysosomes in BMDMs, an overall pro-inflammatory profile was observed after CD treatment, evidenced by increased hepatic inflammation *in vivo* and a strong increase in cytokine release and inflammatory gene expression *in vitro* in murine BMDMs and macrophages cell lines. Nevertheless, this CD-induced pro-inflammatory profile was time-dependent, as short-term exposure to CD did not result in a pro-inflammatory response in BMDM. While CD exerts desired cholesterol-depleting effects, its inflammatory effect is dependent on the exposure time. As such, using CD in the clinic, especially in a metabolic inflammatory context, should be closely monitored as it may lead to undesired, pro-inflammatory side effects.

1. Introduction

As one of the most important steroid alcohols of the human body, cholesterol carries a number of essential functions ranging from acting as a key structural component of cell membranes [1] to serving as precursor for bile acids, steroid hormones and vitamin D metabolites [2]. Due to this key cellular function of cholesterol, a highly coordinated extra- and intracellular transport system maintains cholesterol homeostasis at a whole body as well as at a cellular level [3, 4]. Evidently, deficiencies in this transport system leading to a disproportionate cellular supply of cholesterol result in severe pathogenic profiles. At one hand, these deficiencies can be induced via mutations in genes encoding for receptors and/or enzymes involved with cholesterol homeostasis. Examples of such disorders include Wolman disease [5] and the Niemann-Pick diseases [6] (both lysosomal storage disorders) as well as familial hypercholesterolemia [7]. Though these diseases are rather rare, their pathogenesis is often severe and sometimes results in premature death. Besides genetic predisposition, another and more prevalent manner that influences cholesterol homeostasis is via excessive overnutrition, leading to metabolic syndrome and its associated diseases [8]. In this context, excessive cholesterol accumulates at locations such as the vessel wall (atherosclerosis) and the liver (non-alcoholic fatty liver disease (NAFLD)) subsequently triggering resident macrophages to evolve into foam cells characterized by lysosomal cholesterol accumulation that induces an undesired inflammatory response, also referred to as metabolic inflammation [4, 9]. This inflammatory response is an antecedent for more severe consequences both in the context of atherosclerosis (heart attack, stroke, peripheral vascular disease [10]) as well as NAFLD (advanced liver diseases such as cirrhosis and hepatocellular carcinoma [9]). Taken the prevalence and the severity of these disorders into consideration, efficient therapeutic options aimed at reducing cellular cholesterol levels are needed.

A straightforward and successfully applied approach to reduce cholesterol levels is via pharmacological approaches, which include the use of statins [11], fibrates [12] and more recently antibodies targeting proprotein convertase subtilisin-kexin type 9 (PCSK9) [13]. However, substantial reduction of plasma cholesterol levels cannot be guaranteed for all patients [11], requiring alternative ways to improve cholesterol homeostasis. Relevantly, while the aforementioned interventions focus on reducing plasma cholesterol levels, a relative new approach aims at mobilizing cholesterol at the site of accumulation. This has led to the proposition to use 2-hydroxypropyl- β -cyclodextrin (CD), a substance initially used to solubilize lipophilic pharmaceutical agents, as a compound to treat cholesterol accumulation. Indeed, CD mobilized cholesterol from foam cells [14], promotes atherosclerosis regression [15], improved hepatic cholesterol metabolism [16], reduced lysosomal size of neural stem cells derived from Wolman disease patients [17] and is under evaluation in advanced human clinical trials for Niemann-Pick disease, type C1 (NPC1) [18]. In contrast to these beneficial effects, CD causes massive damage to all cells of the developing [19] and some cells of the adult auditory system [20]. Moreover, while dietary supplementation of β -cyclodextrin in hypercholesterolemic rats improved lipid metabolism, it concomitantly produced hepatotoxic effects characterized by increased plasma aminotransferase levels [21]. Together, though the use of CD has clearly shown its benefits, the previous observations question whether this compound can be directly used in the clinic. Given its potential as therapeutic compound in metabolic inflammatory diseases, in this study, we therefore investigated the inflammatory aspects of CD administration in the context of cholesterol-induced metabolic inflammation *in vivo* and *in vitro*.

For this purpose, we investigated the inflammatory and cholesterol-depleting properties of CD *in vivo* and in a series of *in vitro* experiments for metabolic inflammation. First, we analyzed the effect of CD injection in low-density lipoprotein receptor knockout (*Ldlr*^{-/-}) mice transplanted with *Npc1*^{nih} or *Npc1*^{wt} bone marrow on a high-fat, high-cholesterol (HFC) diet. Due to the absence of LDL receptors, and following an HFC diet, *Ldlr*^{-/-} mice are characterized by high plasma LDL levels, mimicking diet-induced dyslipidemia and serving as an excellent animal model for metabolic diseases. Furthermore, *Npc1*^{nih} bone marrow-transplanted mice feature a dysfunctional NPC1 protein in their immune cells, due to a deleterious frameshift mutation in the *Npc1* allele, leading to lysosomal cholesterol buildup and a pro-inflammatory state. As such, chimeric *Npc1*^{nih} transplanted (*Npc1*^{nih}-tp) *Ldlr*^{-/-} mice given an HFC diet constitute an extreme state of lysosomal cholesterol-induced metabolic inflammation [22]. Next, as CD is able to mobilize lysosomal cholesterol, we assessed the ability of CD to influence lysosomal size of metabolically challenged bone marrow-derived macrophages (BMDM) by employing confocal microscopy. Finally, in a series of *in vitro* experiments we investigated the inflammatory properties of CD in more detail.

2. Materials and methods

2.1 Mice, bone marrow transplant, diet and injections

Throughout the study, mice were housed under standard conditions and had unlimited access to food and water, unless explicitly mentioned otherwise. For one week prior to and up to four weeks after bone marrow transplantation, *Ldlr*^{-/-} mice were housed in filter-top cages and received antibiotics diluted in drinking water to prevent infections following immunosuppression (Neomycin, 100 mg/l, Gibco, Breda, the Netherlands; 6*10⁴ U/L polymycin B sulfate). Six weeks old bone marrow donors *Npc1*^{nih} and *Npc1*^{wt} mice were derived from heterozygous founders of a C57BL/6 genetic background. Genotype of *Npc1*^{nih} and *Npc1*^{wt} mice was determined as previously described [23]. On the day of the bone marrow transplant, *Npc1*^{nih} and *Npc1*^{wt} littermates were sacrificed *via* CO₂ inhalation and their bone marrows were isolated. One day before and on the day of the bone marrow transplant, *Ldlr*^{-/-} mice were subjected to six Gray of g-radiation, thus having received 12 Gray of g-radiation before receiving 1*10⁷ bone marrow cells collected from *Npc1*^{wt} or *Npc1*^{nih} mice *via* intravenous injection. After ten weeks of recovery, transplanted mice were placed on a high-fat, high-cholesterol (HFC) diet [24] for twelve weeks. In the final three weeks (based on [16, 25]), mice received daily, subcutaneous injections of control (saline) or 2-hydroxypropyl- β -cyclodextrin (1800 mg/kg body weight; CD, Sigma-Aldrich), creating four experimental groups: (1) *Npc1*^{wt}-tp NaCl (n = 6), (2) *Npc1*^{wt}-tp CD (n = 9), (3) *Npc1*^{nih}-tp NaCl (n = 6) and (4) *Npc1*^{nih}-tp CD (n = 9). A schematic overview of the experimental set-up is provided in Supplementary Figure S1. All experiments were performed according to Dutch laws and approved by the Animal Experiment Committee of Maastricht University.

Upon sacrifice, all tissues were isolated and snap-frozen in liquid nitrogen and stored at -80°C or fixed in 4% formaldehyde/ PBS. The collection of blood and tissue specimens, biochemical determination of lipids in plasma, RNA isolation, cDNA synthesis and qPCR were determined as described previously [26, 27]. Primer sequences for genes are listed in Supplementary Table 1. Hepatic sterol content was determined by gas-liquid chromatography-mass spectroscopy, as described elsewhere [28].

2.2 Immunohistochemistry

Frozen liver sections (7 μ m) were fixed in acetone and blocked for endogenous peroxidase by incubation with 0.25% of 0.03% H₂O₂ for 5 minutes. Primary antibodies used were against hepatic macrophages (1:100 rat anti-mouse CD68, clone FA11), infiltrated macrophages and neutrophils (1:500 rat anti-mouse Mac-1 (M1/70)) and neutrophils (1:100 rat anti-mouse NIMP (Ly6G)). 3-Amino-9-ethylcarbazole (Vector laboratories, CA, USA) was applied as color substrate and hematoxylin for nuclear counterstain. Sections were enclosed with Faramount aqueous mounting medium. Additional information concerning the immunostainings is also described elsewhere [29].

Pictures were taken with a Nikon digital camera DMX1200 and ACT-1 v2.63 software (Nikon Instruments Europe, Amstelveen, The Netherlands). Infiltrated macrophages and neutrophil cells (Mac-1⁺) and neutrophils (NIMP) were counted by two blinded researchers in six microscopical views (original magnification, 200x) and were indicated as number of cells per square millimeter (cells/mm²). Hepatic macrophages (CD68) were counted in six microscopical views (original magnification, 200x) and indicated as the percentage of CD68 positive area (Adobe Photoshop CS2 v.9.0.).

2.3 Fluorescence confocal microscopy and quantification of lysosomes categorized by size

LAMP1- staining

For assessing lysosomal size, we performed fluorescence confocal microscopy by staining for the lysosomal membrane marker lysosomal-associated membrane protein 1 (LAMP-1) followed by quantification of the number of lysosomes based on size. For this staining, fresh BMDM were fixed in paraformaldehyde (4%) and permeabilized in Triton-X (0.1%)/BSA (0.2%) solution. BMDM were incubated with the primary (1:100, rabbit polyclonal Lamp1, ab24170, Abcam, Cambridge, United Kingdom) and secondary (1:200, Alexa fluor 488 goat anti-rabbit IgG, A11008, Thermo Fisher Scientific, Waltham, Massachusetts, USA) antibodies and finally, sections were enclosed with glycerol mounting medium (DABCO-DAPI). Confocal pictures were taken with a LEICA DMI 4000 microscope (Leica microsystems, Wetzlar, Germany), providing 3D images of BMDM and their lysosomes.

Lysosomal quantification

Step 1: Configuring and adapting the lysosomal pictures

For optimal quantification, one cell remained visible per purview. For this purpose, the original photo was cropped, and then configured into a TIFF file for further processing.

Step 2: Splitting the channels for nucleus and lysosomal quantification

In this study we made use of two fluorescent stainings either for the nucleus (DAPI) or lysosome (LAMP-1). To determine the nucleus to lysosomal distance, the color channels were bifurcated for better differentiation, creating a nucleus channel and lysosomal channel.

Step 3: Coordinates of the nucleus

To determine the lysosome to nucleus distance we first determined the location of the nucleus in the 3D picture. Therefore, we analyzed the nucleus channel with the 3D ObjectCounter.

Step 4: Creating a mask of lysosome channel

To obtain the coordinates of the lysosomes, a mask was created by using the ObjectCounter. The mask was created by calculating the geometrical centers of the lysosomes [30]. Threshold was determined before the picture was analyzed.

Step 5: Watershed

To discriminate between separated or combined lysosomes, the watershed method was used. The watershed method uses the mask to determine the centers of the masses (Parra et al.).

