The entanglement of plasma Cathepsin D and metabolic disturbances

The entanglement of plasma Cathepsin D and metabolic disturbances: relevant insights in the metabolic syndrome

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The entanglement of plasma Cathepsin D and metabolic disturbances: relevant insights in the metabolic syndrome

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General introduction

Chapter 1

Metabolic syndrome

Metabolic syndrome (MetS) has become a global, escalating public health challenge.[1] MetS is not a single disease but rather a constellation of risk factors that increases the risk to develop cardiovascular diseases (CVD) and type 2 diabetes mellitus (T2DM).[2] Despite the controversy regarding the precise definition for MetS, the presence of any three of the five criteria being abdominal obesity, high serum triglycerides, low serum HDL, hypertension and hyperglycemia is the most widely used for defining MetS set by the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) (Figure 1.1).[3,4] Though other abnormalities (i.e., chronic inflammation) are also associated with MetS, the diagnosis criterion mentioned above is considered the standard to diagnose MetS.[2] Currently, it is estimated that 12–37% of the Asian population, 12–26% of the European population and more than 30% of Northern American population suffers from MetS,[5,6] identifying the MetS as a pandemic. Relevantly, the prevalence of Mets is the result of genetic and environmental factors as well as their interactions (Figure 1.1).[7,8,9] Additionally, besides posing an increased risk for developing CVD and T2DM, individuals suffering from MetS are also at increased risk to develop other chronic diseases, such as cancer and fatty liver disease,[2,10-12] suggesting the enormous challenge public health is facing.

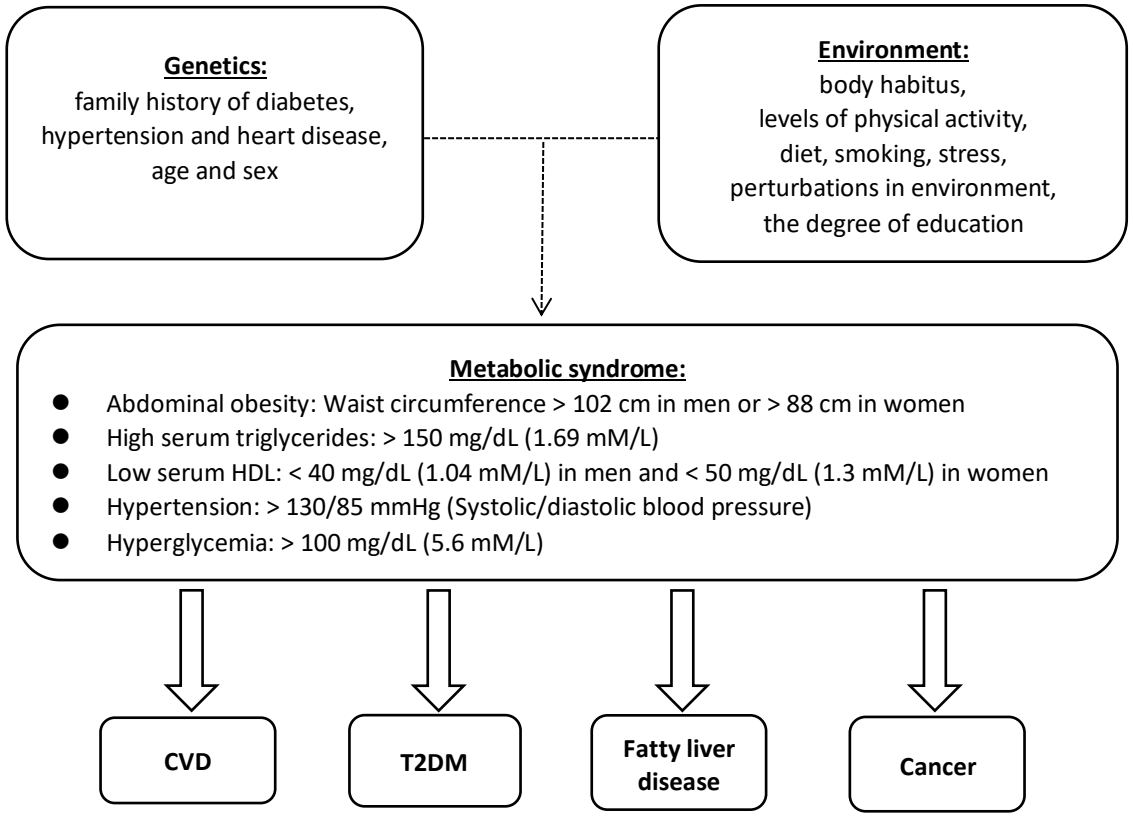

Figure 1.1 **The metabolic syndrome (MetS)**. MetS is a complex metabolic disorder, mediated by genetic and environmental factors. The presence of any three of five criteria (abdominal obesity, high serum triglycerides, low serum HDL, hypertension and hyperglycemia) is used to diagnose MetS. Consequently, individuals suffering from MetS are at increased risks to develop chronic diseases, such as CVD, T2DM, fatty liver disease and cancer.

Chapter 1

Disturbances in metabolic syndrome

Notably, metabolic disturbances are associated with MetS and other diseases that are linked to metabolism. Relevantly, metabolism is a term that is used to describe all chemical reactions involved in maintaining the living state (and energy levels) of cells and the organism and can be divided into two categories:1) catabolism: the breakdown of molecules to obtain energy and 2) anabolism: the synthesis of all compounds thereby storing energy inside cells.[13] Evidently, metabolism is closely linked to nutrition and availability of nutrients, such as carbohydrate, proteins and fats. As these nutrients need to be readily internalized in the human body, they are being 'cut' into smaller building blocks by proteins called enzymes, which therefore carry an essential function in both catabolic and anabolic processes. An example of such enzymes are lysosomal hydrolases that are capable to degrade lipids, carbohydrates, proteins and nucleic acids and also regulate cellular metabolism.[14] It is not surprising that an imbalance between catabolism and anabolism leads to disturbances in glucose and lipid metabolism, which are two characteristics in MetS.

Disturbances in glucose metabolism

Being the most important source of energy in the human body, glucose circulates in the human blood as blood sugar, which is derived from 1) the degradation of carbohydrates from the diet and 2) the glycogenolysis from glycogen stored in liver and muscle. Notably, insulin and glucagon are potent hormones regulating glucose metabolism[15] to maintaining blood glucose concentration in the normal range. Insulin is a hormone secreted by pancreatic β cells, which exerts its actions in controlling blood glucose concentration in three ways: 1) insulin stimulates cells of insulin-sensitive peripheral tissues (primarily skeletal muscle) to increase glucose uptake via a mechanism by which glucose transporters from the intracellular pool are mobilized to the plasma membrane via insulin stimulation. This process is also referred to as insulin-mediated glucose uptake.[16,17] 2) insulin acts on the liver to promote glycogenesis to reduce blood glucose and 3) insulin simultaneously inhibits the secretion of glucagon (a hormone secreted by pancreatic α cells, sustains blood glucose concentration during fasting or blood glucose concentration below normal ranges via promoting glycogenolysis in the liver), thus stopping the liver producing glucose.[15] However, **under MetS conditions**, glucose metabolism is disturbed due to the imbalance of blood glucose clearance (i.e. glucose uptake by skeletal muscle cells and glycogenesis) and glucose production (i.e. glycogenolysis). These disturbed processes are induced by abnormal pancreatic β-cell action in secreting insulin, lack of insulin-stimulated glucose disappearance, poorly regulated hepatic glucose production and the dysfunction of several enzymes that involve with glucose metabolism.[15,18] As a result, the disturbed glucose metabolism leads to increased fasting and postprandial glucose concentrations, that are known as hyperglycemia. Furthermore, hyperglycemia can also result in diabetic ketoacidosis,[19] one type of metabolic acidosis that is a clinical disturbance characterized by an increase in plasma acidity (a reduction of plasma pH).[20] Diabetic ketoacidosis is a serious complication of diabetes, which occurs when the body break down

fat as energy source instead of glucose due to relative insulin deficiency, leading to the excessive production of acidic ketones in the circulation resulting in disturbances of the acid-base balance in the blood. As such, these accumulating evidences suggest that disturbances in glucose metabolism contribute to MetS.

Disturbances in lipid metabolism

Besides disturbances in glucose metabolism, abnormal lipid metabolism also has a major impact on MetS, for instance, central obesity and dyslipidemia, individual risk factors which are strongly associated with MetS.[21] Relevantly, lipids are a group of hydrophobic compounds that serve as a major energy source for the body and act as structural components of cell membranes. Triglycerides (TGs) and cholesterol are the most important lipids.[22] TGs are a key energy source that is made up of free fatty acids (FFAs) that are ester-linked to a glycerol backbone and cholesterol has numerous roles, including being a component of cell membranes, the precursor for steroid hormones and vitamin D, and for oxysterols and bile acids.[22-25] It is therefore of critical importance to maintain normal lipid metabolism. Notably, lipid metabolism is a complex process, which involves multiple steps from synthesis of lipids within the body or dietary intake of lipids to degradation or transformation into several lipid-containing structures in the body.[26] Physiologically, lipid metabolism is regulated by several kinds of enzymes (involved in lipogenesis, lipolysis and lipids transformation) as well as the distribution of lipids over the body by means of lipoproteins which are complexes of lipids and specific proteins.[27] Lipoproteins including chylomicrons, very-low-density lipoproteins (VLDL), low-density lipoprotein (LDL) and high-density lipoproteins (HDL), constitute lipid transport systems, which allow insoluble TGs and cholesterol to be transported throughout the bloodstream. As such, deficiencies in any of these processes or any imbalance in lipid catabolism and/or anabolism lead to serious disturbances of lipid metabolism, creating clinical problems. MetS is often the result of an excessive intake of nutrients, leading to elevated plasma TGs levels, high level of plasma cholesterol and reduced level of HDL, which is referred to as dyslipidemia.[28] Furthermore, ectopic fat accumulation, also known as the excessive accumulation of triglycerides in non-fat storing organs,[29] also arises. For instance, excess fat accumulates in the liver, resulting in fatty liver diseases. Similar to the liver, lipids also accumulate ectopically inside the muscle, a condition referred to as myosteatosis. In this condition, fat infiltrates within the myocytes (intramyocellular fat) and fat within the fascia surrounds the skeletal muscle (intermuscular fat),[30] inducing metabolic disturbances in skeletal muscles. Moreover, the excess amount of lipids present in the human body during MetS is also one of the triggers to induce insulin resistance in adipose tissue, skeletal muscles and liver.[31] Indeed, it has been suggested that lipid infusions designed to increase plasma FFAs concentrations reduce insulin-stimulated glucose disposal in humans,[32,33] implying that accumulation of FFAs contributes to the pathology of insulin resistance. Altogether, considering the influences of disturbed lipid metabolism on MetS, it is therefore of great importance to maintain the balance between lipid catabolism and anabolism.

Chapter 1

Type 2 diabetes mellitus

The prevalence of T2DM has been increasing exponentially. Indeed, it is estimated that 1 in 11 adults aged 20–79 years (463 million adults) had diabetes mellitus globally in 2019,[34] which is expected to rise to 642 million by 2040.[35] T2DM is a type of diabetes mellitus, which accounts for approximately 90% of all cases.[36] Moreover, due to the increasing rates of childhood obesity, T2DM has become more common in children, teenagers and adolescents.[37] Consistent with the rising prevalence of T2DM, the healthcare costs of diabetes are also huge, accounting for 10% of global health care expenditure (USD 760 billion).[34] Considering the high prevalence and healthcare expenditure of T2DM, finding new targets against T2DM is therefore needed for effective treatments.

As previously indicated, MetS is a predictor for the development of T2DM,[38] which is characterized by a relative insulin deficiency and an impaired insulin sensitivity in certain specific target organs.[39,40] Relative insulin deficiency refers to the defect in insulin action resulting from inadequate insulin secretion and/or diminished tissue responses to insulin.[50] Hyperglycemia, or high blood glucose is the main feature of T2DM. Insulin exerts its function in lowering blood glucose concentration by promoting the clearance of blood glucose, whereas, glucagon plays a major role in sustaining blood glucose by stimulating hepatic glucose production.[15,41,42] As such, defects in insulin action induces hyperglycemia which leads to the development of T2DM.

Relevantly, insulin sensitivity refers to how sensitive the body's cells respond to insulin, in which high insulin sensitivity allows cells to use blood glucose more effectively, thus reducing blood glucose or vice versa.[43] In general, insulin sensitivity can be subdivided into whole-body insulin sensitivity and tissue-specific insulin sensitivity, including peripheral and hepatic insulin sensitivity. Whole-body insulin sensitivity is the sum of peripheral and hepatic insulin sensitivity.[44] In the context of the pathogenesis of T2DM, impaired peripheral insulin sensitivity reduces insulin-mediated glucose uptake from blood into peripheral tissues (mainly skeletal muscle), while impaired hepatic insulin sensitivity manifests as the inability of insulin to suppress hepatic glucose production.[45] Given the differences in pathogenesis of tissue-specific insulin sensitivity in T2DM, determining peripheral and hepatic insulin sensitivity is important for T2DM patients as it might be helpful to achieve organ-specific treatment for T2DM.[46]

Non-alcoholic fatty liver disease

Besides T2DM, MetS is also strongly linked with non-alcoholic fatty liver disease (NAFLD).[47] NAFLD covers a disease spectrum, initiating with hepatic steatosis defined by the presence of ≥5% hepatic fat (referred to non-alcoholic fatty liver (NAFL)) in the absence of chronic viral hepatitis and alcohol consumption (21 drinks/week in men and 14 drinks/week in women).[48]

In more advanced stages, hepatic steatosis may develop into nonalcoholic steatohepatitis (NASH), which is characterized by a combination of hepatic steatosis and inflammation with or without fibrosis. The transition from NAFL to NASH is important as this transition predisposes patients to progress into advanced-stage liver diseases such as cirrhosis and hepatocellular carcinoma (HCC).[49] Therefore, it indicates that hepatic inflammation during NASH is a key step in the etiology of NAFLD.

Recently, NAFLD has become the most prevalent chronic liver disease worldwide.[50] Indeed, in the last three decades, the prevalence of NAFLD has increased at a constant rate. Globally, it is currently estimated that NAFLD affects about 25% of the general population and NASH has been calculated at 2–5% of the general population.[51] Additionally, Estes *et al*. predicted the future prevalence of NAFLD population, expecting that the prevalence of NAFLD will increase with 21% in 2030 with a number of 100.9 million NAFLD patients, of which 27.0 million patients will suffer from NASH.[52] In line with the increasing prevalence of NAFLD, the economic costs related to NAFLD are also rising. Studies have indicated that the United States alone spent approximately 103 billion dollars every year on NAFLD-related costs, while in European countries (i.e., France, the United Kingdom, Germany, and Italy), the expenditure of NAFLD-related healthcare was around €35 billion per year.[53] As such, it suggests that NAFLD brings a huge economic burden on the current healthcare systems, emphasizing the urgent need for earlier and faster treatment to prevent further development of this disease.

Multiple pathophysiological processes are involved with NAFLD, including dysregulation of lipid metabolism, increased hepatic inflammation, and the presence of hepatic fibrosis.[54] However, how patients with steatosis develop hepatic inflammation is still unclear, which also makes clinicians face a major challenge in diagnosing and treating NASH. Indeed, NAFL and NASH are often underdiagnosed due to the non-specific nature of symptoms (fatigue and irritation of right upper quadrant) and lack of sensitive non-invasive methods.[55] Though plasma liver enzymes, such as alanine amino transaminase (ALT) and aspartate amino transaminase (AST), are primarily determined, the elevation of these liver enzymes implies liver cell damage, which are not specific for NAFLD and moreover, some NAFLD patients show normal ALT levels.[56] Besides, several imaging methods have also been tested for NAFLD diagnosis, including ultrasound elastography, computed tomography (CT), magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS).[57] Although these imaging techniques can be used to define structural abnormalities of the liver, for instance fatty liver and fibrosis, these techniques lack the ability to detect the inflammatory component (being NASH).[58] So far, the golden standard used to diagnose NASH is the histological examination of a liver biopsy by using a predetermined scoring system to include a mixture of several liver pathologic features (steatosis, hepatocyte ballooning, lobular inflammation, portal inflammation and fibrosis).[59,60] However, this invasive procedure has important limitations, such as risks for complications including pain, bleeding, patient stress and discomfort and even

death.[61] Thus, considering the shortcomings of the current tools to diagnose NASH, non-invasive, specific and sensitive methods are needed to diagnose NASH at an early stage to prevent further progress of this disease.

Currently, while therapies to reduce hepatic steatosis are known, there are no effective therapeutic approaches available for reducing hepatic inflammation.[54] To date, dietary changes and lifestyle interventions resulting in weight reduction are currently the first-line therapy for NAFLD patients.[62] However, for morbidly obese patients, dietary change and lifestyle interventions are not sufficient to achieve sustained weight loss. Therefore, bariatric surgery (more recently termed metabolic surgery) can be considered,[63] typically resulting in massive weight loss and concordant improvements in liver histology.[64] Besides the dietary and lifestyle interventions, pharmaceutical interventions that aim at targeting lipotoxicity, insulin/glucose metabolism, hepatic inflammation and fibrosis, and bile acid metabolism are also utilized for the treatment of NAFLD.[54] Though multiple agents are currently under evaluation in clinical trials for treatment of NASH,[65] none of those therapies are already approved. As such, novel and effective therapeutic strategies against hepatic inflammation are needed.
Recent evidences point toward the link between plasma lysosomal enzymes to the present of hepatic inflammation and MetS in general. In line, we have previously demonstrated that the lysosomal enzyme Cathepsin D (CTSD) is a sensitive and specific marker for the development of hepatic inflammation.[66,67] In the current thesis we have further explored the link between plasma CTSD to different aspect of the MetS.

Lysosomes: location and formation

When in the 1950s, the Belgian cytologist Christian René de Duve and his team aimed to purify and isolate glucose 6-phosphatase, which is an enzyme that hydrolyzes glucose 6-phosphate from cellular extracts they discovered the lysosome. Lysosomes are membrane-enclosed cellular organelles, which are broadly distributed throughout the cytoplasm in nearly all types of eukaryotic cells (cells with a clearly defined nucleus).[68,69] Under the microscope, lysosomes are visualized as dense spherical vacuoles and can display considerable variation in size[70] (varying from 0.1 µm to 1.2 µm) and shape due to different materials that have been taken up.[71] Lysosomes consist of a membrane, hydrolytic enzymes as well as an acidic lumen with pH 4.5~5.0 (Figure 1.2). The formation of lysosomes starts at the cell membrane and occurs in three steps (Figure 1.3). Firstly, with the help of specialized membrane components (for instance, receptors), molecules are taken up from outside the cell into endocytic vesicles, which bud from the plasma membrane and then form early endosomes. Early endosomes then gradually mature into late endosomes. During this process endosome forming specialized membrane components are recycled back to the plasma membrane. Finally, the late endosome then fuses with lysosomes, which are transport vesicles formed and budded from the Golgi apparatus, carrying acid hydrolases that are tagged with the mannose-6-phosphate

(M6P) receptor (transmembrane glycoproteins that target enzymes to lysosomes) in the Golgi apparatus. After the lysosomes fuse with late endosomes, the internal pH of the endosome is reduced to about 5.5. Afterwards, the lower internal pH dissociates the acid hydrolases from the M6P receptor to release the acid hydrolases into the lumen of endosome/lysosome, thus forming lysosomes. Meanwhile, the M6P receptors are recycled to the Golgi apparatus or plasma membrane.[71]

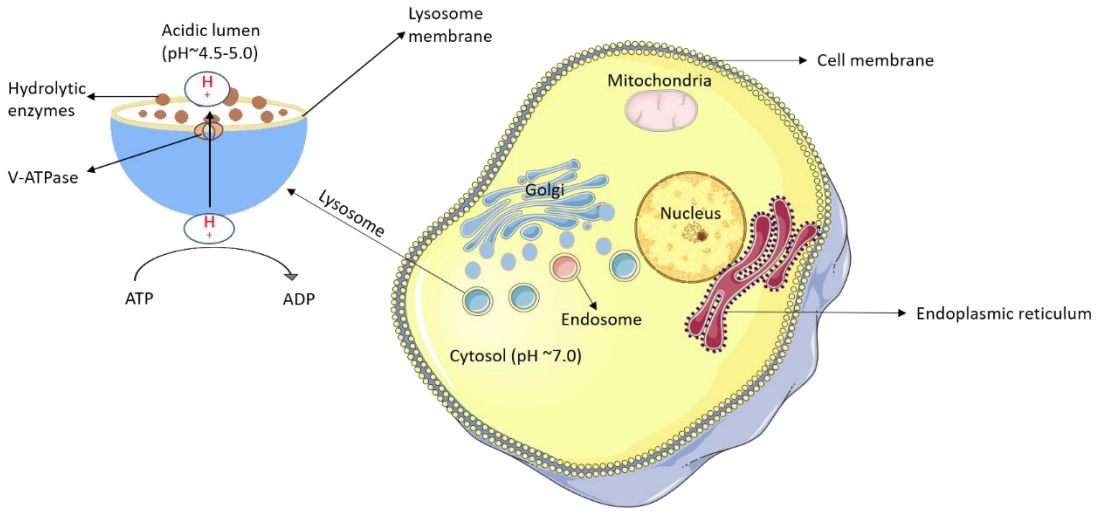

Figure 1.2 **Lysosomal structure**. For the simplest form, lysosomes are visualized as dense spherical vacuoles in cytoplasm. Structurally, lysosomes are characterized by a membrane, hydrolytic enzymes and an acidic lumen. The acidic pH of lysosomal lumen is generated by the action of the vacuolar-type H^+- ATPase (v-ATPase) located at the lysosomal membrane, which imports H^+ ions from the cytosol where ATP is being hydrolyzed. (Figures are adapted from Servier Medical Art)

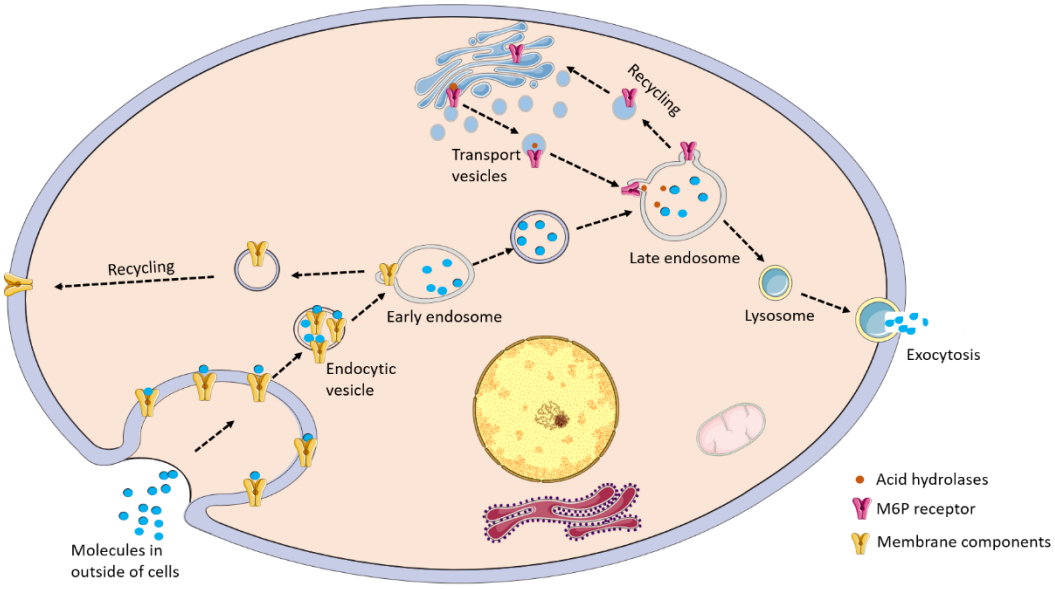

Figure 1.3 **Lysosome formation**. Molecules are taken up from outside the cell in endocytic vesicles, which form early endosomes. As the early endosomes mature into late endosomes, membrane components are recycled to plasma membrane. Transport vesicles carrying acid hydrolases combined with the M6P receptor from the Golgi apparatus then fuse with late endosomes, which mature into lysosomes as the hydrolytic enzymes are released into the lumen of endosome/lysosome. As the acid hydrolases dissociate from the M6P receptor, the receptors are recycled to the Golgi apparatus. Additionally, lysosome can also merge with plasma membrane to release its content into outside of the cells. (Figures are adapted from Servier Medical Art)

Lysosomal enzymes

Lysosomes contain around 60 degradative lysosomal enzymes, that are capable of degrading proteins, lipids, carbohydrates and nucleic acids,[71,72] thereby playing crucial roles in metabolism. According to the different substrates, lysosomal enzymes can be categorized into five classes: 1) phosphatases: including acid phosphatase and acid phosphodiesterase, whose substrates are phosphomono-esters and phosphodiesterase, respectively, 2) nucleases: their substrates are DNA and RNA, including acid deoxyribonuclease and acid ribonuclease, 3) polysaccharides/mucopolysaccharides hydrolyzing enzymes: there are seven subclasses in total, for instance, glucosidase and mannosidase, whose substrates are glycogen and mannosides, respectively, 4) proteases: including cathepsins, collagenase and peptidase, whose substrates are proteins, collagen and peptides, respectively, 5) lipid degrading enzymes: including esterase and phospolipase, whose substrates are fatty acyl esters and phospholipids, respectively.[73] All of the lysosomal enzymes are acid hydrolases, which are optimally active at acidic pH (~4.5-5.0).[74] However, studies have shown that several lysosomal enzymes also maintain reduced activity in neutral pH.[75,76]

Chapter 1

Lysosomal enzyme synthesis

Lysosomal enzymes, which are synthesized in the rough endoplasmic reticulum (ER), move across the ER membrane to the lumen of ER to get N-glycosylation, which are essential to ensure proper function of lysosomal hydrolases, for instance mediating lysosomal sorting, transporting and folding of newly synthesized lysosomal enzymes in ER.[77,78] Subsequently, lysosomal enzymes are transported to *cis*-Golgi apparatus, where lysosomal enzymes acquire the M6P ligand that identifies the protein as destined for the lysosome.[79] After transportation to the *trans*-Golgi apparatus, lysosomal enzymes are recognized by the interaction of M6P ligand with the M6P receptor, which is present in *trans*-Golgi network. As mentioned before, transport vesicles carrying the receptor-protein complex from the Golgi apparatus, fuse with late endosomes, where dissociation of lysosomal enzymes occurs. Eventually, M6P receptors are recycled either to the Golgi or to the plasma membrane once lysosomal enzymes have been delivered to the lysosomes (Figure 1.4).[77] While lysosomal enzymes are primarily targeted to the lysosomal compartment, a fraction can follow the alternative secretion route via which they are released in the extracellular milieu.[80] Indeed, lysosomal enzymes (e.g. cathepsin D) have been detected in the plasma.[46,81] Therefore, lysosomal enzymes form an essential unit that mediate the previously mentioned regulatory lysosomal functions such as maintaining physiological homeostasis, especially in metabolism.[82]

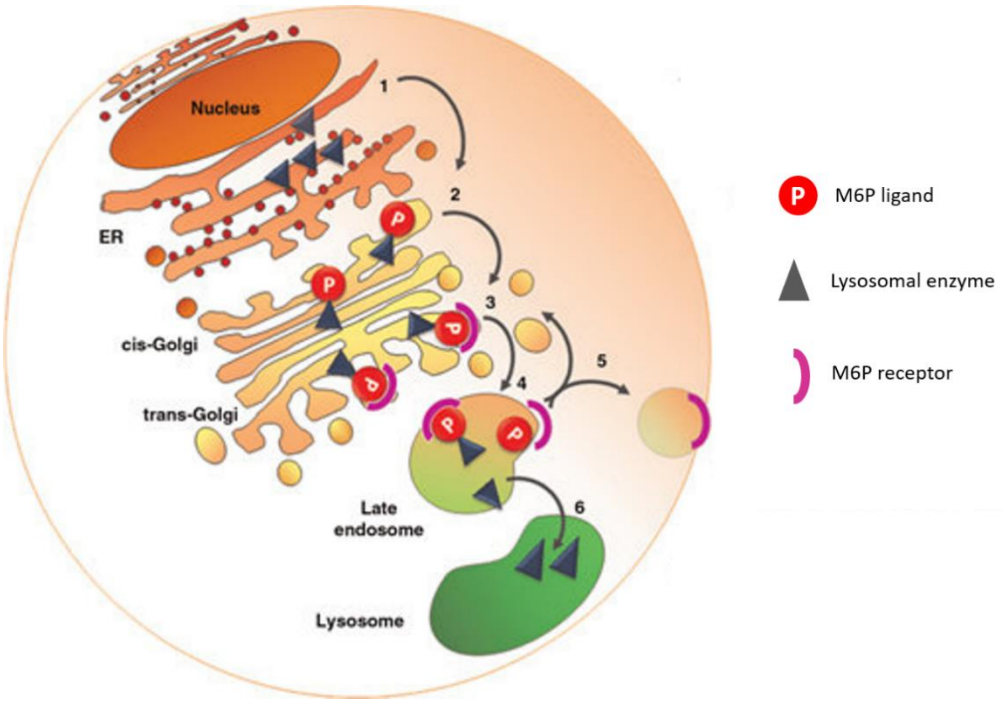

Figure 1.4 **The synthesis of lysosomal enzymes**. 1) Lysosomal enzymes are synthesized in endoplasmic reticulum (ER). 2) Lysosomal enzymes move to *cis*-Golgi apparatus, where lysosomal enzymes acquire the M6P ligand. 3) Lysosomal enzymes are recognized via interaction of M6P ligand and M6P receptor from *trans*-Golgi. 4) The complex of receptor-protein is packaged into transport vesicles, which fuse with late endosome. 5) M6P receptors are recycled either to Golgi apparatus, or to plasma membrane. 6) After dissociation from the M6P receptor, lysosomal enzymes are eventually delivered to lysosomes. (Figure is adapted from *Filocamo et al*[77])

Chapter 1

Lysosomal function

Degradation of macromolecules

An essential characteristic of the lysosome concerns its acidic lumen (pH~4.5–5.0), which serves for the optimal functionality of the lysosomal enzymes. The acidic pH of the lysosomal lumen is generated by the action of the vacuolar-type H^+-ATPase (v-ATPase) located at the lysosomal membrane, which imports H^+ ions from the cytosol where ATP is being hydrolyzed (Figure 1.2).[71] Functionally, the lysosome used to be mainly considered as the key digestion system of the cell, where not only materials from outside the cell are degraded, but also components of cells, for instance, obsolete organelles or un-used material of the cytoplasm are digested.[71] As such, lysosomes digest material derived from three pathways: 1) endocytosis: molecules taken up from outside the cell via endocytic vesicles, 2) phagocytosis: large particles (such as bacteria) are taken up into phagocytic vacuoles or phagosomes and 3) autophagy: the degradation and recycling of the cell's own components.[68,71] After lysosomal degradation, many of the products, such as amino acids and nucleotides, are recycled back to the cell for the synthesis of new cellular components or exocytosed.[69]

Regulating cellular processes to maintain physiological homeostasis

Besides functioning as a digestion system of cells, lysosomes have emerged as crucial regulators of cell homeostasis.[83] Recent studies have demonstrated that lysosomes are also involved with other cellular processes, including killing of intracellular pathogens, antigen presentation, plasma membrane repair, exosome release, cell adhesion and migration, tumor invasion and metastasis, apoptosis, metabolic signaling and gene regulation (Figure 1.5).[68,84,85] These evidences therefore indicate that lysosomes play a pivotal role in cellular function and physiological homeostasis in general.

Lysosomal exocytosis

While lysosomes are located in cytoplasm, their positions can be changed as a result of lysosome movement between the center and the periphery of the cell, which can be influenced by cellular perturbations.[68,86,87] Relevantly, as mentioned above, products after lysosomal degradation can also be exocytosed. Indeed, emerging studies suggest that lysosomes are also involved with cell-to-cell communication in extracellular spaces mainly via lysosomal exocytosis,[88,89] a process via which lysosomes release their lysosomal content outside of cells in response to different stimuli. This is achieved via fusion of lysosomes with the plasma membrane.[90-92] Lysosomal exocytosis is an important function that any cell type performs.[93] Upon release, the content of lysosomes travels through the extracellular fluid for varying times and distances and subsequently interacts with recognized target cells to achieve the intercellular communication.[89] Relevantly, lysosomal exocytosis plays a crucial role in

several physiological processes, such as degranulation in cytotoxic T lymphocytes,[94] bone resorption by osteoclasts,[95] melanocyte function during pigmentation,[96] immune response against parasitic attack,[97,98] hydrolase release by spermatozoa during fertilization[99] and platelet function in coagulation.[100] As such, lysosomal exocytosis appears to play key physiological functions.

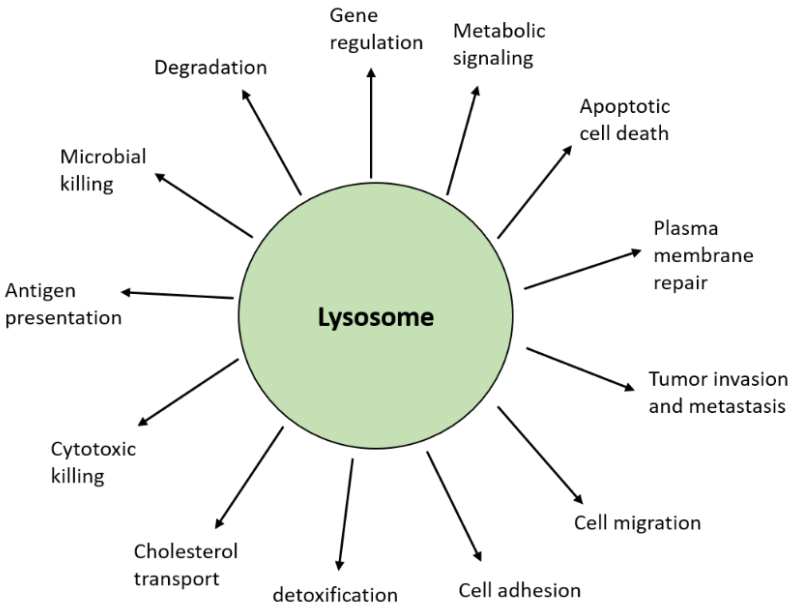

Figure 1.5 **Lysosomal functions**. Besides macromolecular degradation, lysosomes also participate in other important cellular processes, including gene regulation, metabolic signaling, apoptotic cell death, plasma membrane repair, tumor invasion and metastasis, cell migration and adhesion, detoxification, cholesterol transport, cytotoxic and microbial killing as well as antigen presentation. (Figure is adapted from *Pu et al*[68])

Lysosomal enzymes in the context of metabolic syndrome

Lysosomal enzymes in metabolic syndrome

The lysosome has emerged as a central hub for metabolic signaling, in which signaling pathways connect lysosomes to key anabolic and catabolic processes that control many facets of cellular metabolism.[101] Moreover, as previously mentioned, lysosomal enzymes are also involved with metabolism, functioning in degrading lipids (i.e., lysosomal acid lipase), carbohydrates (i.e., β-galactosidase), proteins (i.e., cathepsins) and nucleic acids (i.e., nucleases).[102,103] On one hand, emerging studies have shown that the dysfunction of lysosomal enzymes results in several metabolic disorders, implying the importance of lysosomal enzymes in metabolism. Indeed, studies have observed dysfunction of pancreatic islets lysosomes and reduced islets catalytic activity of several lysosomal enzymes that are involved with glucose metabolism (i.e., acid glucan-1,4-alpha-glucosidase and acid alpha-glucosidase) as an important defect in diabetic rats. Consequently, this defect impairs the transduction mechanisms for nutrient-stimulated insulin release in islets, thus resulting in disturbances of glucose metabolism in diabetic rats.[104,105] On the other hand, metabolic disturbances, as

observed during MetS conditions, can also cause lysosomal enzyme dysfunction. For instance, obesity causes a decline in cathepsin L (one type of cathepsins) maturation likely via oxidative stress and lysosomal pH abnormality. This further results in the downregulation of cathepsin L activity that evokes abnormal activation of another enzyme (ie., cathepsin B), which contributes to lysosomal dysfunction.[18] Similarly, obesity-induced ectopic lipid accumulation in the liver (disordered lipid metabolism) has been shown to associate with abnormality of lysosomal proteases as well as other lysosomal enzymes.[18] These evidences thereby point toward the critical importance of lysosomal enzymes in metabolism in the context of MetS.