Step 6: Coordinates of the lysosomes

After the watershed, the Regions of interest (ROIs) were added to the 3D ROI Manager, where the volume and coordinates were calculated. To quantify the pictures in an efficient manner, a macro was created (Supplementary methods).

Step 7: Calculating the distance

After the coordinates and volumes were obtained, the distance for each lysosome to the nucleus was calculated. For this we used the Pythagoras equation.

$$\text{Distance} = \sqrt{(X_{\text{nucleus}} - X_{\text{lysosome}})^2 + (Y_{\text{nucleus}} - Y_{\text{lysosome}})^2 + (Z_{\text{nucleus}} - Z_{\text{lysosome}})^2}$$

Next, we created a plugin-Macro for ImageJ to identify the amount and volume of lysosomes. Finally, the number of lysosomes was quantified and categorized them by size ($< 0.1 \mu\text{m}^3$; $0.1\text{-}1 \mu\text{m}^3$; $> 1 \mu\text{m}^3$) and expressed the number of lysosomes per size relative to the total amount of lysosomes present inside the BMDM. Representative videos were created using Z-stack images played in series and recorded as AVI files which were trimmed using quick time movie player. DAPI (blue) represents nuclei and LAMP-1 (green) represents lysosomes.

2.4 Statistical analysis

Data were statistically analyzed by performing the unpaired t-test or the two-way ANOVA and Tukey's post hoc test using GraphPad Prism software (version 6 for Windows, GraphPad Software Inc, San Diego, CA, U.S; www.graphpad.com). Data were expressed as the group mean and standard error of the mean.

3. Results

3.1 Administration of 2-hydroxypropyl- β -cyclodextrin mobilizes hepatic cholesterol in *Npc1^{nih}-tp Ldlr^{-/-}* mice

Npc1^{nih} bone marrow-transplanted mice feature a dysfunctional NPC1 protein in their immune cells, due to a deleterious frameshift mutation in the *Npc1* allele, leading to lysosomal cholesterol buildup and a pro-inflammatory state. As such, chimeric *Npc1^{nih}* transplanted (*Npc1^{nih}-tp*) *Ldlr^{-/-}* mice given an HFC diet constitute an extreme state of lysosomal cholesterol- induced metabolic inflammation [22].

To confirm successful injection of 2-hydroxypropyl- β -cyclodextrin (CD) into the mice in the final three weeks of the experiments, plasma and hepatic lipid metabolism were profiled. CD-treated *Npc1^{wt}-tp Ldlr^{-/-}* mice showed reduced plasma triglycerides (Table 1), though this reduction was already apparent before the start of CD treatment (Table 1). While no effects were observed on plasma (Table 1) and hepatic (Figure 1) cholesterol levels, the ratios of the cholesterol oxidation products 7 α -hydroxycholesterol to cholesterol and 27-hydroxycholesterol to cholesterol were both significantly increased in the livers of CD-treated *Npc1^{nih}-tp* and *Npc1^{wt}-tp Ldlr^{-/-}* mice, suggesting increased mobilization of cholesterol (Figures 1B, C). Hepatic gene expression analysis of cytochrome P450 7A1 (*Cyp7a1*), the enzyme responsible for the conversion of cholesterol into 7 α -hydroxycholesterol, was concomitantly increased (Figure 1D), while sterol 27-hydroxylase (*Cyp27a1*) hepatic gene expression levels remained unaffected upon CD-treatment (Figure 1E). Concerning other non-cholesterol sterols, CD-treatment reduced hepatic cholestanol and desmosterol levels in CD-treated *Npc1^{nih}-tp Ldlr^{-/-}* mice (Table 2). No effects were observed on liver and spleen weight (Supplementary Figures 2A, B). Body weight over time seemed to be lower in *Npc1^{nih}-tp Ldlr^{-/-}* mice compared to *Npc1^{wt}-tp Ldlr^{-/-}* mice, but this difference was not significant (Supplementary Figure 2C). Overall, these findings indicate that CD-treatment mobilizes hepatic cholesterol, confirming successful administration of CD.

Figure 1 Hepatic lipid parameters. (A) Hepatic cholesterol levels of *Npc1^{wt}-tp* and *Npc1^{nih}-tp* mice on a HFC diet that received CD or saline treatment. (B-C) Hepatic levels of the cholesterol degradation products 7 α -hydroxycholesterol and 27-hydroxycholesterol. (D-E) Hepatic gene expression

analysis of *Cyp7a1* (D) and *Cyp27a1* (E). $n = 6-9$ mice/group. Gene expression data are set relative to *Npc1^{wt}-tp* mice treated with saline. *, ** and **** indicate $p \leq 0.05$, 0.01 and 0.0001 resp. by means of two-way ANOVA followed by Tukey post-hoc analysis.

Table 1: Plasma total cholesterol and triglyceride levels

	<i>Npc1^{wt}-tp</i>		<i>Npc1^{nh}-tp</i>	
	NaCl	Cyclo	NaCl	Cyclo
Plasma cholesterol (mM)				
T0	9.48 ($\pm$ 0.86)	7.87 ($\pm$ 0.73)	6.372 ($\pm$ 0.30)	5.81 ($\pm$ 0.69)
T9	148.15 ($\pm$ 5.33)	118.3 ($\pm$ 11.77)	66.68 ($\pm$ 8.60)	80.72 ($\pm$ 4.13)
T12	137.15 ($\pm$ 4.73)	120.62 ($\pm$ 9.68)	97.97 ($\pm$ 6.61)	112.76 ($\pm$ 8.48)
Plasma triglycerides (mM)				
T0	1.30 ($\pm$ 0.11)	1.47 ($\pm$ 0.17)	0.96 ($\pm$ 0.11)	0.93 ($\pm$ 0.11)
T9	5.57 ($\pm$ 0.82)	2.99 ($\pm$ 0.44) **	1.17 ($\pm$ 0.25)	1.10 ($\pm$ 0.19)
T12	3.18 ($\pm$ 0.47)	1.88 ($\pm$ 0.46) ***	0.58 ($\pm$ 0.13)	0.64 ($\pm$ 0.11)

** and *** indicates $p < 0.01$ and 0.001 by means of two-way ANOVA followed by Tukey post-hoc analysis.

Table 2: Hepatic non-cholesterol sterol concentrations

	<i>Npc1^{wt}-tp</i>		<i>Npc1^{nh}-tp</i>	
	NaCl	Cyclo	NaCl	Cyclo
Absorption markers				
R_Sitosterol (pg/mg)	0.05 ($\pm$ 0.01)	0.05 ($\pm$ 0.01)	0.13 ($\pm$ 0.01)	0.12 ($\pm$ 0.01)
R_Campestanol (pg/mg)	0.12 ($\pm$ 0.01)	0.12 ($\pm$ 0.01)	0.14 ($\pm$ 0.03)	0.12 ($\pm$ 0.02)
R_Cholestanol (pg/mg)	4.73 ($\pm$ 0.25)	5.00 ($\pm$ 0.16)	3.91 ($\pm$ 0.16)	3.06 ($\pm$ 0.11) ^a
Cholesterol synthesis markers				
R_Lathosterol (pg/mg)	0.49 ($\pm$ 0.06)	0.56 ($\pm$ 0.05)	0.33 ($\pm$ 0.04)	0.28 ($\pm$ 0.01)
R_Desmosterol (pg/mg)	0.29 ($\pm$ 0.02)	0.30 ($\pm$ 0.01)	1.17 ($\pm$ 0.10)	0.84 ($\pm$ 0.09) ^b
R_Lanosterol (pg/mg)	28.6 ($\pm$ 2.2)	30.2 ($\pm$ 2.0)	41.2 ($\pm$ 1.1)	34.7 ($\pm$ 1.9)

Data is given as mean $\pm$ standard error of the mean (S.E.M.).

^{a,b} indicates $p < 0.05$ and $p < 0.01$ respectively compared to NaCl-injected *Npc1^{nh}-tp* mice.

All values are given as the ratio sterol to cholesterol.

3.2 Administration of 2-hydroxypropyl- β -cyclodextrin increases hepatic inflammation and fibrotic markers in *Npc1^{nh}-tp* *Ldlr*^{-/-} mice

After we confirmed successful administration of CD, we further evaluated the impact of three-week CD treatment on hepatic inflammation by staining hepatic cryosections for the inflammatory markers Mac-1 (infiltrated macrophages and neutrophils; directed against Cd11b), NIMP (neutrophils) and CD68 (macrophages) (Figures 2A–C). While CD treatment only induced a minor non-significant increase for these inflammatory markers in *Npc1^{wt}-tp* *Ldlr*^{-/-} mice, this increase was more pronounced in *Npc1^{nh}-tp* *Ldlr*^{-/-} mice (Figures 2A, D). This pro-inflammatory effect of CD was confirmed by histological scoring of HE-staining of livers of the *Npc1^{nh}-tp* *Ldlr*^{-/-} mice (Supplementary Figure S3). To confirm these histological data, hepatic gene expression for the inflammatory markers tumor necrosis factor alpha (*Tnfa*), chemokine (C-C motif) ligand 2 (*Ccl2*), chemokine (C-C motif) ligand 5 (*Ccl5*) and serum amyloid A1 (*Saa1*) was analyzed. In line with the histological data, CD treatment

increased the expression of these hepatic inflammatory markers in the *Npc1^{wt}-tp* or in the *Npc1^{nih}-tp* group (Figures 2E–H). Moreover, the hepatic fibrotic markers matrix metalloproteinase 9 (*Mmp9*) and plasminogen activator inhibitor-1 (*Pai-1*) were concomitantly increased in CD-treated *Npc1^{nih}-tp* *Ldlr^{-/-}* mice, while no significant changes were observed in transforming growth factor beta (*Tgfb*) and tissue inhibitor of metalloproteinase (*Timp1*) (Supplementary Figure S4). Together, these findings indicate that three-week treatment with CD increased hepatic inflammation and mild elevations in fibrosis in our experimental model.

Figure 2 **Parameters of hepatic inflammation:** (A–C) Liver sections were stained for infiltrating macrophages and neutrophils (Mac-1), neutrophils (NIMP) and hepatic macrophages (CD68) and subsequently quantified by counting (Mac-1 and NIMP) or by assessing the positive area of the staining (CD68). (D) Representative images of liver sections stained for infiltrated macrophages and neutrophils (Mac-1). (E–H) Hepatic gene expression analysis of *Tnfa*, *Ccl2*, *Ccl5* and *Saa1*. Gene expression data were set relative to control-treated *Npc1^{wt}-tp* mice. * indicates $p \leq 0.05$ by means of two-way ANOVA followed by Tukey post-hoc analysis.

3.3 Treatment with 2-hydroxypropyl- β -cyclodextrin normalizes lysosomal size of metabolically challenged bone marrow-derived macrophages

As CD treatment mobilized cholesterol, but increased inflammation in our *in vivo* model for lysosomal cholesterol-induced inflammation, we opted to investigate the effect of CD on lysosomal size in metabolically challenged BMDM *via* confocal microscopy. For this purpose, we first compared lysosomal size between wildtype and *Npc1^{nih}* BMDM. While *Npc1^{nih}* BMDM showed a reduced

number of small lysosomes (categorized as $< 0.1 \mu\text{m}^3$), they showed an increase in the number of large lysosomes (categorized as $> 1 \mu\text{m}^3$) as compared to wildtype BMDM, which is likely the result of lysosomal lipid accumulation that is characteristic for the mutation (Figure 3 Comparison A; Supplementary Table 2).