Lysosomal enzymes in T2DM

Lysosomal enzymes play a role in glycogenesis, glycogenolysis and glycosylation and are therefore linked to glucose metabolism overall. Indeed, studies have shown that several abnormal cathepsins are linked to insulin resistance, highlighting the critical importance of lysosomal enzymes in T2DM.[46,106-108] Additionally, another lysosomal enzyme, hexosaminidase A also plays a role in the regulation of insulin sensitivity as it converts GM2 ganglioside (one type of ganglioside that is a molecule composed of a glycosphingolipid) to GM3 ganglioside (that participates to insulin resistance) by removing an N-acetyl-glucosamine residue.[109-111] It is therefore not surprising that lysosomal enzyme dysfunction is one of the triggers to induce disturbances in glucose metabolism, thereby leading to the development of T2DM. Additionally, T2DM can also cause abnormality of lysosomal enzymes. For instance, streptozotocin-induced diabetes leads to decrease in the specific activities of cysteine proteases (especially cathepsin B) in the liver of rats, in which lower gene expression may be one of the mechanisms responsible for lower enzymatic activity.[112] Altogether, these accumulating evidences highlight the important role of lysosomal enzymes in the context of T2DM, thereby providing a new perspective on the pathogenesis of T2DM.

Lysosomal enzymes in NAFLD

Besides their role in T2DM, lysosomal enzymes also appear to be of importance in NAFLD due to their role in regulating lipid metabolism. Indeed, recent studies have revealed the role of lysosomal acid lipase-mediated lipolysis in regulating the production of lipid mediator (i.e., eicosanoids and thromboxane B2, a class of bioactive lipids that are produced locally through specific biosynthetic pathways in response to extracellular stimuli[113]), VLDL secretion, extracellular degradation of aggregated-LDL, and adipose tissue lipolysis,[103,114] suggesting the importance of lysosomal enzymes in lipid metabolism. However, dysfunction of lysosomal enzymes in lipid metabolism is one of pathological triggers to induce the development of NAFLD. For instance, emerging studies have shown that NAFLD is characterized by a specific deficit in lysosomal acid lipase activity, suggesting a pathogenic role of lysosomal acid lipase in NAFLD.[115] Additionally, other studies also have observed decreased expression of hepatic cathepsins (i.e., cathepsin B, cathepsin D and cathepsin L) in NAFLD patients, which is associated with the suppression of autophagic proteolysis.[116] As such, compelling evidences

suggest a link between lysosomal enzymes and NAFLD pathogenesis placing lysosomal enzymes also at the center for understanding the mechanisms of NAFLD progression.

Cathepsin D in metabolism and metabolic syndrome

Cathepsin D

Cathepsin D (CTSD) is a type of cathepsin, which is a family of lysosomal proteases containing several members ranging from cathepsin A to cathepsin Z.[117-119] These cathepsins are roughly classified into three types according to the type of amino acid present in the active center: serine proteases (for example cathepsin A and G), aspartic proteases (for example CTSD and cathepsin E) and cysteine proteases (cathepsin B and L) and many other cathepsins.[120,121] Similar to other lysosomal enzymes, CTSD is also synthesized in rough ER as pre-pro-CTSD, which comprises 412 amino acids (aa) that contain an N-terminal secretion signal peptide (S) of 20 aa and a pro-peptide (P) of 44 aa.[69] This signal peptide is removed during co-translational translocation of CTSD across the ER membrane, thereby generating an inactive pro-CTSD (392 aa, 52 kDa) (Figure 1.6).[76] Following removal of the signal peptide, sugars are attached at two N-linked glycosylation sites at asparagine residues 134 and 263 (pre-pro-CTSD numbering). After glycosylation, pro-CTSD is transported to Golgi, where the two N-linked oligosaccharides of pro-CTSD are covalently modified and their mannose residues are phosphorylated at position six,[122] thus forming M6P ligand. Under the recognition of M6P receptor,[123] pro-CTSD enters the acidic endosomal and lysosomal compartment (Figure 1.6). Firstly, due to the low pH of late endosomes, the pro-CTSD dissociates from M6P receptors and the phosphate group is removed. Then, the pro-peptide from pro-CTSD is removed to generate an active intermediate (48 kDa, 348 aa) single-chain molecule. Further proteolytic cleavage carried out by cathepsin B and L yields the mature active lysosomal protease which is composed of heavy (34 kDa) and light (14 kDa) chains linked by non-covalent interactions.[124-126] Finally, seven amino acid residues between the heavy and light chain are removed[127] and several more amino acids are removed from the carboxyl terminus of the 34 kDa heavy chain,[128] thus forming the mature CTSD.

Functionally, based on the ability of CTSD in cleaving structural and functional proteins and peptides, it has been suggested that CTSD has numerous physiological functions, such as, degrading intracellular proteins, activating and degrading polypeptide hormones and growth factors, activating enzymatic precursors, processing enzyme activators, inhibitors and brain antigen as well as regulating programmed cell death.[76] Moreover, emerging studies demonstrate that CTSD also plays an important role in metabolism.[46,129] As such, it suggests that CTSD has important roles in maintaining physiological homeostasis.

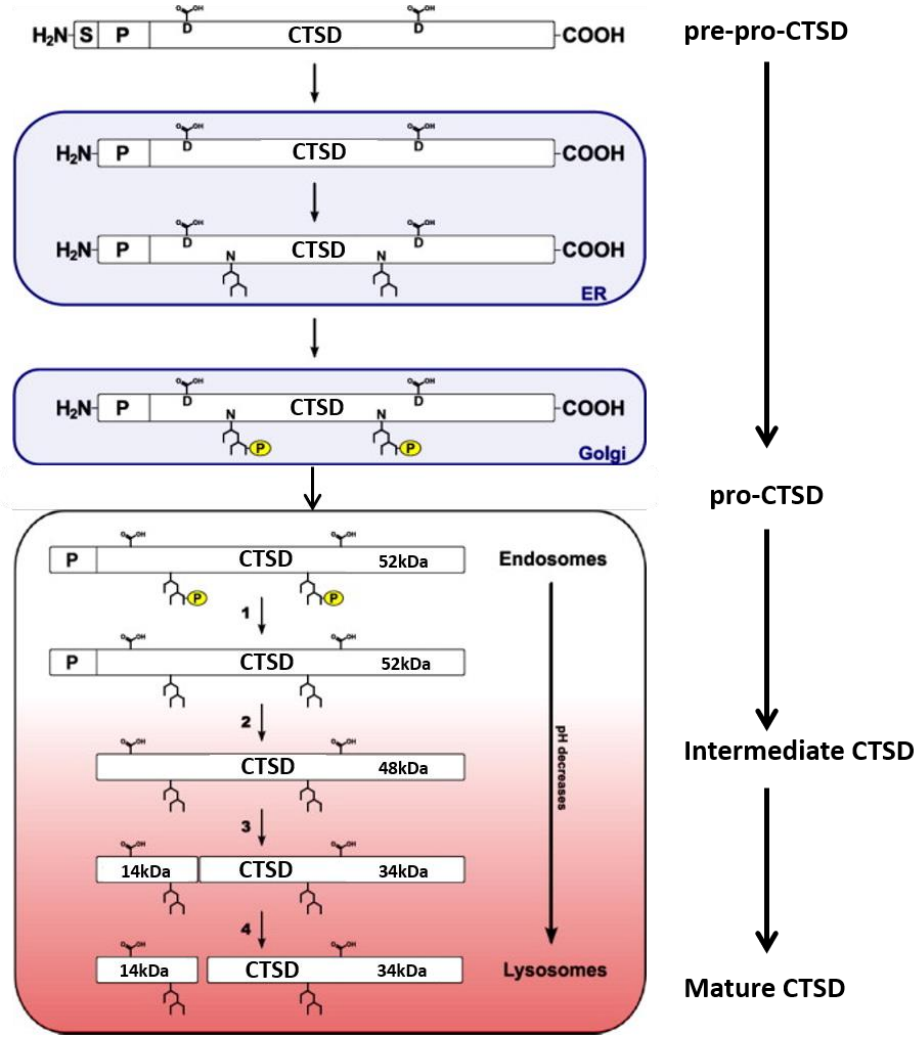

Figure 1.6 **The formation of mature CTSD.** CTSD maturation includes the transformations of pre-pro-CTSD to pro-CTSD, intermediate CTSD and mature CTSD, which occurs in different cellular organelles. CTSD is synthesized as a pre-pro-CTSD (412 aa, human) on rough ER that contains an N-terminal secretion signal peptide (S) of 20 aa and a pro-peptide (P) of 44 aa. The signal peptide is removed during co-translational translocation of pre-pro-CTSD across the ER membrane to generate an inactive pro-CTSD. In ER, sugars are attached at two N-linked glycosylation sites at asparagine residues N134 and N263 (pre-pro-CTSD numbering). After transported to Golgi, the two N-linked oligosaccharides of pro-CTSD are covalently modified and their mannose residues are phosphorylated at position six, thereby forming M6P that targeting pro-CTSD to endosomes/lysosomes. Upon pro-CTSD entering the late endosomes via the recognition of M6P receptors, there are mainly four processes: 1) the low pH of late endosomes dissociates pro-CTSD from their receptors and subsequently the phosphate group is removed. 2) The pro-peptide (44 amino acids) is removed from pro-CTSD to generate an active intermediate (48 kDa, 348 aa) single-chain molecule. The removal of pro-peptide is carried out by cathepsin B and L, independent of CTSD autocatalytic activity. 3) The intermediate CTSD is further processed by cathepsin B or L into mature two-chain form comprising an amino terminal light chain (14 kDa) and a carboxyl-terminal heavy chain (34 kDa). 4) During this conversion, seven amino acids between heavy and light chain and several other residues are removed from carboxyl terminus of the heavy chain to form the mature CTSD. (Figure is adapted from *Zaidi et al*.[130])

CTSD in metabolism

As previously mentioned, CTSD has been reported to play a role in maintaining tissue homeostasis and metabolism,[131] of which glucose metabolism and lipid metabolism are two important aspects. Regarding glucose metabolism, insulin is identified as a potent hormonal regulator of both glucose appearance and disappearance in the circulation, suggesting the pivotal role of insulin in glucose metabolism. Relevantly, CTSD is one of the major lysosomal proteases that contributes to the conversion of proinsulin to insulin in pancreatic Langerhans cells and rat hepatocytes,[132] highlighting the important role of CTSD in insulin production. Likewise, our group previously found that inhibiting CTSD activity reduces plasma insulin levels in steatosis rats.[133] Vice versa, insulin-treated diabetic rats showed increased CTSD activity in liver, kidney, heart and brain,[134] thereby pointing towards the link between CTSD and insulin. Thus, these evidences provide support for a role of CTSD in glucose metabolism via influencing insulin. Besides glucose metabolism, CTSD also plays a role in lipid metabolism. Indeed, our previous study has indicated that CTSD regulates lipid metabolism in murine steatohepatitis, of which our data showed that inhibiting CTSD activity via pepstatin A (an inhibitor of aspartyl proteases) reduces circulating and hepatic lipid level, thus improving lipid metabolism.[129] Similarly, another recently published study from our group also reported that inhibiting CTSD activity by specific extracellular CTSD inhibitor reduces fatty liver and improves hepatic lipid metabolism,[133] highlighting the role of CTSD in lipid metabolism. Altogether, these observations further confirm the role of CTSD in regulating glucose and lipid metabolism.

CTSD in inflammation

Relevantly, besides the role in metabolism, CTSD is also directly linked to inflammation. Indeed, it has been observed that CTSD is up-regulated in macrophages of inflammatory bowel disease (a term used to describe disorders that involve chronic inflammation of the digestive tract).[135] Similarly, another study demonstrated that activation of CTSD contributes to intestinal inflammation in a mouse model for inflammatory bowel disease.[135,136] Additionally, our previous data also point towards a pivotal role for macrophage-derived CTSD in the development of hepatic inflammation.[129] Therefore, these evidences present a clear insight that CTSD is linked to inflammation.

CTSD in MetS

Lipid-mediated damage to lysosomal membranes has been shown to result in the unintended translocation of lysosomal enzymes into the cytosol and/or the extracellular environment,[137-141] for example, in the circulation. Indeed, emerging studies have focused the role of circulating CTSD in the context of MetS. For instance, it has been shown that high level of plasma CTSD is associated with increased risk for future coronary syndromes in 5 years follow-up community cohorts.[142] Similar to MetS, several studies have investigated the role of CTSD in T2DM. For instance, Liu *et al* indicated that circulating plasma CTSD levels are closely linked with the presence of T2DM, and plasma CTSD levels might serve as a novel biomarker for cardiac

dysfunction in newly diagnosed T2DM.[81] Likewise, Velders *et al* also demonstrated that CTSD improves the prediction of undetected diabetes in patients with myocardial infarction, which may be helpful in the a priori risk determination of diabetes as a motivation for confirmatory oral glucose tolerance test (OGTT).[143] Relevantly, our previous studies have shown that plasma CTSD levels also associate with hepatic inflammation in NASH patients, suggesting that plasma CTSD could serve as a non-invasive biomarker for earlier diagnosis of NASH.[66,67] Additionally, our group also demonstrated that inhibition of CTSD activity via pepstatin A reduced hepatic inflammation and improved lipid metabolism in murine steatohepatitis, suggesting that CTSD plays a key role in the development of hepatic inflammation and dyslipidemia. Altogether, given the fact that accumulating evidences have demonstrated CTSD is involved in metabolism and inflammation in the context of MetS, it is important to further investigate the link between plasma CTSD and different aspect of the MetS.

Chapter 1

Thesis aim and outline

While emerging studies have demonstrated that CTSD is involved in metabolism and inflammation, its link to different aspect of the MetS has not been completely understood. In this thesis, we investigated the link between plasma CTSD to MetS related diseases (i.e., NAFLD and T2DM).

Chapter 2 gives an overview of recent knowledge regarding NAFLD. We first describe current epidemiological data and its related clinical and economic costs. We then provide an overview of pathophysiological hepatic processes in NAFLD and highlight the systemic aspects of NAFLD that point toward metabolic crosstalk between organs as an important cause of metabolic disease. Finally, we end by highlighting the currently investigated therapeutic approaches for NAFLD.

Previously, we found that plasma CTSD plays a role in regulating lipid metabolism, which also correlates with hepatic inflammation in adult NASH patients, suggesting the contribution of the liver to plasma CTSD levels. However, pathologies such as T2DM, Alzheimer's disease and inflammatory bowel diseases have also been linked to CTSD, implying that other, non-hepatic organs are also linked to plasma CTSD levels. **Chapter 3** assesses whether, besides the liver, also the muscle associates with plasma CTSD levels. We demonstrate a positive association between myosteatosis (lipid accumulation in the muscle) and plasma CTSD levels, which is independent of sex, age, BMI, waist circumferences and hepatic steatosis.

Recent evidences emerged showing that plasma CTSD is associated with insulin sensitivity and hepatic inflammation. However, it remains unclear whether plasma CTSD is associated with hepatic and/or peripheral insulin sensitivity in humans. In **Chapter 4**, the links between plasma CTSD (levels/activity) and peripheral/hepatic insulin sensitivity as well as systemic inflammation were investigated in overweight and obese humans. We demonstrate that plasma CTSD activity, but not systemic inflammation, inversely related to hepatic insulin sensitivity.

Plasma levels of CTSD, which is optimally active in the acidic environment of lysosomes, has been reported to correlate with insulin resistance in a previous study. As plasma pH is slightly reduced in T2DM and the fact that we have previously shown that plasma CTSD activity is causally linked to insulin levels in vivo, it is likely that the activity of CTSD in plasma will be increased in T2DM compared to healthy individuals. However, currently the interaction between CTSD activity and levels to postprandial metabolic derangements in T2DM is not known. Therefore, **Chapter 5** investigates the link between plasma CTSD (activity/levels) and metabolic parameters of T2DM. In this chapter, we show that despite similar levels of plasma CTSD between T2DM patients and healthy individuals, a metabolically-induced reduction of the plasma pH results in increased plasma CTSD activity in T2DM patients.

Chapter 1

Insulin resistance is a major feature of NAFLD and is one of the multiple hits determining the progression from NAFL to NASH. Previous studies demonstrated that plasma CTSD levels are elevated in NASH patients and that plasma CTSD activity is associated with hepatic insulin resistance in overweight and obese humans. Thus, to increase our understanding regarding the mechanisms by which insulin resistance potentially mediates NAFLD progression, **Chapter 6** mainly focused on the association between plasma CTSD activity and insulin resistance in NAFLD patients. This chapter demonstrates that insulin resistance is independently associated with plasma CTSD activity in NAFLD patients.

Finally, in **Chapter 7** the key findings of this thesis are discussed in the context of these metabolic diseases along with a perspective view on their clinical implications.

Chapter 1

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Chapter 1

Chapter 2

Nonalcoholic fatty liver disease

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Chapter 2

Abstract:

Non-alcoholic fatty liver disease (NAFLD) is considered the hepatic manifestation of the metabolic syndrome (MetS) and comprises one of the largest health threats of the 21st century. In this chapter, we review the current state-of knowledge of NAFLD and underline the striking similarities with atherosclerosis. We first describe current epidemiological data showing the staggering increase of NAFLD numbers and its related clinical and economic costs. We then provide an overview of pathophysiological hepatic processes in NAFLD and highlight the systemic aspects of NAFLD that point towards metabolic crosstalk between organs as an important cause of metabolic disease. Finally, we end by highlighting the currently investigated therapeutic approaches for NAFLD, which also show strong similarities with a range of treatment options for atherosclerosis.

Chapter 2

1. Epidemiology

1.1 Definition, prevalence and incidence of NAFLD

Being the most prevalent chronic liver disease worldwide,[1] nonalcoholic fatty liver disease (NAFLD) covers a diseases spectrum, initiating with hepatic steatosis which is defined by the presence of ≥5% hepatic fat (referred to steatosis) in the absence of any secondary cause of hepatic steatosis such as chronic viral hepatitis and alcohol consumption (21 drinks/week in men and 14 drinks/week in women).[2,3] In a second, more advanced stage, hepatic steatosis may advance into non-alcoholic steatohepatitis (NASH), which is characterized by a combination of hepatic steatosis and inflammation in the presence or absence of fibrosis. Finally, NASH can progress into advanced-stage liver diseases such as cirrhosis and hepatocellular carcinoma (HCC).[2,4-11] Being the hepatic component of the metabolic syndrome (MetS),[3] NAFLD is commonly associated with other metabolic disorders such as obesity, which is also linked to the development of cardiovascular diseases such as atherosclerosis.[2,12] Considering these links between NAFLD, obesity and atherosclerosis, it is no surprise that NAFLD has become the most prevalent liver disease worldwide.[1]

Indeed, in the last three decades, the prevalence of NAFLD has increased at a constant rate. Numbers from a study from Younossi *et al.* showed the evolution of NAFLD prevalence in the United States from 1988 to 2008 ranging from 5.51% (1988-1994) to 9.84% (1999-2004) and 11.01% (2005-2008), indicating a two-fold increase over two decades.[13] At a global level, it is currently estimated that NAFLD affects about 25% of the general population. At the other hand, NASH has been calculated at 2 to 5% of the general population and so far, represents the minority of NAFLD patients (10%-20%).[14] However, while hepatic steatosis seems less harmful for the liver, patients suffering from steatosis are at increased risk for cardiac-related death.[15] Therefore, steatotic patients should be monitored even at an early stage of the disease. For the future, Estes *et al*. predicted the NAFLD population to increase with 21% in 2030, expecting a staggering 100.9 million NAFLD patients, of which 27.00 million patients would also suffer from NASH, the latter indicating a 63% increase in prevalence compared to current numbers.[16]

From a regional perspective, the pooled incidence of NAFLD in the West (being Europe and Northern America) was estimated to be 28 per 1,000 persons per year.[2,17] As mentioned previously, NAFLD has been reported as the most common liver disease in the United States.[18] However, within the Asian population, recent meta-data also indicated the global incidence rate of NAFLD at 50.9 cases per 1000 individuals per year.[1] Indeed, while it was initially perceived as a 'Western disease', NAFLD is now highly prevalent in all continents with the highest rates reported in South America (31%) and the Middle East (32%), followed by Asia (27%), the USA (24%) and Europe (23%), while being less common in Africa (14%).[17]

Taken these numbers into account, and especially those indicating the exponential increase of NASH patients, it is clear that NAFLD poses one of the largest burdens on current health care systems, emphasizing the urge for early and fast treatment to prevent further escalation of this disease.

1.2 Association with other diseases

NAFLD has been reported to be strongly linked to obesity, with a prevalence as high as 80% in obese patients and only 16% in individuals with a normal BMI and without metabolic risk factors.[19,20] Relevantly, obesity seems to play a role in both the initial process leading to simple steatosis, but also to its progression towards NASH.[21] Indeed, it has been demonstrated that the risk of NASH development is lower in lean than overweight/obese individuals.[22] Next to NASH, also patients suffering from hepatic fibrosis tend to be rather obese than non-obese (86% vs. 27%, respectively).[23]

Besides obesity, it has been suggested that NAFLD is also tightly linked to cardiovascular diseases.[24] Recently, clinical observations indicated that NASH increases atherosclerosis and cardiovascular risks by local overexpression of inflammatory mediators, endothelial damage, and regulators of blood pressure.[25,26] Others confirmed that NAFLD is independently associated with atherosclerosis progression.[25] Additionally, other studies demonstrated that NAFLD patients have impaired flow-mediated vasodilatation,[27] increased carotid-artery intimal medial thickness and an increased prevalence of carotid atherosclerotic plaques compared to healthy subjects,[28] independently of obesity and other established risk factors. These observations therefore emphasize the link between NAFLD and atherosclerosis.

Additionally, NAFLD has also been suggested as a risk factor for gastrointestinal tract malignancies, such as colorectal cancer.[29-31] Also, NAFLD is also associated with chronic kidney disease (CKD),[32] which is defined by the presence of kidney damage or a reduced glomerular filtration rate for 3 months or more.[33] Specifically, it has been suggested that the prevalence of CKD in NAFLD patients is between 4%–40%.[34] This association has been partly explained by the NAFLD-associated microvascular alterations that also affect the kidney.[35] Moreover these microvascular alterations have been linked to cerebrovascular disease, potentially contributing to cognitive impairment.[36] Finally, polycystic ovary syndrome (PCOS), a condition that leads to the production of higher-than-normal amounts of male hormones in women, is also associated with NAFLD.[37,38] However, the clinical significance as well as its pathophysiological basis remains to be further investigated.

1.3 Clinical, economic and social burden of NAFLD

In spite of its increased prevalence over the last decades, to our knowledge, the clinical burden of NAFLD has not been characterized in detail.[39-42] Notwithstanding that all stages of NAFLD

contribute to its clinical burden, the more progressive stages of NALFD are expected to have the largest impact.[7] As a progressive form of NAFLD, NASH is currently the second leading cause for liver transplantation in the United States, and even the leading cause for liver transplantation in females.[43] Furthermore, it is known that NAFLD has become one of the leading causes for cirrhosis,[44] the end stage liver disease which is associated with high risks for development of bacterial infections leading to hospitalization.[45-47] Additionally, the presence of advanced fibrosis (stage >=2) in NAFLD has been directly associated with liver-related mortality.[48,49] There is also accumulating evidence that NAFLD is an important risk factor for hepatocellular carcinoma (HCC), which is the fifth most common type of cancer and third most common cause of cancer mortality.[50-52] Translating the latter described observations into exact numbers, a recent study showed that in 2015, 28,000 deaths (2.2% of all deaths in the NAFLD population) were related to cirrhosis, HCC or liver transplantation, while 162,560 deaths (accounting for 12.8% of all NAFLD deaths) were due to cardiovascular diseases.[49,53] Indeed, these data point towards an important role for cardiovascular diseases in NAFLD-related mortality. In line with the previously estimated increased prevalence of NAFLD in the future, reports have indicated that the total number of deaths resulting from NAFLD will increase 44%, reaching 1.83 million deaths at an annual basis by 2030.[16] Regarding to the healthcare expenditure, Lam *et al.* demonstrated that more frequent clinical visits are associated with improved outcomes in pediatric NAFLD patients, substantiating the importance of frequent monitoring and follow-up to manage NAFLD progression.[42] Moreover, Boursier *et al.* evaluated the hospitalization of NAFLD/NASH-related end stage liver disease (ESLD) patients by a 7 years follow-up study in France. This report described that ESLD patients experience more hospitalization per year (over 400%), which are longer (400% increase in length) and are associated with a 300% increase in hospitalization costs.[41] These numbers therefore indicate the gigantic investments that are required to manage NAFLD.

In line with the increasing prevalence of NAFLD, the economic costs related to NAFLD have also been predicted to rise in the future. In the US alone, approximately 103 billion dollars are annually spent on NAFLD-related costs, while in European countries (i.e., France, UK, Germany and Italy) these costs account for approximately €35 billion.[54] Of particular concern is the rising prevalence of obesity as well as the increase in general health-care costs, which contributes to a ten-fold increase in the current economic burden of NAFLD by 2025.[14,54] Several studies also investigated the relationship between NAFLD patients and healthcare utilization and associated costs. These studies demonstrated that the number of outpatient visits for patients with NAFLD significantly doubled over time and underlined the fact that, between 2005 and 2010, the healthcare costs of inpatients and outpatients were increased 5% and 10%, respectively.[55-57] Additionally, a study of the hepatology clinics in the West Suffolk area of the UK showed that the total annual hepatology budget for these specialized clinics was £130,000, including £58,000 for resources and £72,000 for clinic attendances. Moreover, the latest research estimating the economic burden of NASH patients by using a Markov decision

analytic model demonstrated that lifetime costs of all NASH patients was approximately $222.6 billion in the United States in 2017, with $95.4 billion reflecting the advanced NASH population.[58]

From an individual perspective, NALFD patients have to contend with a range of symptoms such as fatigue, decreased physical activity and emotional health impairment which affect their quality of life (health-related quality of life (HR-QOL)).[9,59-61] Several studies have demonstrated that NAFLD patients had poorer HR-QOL compared to other chronic liver diseases and also showed that NAFLD-related fatigue associated with impairments in physical functioning.[62-64] Potential explanations for the decrease of HR-QOL in NAFLD patients that have been raised are related to obesity and psychological processes as well as psychiatric issues such as depression and anxiety.[65-67] Indeed, several studies have demonstrated that depressive disorders, as well as anxiety disorders, are more frequent in patients with NAFLD/NASH and are associated with more advanced liver histological abnormalities, such as severe hepatocyte ballooning.[65,68-70]

Altogether, these numbers substantiate the impact of NAFLD on health care, economy but also on the daily life of individual patients. To prevent further escalation of the disease, it is therefore of utmost importance to increase the understanding of the disease to find approaches to diagnose and treat (and if possible prevent) the progression of NAFLD.

2. Pathophysiology of NAFLD

As NAFLD comprises a spectrum of diseases, multiple pathophysiological processes are involved including dysregulation of lipid metabolism, increased hepatic inflammation and the presence of hepatic fibrosis. However, how patients with steatosis develop inflammation is still unclear, leaving a blind spot in the understanding of how NASH exactly arises. Nevertheless, scientists have succeeded in unraveling several disease processes in NALFD, which appear to show striking similarities with disease processes described in atherosclerosis. Furthermore, increasing evidence links different metabolic organs to NAFLD development, emphasizing the presence of metabolic crosstalk during NAFLD.

2.1 Intrahepatic disturbances during NAFLD

The liver constitutes a key role in regulating whole body metabolism, which involves a complex interplay between different hepatic cell types ranging from hepatocytes as parenchymal cells to Kupffer cells (KCs), stellate cells and liver sinusoidal endothelial cells (LSECs) among other cell types. As such, each of these cell types are influenced by pathophysiological processes during NAFLD, eventually leading to hepatic disturbances at whole organ level.

2.1.1 Lipo- and glucotoxicity

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Due to low physical activity and increased consumption of fats, lipotoxicity has arisen as one of the main players to contribute to NAFLD pathogenesis.[71] Lipotoxicity is defined by the excess generation of cytosolic lipids (mainly triglycerides and subtypes of free fatty acids) that have direct adverse effects on metabolic pathways of the cell.[72] Under normal conditions, triglycerides and free fatty acids are stored in adipose tissue, where they can be employed as energy source during periods of energy deprivation or during extreme exercise.[73] However, in obesity, when the storage capacity of adipose tissue is exceeded, free fatty acids accumulate in ectopic organs, including the liver (but also in the arteries). As such, hepatic steatosis[74] develops, resulting in the formation of adverse metabolites that hamper normal cellular physiology. For example, an excess of free fatty acids such as palmitic or stearic acids induces the generation of toxic metabolites, leading to caspase-dependent apoptosis of hepatocytes,[75,76] but also of cardiomyocytes[77,78] and endothelial cells.[79,80] Indeed, while free fatty acid-induced apoptosis of hepatocytes is a key feature of lipotoxicity in the context of NAFLD,[81] other reports have shown that excess palmitate induces endoplasmic reticulum stress and apoptosis in the context of atherosclerosis as well.[82] Therefore, free fatty acid-induced apoptosis of parenchymal cells appears to be a shared mechanism between NAFLD and atherosclerosis development.

Besides inducing apoptosis, free fatty acid influx into hepatocytes also influences the function of key enzymes and nuclear receptors involved in hepatic *de novo* lipogenesis, fatty acid oxidation and cholesterol metabolism, thereby further disturbing hepatic lipid metabolism. Indeed, expression levels of acetyl-coenzyme-A carboxylase 1, a key enzyme in fatty acid metabolism,[83] were shown to decrease in advanced stages of NASH compared to individuals with steatosis.[84] Moreover, the transcriptional levels liver X receptor (LXR), a nuclear receptor involved with regulation of cholesterol, fatty acid and glucose metabolism,[85] correlated with intrahepatic inflammation and fibrosis in NAFLD patients.[86,87] In line, macrophage-targeted delivery of LXR agonist inside the atherosclerotic plaque reduced atherosclerosis progression,[88] pointing towards a key function of LXR in both NAFLD and atherosclerosis development.

Lipotoxic responses also affect LSECs, a type of non-parenchymal cell that are specifically involved in maintaining hepatic vascular tone and quiescence of hepatic stellate cells that are responsible for the fibrotic response. Upon treatment with oxidized lipids[89] or palmitic acid,[90] LSCEs directly or indirectly triggered the release of reactive oxygen species (ROS) production,[91] which influences mechanisms related to inflammation and fibrosis.[87] Increased hepatic fat accumulation has also been associated with reduced levels of high-density lipoproteins (HDL) and increased levels of total plasma cholesterol, low-density lipoproteins (LDL) and very low-density lipoprotein particles,[92] the latter being involved with hepatic lipid export. Moreover, besides triglycerides and fatty acids, it has become evident that cholesterol is a key player in inducing hepatic inflammatory responses.[93-95] Indeed, in the context of obesity-associated

diseases, it was shown that cholesterol levels associated with hepatic inflammation[96,97] and atherosclerosis[98] in humans. In agreement with these data, it has previously been shown that omitting cholesterol from the diet was able to prevent liver inflammation in hyperlipidemic and atherosclerosis-prone mice,[99] pointing towards cholesterol as a significant risk factor for early onset of NASH and progression of atherosclerosis.

In addition to lipotoxicity, glucotoxicity is a metabolic condition linked to increased intake of dietary sugars, resulting in hyperglycemia which may cause hepatotoxic effects by increasing steatosis.[100] For instance, it was shown that high carbohydrate intake plays a role in *de novo* lipogenesis and hepatic steatosis,[101,102] presumably via activation of lipogenic enzymes such as fatty acid synthase and stearoyl-CoA desaturase-1.[103] In addition, high-fructose intake was shown to correlate with the severity of fibrosis in NAFLD patients,[100,104] and carbohydrate intake associated with the progression of coronary atherosclerosis.[105] Glucotoxic and lipotoxic products, including free fatty acids, cholesterol and ceramides, amongst others,[106] are also involved in the activation of cellular stress responses. For instance, it has been shown that saturated long-chain fatty acids can disturb metabolic fluxes, thereby increasing the production of harmful lipid intermediates[76]. These intermediates can promote the release of ROS, leading to oxidative stress and hence the progression from steatosis to NASH[107,108] and the development of atherosclerosis.[109]

Overall, these evidences show that triglycerides, fatty acids and cholesterol overload disturb essential processes in the liver that result in NAFLD features, placing lipids at the center of NAFLD development. Moreover, as these processes show striking similarities with disturbances present in atherosclerosis, lipotoxicity is denominator linking NAFLD to atherosclerosis.

2.1.2 Oxidative stress and mitochondrial dysfunction

As mentioned in the previous paragraph, part of the lipotoxic response involves the generation of ROS, resulting in oxidative stress. Oxidative stress comprises a state during which there is an imbalance between generation of ROS at one hand and an inability to detoxify (*i.e.* via antioxidant mechanisms) these oxygenated intermediates.[110] As a consequence, free radicals, peroxides and related products are generated and react with biological components such as proteins, DNA but also lipids.[111] Indeed, considering the increased amount of lipids present during NAFLD conditions, larger quantities of lipid peroxidation products are present in the liver of NAFLD patients[112] and contribute to the transition towards more serious stages of NAFLD.[113,114] In addition, ROS is known to mediate endoplasmic reticulum stress, thereby causing the formation of misfolded proteins, which is a critical factor in NAFLD[115] as well as the progression of atherosclerosis.[116,117] Moreover, cholesterol oxidation products that are part of oxidized low-density lipoproteins (oxLDL) are majorly involved in inflammatory and fibrotic

responses in the liver (also further discussed in next section).[118]

Considering the key role of mitochondria in cellular oxygen consumption and production of ROS, lipotoxic influences on mitochondria have the potential to further aggravate oxidative stress.[119] Under physiological conditions, fatty acid transport into the mitochondria is mediated via carnitine palmitoyl transferase 1 (CPT-1) in order to stimulate beta-oxidation. Nevertheless, the expression of *Cpt-1* was shown to be reduced in NAFLD,[120] findings that were further supported by Francque *et al.*, showing that peroxisome proliferator-activated receptor alpha (*PPARα)*, an important nuclear receptor regulating CPT-1, inversely correlated with disease severity in patients with NASH.[121] By using isolated mitochondria, it was also shown that short chain ceramides increase mitochondrial permeability due to the generation of ceramide channels and increased cytochrome C release,[122] thereby mediating toxic effects. Moreover, mitochondrial cholesterol accumulation caused mitochondrial dysfunction,[123] and based on studies in the context of neurotoxicity,[124] mitochondrial cholesterol may play a role in endoplasmic reticulum stress and subsequent apoptosis.