As internalization of oxidized cholesterol-rich low-density lipoprotein (oxLDL) in BMDM also leads to lysosomal accumulation, we next compared lysosomal size of oxLDL (25 $\mu\text{g}/\text{ml}$; 72hr)-incubated wildtype BMDM to control- incubated wildtype BMDM. Incubating wildtype BMDM with oxLDL similarly reduced the number of small lysosomes and increased the number of large lysosomes, confirming the effect of oxLDL on lysosomal size (Figure 3 Comparison B; Supplementary Table 3) as well as our method to quantify the number of lysosomes based on their size. Representative videos of all indicated conditions are shown in Supplementary Videos 1–5. Next, to assess the impact of CD treatment, we compared lysosomal size of oxLDL-incubated wildtype BMDM that were treated with or without CD (1.95 mM; 4hr). As expected, CD treatment increased the number of small lysosomes and reduced the level of large lysosomes, thereby reversing the oxLDL- induced effects on lysosomal size (Figure 3 Comparison C; Supplementary Table 4). Furthermore, similar findings were observed when *Npc1^{nih}* BMDM were treated with CD (Figure 3 Comparison D; Supplementary Table 5). Overall, these findings confirm the beneficial effect of CD treatment on lysosomes of metabolically challenged BMDM.

Figure 3 Lysosomal volume of metabolically-challenged bone marrow-derived macrophages with and without intervention: We quantified the number of lysosomes, categorized them by size ($< 0.1 \mu\text{m}^3$; $0.1-1 \mu\text{m}^3$; $> 1 \mu\text{m}^3$) and expressed the number of lysosomes per size relative to the total amount of lysosomes present inside the BMDM. *Comparison A:* Relative comparison of the distribution of lysosomal volumes between *Wt* and *Npc1^{nih}* BMDM. *Comparison B:* Relative comparison of the distribution of lysosomal volumes between control and oxLDL (25 $\mu\text{g}/\text{ml}$; 72hr)-stimulated *Wt* BMDM. *Comparison C:* Relative comparison of the distribution of lysosomal

volumes between oxLDL-stimulated *Wt* BMDM treated with or without CD (1.95 mM; 4hr). *Comparison D*: Relative comparison of the distribution of lysosomal volumes between *Npc1^{nih}* BMDM treated with or without CD. *, ** and *** indicate $p < 0.05$, 0.01 and 0.001 resp. by means of unpaired t- test. Data are the result of analysis of $n = 5-7$ BMDM per experimental condition.

3.4 Incubation with 2-hydroxypropyl- β -cyclodextrin exerts pro-inflammatory effects in metabolically challenged bone marrow derived macrophages

To increase our insight into the inflammatory effects of CD, we performed a series of *in vitro* experiments in BMDM, framed within the context of metabolic inflammation. First, we incubated wildtype and *Npc1^{nih}* BMDM with CD for 4hr followed by 4hr lipopolysaccharide (LPS) stimulation. Four-hour CD treatment increased TNF α protein secretion in both wildtype and *Npc1^{nih}* BMDM, suggesting an acute pro-inflammatory effect of CD also in wildtype and *Npc1^{nih}* BMDM (Figure 4A; Supplementary Figure S5A). To provide stronger evidence for this inflammatory effect of CD, *Npc1^{nih}* BMDM were challenged with oxLDL, followed by 4hr CD incubation and 4hr LPS stimulation, thereby creating a more severe model of metabolic inflammation. Similar to our previous findings, CD treatment increased TNF α protein secretion in both oxLDL- and control-challenged *Npc1^{nih}* BMDM (Figure 4B; Supplementary Figure S5B). This was confirmed at gene expression level, showing increased levels of pro-inflammatory markers (*Tnfa*, *iNos/Arg1* ratio) (Figure 4B; Supplementary Figure S5B) and decreased levels of anti-inflammatory markers (*IL-10*; Figure 4B; Supplementary Figure S5B) after CD treatment. These pro-inflammatory findings of CD were also confirmed in a separate BMDM experiment with similar set-up in wildtype BMDM (Supplementary Figure S6A) and without the final LPS stimulus (Supplementary Figure S6B).

As another model for metabolic disturbance, we investigated the inflammatory effects of CD on *Ldlr^{-/-}* BMDM, which is a mutation causing disturbances in cholesterol metabolism. Twenty-four-hour incubation with oxLDL followed by 4hr CD treatment resulted in increased gene expression levels of *Tnfa*, *IL-1b*, and *IL-18*, confirming the pro-inflammatory effects of CD also in *Ldlr^{-/-}* BMDM (Figure 4C; Supplementary Figure S4C). Nevertheless, CD treatment increased *Lxra* and *Npc2* expression, confirming the cholesterol-mobilizing properties of CD. To further confirm these findings, a similar experiment was conducted with the addition of a final stimulation with Pam3Cys or LPS for 4hr. Stimulation with Pam3Cys (Figure 4D; Supplementary Figure S4D) and LPS (Figure 4E; Supplementary Figure S4E) showed similar results as previously described, showing pro-inflammatory and cholesterol-mobilizing effects of CD treatment in *Ldlr^{-/-}* BMDM. Together, these findings show that while CD maintains its effect on cholesterol metabolism, pro-inflammatory effects become apparent after 4hr incubation in metabolically challenged BMDM.

Figure 4 Metabolically challenged BMDM parameters of inflammation and cholesterol metabolism after four-hour incubation with 2-hydroxypropyl- β -cyclodextrin: (A) TNF α protein levels of 2-hydroxypropyl- β -cyclodextrin (1.95 mM; 4hr)-treated Wt or Npc1^{nh} BMDM that were terminally stimulated with LPS (100ng/ml; 4hr). (B) TNF α protein levels and *Tnfa*, *IL10* and *iNOS/Arg1* ratio gene expression data of oxLDL (25 μ g/ml; 24hr)-exposed Npc1^{nh} BMDM treated with or without CD (4hr) that were terminally stimulated with LPS (100ng/ml; 4hr). Gene expression data were set relative to control exposed Npc1^{nh} BMDM treated with saline and stimulated with LPS. (C–E) Gene expression analysis of *Tnfa*, *IL-1b*, *IL-18*, *Lxr α* and *Npc2* of oxLDL (25 μ g/ml; 24hr)-exposed Ldlr^{-/-} and Wt BMDM treated with or without CD (1.95 mM; 4hr) and terminally stimulated with control (C), Pam3Cys (4hr) (D) or LPS (4hr) (E). Gene expression data were set relative to oxLDL-exposed Wt BMDM treated with saline. Colored (red or blue) boxes are compared to the box directly at their left via two-way ANOVA followed by Tukey post-hoc analysis, indicating the effect of CD. Data are the result of 2 or 3 independent experiments.

3.5 Incubation with 2-hydroxypropyl- β -cyclodextrin exerts pro-inflammatory effects in macrophage-derived cell lines

To validate the inflammatory effect of CD in non-primary cells, we investigated the impact of CD treatment on the macrophage-derived mouse RAW 264.7 and human THP-1 cell lines. Firstly, RAW 264.7 cells were incubated with CD for 4 or 8hr, followed by 4hr LPS stimulation. While 4hr incubation did not show any inflammatory effect, CD treatment for 8hr increased TNF α protein secretion (Figures 5A, B; Supplementary Figures S7A, B). Furthermore, THP-1-derived macrophages were challenged with sphingomyelinase-aggregated LDL (smLDL), native LDL or control for 24hr and incubated with CD for 4hr. Secreted protein levels of TNF α increased in all three experimental groups

after CD incubation (Figures 6A–C). Together, these results indicate that CD also shows pro-inflammatory properties in macrophage-derived cell lines.

Figure 5 RAW cell line-related parameters of inflammation after prolonged exposure to 2-hydroxypropyl- β -cyclodextrin: (A, B) TNF α protein levels of 2-hydroxypropyl- β -cyclodextrin (1.95 mM; 4 or 8hr)-treated RAW cells that were terminally stimulated with LPS (100ng/ml; 4hr). Data represent n = 3 for each experimental group. * indicates $p < 0.05$ by means of unpaired t-test.

Figure 6 THP1 cell line-related parameters of inflammation after prolonged exposure to 2-hydroxypropyl- β -cyclodextrin: (A–C) TNF α protein levels of control, nLDL or smLDL (24hr)-exposed THP1 cells treated with or without 2-hydroxypropyl- β -cyclodextrin (1.95 mM; 4hr). *, ** indicates $p < 0.05$ and 0.01 by means unpaired t-test. Data represent n = 3 for each experimental group.

3.6 The pro-inflammatory effect of 2-hydroxypropyl- β -cyclodextrin on bone marrow-derived macrophages is time-dependent and concentration-dependent

As increased depletion of cholesterol from the plasma membrane was shown to result in pro-inflammatory responses [31], we first investigated whether incubation time influenced the pro-inflammatory effect of CD. For this purpose, wildtype BMDMs were incubated with CD for 5, 10 and 30 min as well as for 1 and 4hr. Protein secretion of TNF α showed a marked time- dependent response after CD treatment (Figure 7A). This time-dependent, pro-inflammatory effect of CD was confirmed at gene expression levels for the inflammatory markers *Tnfa*, *IL-1b* and *Ccl2*, only showing increased expression after 1 and 4hr incubation (Figures 7B–D). *Lxra* showed a similar increase after 4 hours of CD incubation, *Npc2* only showed a trend (Figures 7E, F). Expression levels of *IL-18*, *Arg1*, *Abca1*, *Abcg1* and *Npc1* remained similar (Supplementary Figure S8).

Figure 7 Time dependency of the inflammatory effect of 2-hydroxypropyl- β -cyclodextrin: (A) TNF α protein levels of *Wt* BMDM treated with 2-hydroxypropyl- β -cyclodextrin for different time durations. (B–D) Gene expression analysis of *Tnf- α* , *IL-1 β* , *Ccl2*. (E, F) Gene expression analysis of *Lxr- α* and *Npc2*. Gene expression data were set relative to *Wt* BMDM treated with saline. Data are the result of 2 independent experiments. *, ** indicates $p < 0.05$ and 0.01 by means of unpaired t-test.

To further confirm our hypothesis, we also performed a dose- response curve with CD (4hr incubation) in wildtype BMDMs. As indicated in Figure 8, CD incubation showed a clear concentration-dependent effect on TNF α secretion, adding fuel to our argument that the pro-inflammatory effects of CD are related to cholesterol depletion from the plasma membrane.

Figure 8 Concentration dependency of the inflammatory effect of 2- hydroxypropyl- β -cyclodextrin: Dose response analysis of TNF α protein levels of Wt BMDM treated with 2-hydroxypropyl- β -cyclodextrin for 4hrs.

4. Discussion

New perspectives to reduce cholesterol levels aim at improving cellular instead of plasma cholesterol levels, raising the argument to use CD as a pharmacological compound to improve cholesterol homeostasis. Here, we confirm the cholesterol- depleting effects of CD in a metabolic inflammatory context, but concomitantly show a detrimental time- and concentration- dependent inflammatory effect of CD treatment. Therefore, while CD is able to decrease cellular cholesterol levels, our findings demonstrate that its use in the clinic should be closely monitored especially in patients with a metabolic inflammatory background.