2.1.3 Hepatic inflammation and fibrosis

An essential pathophysiological process during NAFLD that also unites the lipotoxic response with the generation of oxidative stress, is the presence of hepatic inflammation which can progress into hepatic fibrosis. In contrast to the uptake of non-modified LDL, it has been established that the uptake of oxLDL contributes to cholesterol-induced foam cell formation and metabolic inflammation[125] in NASH,[126] but also in the context of atherosclerosis.[127] Moreover, the accumulation of oxidized lipids into the lysosomal compartment of macrophages activates inflammatory cascades including inflammasome complexes and apoptosis.[118,128-130] Indeed, recent studies show that specific inhibition of the NLRP3 inflammasome not only reverses hepatic inflammation and fibrosis,[131] but also reduces atherosclerotic lesion development,[132] pointing towards an important role for the inflammasome in chronic inflammatory diseases.[133-135] Furthermore, cholesterol-mediated activation of inflammasomes decreases cholesterol efflux, thereby disturbing the regulation of bile acid metabolism. Previously, it was indeed shown that mice lacking the bile acid receptor farnesoid X receptor (FXR) had pro-atherogenic lipoproteins[136] and increased hepatic bile acid levels,[137] pointing towards a potential role for FXR in cholesterol-induced liver inflammation. Indeed, improving cholesterol efflux in hepatic macrophages by overexpressing *Cyp27a1,* an enzyme responsible for the conversion of cholesterol into bile acids*,* reduced hepatic inflammation and fibrosis in an experimental model.[138] Via accumulation of oxidized lipids into lysosomes, also disturbances in autophagy contribute to increased levels of inflammation both during NAFLD[139] and atherosclerosis.[140] Besides cholesterol, also other lipids such as phospholipids[141] and fatty acids[142] can (non)enzymatically interact with free radicals, triggering inflammation by a wide variety of underlying processes.[126]

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While macrophages play a key role in the inflammatory response, hepatic stellate cells are the main drivers of the fibrotic response.[143] After a damaging insult, stellate cells are activated, thereby secreting collagens and related matrix proteins that lead to generation of scar tissue or fibrosis,[91,144] a pathological process also described in atherosclerosis.[145] Relevantly, Chu *et al.* recently demonstrated that exposing hepatic stellate cells to fatty acids resulted in an increased secretion of CCL20, resulting in a switch from a quiescent to an activated hepatic stellate cell. These findings were further confirmed in humans, showing increased circulating CCL20 protein levels in patients with NAFLD-related fibrosis.[146] Further data based on an elegant co-culturing system using primary liver cells pointed towards CCL5 as an important hepatic stellate cell-derived chemokine capable of mediating steatosis and pro-inflammatory responses in initially healthy hepatocytes.[147] Moreover, *in vivo* induction of CCL5 in response to high fat diet was also shown to serve as an important regulator of vascular remodeling, revealing a role for CCL5 and its receptor in atherogenesis.[148] Therefore, multiple reports indicate that lipids enable fibrotic responses by influencing hepatic stellate cells.

2.2 Metabolic crosstalk in NAFLD

As previously mentioned, the capacity of adipose tissue to store lipids determines the quantity of free fatty acids to be released into the circulation under high lipid conditions. However, besides its storage capacity, adipose tissue is known as a 'secretory' organ, releasing adipokines and adipocytokines that influence other organs.[149] For this reason, lipid-induced adipose tissue function increases the release of adipocytokines such as TNFα, IL6, IL18 and ANGPTL, leading to inflammatory responses in other metabolic organs such as the liver.[149,150] Moreover, the release of these adipokines also influence circulating immune cells, contributing to a state of chronic inflammation.[151-153] Due to this systemic impact, it is not surprising that adipokines also influence atherosclerosis development. Indeed, adipose tissue-release TNFα directly influenced atherosclerosis development.[154] Besides modulating inflammation, the increased release of free fatty acids also hinders the anti-lipolytic role of insulin, aggravating insulin resistance.[155,156]

Another extrahepatic organ that has been linked to NAFLD development is the thyroid. Being an endocrine organ, the thyroid secretes hormones that have a role in the regulation of energy homeostasis including the metabolism of cholesterol and fatty acids.[157] Specifically, hypothyroidism is characterized by increased serum LDL and HDL levels and decreased triglyceride levels.[158] Besides indirectly influencing hepatic lipid metabolism by modulating circulating lipid levels, thyroid hormones also directly affect hepatic lipid metabolism mainly via the presence of hepatic thyroid hormone receptors (THR).[157] THRs are nuclear hormone receptors that function as ligand-dependent transcription factors influencing downstream metabolic genes,[159] but also disturb other metabolic transcription factors such as PPARγ, LXR and Sterol regulatory element-binding protein 1 (SREBP1c).[160,161] For this regulatory role on

hepatic lipid metabolism, THR agonists were also considered for the management of hepatic steatosis,[162] but later observed adverse effects resulted in discontinuation of these clinical trials.[163] Nevertheless, thyroid hormones analogues (rather than THRs) are still considered as potential future NAFLD treatment.[164]

Another organ that has gained attention in the context of NAFLD, is the brain. At one hand, NAFLD-related inflammation has been demonstrated to influence microglia in the brain, leading to alterations in microvasculature of the brain.[165,166] Furthermore, NAFLD-associated endothelial dysfunction and the procoagulant state were linked to the same microvascular alterations, which may contribute to disturbances in brain circulation, damage and cognitive impairment.[36] Besides the link to the aforementioned cerebrovascular diseases,[167] other brain-related associations have been established to NAFLD. Firstly, a recent report from Horwath *et al.* demonstrated that endoplasmic reticulum stress in the subfornical organ of the brain, a brain region previously linked to appetite,[168] directly mediated hepatic steatosis, thereby directly linking the brain to the liver in the context of NAFLD. Moreover, Weinstein *et al.* recently linked NAFLD to lower cerebral brain volume hinting at a more profound role for the brain in NAFLD.[169] Finally, as a regulation center for energy metabolism, brain regions such as the arcuate nucleus in the hypothalamus sense the metabolic status and govern food intake, [170] making an obvious link to obesity-related NAFLD. An essential hormone involved with the homeostatic regulation of energy and acting via the hypothalamus is leptin.[171] Notably, variants of leptin receptors associated with increased NAFLD susceptibility, pointing towards a potential role for hypothalamic leptin sensitivity in NAFLD.[172] Additional evidence linking hypothalamic inflammation to hepatic steatosis further substantiated the potential involvement of the hypothalamus in NAFLD.[173]

To end, multiple reports have indicated the involvement of the gastrointestinal tract to play a role in NAFLD development. Under physiological circumstances, the intestinal lining serves as a physical barrier that separates the host from contents in the gut. Disruption of this barrier leads to intestinal permeability,[174] allowing for leakage of intestinal bacteria and other products into the circulation. Indeed, leakage of lipopolysaccharide (LPS) derived from intestinal bacteria into the circulation[175] can activate KCs in the liver,[176] thereby directly resulting in NASH development.[175,177] Relevantly, gut-derived serum LPS was similarly associated with atherosclerosis development, reaffirming the tight link between NASH and atherosclerosis.[178] Though there are limited studies providing a causal role of the gut microbiome in NAFLD pathogenesis, the current amount of evidence suggests that the gut microbiota are at least involved with the development of NAFLD.[179-182,183] Other well-known factors linking the gut to NAFLD are bile acid metabolism,[184,185] bacterial-derived short-chain fatty acids[186] and the toxic compounds dimethylamine and trimethylamine that were converted by bacteria from choline.[187,188] In line with our other descriptions, each of these compounds have also been associated with atherosclerosis development.[189-191]

Based on these evidences, it is clear that the development of NAFLD is linked to pathophysiological processes that arise in other (metabolic) organs. This information fuels a view of NAFLD being a complex, systemic disease influenced by a range of other organs. It is therefore likely that future management of NAFLD will require a systemic rather than a liver-specific approach.

2.3 Genetic predisposition to NAFLD

NAFLD is considered a polygenic disease, implying the involvement of a variety of genetic factors in predisposing individuals to disease onset. While mutations in the patatin-like phospholipase domain-containing 3 (PNPLA3) gene were initially associated with hepatic steatosis,[192] other reports have also correlated the PNPLA3 variation to NASH progression.[193,194] Similarly, PNPLA3 genetic variants also associated with carotid atherosclerosis in younger patients NAFLD.[195] Though PNPLA3 variants were recently linked to the ubiquitylation processes,[196] the exact underlying mechanism explaining the onset of NAFLD is still unclear.

Additionally, based on several population studies, it was recently described that mutations in the transmembrane 6 superfamily 2 (TM6SF2), a key regulator of very low density lipoprotein (VLDL) export, correlated with NASH progression[197] and cardiovascular disease,[45] most likely via changes in plasma lipids. Indeed, plasma lipids appear to be one of the common denominators predicting severity of both NAFLD and coronary artery disease.[198] Further genetic screenings for NAFLD revealed that glucokinase regulator (*GCKR*)[199] and lysophospholipid acyltransferase 7 (known as MBOAT7),[200] key enzymes for glucose metabolism and reacetylation of phospholipids, respectively, as well as neurocan were associated with NAFLD development.[201] Yet, a more recent study focusing on the aforementioned NAFLD-risk alleles (*PNPLA3, TM6SF2, GCKR*, and *LYPLAL1*) substantiated the heterogeneity of the NAFLD phenotype between patients, emphasizing the complexity of the disease.[202] As such, though genetic predisposition may influence disease onset, other pathophysiological factors that are independent of genetic predisposition are likely a stronger contributor to explain NAFLD development.

A line of research that has received increased attention is the influence of epigenetic changes on NAFLD development.[203] Epigenetic changes are induced by modifications in the regulators of DNA such as DNA methylation reactions, histone proteins, chromatin structure and RNA-based mechanisms resulting in changes in genes expression.[203] These epigenetic modifications influence ageing related processes which contribute to NALFD,[204] but can also be transmitted to the progeny, thereby combining genetic and environmental factors involved in the development of disease. Mice that were rechallenged with a high-fat diet after being exposed to this diet during fetal life showed more several hepatic steatosis, inflammation and fibrosis.[205] This influence of a detrimental fetal environment on NAFLD has been further substantiated by studies linking intra-uterine growth retardation to increased risk of

developing NAFLD.[206-208] Furthermore, methylation patterns of genes involved insulin signaling associated with the presence of NASH, which disappeared after bariatric surgery.[209] As such, though being in its infancy, epigenetic modifications are expected to have an important role on NAFLD progression.[203]

3. Therapeutics

The involvement of different mechanisms in the pathogenesis of NAFLD also adds a level of complexity in finding appropriate therapeutic options to improve the different aspects of NAFLD. While therapies to reduce hepatic steatosis are known, a major problem is reversing the inflammatory component in the liver. Indeed, at present, no effective therapeutic approaches exist for reducing hepatic inflammation.[126] From market size perspective, NASH-related therapeutics generated $1,179 million in 2017, and is estimated to reach $21,478 million by 2025,[210] pointing towards the huge demand for NASH treatments. Due to the magnitude of this health concern and its potential impact on health care, multiple treatments are currently being investigated with the aim to decrease inflammation and fibrosis.[211] In this section, we provide a selection of currently investigated therapeutic approaches for NAFLD and demonstrate that these approaches are also investigated in the context of atherosclerosis (see figure 2.1). From this perspective, we further highlight the link between NAFLD and atherosclerosis.

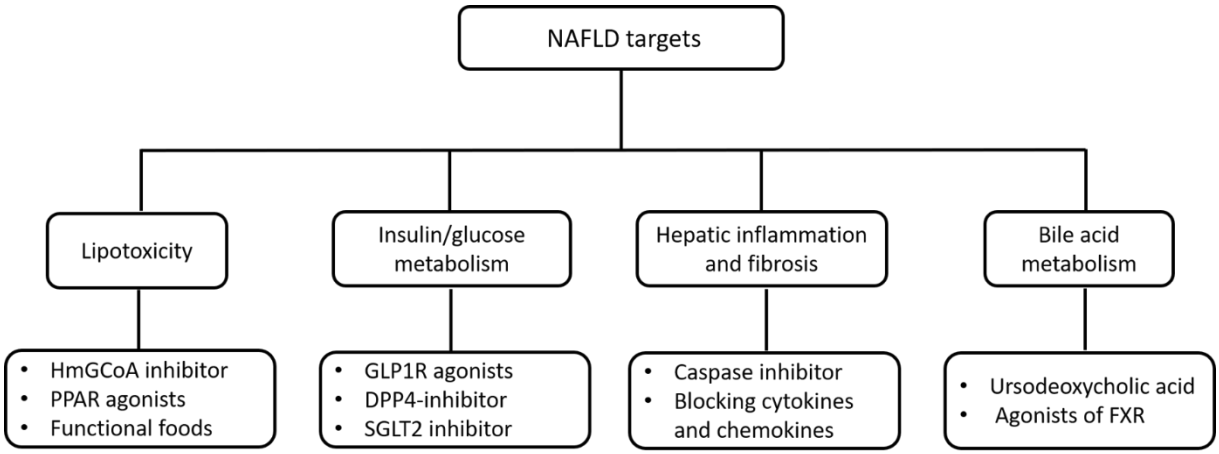

Figure 2.1 **Targets for NAFLD therapy**. Besides exercise, changing the dietary pattern or surgical intervention,pharmacological intervention to improve NAFLD target the pathological mechanisms of lipotoxicity, insulin/glucose metabolism, hepatic inflammation,fibrosis as well as bile acid metabolism. HmGCoA: 3-hydroxy-3-methylglutarylcoenzyme A; PPAR: peroxisome proliferator-activated receptor; GLP1R: glucagon-like peptide-1 receptor (GLP1R); DPP4: Dipeptidyl peptidase-4; SGLT2: sodium-glucose transport protein 2; FXR: farnesoid X receptor.

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3.1 Dietary/life style intervention and bariatric surgery

Dietary changes and life style interventions resulting in weight reduction are currently the first-line therapy for NAFLD patients.[212] Indeed, dietary restriction is the most effective way to reduce liver fat.[213,214] Furthermore, it has been suggested that hepatic triglycerides content normalizes after a few weeks under a strictly hypocaloric diet,[214] *i.e.* low-fat and low carbohydrate, which has been proposed as the optimal composition of a diet for NAFLD patients.[215] Apart from dietary changes, life style modification is another way to lose weight, for instance via physical activity instead of sedentariness.[216] However, compared to dietary restriction, physical activity is less effective in losing weight due to reduced caloric consumption as compared with dietary restriction.[213,217] While dietary change and life style intervention are able to reduce body weight, many patients cannot adhere to these interventions. Therefore, bariatric surgery, and more recently termed metabolic surgery,[218] typically results in massive weight loss and in concordant improvements in liver histology.[219] Indeed, Mummadi *et al.* reported that the resolution rates of steatosis, steatohepatitis, and fibrosis were 91.6%, 81.3% and 65.5% in 15 studies using paired liver biopsies after bariatric surgery.[220] Recently, a 1 year follow-up study by Nickel *et al.* also supported bariatric surgery as an effective treatment for NAFLD.[221] However, as not all NAFLD patients qualify for bariatric surgery other interventions are necessary to combat NAFLD and related symptoms.

3.2 Targeting lipotoxicity

As accumulation of lipids inside the liver comprises an essential component in the development of NAFLD, multiple therapeutic approaches have aimed to reduce hepatic lipids with the objective to concordantly reduce hepatic inflammation and fibrosis. The best known example of cholesterol-reducing agents are statins, which are drugs aimed at inhibiting 3-hydroxy-3-methylglutarylcoenzyme A (HmGCoA) reductase, the rate limiting enzyme in the cholesterol biosynthesis pathway.[222] Showing beneficial results in the context of atherosclerosis,[223] statins were also investigated in NAFLD progression. Though some improvements were observed in hepatic damage and inflammation,[224] other reports declare only minor improvements or even increasing levels of inflammation and fibrosis when statins are administered over a longer period of time.[225] Moreover, recent observations pointing towards the detrimental effects of statins on ageing and associated processes[226,227] raises drawbacks for using these drugs under certain conditions.

Finally, and potentially the most promising pharmacological compound currently under investigation to regress NASH, are agonists of PPAR. PPARs are nuclear receptor proteins exerting key regulatory functions as transcription factors on metabolism, among other physiological processes.[228] Currently, three types of PPARs (being PPARα, PPARβ/δ and PPARγ) are known and used as targets to improve MetS-related symptoms. In the context of NAFLD, and specifically NASH, the PPARγ agonist class thiazolidinediones has been shown to improve

hepatic inflammation and advanced fibrosis.[229,230] Furthermore, a new agonist, named elafibranor (or GFT505) that targets PPARα and PPARδ, was recently shown to improve hepatic inflammation and fibrosis, along with improvements in systemic inflammation, lipid and glucose metabolism.[231,232] Due to these positive results, both thiazolidiniediones and elafibranor are currently under clinical investigation for the treatment of NASH.[233] With regard to their application in atherosclerosis, thiazolidiniediones have also been proven to slow progression of atherosclerosis in patients,[234] while elafibranor was so far not tested in this context. However, preliminary results in an atherosclerotic mouse model suggests that this latter PPARα/δ dual agonist might also be beneficial in the context of atherosclerosis.[235]

Besides pharmacological intervention, a more convenient manner of reducing lipids is by means of dietary intervention. Apart from following dietary regimens in which the composition of lipids, protein and carbohydrates is modulated and caloric intake is minimized.[236] in order to achieve improvements in energy metabolism,[237,238] another approach is to increase the intake of food components named functional foods. Plant sterol and stanol esters are examples of such functional foods that have been proven to reduce serum total and LDL cholesterol,[239,240] leading to improvements in atherosclerosis[241] and NAFLD.[95] However, more studies are necessary to prove the potential benefit of plant sterol and stanol esters in NAFLD patients.