Our observation that CD treatment promotes cellular cholesterol mobilization but induces a time- and concentration-dependent inflammatory effect implies the importance of the subcellular distribution of cholesterol and the subsequent impact of cholesterol depletion from these specific locations. Unesterified cholesterol serves a key structural function in the plasma membrane as it is critical for the formation of liquid-ordered rafts, which determine membrane fluidity. The membrane fluidity is therefore coupled to the free cholesterol/phospholipid ratio of the plasma membrane as this ratio determines the formation of the aforementioned rafts [32, 33]. Under hyperlipidemic conditions, the plasma membrane serves as the first pool to deposit free cholesterol from external sources [34]. However, accumulation of free cholesterol in the plasma membrane above an optimal free cholesterol/phospholipid ratio negatively impacts plasma membrane fluidity [33]. Indeed, Yvan-Charvet et al. showed that increased accumulation of free cholesterol in the plasma membrane (induced by *Abca1* or *Abcg1* knockout) directly results in pro-inflammatory responses, supporting our current notion of the limited physiological capacity of the plasma membrane to harbor free

cholesterol [31]. Therefore, exceeding the plasma membrane's capacity to harbor cholesterol directly results in intracellular cholesterol accumulation (mainly in lysosomes), which is considered a severe pathological phenomenon for mediating inflammatory responses [4]. Evidently, under conditions that generate severe accumulation of intracellular cholesterol (exemplified by the NPC1 mutation and LDLR knockout), CD-mediated depletion of cholesterol is highly desirable. In line with this concept, the increased lysosomal size in metabolically challenged macrophages was reverted after incubation with CD treatment. This advantageous cholesterol-depleting effect of CD has been confirmed in multiple previous reports [15, 18, 31, 35]. In contrast, in the current study, we consistently show a harmful, pro-inflammatory (and even mild pro-fibrotic) effect of CD under cholesterol-induced inflammatory conditions *in vivo* and *in vitro*. This pro-inflammatory effect can be rationalized by the prolonged cellular exposure to CD, which influences plasma membrane cholesterol combined with intracellular cholesterol levels. Indeed, given the key role of cholesterol in the formation of liquid-ordered rafts, excessive depletion of plasma membrane cholesterol (as is induced with CD treatment) is highly undesirable as it disrupts these rafts, affecting membrane fluidity. This rationale is further supported by findings showing that cholesterol extraction of the plasma membrane is a primary location where CD exerts its function [36, 37]. Therefore, excessive depletion of cholesterol from the plasma membrane by CD might be an explanation for the observed pro-inflammatory findings in our study as well as the time- and concentration-dependent character of our findings. The time-dependent findings are also in line with experiments reported by Pilely *et al.* and Ding *et al.*, that show that while incubation of different types of cyclodextrin for a short period of time (10 and 30 min) is anti-inflammatory [35], incubation for 24 and 48 hr lead to ototoxicity [19]. Together, these observations indicate that while CD has potential in advantageously depleting cellular cholesterol, it is essential to monitor the quantity of cellular cholesterol and adjust the therapeutic dose/time accordingly to prevent undesired pro-inflammatory side-effects.

Notably, while neutrophils generally constitute the major immune cell population under inflammatory conditions, in the current study we only focused on the effect of CD on macrophages. This choice is based on previous findings by our group that demonstrate macrophages to be the most important immune cell in the *in vivo* model here described (as evidenced by changes in hematopoiesis [38] and organ-specific inflammation [22]). Nevertheless, as neutrophil aberrations have also been described in NPC1 disease [39], future research should further explore the involvement of neutrophils in CD-induced inflammatory responses in a metabolic inflammatory context.

These dichotomic characteristics of CD raise the question whether structural manipulation of cyclodextrins can reduce the harmful pro-inflammatory effects, while maintaining the advantageous cholesterol-depleting effects of CD. Cyclodextrins are composed of cyclic oligosaccharides of 6, 7 or 8 glucose units (referred to as α -, β - and γ -cyclodextrins respectively), providing cyclodextrin a polar and hydrophilic surface combined with a non-polar cavity [40]. This dual-property structure of CD grants itself for modification *via* polymerization, creating so-called polycyclodextrins. Indeed, various modifications have been performed on cyclodextrin, creating more efficient and effective cyclodextrins for a plethora of purposes [40]. In line, Kulkarni *et al.* adapted the structure CD (the type we employed in our study) and showed that their linear degradable, high molecular weight polymer variation improved the pharmacokinetic profile and bioavailability in NPC mice [41]. Moreover, using polyrotaxanes enabled specific release of CD inside lysosomes, thereby minimizing the effect on plasma membrane cholesterol [42]. Based on these reports, it is anticipated that

designing polycyclodextrins is a promising approach that can have a considerable clinical impact due to its ability to reduce the pro-inflammatory properties of CD described in our study.

In conclusion, though we confirm its cholesterol-depleting effects, we here demonstrate a time- and concentration- dependent harmful pro-inflammatory effect of CD under metabolic inflammatory conditions. As such, we suggest that clinical use of CD, in particular in a metabolic inflammatory context, should be closely monitored to prevent undesired side effects.

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Supplemental material

Figure S1 Overview of experimental set-up of *in vivo* experiment

Figure S2 Relative organ weights

Figure S3 Quantification of hepatic HE staining of *Npc1^{nih}-tp* mice

Figure S4 Hepatic gene expression of fibrotic markers

Figure S5 Experimental set-ups related to Figure 4

Figure S6 Heatmap of inflammatory mediators of CD-treated Wt BMDM. (A-B) TNF α protein levels and *Tnf α* , *IL1b*, *IL-10*, *Ccl2*, *Lxra* and *Npc2* gene expression of oxLDL (24hr)-exposed Wt BMDM treated with or without CD (4hr) that were terminally stimulated with LPS (A) or without (B) LPS for 4 hr. Gene expression data were set relative to control-exposed Wt BMDM treated with saline and stimulated with (A) or without (B) LPS. Colored (red or blue) boxes are compared to the box directly at their left via two-way ANOVA followed by Tukey post-hoc analysis, indicating the effect of CD. Data are the result of 2-4 independent experiments.

Figure S7 Experimental set-ups related to Figure 5

Figure S8 Gene expression analysis of BMDM exposed to different time intervals of CD

Table S1: Primer sequences of genes used for quantitative RT-PCR.

Gene name	Forward primer 5'-3'	Reverse primer 5'-3'
Cyp7a1	CATTACAGAGTGTGGCCAAGA	CGCAGAGCCTCTTGATGAT
Cyp27a1	CTGCACTTCTCTGCTGACCAAT	AGGGCCCATGTCAAGTGTGT
Tnfa	CATCTTCTCAAAATTCAGGTGACAA	TGGGAGTAGACAAGGTACAACCC
Ccl2	GCTGGAGAGCTACAAGAGGATCA	TCTCTCTTGAGCTTGGTGACAAAA
Ccl5	GGAGTATTTCTACACGACGCAA	GCGGTTCTTCGAGTGACA
Saa1	GGCTGCTGAGAAATCAGTGATG	TCAGCAATGGTGTCTCATGTC
iNOS	GCAAACCCAAGGTCTACGTTCA	CCTCATTGGCCAGCTGCTT
Arg1	CATGGGCAACCTGTGTCTT	CGATGTCTTTGGCAGATATGCA
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
IL-18	AAAGAATCTATACCTGTCTGTGAATGAAA	GGTATTGCTTGGGATCCCACT
IL-18	GACTCTTGCGTCAACTTCAAGG	CAGGCTGTCTTTGTCAACGA
Lxr-a	CAACAGTGTACACGCGCT	TGCAATGGGCCAAGGC
Npc2	CGGAGCCCCTGCACCTC	ACAGGGATCGGTGGGACAT
Abca1	CCCAGAGCAAAAAGCGACTC	GGTCATCATCACTTTGGTCCTTG
Abcg1	TCGGACGCTGTGCGTTTT	CCCACAAATGTCGCAACCT
Npc1	ATATAACGAGAGCATTACCATGAGTA	TAACACCACGATCCCTCCAAA
Cyclophilin (housekeeping)	TTCTCTTTTACAGAATTATTCCA	CCGCCAGTGCCATTATGG
Hprt (housekeeping)	TCAGTCAACGGGGACATAAA	GGGGCTGTACTGCTTAACAG

Table S2: Raw data related to confocal quantification of conditions Wt control vs. NPC1 control. Blue numbers are used for statistical analysis.

WT control								NPC1 control							
Absolute numbers				Fractions (%)				Absolute numbers				Fractions (%)			
Bmdm	<0.1	0.1-1	>1	Sum	<0.1	0.1-1	>1	Bmdm	<0.1	0.1-1	>1	Sum	<0.1	0.1-1	>1
1	231	46	9	286	80,77	16,08	3,15	1	157	28	40	225	69,78	12,44	17,78
2	175	30	16	221	79,19	13,57	7,24	2	77	13	22	112	68,75	11,61	19,64
3	137	49	18	204	67,16	24,02	8,82	3	378	113	66	557	67,86	20,29	11,85
4	187	56	12	255	73,33	21,96	4,71	4	505	152	90	747	67,60	20,35	12,05
5	222	55	14	291	76,29	18,90	4,81	5	237	61	46	344	68,90	17,73	13,37
6	140	41	19	200	70,00	20,50	9,50	6	137	23	33	193	70,98	11,92	17,10
7	346	95	21	462	74,89	20,56	4,55								
Avg	1438	372	109	1919	74,52	19,37	6,11		1491	390	297	2178	68,98	15,72	15,30

Table S3: Raw data related to confocal quantification of conditions Wt control vs. Wt oxLDL. Blue numbers are used for statistical analysis.

WT control								WT oxLDL							
Absolute numbers				Fractions (%)				Absolute numbers				Fractions (%)			
Bmdm	<0.1	0.1-1	>1	Sum	<0.1	0.1-1	>1	Bmdm	<0.1	0.1-1	>1	Sum	<0.1	0.1-1	>1
1	231	46	9	286	80,77	16,08	3,15	1	207	53	31	291	71,13	18,21	10,65
2	175	30	16	221	79,19	13,57	7,24	2	161	52	33	246	65,45	21,14	13,41
3	137	49	18	204	67,16	24,02	8,82	3	209	42	61	312	66,99	13,46	19,55
4	187	56	12	255	73,33	21,96	4,71	4	311	88	47	446	69,73	19,73	10,54
5	222	55	14	291	76,29	18,90	4,81	5	835	186	123	1144	72,99	16,26	10,75
6	140	41	19	200	70,00	20,50	9,50								
7	346	95	21	462	74,89	20,56	4,55								
Avg	1438	372	109	1919	74,52	19,37	6,11		1723	421	295	2439	69,26	17,76	12,98

Table S4: Raw data related to confocal quantification of conditions Wt-oxLDL-control vs. Wt-oxLDL-CD. Blue numbers are used for statistical analysis.

WT oxLDL - control								WT oxLDL - CD							
Absolute numbers				Fractions (%)				Absolute numbers				Fractions (%)			
Bmdm	<0.1	0.1-1	>1	Sum	<0.1	0.1-1	>1	Bmdm	<0.1	0.1-1	>1	Sum	<0.1	0.1-1	>1
1	207	53	31	291	71,13	18,21	10,65	1	489	104	2	595	82,18	17,48	0,34
2	161	52	33	246	65,45	21,14	13,41	2	301	43	2	346	86,99	12,43	0,58
3	209	42	61	312	66,99	13,46	19,55	3	509	123	21	653	77,95	18,84	3,22
4	311	88	47	446	69,73	19,73	10,54	4	368	81	8	457	80,53	17,72	1,75
5	835	186	123	1144	72,99	16,26	10,75	5	271	76	15	362	74,86	20,99	4,14
								6	383	107	51	541	70,79	19,78	9,43
Avg	1723	421	295	2439	69,26	17,76	12,98		2321	534	99	2954	78,88	17,87	3,24

Table S5: Raw data related to confocal quantification of conditions NPC1 control vs. NPC1 CD. Blue numbers are used for statistical analysis.

NPC1 - control								NPC1 -CD							
Absolute numbers				Fractions (%)				Absolute numbers				Fractions (%)			
<i>Bmdm</i>	<0.1	0.1-1	>1	<i>Sum</i>	<0.1	0.1-1	>1	<i>Bmdm</i>	<0.1	0.1-1	>1	<i>Sum</i>	<0.1	0.1-1	>1
1	157	28	40	225	69,78	12,44	17,78	1	157	51	7	215	73,02	23,72	3,26
2	77	13	22	112	68,75	11,61	19,64	2	428	103	23	554	77,26	18,59	4,15
3	378	113	66	557	67,86	20,29	11,85	3	205	54	11	270	75,93	20,00	4,07
4	505	152	90	747	67,60	20,35	12,05	4	208	58	9	275	75,64	21,09	3,27
5	237	61	46	344	68,90	17,73	13,37	5	248	66	34	348	71,26	18,97	9,77
6	137	23	33	193	70,98	11,92	17,10	6	149	28	18	195	76,41	14,36	9,23
Avg	1491	390	297	2178	68,98	15,72	15,30		1395	360	102	1857	74,92	19,45	5,63

Supplementary video 1: Wildtype control**Supplementary video 2: *Npc1^{niH}* control****Supplementary video 3: Wildtype oxLDL****Supplementary video 4: Wildtype oxLDL CD****Supplementary video 5: *Npc1^{niH}* CD**

Supplemental methods

Macro created to quantify volumes and coordinates of lysosomes

```

function isTIFF(filename) {
    extensions=newArray("tif","tiff");
    result=false;
    for(i=0;i<extensions.length;i++) {
        if(endsWith(toLowerCase(filename),"." + extensions[i]))
            result = true;
    }
    return result;
}
setBatchMode(true);
inputdir=getDirectory("Choose directory containing TIFF images.");
imagefiles=getFileList(inputdir);
outputdir=getDirectory("Choose directory to save results.");
distance_between_centers=10 distance_max_contact=1.80");
for(i=0;i<imagefiles.length;i++) {
    imagepath=inputdir + imagefiles[i];
    imagename=replace(imagefiles[i],".tif","");
    if(isTIFF(imagepath)) {
        print("Processing file: " + imagepath + "...\\n");
        open(imagepath);
        n=nSlices;
        selectWindow(imagefiles[i]);
        run("Properties...", "channels=1 slices=n frames=1 unit=micron pixel_width=0.169 pixel_height=0.169
        voxel_depth=0.169 frame=[0 sec] origin=0,0");
        run("Split Channels");
        selectWindow(imagefiles[i] + " (red)");
        run("Close");
        NucleusChannel=imagefiles[i] + " (blue)";
        LysosomeChannel=imagefiles[i] + " (green)";
    }
}

```

```

    print("... nucleus...\n");
    selectWindow("NucleusChannel");
    run("Object Counter3D", "threshold=80 slice=10 min=10 max=20971520 new_results geometrical dot=3
numbers font=12 summary");
    selectWindow("Results from "+NucleusChannel);
    saveAs("Results", outputdir + imagename + "_results_nucleus.xls");
    run("Close");
    selectWindow("Geometrical Centres "+NucleusChannel);
    close();
    selectWindow(NucleusChannel);
    close();
    print("... lysosome...\n");
    selectWindow(LysosomeChannel);
    run("Object Counter3D", "threshold=40 slice=10 min=10 max=20971520 new_results geometrical dot=3
numbers font=12 summary");
    run("3D Watershed", "seeds_threshold=1 image_threshold=20 image=" + imagename + "
seeds=Geometrical radius=2");
    selectWindow("watershed");
    run("Properties...", "channels=1 slices=n frames=1 unit=micron pixel_width=0.169 pixel_height=0.169
voxel_depth=0.169 frame=[0 sec] origin=0,0");
    run("3D Manager Options", "volume surface centroid_(pix) centroid_(unit) distance_between_centers=10
distance_max_contact=1.80");
    run("3D Manager");
    Ext.Manager3D_AddImage();
    selectWindow(LysosomeChannel);
    Ext.Manager3D_MultiSelect();
    Ext.Manager3D_SelectAll();
    Ext.Manager3D_Select(0);
    Ext.Manager3D_Measure();
    selectWindow("3D Measure");
    saveAs("Results", outputdir + imagename + "_results_lysosomes.xls");
    run("Close");
    selectWindow("Results from " + LysosomeChannel);
    run("Close");
    selectWindow(LysosomeChannel);
    close();
    selectWindow("watershed");
    close();
    selectWindow("Geometrical Centres " + LysosomeChannel);
    close();
    print("... finished image.\n");
}
}
setBatchMode(false);

selectWindow ("Log");
saveAs("Text", outputdir + "Log.txt");
run("Quit");

```

Chapter 7

General discussion

Inflammation is considered a strong risk factor in the development of many diseases related to the metabolic syndrome such as NAFLD, diabetes, cardiovascular diseases and cancer. However, the exact cause of inflammation and the underlying mechanisms in these diseases are not yet completely understood. In the current thesis, we aimed to characterize the mechanisms linking lysosomal lipid overload and inflammation and investigated the effects of different intervention strategies to treat metabolic inflammation. One of such interventions include the potential of the lysosomal protease, cathepsin D, as a safe therapeutic target in metabolic inflammatory diseases. Here, we bring together the results of the chapters to discuss the implications of our findings on the future potential of different therapeutic approaches in modulating inflammation.

Secretion of Cathepsin D is increased upon various external and internal stimuli and induces inflammation

Metabolic and inflammatory pathways are tightly connected, meaning that metabolic burden induces lipid metabolism regulators that influence inflammatory processes [1,2]. Under normal conditions, excess cholesterol is taken up by macrophages and is processed inside the lysosome. Re-esterified cholesterol is transported back to the cytoplasm where it is stored or exported out of the cell by cholesterol efflux transporters [3,4]. Cholesterol efflux is regulated by liver X receptors (LXRs) that not only detect intracellular cholesterol overload but also activate genes involved in anti-inflammatory response [5]. Dysregulation of metabolism results in loss of lipid control leading to cholesterol accumulation, oxidative stress and insulin resistance [6,7]. Relevantly, macrophage lipid accumulation and defective lysosomal function are indicated as central events in many metabolic-inflammatory disorders. Unfavorable accumulation of lipids in tissues and high levels of circulating lipids, which are prone to oxidation, are commonly associated with inflammatory conditions [8,9]. Specifically, oxidation of Low-Density Lipoprotein (oxLDL) particles is considered as a marker of inflammation [10]. In line, we have formerly shown that intracellular accumulation of oxLDL in hepatic macrophages causes inflammation in non-alcoholic steatohepatitis (NASH) [11]. Importantly, recent data showed that plasma levels of the lysosomal protease, cathepsin D (CTSD) correlated with total cholesterol levels and severity of hepatic inflammation in patients [12]. This observation suggests a link between lipid excess, secretion of CTSD and inflammation.

In **Chapter 2**, we have extensively reviewed the triggers and respective mechanisms through which lysosomal cathepsins are released into cytosol and extracellular space. Progressive accumulation of modified lipids such as oxLDL in macrophages are known to cause trafficking defects that disrupt the lysosomal membrane integrity leading to release of lysosomes enzymes into the plasma [13,14]. The released lysosomal enzymes and undigested lipids can trigger immune responses. The discharge of CTSD has been reported to affect various pathways which can result in the initiation of inflammatory responses. Secreted pro-CTSD by its proteolytic activity on extra- and/or transmembrane proteins [15], by disturbed autophagy [16] or by initiating apoptosis [17,18], as well as by damaging extracellular matrix (ECM) [19] can lead to inflammation. For instance, macrophages are capable of responding to external signals by secretion of CTSD which is associated with inflammatory responses in IBD [20], cancer [21,22] and cardiovascular diseases [23]. However, despite the numerous associations of CTSD, in particular extracellular CTSD with inflammatory processes, it has not yet been demonstrated whether extracellular CTSD has a direct effect on inflammation. In **Chapter 3 & 4** this was investigated through the use of specific inhibition of extracellular CTSD which resulted in

improved lipid metabolism and reduced inflammation in high fat-fed rodent models suggesting that pro-CTSD secretion is involved with mediating metabolic inflammatory responses.

Apart from lipid mediators, acute and chronic stress are also associated with increased inflammatory activity. In **chapter 5**, we have described in detail the relation between stress/gut microbiota/brain interplay in intestinal barrier dysfunction and inflammation. Intestinal macrophages of inflamed mucosa are known to induce cathepsin secretion resulting in further tissue destruction and inflammation [24]. To note, the impact of stress and gut microbiota on CTSD function was not tested in the current thesis and future studies measuring the levels and activity of CTSD upon these stimuli are needed to confirm these observations. Collectively, the findings of this thesis suggest that CTSD release from cells upon external and internal stimuli seems to be a key process in inflammatory signaling.

Extracellular cathepsin D has a distinct pathophysiological role in inflammation compared to its lysosomal counterpart

In **chapter 2 & 4**, we have described that the intracellular and extracellular CTSD fractions have distinct cellular and molecular functions. Our findings demonstrated that the respective fractions of CTSD exhibit a different proteome profile and thereby regulate distinct cellular pathways. CTSD is an aspartic protease which is synthesized and translocated into the endoplasmic reticulum as an inactive pre-proCTSD (52 kDa) which is converted into an enzymatically active, intermediate pro-CTSD (48 kDa) that is finally processed into the mature CTSD form of 32 kDa in the lysosomes [15,25]. Lysosomal CTSD is involved in the processing of antigenic peptides [26], hormones [27,28], and growth factors [29]. Failure of such functions either by mutations or partial loss of CTSD activity is known to cause abnormal accumulation of cholesterol esters [30], lysosomal malfunction [31], neurodegeneration [32], developmental regression, visual loss [33]. CTSD knock-out mice are thus short-lived [34,35]. Under physiological conditions, extracellular CTSD is mainly involved in ECM degradation [36] and hydrolysis of protein components of LDL [37]. A small fraction of pro-CTSD is secreted under normal conditions and is found in biological fluids such as blood, sweat [38] and urine [39]. However, pro-CTSD is secreted in large quantities in several types of cancer [40], NASH [12], diabetes [41] and IBD [20], implying its role in metabolic pathologies.

The observed differences in the functionalities of intracellular and extracellular CTSD fractions might stem from a variety of factors including the pH of the microenvironment, availability of interacting partners or differences in the post-translational modifications of extracellular pro-CTSD and active lysosomal CTSD fractions. For instance, secreted pro-CTSD from MCF-7 cells harbored different glycosylation patterns rendering it sensitive to Endo H (glycosidase) digestion, whereas lysosomal CTSD from healthy tissues is partially Endo H-resistant which might affect its functions [25]. Moreover, there is evidence that CTSD displays both proteolytic-dependent and independent actions. Indeed, pro-CTSD has been shown to have the potential to act as a protease by cleaving various substrates as well as a ligand (binding function) for membrane receptors [42-44]. A CTSD variant (mutation at D231N) that lacks the catalytic function was still shown to be mitogenic in fibroblasts, indicating a pathway involving protein-protein interaction [45]. Thus, pro-CTSD might govern a greater number of functions than the lysosomal fraction due to its dual function as a ligand and a protease. Given the site-specific role for CTSD, inhibitors specifically targeting extracellular CTSD, and/or intracellular CTSD and tools that can block its protein binding functions are favored.