3.3 Targeting insulin/glucose metabolism

As diabetes has been associated with several stages of NAFLD,[242] researchers have investigated the impact of improving insulin and glucose metabolism in order to improve aspects of NAFLD. Firstly, glucagon-like peptide receptor (GLP1R) agonists, which mimic the function of incretins, are currently investigated in NAFLD.[243] GLP is a peptide derived from the L cells of the lower gastrointestinal tract (small intestine and proximal colon) and known to enhance insulin secretion from pancreatic β cells and inhibit glucagon release from pancreatic α cells.[236,244] Whereas the GLP1R agonist exenatide enhanced hepatic steatosis,[245] hepatic oxidative stress, hepatic inflammation[246] and improved adipose tissue lipolysis in different *in vivo* models, the application of dulaglitide, lixisenatide, liraglutide and, recently, semaglutide also show promising results in terms of improvements in hepatic fat, damage, inflammation and fibrosis.[247-252] As such, liraglutide[247] and semaglutide were under extensive clinical investigation. While liraglutide will not be further evaluated in phase 3 development, Novo Nordisk has initiated a phase 2b trial (NCT02970942) evaluating semaglutide versus placebo in 372 participants with stage F2-F3 fibrosis and NAS ≥4 with a score of at least 1 for each of the components (steatosis, ballooning, and lobular inflammation).[233] Relevantly, as diabetes has also been linked to formation and progression of the atherosclerotic plaque,[253-255] several GLP1R agonists have also been shown to improve atherosclerosis including exenatide, liraglutide and semaglutide.[252,256-258]

Besides the GLRP1 agonists, another approach to improve the GLP1-related effects on insulin and glucose metabolism is by administration of dipeptidyl peptidase (DPP4)-inhibitors. DPP4 (also referred to as CD26) is an enzyme known to degrade GLP1. Hence, inhibition of DPP4 enhances the activity of GLP1. While administration of the DPP4-inhibitor sitagliptin has been successfully applied in diabetic patients,[259,260] several reports demonstrated only minor to no beneficial effects on hepatic fat content or hepatic fibrosis.[261,262] Relevantly, these negative results in the context of NAFLD were also confirmed in atherosclerosis, showing only minor effects on coronary artery plaque improvement.[263,264] Another class of pharmacological compounds that specifically improve glucose metabolism are inhibitors for sodium-glucose transport protein 2 (SGLT2), a transporter protein in the kidney responsible for the reabsorption of glucose.[265-267] In contrast to the minor effects of the DPP4-inhibitors on NAFLD, the SGLT2 inhibitors canagliflozin, ipragliflozin and luseogliflozin all show substantial improvements in hepatic steatosis, apoptosis and fibrosis in *in vivo* models and NAFLD patients.[268-272] In line with the previously described similarities between NAFLD and atherosclerosis, SGLT2 inhibitors were shown to also positively impact atherosclerosis progression and development.[273-276]

Together, several treatments aimed at improving insulin or glucose metabolism have positive effects on several aspects of NAFLD, substantiating the role of insulin and glucose metabolism in the progression of NAFLD. Moreover, therapeutic products that improve features of NAFLD also positively impact atherosclerosis, providing further evidence for the similarities between NAFLD and atherosclerosis.

3.4 Targeting hepatic Inflammation and fibrosis

Another therapeutic approach to ameliorate NAFLD is to directly target components of the inflammatory and/or fibrotic pathway, as these features are the main cause for hepatic symptoms in NAFLD patients and are also responsible for the development towards advanced liver diseases.[277] The caspase inhibitor, emricasan, is one of those investigated compounds targeting the inflammatory aspect of NAFLD. Specifically, caspases are enzymes involved with several physiological processes including inflammation, making them an attractive inflammatory drug target. Administration of emricasan to NAFLD patients showed improvements in hepatic damage (as evidenced by reductions in alanine transaminase (ALT) levels).[278] However, recent negative results with this compound have questioned its continuation for further clinical investigation.[279] Another potential caspase-related target for inflammatory drugs is blocking the activation of inflammasomes.[277] Indeed, inhibition of the P2X7 receptor, which is known to activate the NLRP3 inflammasome,[280] via SGM-1019 resulted in improvements in hepatic inflammation and fibrosis in mouse models and NASH patients,[281] substantiating its further clinical investigation in NAFLD.

Another way to reduce inflammation and fibrosis is by blocking the effect of cytokines and

chemokines that propagate the inflammatory reaction. With this regard, the C-C chemokine receptor type 2/C-C chemokine receptor 5 (CCR2/CCR5) inhibitor cenicriviroc has been successfully created. Specifically, cenicriviroc reduced hepatic fibrosis, inflammation as well as systemic inflammatory parameters in NAFLD patients and animal models.[282-284] Currently, cenicriviroc is being evaluated in phase 3 trials, targeting patients with F2-F3 fibrosis and having an anticipated enrollment of 2000 participants.[233] Additionally, inhibition of galectin-3, a protein belonging to the lectin family and previously linked to NASH severity, has provided promising first results,[285] which need to be further validated. Finally, inhibition of apoptosis signal-regulating kinase 1 (ASK1) has also been investigated as drug target to reduce hepatic inflammation and fibrosis. ASK1 is part of the mitogen-activated protein kinase family and has been shown an essential role in NASH development in patients and mouse models.[286-288] In line with this observation, inhibition of ASK1 using selonsertib has shown impressive improvements in hepatic inflammation and fibrosis.[289,290] Selonsertib is currently under evaluation in two phase 3 clinical trials (STELLAR-3 [NCT03053050] and STELLAR-4 [NCT03053063]) for the treatment of NASH.[233]

Similar to the dual therapeutic effects of approaches targeting insulin and glucose metabolism, therapeutic approaches targeting inflammation and fibrosis also show dual positive influences in atherosclerosis and NASH. Galectin-3 has for example been linked atherosclerotic plaque progression[291] and its inhibition results in reductions of atherosclerotic lesion size *in vivo*.[292] However, compared to drugs targeting insulin and glucose metabolism, targeting inflammation and fibrosis pathways are less investigated in the context of atherosclerosis, as exemplified by no described clinical studies for selonsertib, emricasan, ASK1 inhibitors or cenicriviroc.

3.5 Targeting bile acid metabolism

Hepatic components that have been extensively linked to different aspects of NAFLD include bile acids. Bile acids have regulatory functions on lipid and glucose metabolism, impact gut microbiota composition and influence hepatic inflammation and damage,[277] explaining why modulation of bile acid metabolism has been an attractive therapeutic target for NAFLD. Firstly, the hepatoprotective natural bile acid ursodeoxycholic acid (UDCA) has been shown to exert beneficial effects on immune function, has anti-apoptotic and insulin-sensitizing effects and reduces harmfull effects of reactive oxygen species,[293-295] all aspects present during NAFLD. Indeed, besides improvements in hepatic steatosis, inflammation and damage in NASH animal models, two randomized controlled trials showed improvements in lobular inflammation and hepatic fibrosis along with reductions in ALT levels upon UDCA administration.[296,297] However, other studies showed no effect of UDCA administration in NASH patients,[298] emphasizing the need for further investigation. In addition, agonists of FXR, a nuclear receptor that has been linked to NAFLD,[299] have also been tested in NAFLD. Obeticholic acid (OCA), a semi-synthetic variant of chenodeoxycholic acid, showed reductions in steatosis and fibrosis[300,301] and recently, the first promising results were published from the FLINT study, investigated OCA in

NAFLD patients.[302] Currently, OCA is being evaluated in the phase 3 study REGENERATE (NCT02548351) for the treatment of NASH.[233]

Strikingly, though bile acids are produced by the liver, multiple evidences have pointed towards their systemic effects on inflammation, cell death and apoptosis.[303] As such, recent evidences have also shown beneficial effects of UDCA[304] and OCA[305,306] in atherosclerotic models.

Conclusion

As the hepatic component of the MetS, NAFLD comprises one of the largest global health threats of the 21st century. Though the exact aetiology of why NAFLD patients progress from hepatic steatosis to hepatic inflammation and fibrosis is unclear, several studies have established key pathophysiological processes contributing to hepatic inflammation. Considering this large amount of processes involved with NAFLD (which have intra- and extrahepatic origins), it is clear that NAFLD is a complex, systemic disease with high interindividual variation, pointing towards combination therapies or personalized medicine as potential future directions for NAFLD. Moreover, due to this systemic nature, it is clear that NAFLD and atherosclerosis are very closely linked,[307] implying the liver as a potential target to manage atherosclerosis.

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Chapter 2

Chapter 3

Myosteatosis in NAFLD patients correlates with plasma Cathepsin D

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Chapter 3

Abstract

Background: Nonalcoholic fatty liver disease (NAFLD) covers a disease spectrum ranging from hepatic steatosis to nonalcoholic steatohepatitis (NASH), leading to fibrosis and ultimately hepatocellular carcinoma and liver failure. Previously, we have shown that hepatic lipid accumulation induces the secretion of cathepsin D (CTSD), and that plasma CTSD levels are associated with increased inflammation and disease severity in NAFLD. it is unknown whether other metabolically active organs such as the muscle, also associate with plasma CTSD levels in NAFLD patients. Therefore, the aim of this study was to explore the relation between lipid accumulation in the muscle (myosteatosis) and plasma CTSD levels.

Methods: Forty-five NAFLD patients were recruited in our study. Muscle fat fraction (myosteatosis) was determined by muscle signal intensity loss on in- and opposed-phase imaging (chemical shift MRI). In addition, hepatic steatosis was assessed with MRI and liver stiffness with transient elastography. Afterwards, blood samples were analyzed for the measurement of glucose- and lipid-related parameters, liver enzymes as well as plasma CTSD levels. Then, multiple linear regression was performed to determine whether myosteatosis associated with plasma CTSD levels.

Results: Firstly, the hepatic fat fraction (hepatic steatosis) positively associated with plasma CTSD levels, confirming the previously established link between plasma CTSD and the liver. Furthermore, a positive association between myosteatosis and plasma CTSD levels was observed, which was independent of sex, age, BMI, waist circumferences and hepatic steatosis.

Conclusion: By establishing a positive association between myosteatosis and plasma CTSD levels, our findings suggest that, in addition to the liver, the muscle is also linked to plasma CTSD levels in NAFLD patients. The observed link between myosteatosis and plasma CTSD levels supports the concept of a significant role of the skeletal muscle in metabolic disturbances in metabolic syndrome-related disorders.

Key words: Plasma CTSD levels, myosteatosis, steatosis, ectopic lipid accumulation, NAFLD

Chapter 3

Introduction

Due to the growing prevalence of metabolic syndrome (MetS), nonalcoholic fatty liver disease (NAFLD) is an increasing worldwide health problem. Being the most common cause of chronic liver diseases (CLDs) in the Western world[1], NAFLD is a progressive disease ranging from liver steatosis to nonalcoholic steatohepatitis (NASH), hepatic fibrosis, and ultimately hepatocellular carcinoma and liver failure [2,3]. Given the disease burden associated with NAFLD, more research is needed to elucidate the underlying disease mechanisms as a basis to develop novel therapies.

Previously, our group has shown that, following hepatic steatosis, increased secretion of cathepsin D (CTSD, a lysosomal enzyme) is associated with higher levels of inflammation, disturbed lipid metabolism and disease severity in in vitro and in vivo models for NAFLD, suggesting a pathophysiological role of CTSD in NAFLD [4,5]. In addition, we observed that plasma CTSD levels are also elevated in NASH patients [6], further emphasizing the association between plasma CTSD levels and NAFLD progression. Of note, studies have shown increased plasma CTSD levels in type 2 diabetes [7], Alzheimer's disease [8] and inflammatory bowel disease [9], suggesting that extra-hepatic organs are also linked to plasma CTSD under pro-inflammatory conditions. Importantly, extra-hepatic organs such as the gut, adipose tissue and muscle also induce metabolic disturbances in MetS and NAFLD, raising the possibility that next to the liver, also other extra-hepatic organs are linked to plasma CTSD in MetS and NAFLD patients [10-12]. Among the aforementioned extra-hepatic organs, the muscle is increasingly being implicated in the progression of MetS. Accumulating evidence has shown that ectopic fat infiltration in skeletal muscle, referred to as myosteatosis, includes intramuscular and intermuscular lipid accumulation, and is strongly associated with obesity, diabetes and MetS [13,14]. For instance, intermuscular fat has been shown to be a predictor of insulin sensitivity [15,16], highlighting the link between myosteatosis and systemic metabolism. In addition, intramuscular fat is a metabolically active component of the muscle, which contributes to the secretion of inflammatory cytokines that induce systemic inflammation [14]. Whether the muscle is also linked to plasma CTSD in NAFLD patients has yet to be explored.

Therefore, our study aim was to investigate whether, besides the liver, also the muscle correlates with plasma CTSD levels in NAFLD patients. For this reason, forty-five NAFLD patients were enrolled in this study, in which muscle signal intensity loss on in-phase and opposed-phase imaging (chemical shift MRI) was assessed to determine myosteatosis based on the formula as previously described [17]. Furthermore, fasting blood samples were analyzed for the measurement of glucose- and lipid-related parameters, liver enzymes as well as plasma CTSD levels. Our findings demonstrate a positive association between the degree of myosteatosis and plasma CTSD levels independent of sex, age, BMI, waist and hepatic steatosis in NAFLD patients. Therefore, our data suggest that in addition to the liver, also the muscle is

linked to plasma CTSD levels. Taking into account that plasma CTSD aggravates metabolic disturbances, the observed link between myosteatosis and plasma CTSD levels supports the concept of a significant role of the skeletal muscle in metabolic disturbances in metabolic syndrome-related disorders.

Methods and materials

Subjects characteristics

Forty-five subjects were included in the present study as previously described [16]. All participants had proven NAFLD via liver biopsy or chemical shift magnetic resonance imaging (MRI). Metabolic syndrome was diagnosed based on the International Diabetes Federation (IDF) definition [17]. Exclusion criteria were excessive ethanol consumption (male more than 14 and female more than 7 standard beverages per week), causes for secondary hepatic fat accumulation (medication, Wilson's disease, viral infections, starvation or parenteral nutrition and microvesicular steatosis on liver biopsy), pregnancy and breastfeeding, a history of bariatric surgery, liver cirrhosis and/or hepatocellular carcinoma, malignancy(s) within the last 5 years and individuals about to undergo or recovering from a surgical or otherwise medical procedure. All subjects were recruited from Maastricht University Medical Centre (MUMC+) and CO-EUR (a second line eating disorder clinic) between September 2015 and October 2018. These participants gave written informed consent before entering this study. The study was approved by the Medical-Ethical Committee of Maastricht University (ClinicalTrials.gov Identifier: NCT02422238), and was performed in accordance with the principles of the Declaration of Helsinki, as revised in 2008.

Biochemical analyses

After overnight fasting, venous blood was collected into EDTA tubes that were put on ice after blood collection at Maastricht University Medical Center (MUMC+). After centrifuging (1000 x g; 10 min; 4°C), plasma was snap-frozen in liquid nitrogen and then stored at -80°C until analyses. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (GGT), bilirubin, Alkaline Phosphatase, total cholesterol, low density lipoprotein cholesterol (LDL-cholesterol), high density lipoprotein cholesterol (HDL-cholesterol), triglyceride, fasting plasma glucose and hemoglobin A1c (HbA1C) were determined using routine analyses at the clinical chemistry department of the Maastricht UMC+ hospital. Plasma CTSD levels were determined using the CTSD enzyme-linked immunosorbent assay according to the manufacturer's protocol (Uscn Life Science, Wuhan, China). The absorbance for CTSD levels was measured on a Benchmark 550 microplate reader (Bio-Rad, Hercules, CA). Skeletal muscle mass index (SMI) (appendicular skeletal mass/height^2) were calculated with dual-energy X-ray absorptiometry (DXA).

Chapter 3

Magnetic resonance imaging (MRI) – hepatic fat fraction

Hepatic fat fraction (hepatic steatosis) was assessed as previously described [17 19]. In brief, four circular regions of interest (ROI) of 5 cm^2 from each MRI section (in total 3 MRI sections) were drawn in the liver, where artefact, vascular and biliary structures were avoided. Then, the ROI was copied from the in-phase (IP) image to the opposed-phase (OP) image. The mean signal intensity (SI) loss of all 12 ROIs was calculated as previously described [17,20].

Magnetic Resonance Imaging (MRI) – muscle fat fraction

The MRI technique (chemical shift MRI) was performed to determine muscle fat fraction (myosteatosis) in all participants after a minimal fasting period of 3 hours within one month of the other measurements. MRI images were obtained with a 1.5T or 3T Achieva MRI system (Philips, Best, The Netherlands). The muscle fat fraction was measured with the simultaneously obtained 3D fast gradient echo mDixon images as previously described [17]. Parameters employed for 3D fast gradient echo mDixon sequence were for 1.5T and 3T respectively: flip angle = 15° and 10°, TR = 5.8 and 3.4 ms, TE1/TE2 = 2.37/4.75 and 1.19/2.37ms, field-of-view (FOV) = 320 x 320 and 384 x 384 mm, acquisition matrix = 288 x 227 and 252 x 209, slice thickness 5 and 3 mm. The multifidus and erector spinae muscle were segmented bilaterally at the lumbar 1 (L1) level using Weasis software and muscle fat fraction (chemical shift MRI) was calculated with the formula: (SI IP − SI OP)/SI IP * 100% as previously described [17].

Transient elastography

Liver stiffness measurements (LSM) were performed in all participants using the FibroScan® (touch 502, Echosens, Paris, France). Both the M probe (3,5 MHz) and the XL probe (2,5 MHz) were available for this study. At least 10 valid measurements, a 60% success rate and an interquartile range of less than 30% of the median elasticity were accepted for further analysis. The final result of LSM is the median of the valid (at least 10) individual measurements. The cut-off value for significant fibrosis (histological grade 2 fibrosis (F2)) and advanced fibrosis (F3) were 7.0 kPa and 8.7 kPa, respectively [21]. In the database, we divided fibrosis into no fibrosis (<7.0 kPa) and significant fibrosis (≥7.0 kPa) groups for further analysis according to the cut-off value of 7.0 kPa.