The small-molecule inhibitor used in our experiments does not pass through the cell membrane and therefore is retained extracellularly. With this approach, extracellular action of CTSD is selectively inhibited, but the crucial intracellular function of CTSD was undisturbed.

In **Chapter 3 & 4**, we showed reduced hepatic steatosis, insulin resistance, decreased expression of pro-inflammatory cytokines and induction of an anti-inflammatory state in rodent models upon selectively blocking the activity of extracellular CTSD. Extracellular CTSD is known to process several lipoprotein receptors and lipid transporters and thereby regulate lipid metabolism. It modifies apolipoprotein B-100 (apoB) component of the LDL molecules leading to their accumulation [46] and directly interacts with low-density lipoprotein receptor-related protein 1 (LRP1) [47] thereby modulating ATP-binding cassette transporter A1 (ABCA1) expression [48]. Xian and co-workers proposed an elegant model through which they demonstrated that macrophage LRP1 not only controls ABCA1 expression to sustain cholesterol efflux, but also incorporates cellular cholesterol homeostasis with inflammation [49]. In the setting of lipid overload, binding of oxLDL to CD-36/TLR-4 complex initiates MyD88 dependent inflammation. To counteract this, LRP1 promotes ABCA1 gene expression by Shc1/PI3K/Akt/Ppar- γ /Lxr- α axis to mediate cholesterol export. Besides cholesterol efflux, ABCA-1 modulates TLR-4 trafficking to membrane and induces LXRs to inhibit inflammatory pathways downstream of TLRs [50,51]. In our experiments, extracellular CTSD inhibition altered the expression of CD36 and SR-A, reduced pro-inflammatory cytokine expression in macrophages, upregulated PPAR signaling and downregulated the TLR-4 pathway, suggesting that extracellular CTSD disrupts the LRP1/TLR-4 axis to propagate inflammation (**Chapter 4**) as illustrated in **Figure 1**.

Figure 1 **Proposed mechanism of extracellular CTSD action.** Under normal conditions, oxLDL is endocytosed by the cells using CD36/SR-A receptors and is sent to lysosomes for subsequent degradation. During hyperlipidemic conditions, excess oxLDL bound to CD-36 can elicit TLR-4 - MYD88 dependent inflammatory responses. To suppress this, LRP1 mediates crosstalk between ABCA1 and TLRs to increase cholesterol efflux and cease inflammation respectively. Accumulation of oxLDL inside lysosomes leads to lysosomal membrane permeabilization and release of CTSD. The interaction of extracellular CTSD with LRP1 blocks LRP1 signaling leading to increased inflammatory gene expression. In our experiments, inhibition of extracellular CTSD altered the expression of CD36, SR-A and TLR4 suggesting that extracellular CTSD acts as a master regulator of CD36/TLR-4/LRP1 axis in promoting inflammation. Several elements of the figure are taken from Servier Medical Art image bank.

Moreover, it is possible that increased CTSD activity is both a cause and consequence of inflammatory activity, i.e., that the relationship between CTSD and inflammation is bi-directional where one factor activates the other. In line, inflammatory cytokines are known to cause the secretion of pro-CTSD in endothelial cells [52]. An additional mechanism through which extracellular CTSD may participate in inflammation is via degradation of ECM. The inflammatory process involved in IBD, cardiovascular diseases and cancer appear to converge at the step of ECM degradation by extracellular cathepsins [53]. In IBD patients, increased production and secretion of CTSD participates in ECM degradation of the mucosa leading to damage of intestinal barrier [20,54]. CTSD from the tumor-conditioned media was able to degrade ECM proteins exacerbating tumorigenesis [55,56]. In line, our proteomics data showed the downregulation of ECM proteins such as collagen, prolargin and xylosyl transferase 2 upon extracellular CTSD inhibition, suggesting that CTSD might mediate inflammatory responses by

degrading ECM components (**Chapter 4**). Although this thesis demonstrated a clear effect of extracellular CTSD on inflammation/lipid metabolism, the exact downstream effectors of CTSD are unknown. Therefore, future studies using yeast two-hybrid screening or mutagenesis approaches are needed to identify the binding partners of CTSD in the extracellular space.

Alternative therapeutic targets for modulating cholesterol-induced metabolic inflammation

The involvement of lysosomal cholesterol accumulation as one of the primary causal factors in inflammatory conditions, makes it an attractive therapeutic target. Therefore, **in chapter 6**, we evaluated the potential effects of 2-hydroxypropyl- β -cyclodextrin (CD), a cholesterol-mobilizing compound in models of metabolic inflammation. We found that CD intervention in both *in vitro* and *in vivo* models of metabolic inflammation led to cholesterol mobilization, but with an accompanying unexpected increase in inflammation. The observed pro-inflammatory effects of CD could be the result of its cholesterol-depleting properties. CDs normalize cholesterol homeostasis by releasing the trapped unesterified cholesterol from membranes and also facilitate the cholesterol egress from lysosomal compartments by enhancing lysosomal exocytosis [57,58]. Depletion of plasma membrane cholesterol can lead to dispersal of lipid rafts and associated plasma membrane proteins [59] and affect the function of membrane receptor complexes [60] which are processes known to induce inflammation. This observation is also in line with our observed time-dependent pro-inflammatory effect of CD, suggesting that short exposure to CDs is still beneficial. As discussed in the previous section, lipid raft dispersal can initiate MyD88/NF- κ B activation and subsequent inflammation. Indeed, CD was shown to activate the innate immune response through these lipid-dependent biological processes [61]. Besides cholesterol depletion, lysosomal exocytosis can also lead to propagation of inflammatory responses as discussed in **chapter 2**. Hence, it is plausible to assume that CD treatment would change the mechanical properties of the membranes leading to the pro-inflammatory responses. Therefore, it is important to monitor the clinical application of CD to prevent unwanted side effects. Altogether, our findings demonstrate a clear and well-defined connection between lysosomal cholesterol accumulation and inflammation. The summary of the main findings of this thesis are represented in **Figure 2**.

Figure 2 **Summary of the main findings.** Consumption of high fat diet can lead to lysosomal cholesterol accumulation and subsequent destabilization of the lysosomal membrane leading to release of CTSD into extracellular space. Inhibition of oxLDL-induced CTSD decreased hepatic steatosis and inflammation (chapter 3 & 4). Alternatively, lysosomal cholesterol accumulation was reduced by CD intervention (chapter 6). In addition to dietary factors, stress can have impact on gut microbiota composition leading to inflammatory responses in IBD by releasing lysosomal enzymes or by disturbing intestinal barrier and therefore stress management options can help alleviate IBD symptoms (chapter 5). Figure is created with permission from Servier Medical Art image bank.

Clinical implications of the findings

Dietary supplements and life-style interventions including exercise and stress management have produced mixed results in treating inflammation in patients with NAFLD [62] and IBD [63, 64]. In addition, the anti-inflammatory effects of various medications are inconclusive, possibly due to partial effects on metabolic dysregulation or due to the fact that the cause of inflammation can be multifactorial in these diseases [65]. Moreover, the complex pathophysiology and heterogenous patient populations makes the drug development particularly challenging. This further rationalizes the development of innovative pharmacologic therapeutics aimed at specific molecular targets in inflammatory disorders.

Although cathepsins have proven to be appealing targets in osteoporosis, atherosclerosis and cancer, their clinical adaption is often hindered by the 'off-target' effects and adverse responses [66]. However, progress in understanding the structure of the cathepsins' active sites with parallel advances in computational and *in silico* modelling allowed the development of potent selective cathepsin inhibitors with minimal or zero negative effects. In the current thesis, we demonstrated that targeted inhibition of extracellular CTSD activity restores dysregulated lipid metabolism and reduces inflammation both *in vitro* and *in vivo* (**Chapter 3 & 4**). However, validation of these findings

using human samples is needed to successfully translate these pre-clinical findings to the clinic. In addition, owing to their emerging role as plasma biomarkers, extracellular cathepsins are being considered as potential targets for non-invasive **diagnostics** [67]. In fact, we have successfully demonstrated the potential of plasma CTSD as a biomarker in NASH [12] as well as diabetic patients [68]. Next to extracellular cathepsins, in the current thesis we focused on alternate strategies to counteract cholesterol mediated- inflammation. When we employed a cholesterol mobilizing agent, CD, we observed time-dependent pro-inflammatory effects despite beneficial effects on cholesterol sequestration (**Chapter 6**). Therefore, clinical application of CD, should be closely scrutinized and future studies should focus on optimizing therapeutic dose/time of CD to prevent any pro-inflammatory side-effects.

Gut microbiota dysbiosis plays a crucial role not only in the pathogenesis of IBD but also in metabolic disorders such as NAFLD through its interactions via ‘gut-liver’ axis [65,69]. Consequently, microbiota intervention strategies such as supplementation of pre/probiotics and fecal microbiota transplantation (FMT) are being widely employed as treatment options for inflammatory disease therapy. FMT and probiotics were shown to improve lipid metabolism [70], insulin sensitivity and alleviation of steatohepatitis in rodent models [71]. Furthermore, adjuvant therapy targeting stress, particularly stress-induced inflammation, would be advantageous for the treatment of these diseases (**Chapter 5**).

Taken together, in this thesis we demonstrated various pharmacological and non-pharmacological treatment options aimed at modulating inflammation. We mechanistically demonstrated that pharmacological inhibition of extracellular CTSD activity might be a novel and safe drug target for NASH. Furthermore, we tested the cholesterol mobilizing CD as an alternative therapeutic strategy for metabolic inflammation and demonstrated some undesirable pro-inflammatory effects. Finally, we also reviewed the non-pharmacological stress management strategies in inflammatory disorders.

Limitations and future perspectives

In this thesis we validated putative targets such as extracellular CTSD and CD in preclinical rodent models of metabolic inflammation. While we found promising beneficial effects of these targets on disease outcomes in our studies, limitations include lack of validation of the findings in more pre-clinical models and in human samples. In view of the possible challenges in translating the pre-clinical findings to humans [72], the inhibitors need to be tested for their safety and tolerability for long-term use in humans. Therefore, further studies including human trials or/ and *ex vivo* models such as organ-on-chip platforms that reiterates the histologic endpoints in a single chip can serve as a robust non-invasive, humanized platform to study the disease course and to formulate effective therapeutics [73].

Novel findings of this thesis

In the present thesis, we aimed to provide translational insight into the underlying mechanisms that trigger inflammation in multiple diseases. The findings of the studies described in this thesis are highlighted below and summarized in **Table 1**.

- Cathepsins exhibit both intra and extracellular functions in health and disease (Chapter 2).
- Extracellular cathepsin D plays a major role in lipid metabolism and insulin resistance during NAFLD progression (Chapter 3).
- Extracellular cathepsin D rather than intracellular fraction plays a key role in NASH mice with human-like lipoprotein profile and can be a novel target for modulating inflammation (Chapter 4).
- Stress impacts the inflammatory responses in IBD by acting on the complex gut/microbiota/brain axis (Chapter 5).
- 2-hydroxypropyl- β -cyclodextrin helps in cholesterol mobilization but shows time-dependent pro-inflammatory effects (Chapter 6).

Table 1: Summary of the major findings of this thesis along with limitations

Chapter	Main findings	Limitations	Future directions
Chapter 3	Inhibition of extracellular CTSD reduced hepatic steatosis and inflammation both <i>in vitro</i> and <i>in vivo</i>	The steatotic rat model used in this study is an early model of inflammation and does not mirror hyperlipidemic profile as seen in humans.	To investigate the effects on metabolic inflammation, a more severe model of NAFLD needs to be used. Toxicity studies in pre-clinical models need to be performed.
Chapter 4	Extracellular and intracellular cathepsin D regulate distinct metabolic pathways in NASH and specific inhibition of extracellular CTSD can be a safe therapeutic target for NASH	Small sample size for proteomics and FACS. Limited effects on inflammation. Lack of validation in human models.	Optimizing dose/time of inhibitors. Validation of the findings in pre-clinical/ human models. Pharmacokinetic studies.
Chapter 6	2-hydroxypropyl- β -cyclodextrin helps in cholesterol mobilization but elicits pro-inflammatory responses in a time-dependent fashion	Lack of time-course experiments in models of metabolic inflammation. Lack of validation in human samples	Perform dose/time dependent experiments in human samples.

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Summary

Summary

The focus of this thesis is to increase the understanding of metabolic inflammatory processes underlying chronic inflammatory disorders with a goal to identify novel and safe therapeutic approaches.

Chapter 1 provides a comprehensive summary on the role of inflammation in the context of chronic inflammatory diseases such as NAFLD and IBD. Thereafter, the relationship between disturbed lipid metabolism and lysosomal enzymes in inflammation is described. Subsequently, the significance of non-pharmacological and pharmacological intervention strategies for treating inflammatory disorders are discussed. Finally, the aim and the outline of the thesis are summarized.

Chapter 2 gives an overview on the expression and functional aspects of lysosomal cathepsins. Next, the triggers and the respective mechanisms involved in the secretion of cathepsins into cytosol and extracellular space are described. In addition, detailed pathophysiological role of cathepsins in and outside of the lysosomes are discussed, followed by comprehensive information on the innovative experimental and clinical trials involving extracellular cathepsins.

Chapter 3 investigates the role of extracellular cathepsin D in lipid metabolism and insulin resistance associated with NAFLD. In this chapter, we tested a specific small-molecule extracellular cathepsin D inhibitor in both *in vitro* and *in vivo* models. Extracellular inhibitor-treated macrophages and HepG2 cells showed reduced levels of inflammation and improved cholesterol metabolism. Upon administration of the aforementioned inhibitor, high-fat fed Sprague-Dawley rats displayed reduced hepatic steatosis, inflammation and insulin resistance compared to controls. Collectively, this chapter demonstrates the causal role of extracellular CTSD in pathogenesis of NAFLD.

Chapter 4 offers translational insight into the respective roles of intra and extracellular fractions of cathepsin D in NASH. To mimic a human hyperlipidemic profile, *Ldlr*^{-/-} mice were fed a high- fat, high-cholesterol diet for a period of 10 weeks. Simultaneously, these mice were injected with either control/ intra or extracellular small-molecule inhibitors of cathepsin D. Extracellular CTSD inhibitor-treated mice showed reduced hepatic lipid accumulation, increased faecal bile acid levels and decreased inflammatory responses compared to intracellular CTSD inhibitor-treated mice. Proteomic analysis revealed that extra- and intracellular CTSD fractions regulate distinct metabolic pathways. We concluded that specific inhibition of extracellular CTSD inhibition is beneficial over intracellular CTSD in treating NASH.

Chapter 5 surveys the link between inflammation and gut/microbiota/brain axis in the context of IBD. Using evidence from several pre-clinical and clinical studies, the two-way relationship between gut microbiota and stress is addressed. Impact of non-pharmacological stress reduction strategies on the quality of life in IBD patients is evaluated.

Chapter 6 assesses the function of cholesterol-mobilizing agent, 2-hydroxypropyl- β -cyclodextrin (CD) in treating metabolic inflammation. To obtain an extreme model of inflammation, *Ldlr*^{-/-} mice were transplanted with bone marrow from *Npc1*^{nih} mice and were a fed high- fat, high-cholesterol diet for a period of 12 weeks. These mice were injected with CD in the final three weeks. Compared to control mice, *Npc1*^{nih} -tp mice that received CD showed increased cholesterol mobilization with an unexpected increase in hepatic inflammation. In line with these *in vivo* results, a series of *in vitro* results confirmed the inflammatory effect of CD, which turned out to be time dependent.

Chapter 7 discusses the major outcomes of this thesis, with respect to the current knowledge in the field and highlights the clinical implications of the results.

Nederlandse Samenvatting

De focus van dit proefschrift is het vergroten van het begrip van metabole inflammatoire processen die ten grondslag liggen aan chronische inflammatoire aandoeningen met als doel het identificeren van nieuwe en veilige therapeutische benaderingen.

Hoofdstuk 1 geeft een uitgebreide samenvatting van de rol van ontsteking in de context van chronische ontstekingsziekten zoals NAFLD en IBD. Daarna wordt de relatie tussen verstoord lipidenmetabolisme en lysosomale enzymen bij ontstekingen beschreven. Vervolgens wordt het belang van niet-farmacologische en farmacologische interventiestrategieën voor de behandeling van inflammatoire aandoeningen besproken. Tot slot worden het doel en de omvang van het proefschrift samengevat.

Hoofdstuk 2 geeft een overzicht van de expressie en functionele aspecten van lysosomale cathepsinen. Vervolgens worden de triggers en de respectievelijke mechanismen die betrokken zijn bij de uitscheiding van cathepsinen in het cytosol en de extracellulaire ruimte beschreven. Daarnaast wordt de gedetailleerde pathofysiologische rol van cathepsinen in en buiten de lysosomen wordt besproken, gevolgd door uitgebreide informatie over de innovatieve experimentele en klinische onderzoeken met extracellulaire cathepsinen.

Hoofdstuk 3 onderzoekt de rol van extracellulair cathepsine D in het lipidenmetabolisme en insulineresistentie geassocieerd met NAFLD. In dit hoofdstuk hebben we een specifieke kleinmoleculaire extracellulaire cathepsine D-remmer getest in zowel *in vitro* als *in vivo* modellen. De met extracellulaire remmer behandelde macrofagen en HepG2-cellen vertoonden verminderde ontstekingsniveaus en verbeterd cholesterolmetabolisme. Na toediening van de bovengenoemde remmer vertoonden de met een hoog vetgehalte dieet gevoerde Sprague-Dawley-ratten verminderde leversteatose, ontsteking en insulineresistentie in vergelijking met controles. Gezamenlijk demonstreert dit hoofdstuk een causale rol van extracellulaire CTSD in de pathogenese van NAFLD.

Hoofdstuk 4 biedt translationeel inzicht in de respectievelijke rollen van intra- en extracellulaire fracties van cathepsine D in NASH. Om een humaan hyperlipidemisch profiel na te bootsen, *kregen Ldlr*^{-/-} muizen gedurende een periode van 10 weken een vetrijk dieet met een hoog cholesterolgehalte. Tegelijkertijd werden deze muizen geïnjecteerd met ofwel controle / intra- of extracellulaire kleinmoleculaire remmers van cathepsine D. De met extracellulaire CTSD-remmer behandelde muizen vertoonden verminderde hepatische accumulatie van lipiden, verhoogde fecale galzuur niveaus en verminderde inflammatoire responsen in vergelijking met intracellulaire CTSD-remmer behandelde muizen. Proteomische analyse onthulde dat extra- en intracellulaire CTSD-fracties verschillende metabole routes reguleren. We concludeerden dat specifieke remming van extracellulaire CTSD-remming gunstig is boven intracellulaire CTSD bij de behandeling van NASH.

Hoofdstuk 5 onderzoekt het verband tussen ontsteking en darm/microbiota/hersen-as in de context van IBD. Met behulp van bewijs uit verschillende preklinische en klinische onderzoeken wordt de tweerichtingsrelatie tussen darmmicrobiota en stress aangepakt. De impact van niet-farmacologische stressreductiestrategieën op de kwaliteit van leven bij IBD-patiënten wordt geëvalueerd.

Hoofdstuk 6 beoordeelt de functie van het cholesterol-mobiliserend middel, 2-hydroxypropyl- β -cyclodextrine (CD) bij de behandeling van metabole ontsteking. Om een extreem ontstekingsmodel te verkrijgen, werden *Ldlr*^{-/-} muizen getransplanteerd met beenmerg van *Npc1*^{mtb} muizen en kregen ze gedurende een periode van 12 weken een dieet met een hoog vetgehalte en een hoog cholesterolgehalte. Deze muizen werden in de laatste drie weken met CD geïnjecteerd. Vergeleken met controlemuizen *vertoonden* *Npc1*^{mtb}-tp-muizen die CD kregen een verhoogde cholesterolmobilisatie met een onverwachte toename van leverontsteking. In lijn met deze *in vivo* resultaten bevestigde een reeks *in vitro* resultaten het inflammatoire effect van CD, dat tijdsafhankelijk bleek te zijn.

Hoofdstuk 7 bespreekt de belangrijkste resultaten van dit proefschrift, met betrekking tot de huidige kennis in het veld en benadrukt de klinische implicaties van de resultaten.

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Social-economical and clinical relevance

Chronic low-grade inflammation is a critical factor in progression of metabolic diseases including type 2 diabetes, NASH and cardiovascular diseases. Chronic inflammatory disorders have been identified as the leading cause of death worldwide today and their prevalence is going to steadily increase in the coming years. In European countries, the forecasted prevalence of NASH is 29.5% by 2030 [1]. Moreover, NASH and IBD patients incur substantial financial costs besides poor quality of life. In Europe, the annual healthcare costs incurred by IBD patients were estimated to be around €5.6 billion [2], while those related to NAFLD were estimated to be €35 billion [3] placing high economic burden on the society.

The shortage of early markers that can accurately differentiate NASH from steatosis and the lack of detailed mechanistic knowledge on NASH progression are the main obstacles in diagnosing and treating NASH, making liver transplantation the only therapeutic resort [4]. Although recent years have shown advancements in non-invasive imaging, to date, no (cost)effective methods are available to establish novel non-invasive diagnostic tools and therapeutic targets in the context of NAFLD. New pipelines of drugs should not only focus on reversing steatohepatitis but should also prove to be non-toxic and economical.

The current thesis demonstrated the specific inhibition of extracellular CTSD activity to be a novel strategy for the prevention and treatment of NASH. Even though cathepsin inhibitors are being extensively investigated for their therapeutic application in cancer and bone disorders, most clinical trials involving these inhibitors are abandoned due to their off-target effects [5]. In contrast to this, the targeted cathepsin inhibition followed in this thesis is promising since it specifically blocks the extracellular cathepsin fraction while preserving the essential physiological functions of intracellular cathepsin fraction, thus limiting toxic side effects. This approach thus can decrease the costs spent in clinical development and increase the chances of success in clinical trials. Furthermore, these small molecules are valuable for both liver disease and obesity-related pathologies since extracellular CTSD inhibition improved metabolic parameters including insulin resistance and dyslipidemia. Since extracellular CTSD activity is markedly impaired in atherosclerosis, cancer and lysosomal storage diseases such as NPC1, the findings from the current thesis can be extended to such co-morbidities thereby further increasing the clinical relevance. In addition to its therapeutic role, plasma CTSD has enormous potential as an early non-invasive biomarker for NASH, thus helping in early diagnosis and treatment of NASH.