Data statistics

Statistical analyses were performed using SPSS 25.0 (IBM, Armonk, NY, USA) and Graphpad Prism 6.0 for Microsoft Windows. All data were expressed as means ± SEM. The differences of myosteatosis between males and females were tested using unpaired *t-test* in Graphpad Prism

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6.0. Pearson's correlations were performed to determine simple correlations between plasma CTSD levels and other parameters. Subsequently, multiple linear regression analyses were performed to analyze the association between plasma CTSD levels and hepatic fat fraction/fibrosis/myosteatosis, in which plasma CTSD levels were added as dependent variable and either hepatic fat fraction, fibrosis or myosteatosis as independent variables, resulting in model 1 (simple regression), model 2 (model 1 + adjustment for sex), model 3 (model 2 + adjustment for age), model 4 (model 3 + adjustment for BMI) and model 5 (model 4 + adjustment for waist circumference). Additionally, the association between plasma CTSD levels and myosteatosis was also investigated in a multiple linear regression analysis adjusted for sex, age, BMI and waist circumference, using myosteatosis as dependent variable and plasma CTSD levels as independent variable. Only in the multiple linear regression of plasma CTSD levels and myosteatosis, hepatic fat fraction was adjusted in the model 6 (model 5+hepatic fat fraction). *p*-value <0.05 was considered statistically significant.

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Results

Anthropometric and clinical characteristics of the study participants

The baseline characteristics of participants are summarized in Table 3.1. As previously described [16], forty-five NAFLD patients participated in our study, of which 24 subjects were males and 21 individuals were females, with an average age of 51.1 ± 1.7 years old and a mean BMI of 32.9 ± 0.8 Kg/m^2. The average of myosteatosis was 35.57 ± 1.65%, ranging from 8.08% to 54.31%. Finally, plasma CTSD levels ranged from 40150.00pg/mL to 1364000.00pg/mL, with a mean of 440776.82 ± 43143.42 pg/mL.

Table 3.1. Baseline clinical characteristics of the study participants

	Mean ± SEM	Ranges
Age, yrs	51.1± 1.7	20~65
Sex (M/F)	24/21	-
BMI (kg/m^2)	32.9 ± 0.8	24.6~46.3
Waist circumferences (cm)	108.9 ± 1.8	85.8~146.9
Hip (cm)	114.0 ± 1.8	96.0~154.3
WHR	0.96 ± 0.01	0.73~1.10
SBP (mmHg)	134.5 ± 2.2	107~177
DBP (mmHg)	81.0 ± 1.4	62~115
Heart rate (bpm)	66.2 ± 1.4	49~97
HbA1c (%)	5.87 ± 0.14	4.50~9.10
Fasting glucose (mmol/L)	6.3 ± 0.3	4.8~13.0
Total Cholesterol (mmol/L)	5.2 ± 0.1	3.6~7.7
Triglycerides (mmol/L)	2.15 ± 0.34	0.65~15.58
HDL Cholesterol (mmol/L)	1.26 ± 0.06	0.50~2.20
LDL Cholesterol (mmol/L)	3.2 ± 0.1	1.8~5.4
GGT (U/L)	54.9 ± 7.6	8~256
ALT (U/L)	46.2 ± 6.8	7~272
AST (U/L)	32.0 ± 3.0	11~118
Bilirubin (umol/L)	8.5 ± 0.6	3.7~23.1
Alkaline phosphatase (IU/L)	95.4 ± 4.7	52~186
Hepatic fat fraction (%)	19.51 ± 1.61	6.62~45.49
Fibroscan liver stiffness (Kpa)	6.9 ± 0.4	3.6~12.2
Fibrosis (Yes/No)	18/25	-
Myosteatosis (%)	35.57 ± 1.65	8.08~54.31
Skeletal muscle mass index (kg/m^2)	8.64 ± 0.19	5.69~11,20
CTSD levels (pg/mL)	440776.82 ± 43143.42	40150.00~1364000.00

Data are mean ± SEM. BMI, body mass index; WHR, waist-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, hemoglobin; HDL, high density lipid protein; LDL, low density lipid protein; GGT, gamma-glutamyl transpeptidase; ALT, alanine transaminase; AST, Aspartate transaminase.

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Hepatic steatosis rather than fibrosis is positively associated with plasma CTSD levels

To validate our previous finding that the liver contributes to plasma CTSD levels, we assessed whether hepatic steatosis and fibrosis associated with plasma CTSD levels in our current NAFLD patients. As shown in Table 3.2, linear regression analyses were performed by adjusting for sex, age, BMI and waist. Our data showed that hepatic fat fraction positively associated with plasma CTSD levels (standardized coefficient β=0.300, 95% CI: 0.003~0.595, p=0.048) in model 1. Though the association was nearly significant after adjustment for sex (model 2: standardized coefficient ß=0.288, 95% CI: -0.012~0.586, p=0.059), and age (model 3: standardized coefficient ß=0.308, 95% CI: -0.002~0.617, p=0.051), hepatic fat fraction was still significantly associated with plasma CTSD levels after correcting for BMI (model 4: standardized coefficient ß=0.316, 95% CI: 0.019~0.610, p=0.037) and further adjustment for waist circumference (model 5: standardized coefficient ß=0.325, 95% CI: 0.024~0.623, p=0.035) (Table 3.2). These data demonstrate that hepatic steatosis is positively associated with plasma CTSD levels independent of sex, age, BMI and waist circumference. As displayed in supplementary figure 3.1, plasma CTSD levels are not significantly different between 'no fibrosis' and 'significant fibrosis' groups. Similarly, we didn't observe associations between plasma CTSD levels and fibrosis (as shown in table 3.2). Altogether, these data demonstrate that hepatic steatosis, but not fibrosis, is independently associated with plasma CTSD levels, confirming the link of the liver to plasma CTSD levels.

Table 3.2. Hepatic fat fraction (steatosis) is positively associated with plasma CTSD levels.

Dependent variable: plasma CTSD levels						
	Hepatic fat fraction			Fibrosis		
Models	**Adjusted R square**	**Standardized coefficient β (95% CI)**	**p value**	**Adjusted R square**	**Standardized coefficient β (95% CI)**	**p value**
Model 1	0.068	0.300 (0.003~0.595)	0.048	0.009	0.182 (-0.135~0.504)	0.250
Model 2 (Model 1+Sex)	0.062	0.288 (-0.012~0.586)	0.059	0.019	0.189 (-0.126~0.510)	0.229
Model 3 (Model 2+Age)	0.047	0.308 (-0.002~0.617)	0.051	-0.005	0.187 (-0.133~0.513)	0.242
Model 4 (Model 3+BMI)	0.134	0.316 (0.019~0.610)	0.037	0.050	0.073 (-0.267~0.414)	0.663
Model 5 (Model 4+Waist circumference)	0.120	0.325 (0.024~0.623)	0.035	0.026	0.094 (-0.274~0.466)	0.603

Data were analyzed by multiple linear regression models: Model 1, simple regression; Model 2, model 1 + adjustment for sex; Model 3, model 2 + adjustment for age; Model 4, model 3 + adjustment for BMI; Model 5, model 4 + adjustment for waist circumference. $p<0.05$ is statistically significant.

The positive association between myosteatosis and plasma CTSD levels independent of sex, age, BMI, waist circumference and hepatic fat fraction

To investigate whether the muscle is also involved with plasma CTSD levels, the association between myosteatosis and plasma CTSD levels was analyzed by Pearson's correlation and multiple linear regression analyses. As shown in Figure 3.1A, we observed that plasma CTSD levels positively correlated with myosteatosis ($r=0.358$, $p=0.017$). Moreover, in figure 3.1B, we also found that myosteatosis was gender-dependent, showing higher values for myosteatosis in females than males ($p<0.01$). To further evaluate whether myosteatosis was dependently or independently associated with plasma CTSD levels, multiple linear regression analysis was performed by adjusting for sex, age, BMI, waist and hepatic steatosis. As displayed in table 3.3, myosteatosis positively associated with plasma CTSD levels (model 1: standardized coefficient β=0.358, 95% CI: 0.067~0.644, p=0.017), even after adjustment for sex (model 2: standardized coefficient β=0.525, 95% CI: 0.219~0.822, p=0.001), age (model 3: standardized coefficient β=0.537, 95% CI: 0.223~0.843, p=0.001), BMI (model 4: standardized coefficient β=0.471, 95% CI: 0.136~0.799, p=0.007), waist circumference (model 5: standardized coefficient β=0.474, 95% CI: 0.129~0.812, p=0.008) and further correcting for hepatic fat fraction (model 6: standardized coefficient ß=0.488, 95% CI: 0.165~0.805, p=0.004). Vice versa, and shown in Supplementary table 3.1, we also observed that plasma CTSD levels independently associated with myosteatosis independent of sex, age, BMI, waist circumstances and hepatic steatosis.

As such, these data indicate an independent positive association of myosteatosis with plasma CTSD levels in NAFLD patients, suggesting that, in addition to the liver, also the muscle interacts with plasma CTSD levels.

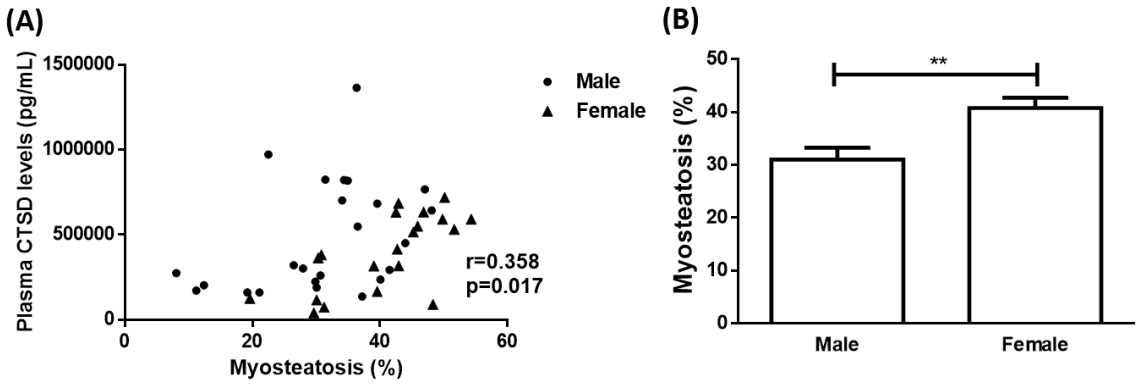

Figure 3.1. Myosteatosis is gender-dependent and positively correlates with plasma CTSD levels in NAFLD patients. Pearson correlation was performed to analyse correlation. $p<0.05$ is statistically significant. **$p<0.01$

Table 3.3. Myosteatosis is positively associated with plasma CTSD levels independent of sex, age, BMI, waist circumference and hepatic fat fraction (steatosis).

Dependent variable: plasma CTSD levels			
		Myosteatosis	
Models	**Adjusted R square**	**Standardized coefficient β (95% CI)**	**p value**
Model 1	0.108	0.358 (0.067~0.644)	0.017
Model 2 (Model 1+Sex)	0.210	0.525 (0.219~0.822)	0.001
Model 3 (Model 2+Age)	0.194	0.537 (0.223~0.843)	0.001
Model 4 (Model 3+BMI)	0.198	0.471 (0.136~0.799)	0.007
Model 5 (Model 4+Waist circumference)	0.177	0.474 (0.129~0.812)	0.008
Model 6 (Model 5+Hepatic fat fraction)	0.280	0.488 (0.165~0.805)	0.004

Data were analyzed by multiple linear regression models: Model 1, simple regression; Model 2, model 1 + adjustment for sex; Model 3, model 2 + adjustment for age; Model 4, model 3 + adjustment for BMI; Model 5, model 4 + adjustment for waist circumference; Model 6, model 5 + adjustment for hepatic fat fraction (steatosis). $p<0.05$ is statistically significant.

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Discussion

The exact mechanisms underlying NAFLD development and progression are not yet fully elucidated. Previously, our studies have demonstrated that plasma CTSD levels are associated with the development of hepatic inflammation and dyslipidemia, suggesting that plasma CTSD is a key player in metabolic disturbances in NAFLD [4,5]. In the current study, our data shows the positive and independent association between myosteatosis and plasma CTSD levels in NAFLD patients, identifying a link between plasma CTSD levels and the muscle. This observed link supports the concept of a significant role of the skeletal muscle in metabolic disturbances in metabolic syndrome-related disorders.

The consumption of highly caloric, lipid-rich diets is one of the major propellers of MetS and the development of metabolic syndrome associated disorders [22,23]. Previously, we established lysosomal lipid accumulation as a link between increased cellular uptake of lipids, metabolic disturbances and hepatic inflammation in mouse models for NASH [4]. In addition to interfering with proper lysosomal function and thus triggering pro-inflammatory pathways within cells, lysosomal lipid accumulation causes the leaking of lysosomal contents, culminating in increased secretion of CTSD into the plasma [24 25,26]. In line, we have previously shown that NAFLD progression is positively associated with plasma CTSD levels [6], indicating that increased metabolic dysfunction and inflammation are accompanied by increased plasma CTSD levels. Furthermore, our studies show that enhanced plasma CTSD levels in adult NASH patients are likely derived from Kupffer cells [6], thus pinpointing the liver as a source of plasma CTSD following hepatic steatosis and inflammation. In the current study, the observation that myosteatosis in NAFLD patients independently associates with plasma CTSD levels raises the possibility that the muscle, as a metabolic organ, also contributes to plasma CTSD levels. Mechanistically, the increased secretion of CTSD induced by lipid overload in the muscle can be explained by lysosomal exocytosis, a process that is regulated by intracellular Ca^{2+} channels [25]. Moreover, lipid accumulation in cells is able to induce chronic intracellular Ca^{2+} overload, subsequently stimulating lysosomes to fuse with the plasma membrane and thus releasing lysosomal enzymes (i.e., CTSD) into the circulation via exocytosis [27,28]. Vice versa, and potentially even more interesting, our observed link also raises the possibility that plasma CTSD levels increase myosteatosis, considering plasma CTSD as a mediator inducing ectopic lipid accumulation. Whether myosteatosis leads to plasma CTSD levels, vice versa, or both is a matter of future debate. Nevertheless, our data show a link between myosteatosis and plasma CTSD in NAFLD patients, implying an involvement of the skeletal muscle in MetS-associated disorders.

Previously, myosteatosis has been strongly associated with obesity, diabetes and MetS, suggesting that myosteatosis plays an important role in metabolic disturbances in the context of metabolic syndrome-related disorders [13]. Of note, ample evidence has demonstrated that

myosteatosis induces the secretion of local and systemic pro-inflammatory cytokines [14], which are key mediators in the pathogenesis of NAFLD [29]. In addition to pro-inflammatory cytokines, our current study suggests that myosteatosis may further contribute to systemic metabolic dysfunction potentially via increased secretion of CTSD. How plasma CTSD further fuels this metabolic dysfunction is a topic for future research, though interactions with insulin and lipid metabolism appear to be involved players [4,30].

Furthermore, myosteatosis has been shown to contribute to the aetiology of insulin resistance through the impairment of insulin signalling [31,32], modulation of adipokine (e.g., adiponectin) secretion [33] and inhibiting nutritive muscle blood flow [34]. Importantly, insulin resistance is recognized as a critical pathophysiological factor in NAFLD [35]. As such, the aforementioned findings suggest that myosteatosis is potentially involved in the pathophysiology of NAFLD by modulating insulin resistance. In line, as previously shown in this cohort, myosteatosis is positively associated with insulin resistance in NAFLD patients [17]. Indeed, our previous studies have demonstrated that inhibiting circulating CTSD reduces plasma insulin levels and improves insulin resistance, suggesting that plasma CTSD aggravates insulin resistance [5,36]. As such, these findings provide support for the possibility that myosteatosis contributes to insulin resistance in NAFLD via increased CTSD secretion.

Although the cohort that we used is relatively small, we demonstrate for the first time a positive and independent association between myosteatosis and plasma CTSD levels in NAFLD patients. On one hand, our findings indicate that the muscle, in parallel with the liver, associates with plasma CTSD levels, which may further trigger and exacerbate metabolic dysfunction in NAFLD patients. In addition, whether other metabolically active organs, for instance, adipose tissue also link to plasma CTSD levels in NAFLD should be further addressed in the future and studies on larger cohorts should be conducted to validate our findings. Furthermore, more research is necessary to better understand the underlying mechanism between the interaction of myosteatosis and plasma CTSD levels in the context of MetS.

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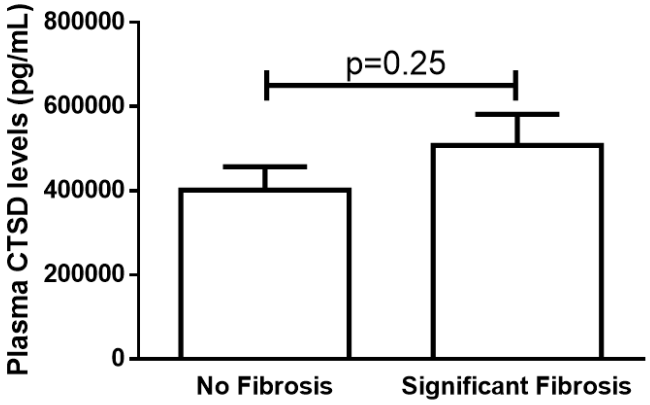
Supplementary data



Supplementary Figure 3.1. **Plasma CTSD levels are not significantly different between no fibrosis and significant fibrosis groups.** p<0.05 is statistically significant.

Supplementary table 3.1.
Plasma CTSD levels are independently associated with myosteatosis in NAFLD patients

Dependent variable: Myosteatosis			
		Plasma CTSD levels	
Models	**Adjusted R square**	**Standardized coefficient β (95% CI)**	**p value**
Model 1	0.108	0.358 (0.068~0.654)	0.017
Model 2 (Model 1+Sex)	0.344	0.435 (0.184~0.693)	0.001
Model 3 (Model 2+Age)	0.351	0.432 (0.182~0.689)	0.001
Model 4 (Model 3+BMI)	0.376	0.367 (0.108~0.631)	0.007
Model 5 (Model 4+Waist circumference)	0.378	0.359 (0.099~0.624)	0.008
Model 6 (Model 5+Hepatic fat fraction)	0.387	0.416 (0.142~0.695)	0.004

Data were analyzed by multiple linear regression models: Model 1, simple regression; Model 2, model 1 + adjustment for sex; Model 3, model 2 + adjustment for age; Model 4, model 3 + adjustment for BMI; Model 5, model 4 + adjustment for waist circumference; Model 6, model 5 + adjustment for hepatic fat fraction (steatosis). p<0.05 is statistically significant.