Another exciting approach to alleviate lipid storage problems is to use cholesterol extracting compounds such as cyclodextrins, which are already being extensively evaluated as treatment options in Alzheimer's, atherosclerosis, Krabbe and Niemann-Pick type C1 diseases [6]. Concomitant with the beneficial cholesterol-depleting effects, the current thesis uncovered the time-dependent pro-inflammatory effects of 2-hydroxypropyl- β -cyclodextrin (CD) in several models of metabolic inflammation. While CD can help to reduce the disease burden in patients, our results warrant careful monitoring of CD in clinic to prevent unwanted effects. These results thus have high clinical impact and pave a way to optimize time and dose regimes for future use of cyclodextrins. Greater affordability and improved safety of CDs can further augment their medical potential.

Besides the above-mentioned contemporary therapeutic approaches, this thesis also discussed the importance of non-pharmacological stress-reduction strategies which can help in societal cost saving. Effective translation of these findings in larger patient populations can help draw more definite and detailed conclusions. Altogether, this endeavor can lead to reduction in socioeconomic burden and consequently improve quality of life in patients suffering from chronic inflammatory disorders.

Novelty of the concept

In this thesis, we investigated novel intervention strategies to treat metabolic inflammation. Along with our previous findings, the current thesis mechanistically demonstrated the pivotal role of cathepsin D in lipid-induced hepatic inflammation. Using the state of the art *in silico* approach, we designed and validated specific small-molecule inhibitors against cathepsin D in a preclinical setting. Here, we show for the first time that specific inhibition of extracellular CTSD has beneficial effects in diminishing hepatic steatosis, inflammation and insulin resistance associated with NAFLD progression. Besides exploiting the function role of CTSD, we uncovered the potential mechanisms regulated by intra and extracellular CTSD fractions in the content of metabolic inflammation.

The benefits of suggested pharmacotherapies can be maximized by adhering to healthy lifestyle habits. While the information on lifestyle interventions for metabolic diseases is mostly limited to diet and exercise, this thesis consolidates the preclinical and clinical evidence on the role of stress reduction therapies in modulating inflammation that have enormous benefits in improving the quality-of-life in patients and reducing the healthcare burden. Also, this thesis provided critical data on the harmful pro-inflammatory effects of CD in the context of cholesterol-induced metabolic inflammation, which provides a novel view on CD as therapeutic compound. As CD shuttles cholesterol directly from the trapped sites and has effective renal clearance, it is currently considered promising for patients who are intolerant to statin therapy or cannot maintain a low-calorie diet. As such, our novel observations challenge the clinical use of CD and call for a careful reassessment of the drug's safety profile.

Future perspectives and potential application

Although the results in this thesis provide new diagnostic and therapeutic approaches for chronic inflammatory disorders, further work needs to be done to ensure that our findings are clinically translated to patients and the general public. In addition to other pre-clinical models, the results of the current project will be validated in blood-derived monocytes from healthy volunteers and samples from NASH. Since NASH patients have high risk of developing hepatocellular carcinoma (HCC), we planned to examine the cathepsin inhibitors in experimental models of HCC. As NASH shares the common pathogenic link with atherosclerosis [7], it is worthwhile to confirm and broaden the findings of the current thesis in atherosclerosis models. The next important step in human cathepsin research would be designing a personalized therapy with cathepsin inhibitors. For instance, optimizing the dosage of inhibitors by evaluating them in different cohorts would help a great deal in formulating efficient treatment regimens with even less side-effects [8].

Besides the role of CTSD, investigating the role of other lysosomal enzymes in the context of inflammation seems attractive. Several extracellular cathepsins are shown to mediate inflammatory

responses by means of their proteolytic activity on the extracellular matrix components, chemokines, cytokines and different membrane receptors. In line, overexpression and secretion of cathepsins is one of the common denominators in several inflammation related pathologies including cancer, atherosclerosis, auto-immune diseases, and arthritis [9]. For instance, cathepsin B is a well-known prognostic marker for several cancers [10]. Moreover, CTSD secreted by breast cancer cells is known to degrade endogenous cysteine protease inhibitor, cystatin C which subsequently leads to increased proteolytic activity of cathepsin B, thus exacerbating tumor progression and metastasis. Besides, blocking CTSD as well as CTSB/CTSL reduced inflammation in experimental colitis [11]. Therefore, it is reasonable to infer that extracellular cathepsins might synergistically contribute to the inflammatory responses. Future experiments with extracellular inhibitors of CTSB/L along with CTSD can clarify such speculations.

Lifestyle-induced weight loss and physical activity are highly recommended first line treatments for NASH patients. However, persistent lifestyle habits and weight loss are hard to reach and, unfortunately, lifestyle modifications alone are not effective in every individual. Nevertheless, it would be interesting to use combination treatment of the proposed pharmacological targets with the lifestyle interventions.

Lastly, instead of interfering with the inflammatory pathways, it is worthy to directly target the origin sites of inflammation, for example the gastrointestinal tract. High fat diet and obesity are known to disrupt the intestinal microflora composition and induce intestinal barrier dysfunction. Ultimately, these modifications lead to leaky mucosal barrier and release of bacterial metabolites that trigger a pro-inflammatory response leading to metabolic inflammation as seen in type 2 diabetes, NASH and atherosclerosis [12]. Therefore, it is worthwhile to study the gut microbiota–metabolism axis in the context of NASH. Collectively, the intricate relationship between lysosomal lipid metabolism and inflammation is gaining momentum and the therapeutic route for this field looks very promising.

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Curriculum Vitae

Curriculum Vitae

Tulasi Yadati was born in 1990 in Kadapa, India. In 2010. She obtained her Bachelors degree from Osmania University (Hyderabad, India) with specialization in genetics and microbiology. Later, she completed the Level-II certification in Protein modelling and rational drug designing from GVK Biocampus and Accelrys. In 2011, she joined the Masters program at the Department of Animal Biotechnology, University of Hyderabad, India. Her senior year project was focused on characterizing the three-dimensional organization of Whey acidic protein (Wap) gene locus in mouse mammary epithelial cells. She graduated in 2013 as university topper for which she was awarded the INSPIRE-Junior Research Fellowship from Department of Science and technology, India which allowed her to pursue research studies in the lab of Prof. Aparna Dutta Gupta for a period of two years where she was engaged in investigating the functional role of Hexamerin proteins in the rice moth morphogenesis.

In July 2017 she joined the group of Prof. Ronit Shiri-Sverdlov for her doctoral studies at Maastricht University. Her research mainly focused on investigating the mechanistic role of lysosomal enzymes in lipid-induced metabolic inflammation and preclinical validation of small-molecule drugs targeted at several aspects of metabolic inflammation. The results of the work are published in this thesis.

Tulasi is currently working as a postdoctoral researcher under the supervision of Prof. dr. Stan Van de Graaf, at Tytgat Institute for Liver and Intestinal Research, Amsterdam University Medical Center, The Netherlands.

List of Publications

1. Khurana P, **Yadati T**, Goyal S, Dolas A, Houben T, Oligschlaeger Y, Agarwal AK, Kulkarni A, Shiri-Sverdlov R (**shared first author**). Inhibiting Extracellular Cathepsin D Reduces Hepatic Steatosis in Sprague-Dawley Rats. *Biomolecules*. 2019 May 4;9(5):171. doi: 10.3390/biom9050171.
2. Oligschlaeger Y, **Yadati T**, Houben T, Condello Oliván CM, Shiri-Sverdlov R. Inflammatory Bowel Disease: A Stressed "Gut/Feeling". *Cells*. 2019 Jun 30;8(7):659. doi: 10.3390/cells8070659.
3. **Yadati T**, Houben T, Bitorina A, Shiri-Sverdlov R. The Ins and Outs of Cathepsins: Physiological Function and Role in Disease Management. *Cells*. 2020 Jul 13;9(7):1679. doi: 10.3390/cells9071679.
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5. Houben T, **Yadati T**, de Kruijf R, Gijbels M, Luiken J, Zandvoort M, Kapsokalyvas D, Lütjohann D, Westerterp M, Plat J, Leake D, Shiri-Sverdlov R (**shared first author**); Pro-Inflammatory Implications of 2-Hydroxypropyl- β -cyclodextrin Treatment. *Frontiers in Immunology*. DOI=10.3389/fimmu.2021.716357
6. Bitorina AV, Oligschlaeger Y, Ding L, **Yadati T**, Westheim A, Houben T, Vaes RDW, Olde Damink SWM, Theys J, Shiri-Sverdlov R. OxLDL as an Inducer of a Metabolic Shift in Cancer Cells. *J Cancer*. 2021 Aug 3;12(19):5817-5824. doi: 10.7150/jca.56307.
7. Ahmad F, Mitchell RD, Houben T, Palo A, **Yadati T**, Parnell AJ, Patel K, Shiri-Sverdlov R, Leake DS. Cysteamine Decreases Low-Density Lipoprotein Oxidation, Causes Regression of Atherosclerosis, and Improves Liver and Muscle Function in Low-Density Lipoprotein Receptor-Deficient Mice. *J Am Heart Assoc*. 2021 Sep 21;10(18):e017524. doi: 10.1161/JAHA.120.017524.

Oral Presentations

1. February 2018: Dutch Liver Science Retraite (Nederlandse Vereniging voor Hepatologie), Spier, the Netherlands
Title: *Inhibiting extracellular Cathepsin D reduces hepatic inflammation in steatohepatitis.*
2. February 2018: Research Line 2 lunch meeting, Maastricht, the Netherlands
Title: *Inhibiting extracellular Cathepsin D reduces hepatic inflammation in steatohepatitis.*
3. March 2018: European Fatty Liver Conference, Vaals, The Netherlands (**awarded the 2nd best pitch presentation**)
Title: *Unconventional treatment for a conventional disease: role of extracellular cathepsin D in NASH.*
4. March 2018: Nederlandse Lipoproteinen Club, Amsterdam, The Netherlands
Title: *Inhibiting extracellular Cathepsin D reduces hepatic inflammation in steatohepatitis.*
5. June 2018: Seminar meeting, Genetics and Cell Biology, Maastricht, The Netherlands
Title: *"Therapeutic potential of extracellular cathepsin D in Non-alcoholic steatohepatitis (NASH)"*
6. February 2019: Dutch Liver Science Retraite (Nederlandse Vereniging voor Hepatologie), Spier, the Netherlands (**awarded the 3rd best oral presentation**)
Title: *Extracellular Cathepsin D as a novel target for NAFLD*
7. October 2020: Research Line 2 lunch meeting, Maastricht, the Netherlands
Title: *Extracellular cathepsin D as a target for Non-alcoholic steatohepatitis (NASH)*

Poster Presentations

1. November 2018: Annual NUTRIM symposium, Maastricht University, The Netherlands.
Title: *"Extracellular cathepsin D: A new target for NAFLD?"*
2. November 2019: Annual NUTRIM symposium, Maastricht University, The Netherlands.
Title: *"Circulating cathepsin D improves hepatic lipid metabolism in murine NASH."*