Chapter 4

Plasma cathepsin D activity is negatively associated with hepatic insulin sensitivity in overweight and obese humans

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Chapter 4

Abstract

Aims/hypothesis Insulin resistance in skeletal muscle and liver plays a major role in the pathophysiology of type 2 diabetes. The hyperinsulinaemic–euglycaemic clamp is considered the gold standard for assessing peripheral and hepatic insulin sensitivity, yet it is a costly and labour-intensive procedure**.** Therefore, easy-to-measure, cost-effective approaches to determine insulin sensitivity are needed to enable organ-specific interventions. Recently, evidence emerged that plasma cathepsin D (CTSD) is associated with insulin sensitivity and hepatic inflammation. Here, we aimed to investigate whether plasma CTSD is associated with hepatic and/or peripheral insulin sensitivity in humans.

Methods 94 overweight and obese adults (BMI, 25–35kg/m^2) underwent a two-step hyperinsulinaemic–euglycaemic clamp (using [6,6-$^2H_2$] glucose) to assess hepatic and peripheral insulin sensitivity (per cent suppression of endogenous glucose output during the low-insulin-infusion step, and the rate of glucose disappearance during high-insulin infusion [40 mU/(m^2 × min)], respectively). Additionally, plasma CTSD levels, CTSD activity and plasma inflammatory cytokines were measured.

Results Plasma CTSD levels were positively associated with the proinflammatory cytokines IL-8 and TNF-α (IL-8: standardised β=0.495, p<0.001; TNF-α: standardised β=0.264, p=0.012). Plasma CTSD activity was negatively associated with hepatic insulin sensitivity (standardised β=−0.206, p=0.043), independent of age, sex, BMI and waist circumference, but plasma CTSD activity was not associated with peripheral insulin sensitivity. However, plasma IL-8 and TNF-α were not significantly correlated with hepatic insulin sensitivity.

Conclusions/interpretation We demonstrate that plasma CTSD activity, but not systemic inflammation, is inversely related to hepatic insulin sensitivity, suggesting that plasma CTSD activity may be used as a non-invasive marker for hepatic insulin sensitivity in humans.

Chapter 4

Introduction

Due to the obesity epidemic, the incidence and prevalence of type 2 diabetes mellitus continues to rise globally.[1] Type 2 diabetes is characterised by a relative insulin deficiency and an impaired insulin sensitivity in certain specific target organs.[2] Insulin sensitivity can be subdivided into whole-body and tissue-specific insulin sensitivity, including peripheral and hepatic insulin sensitivity. Impaired peripheral insulin sensitivity reduces insulin-mediated glucose uptake from blood into peripheral tissues, mainly skeletal muscle, while impaired hepatic insulin sensitivity manifests as the inability of insulin to suppress hepatic glucose production.[3]

Interventions that improve insulin sensitivity may be organ-specific. Individuals with impaired peripheral insulin sensitivity are most likely to respond to interventions that improve skeletal insulin sensitivity, for instance, treatment with a peroxisome proliferator-activated receptor-γ agonist, exercise or a specific diet composition.[4,5] On the other hand, individuals with impaired hepatic insulin sensitivity most likely benefit from interventions that specifically improve hepatic insulin sensitivity, such as metformin treatment.[4] Thus, determination of peripheral and hepatic insulin sensitivity is essential in the decision for a targeted therapeutic regimen for individuals. Indices for whole-body or tissue-specific insulin sensitivity can be derived from plasma insulin and glucose values. For instance, based on an oral glucose tolerance test (OGTT), whole-body and tissue-specific insulin sensitivity can be estimated.[6] However, the two-step hyperinsulinaemic–euglycaemic clamp, in conjunction with the use of a glucose tracer, is considered the gold-standard approach to assessing peripheral and hepatic insulin sensitivity; yet, this procedure is a difficult, labour-intensive and costly.[7] Therefore, there is a need for easier, cost-effective ways to assess hepatic and peripheral insulin sensitivity.

Cathepsin D (CTSD), a lysosomal aspartyl protease, is ubiquitously distributed[8] but the liver is one of the organs with the highest levels of CTSD protein.[9] Further, the liver comprises the largest population of macrophages,[10] which are known to contain high levels of lysosomal enzymes, including CTSD.[11,12] Recently, it has been demonstrated that plasma CTSD is negatively associated with insulin sensitivity, suggesting a link between plasma CTSD and insulin sensitivity.[13,14] Moreover, previous findings from our group indicate that plasma CTSD correlates with hepatic inflammation in individuals with non-alcoholic fatty liver disease (NAFLD),[15,16] linking plasma CTSD to metabolic alterations in the liver. Furthermore, we recently found that inhibiting extracellular CTSD activity reduces plasma insulin levels and fatty liver features in steatotic rat livers.[17] Likewise, another study from our group demonstrated improved hepatic lipid metabolism in response to CTSD inhibition,[18] pointing towards a mechanistic role for CTSD in hepatic metabolism. However, it remains to be elucidated whether plasma CTSD is linked to hepatic insulin sensitivity.

Chapter 4

Considering the inverse association between plasma CTSD and whole-body insulin sensitivity, on one hand,[13,14] and the positive correlation between plasma CTSD and hepatic inflammation on the other hand,[15,16] we hypothesised that plasma CTSD is inversely correlated with hepatic insulin sensitivity in humans.

Methods

Participant characteristics

Ninety-four overweight and obese white men and women were included in the present study (age, 19–69 years; BMI >25 kg/m^2). These individuals participated in two larger clinical trials designed to primarily investigate the effects of antibiotics[7] and polyphenol supplementation[19] on insulin sensitivity in humans (Clinicaltrials.gov registration no. NCT02241421 and NCT02381145, respectively). Participants had a low physical activity level (<3 h of organised sports activities per week) and were weight-stable (<2 kg body-weight change in the 3 months prior to inclusion). The participants either had a normal glucose tolerance (fasting glucose <6.1 mmol/l, 2 h glucose <7.8 mmol/l; *n*=38) or impaired glucose metabolism (impaired fasting glucose: plasma glucose >5.6 mmol/l; impaired glucose tolerance: 2 h glucose 7.8–11.1 mmol/l; *n*=56). Furthermore, participants were not allowed to use lipid- and glucose-lowering drugs, anti-oxidants, corticosteroids or supplements that might impact glucose homeostasis for 3 months before entering the study. Exclusion criteria were pregnancy, menopause, lactation, cancer and any reported history of chronic inflammatory, cardiovascular, hepatic, pulmonary, renal or gastrointestinal diseases.

All participants gave written informed consent before entering this study, which was reviewed and approved by the Medical Ethical Committee of Maastricht University, and was carried out in accordance with the principles of the Declaration of Helsinki, as revised in 2008.

Baseline clinical investigations

Participants were provided with a standardised low-fibre, low-fat evening meal and were instructed to come to the university after an overnight fast (10–12 h), Anthropometric measures included height, weight and waist circumference-to-hip circumference ratio (WHR). After inserting a cannula into the antecubital vein, blood samples were taken during the post-absorptive state.

Biochemical analyses

After overnight fasting, blood collection was conducted at Maastricht University. Blood was collected into pre-chilled tubes and centrifuged (1000 *g* for 10 min at 4°C) and plasma was snap-frozen in liquid nitrogen and stored at −80°C until analyses. Being commonly used as inflammatory markers in the context of obesity,[20] insulin resistance[21] and hepatic

inflammation,[16] plasma TNF-α, IL-6 and IL-8 were measured. In addition to the measurement performed in the previous studies,[7,19] we also measured plasma CTSD levels and activity. Plasma samples were diluted and CTSD levels were determined by the CTSD ELISA, according to the manufacturer's protocol (USCN Life Science, Wuhan, China). Absorbance was measured on a Benchmark 550 microplate reader (Bio-Rad, Hercules, CA, USA); the detection limit ranged from approximately 46.88 to 3.000 pg/ml. Plasma CTSD activity was measured using a CTSD activity assay kit according to the manufacturer's protocol (MBL International, Woburn, MA, USA). The concentrations of plasma inflammatory markers (IL-6, IL-8 and TNF-α) were determined using a multiplex ELISA (Human Proinflammatory II 4-Plex Ultra-Sensitive Kit; Meso Scale Diagnostics, Rockville, MD, USA). Alanine amino transferase (ALT) was determined using routine analyses at the clinical chemistry department of the Maastricht UMC+ hospital. Fasting and 2 h plasma glucose levels during a 75g OGTT, non-esterified fatty acids (NEFA) and triacylglycerol (TAG) were analysed with an automated spectrophotometer (ABX Pentra 400 autoanalyser; Horiba ABX, Montpellier, France), using enzymatic colourimetric assays.

Two-step hyperinsulinaemic–euglycaemic clamp

A two-step hyperinsulinaemic–euglycaemic clamp combined with a [6,6-$^2H_2$] glucose tracer (Cambridge Isotope Laboratories, Tewksbury, MA, USA) was performed to measure rate of disappearance (R_d) and endogenous glucose production (EGP).[22] The first cannula was inserted into the antecubital vein. A second cannula was inserted into a superficial dorsal hand vein for the sampling of arterialised blood (by using a hot box with air circulating at ~50–55°C). After the administration of a bolus injection of 2.4 mg [6,6-$^2H_2$] glucose/kg, a continuous [6,6-$^2H_2$] glucose infusion was started at 0.04 mg/(kg × min) and continued throughout the measurement. After 2 h, insulin infusion was started at 10 mU/(m^2 × min) for 3 h to assess hepatic insulin sensitivity (%EGP suppression), followed by 40 mU/(m^2 × min) insulin for the last 2.5 h to assess peripheral insulin sensitivity (R_d40: rate of glucose disappearance during high-insulin-infusion (40 mU/(m^2 × min)). By variable co-infusion of a 20% glucose solution (wt/vol), enriched to 1.92 mg tracer/ml, blood glucose concentrations were maintained at around 5.0 mmol/l. During the last 30 min of the baseline period and during each insulin-infusion step [0, 10, and 40 mU/(m^2 × min)], three blood samples were collected. Kinetics of R_d were calculated during 0 and 40 mU/(m^2 × min) insulin infusion as absolute increases between these steps [Δ μmol/(kg × min)]. In the meantime, insulin-mediated suppression of EGP was calculated during 0 and 10 mU/(m^2 × min) insulin infusion, which was regarded as relative suppression during 10 vs 0 mU/(m^2 × min) insulin.

Statistical analysis

Statistical analysis was performed by using IBM SPSS Statistics for Windows, version 24 (IBM Corp., Armonk, N.Y., USA). All data are expressed as mean ± SEM. A univariate general linear model was used to analyse associations between plasma CTSD levels and activity, R_d40 and %EGP suppression as dependent variables, respectively, and other parameters as independent variables. Subsequently, multiple linear regression analyses were performed with plasma CTSD levels and activity separately added as independent variables and either R_d40 or %EGP suppression as dependent variables, resulting in Model 1 (simple regression), Model 2 (Model 1 + adjustment for age), Model 3 (Model 2 + adjustment for sex), Model 4 (Model 3 + adjustment for BMI) and Model 5 (Model 4 + adjustment for waist circumference). In addition, multiple linear regression analyses were performed with TNF-α or IL-8 as independent variables (with adjustment for age, sex, BMI and waist circumference in similar models as those mentioned above), and plasma CTSD levels or activity as dependent variables, respectively. Interaction between co-variates in the multiple regression analyses was also tested but no significant interactions were found (data not shown).

For the present study, we calculated that 52 individuals would be needed to demonstrate a significant association between CTSD activity and hepatic insulin sensitivity with a power of 80% ($\alpha = 0.05$, $\beta = 0.8$, effect size $f^2 = 0.20$). Since we included 94 overweight and obese white men and women that had previously participated in two larger clinical trials,[7,19] this study was well powered.

Results

Anthropometric and clinical characteristics of the study population

Participant characteristics are summarised in Table 4.1. Ninety-four overweight and obese individuals (74 men and 20 women) were involved in this study. Age ranged from 19 to 69 years and BMI ranged from 25.4 to 38.6 kg/m^2. The mean plasma CTSD levels and activity were 6586.2 ± 598.9 pg/ml and 237.5±11.0 RFU/µl, respectively. Additionally, the correlation between plasma CTSD levels/activity and other parameters related to overweight and obesity were determined (as shown in supplementary Table 4.1). We found that plasma CTSD levels were significantly correlated with hip measurements, WHR, NEFA and TAG, whereas plasma CTSD activity significantly correlated with TAG only. No significant correlation was observed between plasma CTSD levels/activity and ALT.

Table 4.1 Baseline clinical characteristics of the study participants

	Mean ± SEM	Range
Age, years	50.4 ± 1.4	19–69
Sex, M/F	74/20	
BMI, kg/m^2	30.6 ± 0.3	25.4–38.6
Waist circumference, cm	103.3 ± 1.3	77.0–126.0
Hip circumference, cm	106.2 ± 0.8	89.0–125.0
WHR	0.98 ± 0.01	0.70–1.22
Fasting glucose, mmol/l	5.69 ± 0.07	4.39–7.47
2 h glucose, mmol/l[a]	6.53 ± 0.19	3.35–11.21
IL–6, pg/ml	0.92 ± 0.06	0.07–4.10
IL–8, pg/ml	6.99 ± 0.34	1.90–18.37
TNF–α, pg/ml	2.66 ± 0.07	1.60–5.37
R_d40, µmol kg^{-1} min^{-1}	27.2 ± 1.1	9.8–54.0
EGP suppression, %[b]	50.3 ± 2.1	5.2–97.9
CTSD levels, pg/ml	6586.2 ± 598.9	292.7–32,962.8
CTSD activity, RFU/µL	237.5 ± 11.0	18.3–628.5

Data are mean ± SEM.[a]Plasma glucose concentration 2 h after ingestion of 75g of glucose. [b]Per cent of endogenous glucose production suppression. F, female; M, male; RFU, relative fluorescence units

Plasma CTSD activity is negatively associated with hepatic insulin sensitivity

Next, we tested our hypothesis that plasma CTSD associates specifically with hepatic insulin sensitivity. Plasma CTSD levels were not significantly associated with hepatic insulin sensitivity (per cent of EGP suppression; Table 4.2), neither in the unadjusted model (Model 1: standardised β, 0.171 [95% CI −0.048, 0.410]; p=0.120) nor after adjustment for age (Model 2), sex (Model 3), BMI (Model 4) and waist circumference (Model 5). In contrast, plasma CTSD activity was nearly associated with hepatic insulin sensitivity (Model 1: standardised β, −0.200 [95% CI −0.416, 0.013]; p=0.065), which was statistically significant after adjustment for age (Model 2: standardised β, −0.269 [95% CI −0.487, −0.059]; p=0.013), sex (Model 3: standardised β, −0.221 [95% CI −0.428, −0.020]; p=0.031), BMI (Model 4: standardised β, −0.216 [95% CI −0.422, −0.016]; p=0.035) and waist circumference (Model 5: standardised β, −0.206 [95% CI −0.411, −0.007]; p=0.043), demonstrating that hepatic insulin sensitivity was negatively associated with plasma CTSD activity, independently of age, sex, BMI and waist circumference.

In contrast, peripheral insulin sensitivity (R_d40) was only associated with plasma CTSD levels in the unadjusted model (Model 1: standardised β, 0.231 [95% CI 0.023, 0.425]; p=0.030); after adjustment for age, sex, BMI and waist circumference, R_d40 was no longer associated with plasma CTSD levels (Table 4.3). Plasma CTSD activity was not associated with R_d40 in any of the models (Table 4.3). Collectively, these findings demonstrate that hepatic insulin sensitivity is negatively associated with plasma CTSD activity, independently of age, sex, BMI and waist circumference.

Table 4.2 Plasma CTSD activity is independently negatively associated with hepatic insulin sensitivity (%EGP suppression)

CTSD levels				CTSD activity			
Model	**%EGP suppression**			**Model**	**%EGP suppression**		
	***p* value**	**β (95% CI)**	**Adjusted R^2**		***p* value**	**β (95% CI)**	**Adjusted R^2**
Model 1			0.017	**Model 1**			0.029
CTSD levels	0.120	0.171 (−0.048, 0.410)		**CTSD activity**	0.065	−0.200 (−0.416, 0.013)	
Model 2			0.048	**Model 2**			0.116
CTSD levels	0.654	0.056 (−0.203, 0.322)		**CTSD activity**	0.013 *	−0.269 (−0.487, −0.059)	
Age	0.062	−0.236 (−0.473, 0.011)		**Age**	0.003 **	−0.321 (−0.525, −0.109)	
Model 3			0.180	**Model 3**			0.215
CTSD levels	0.798	0.030 (−0.213, 0.276)		**CTSD activity**	0.031 *	−0.221 (−0.428, −0.020)	
Age	0.827	0.030 (−0.236, 0.294)		**Age**	0.583	−0.069 (−0.314, 0.178)	
Sex	0.000 ***	0.466 (0.207, 0.693)		**Sex**	0.001 **	0.408 (0.160, 0.631)	
Model 4			0.182	**Model 4**			0.218
CTSD levels	0.836	0.024 (−0.219, 0.270)		**CTSD activity**	0.035 *	−0.216 (−0.422, −0.016)	
Age	0.790	0.036 (−0.229, 0.300)		**Age**	0.641	−0.059 (−0.304, 0.188)	
Sex	0.001 **	0.448 (0.188, 0.677)		**Sex**	0.002 **	0.391 (0.142, 0.616)	
BMI	0.288	−0.111 (−0.318, 0.096)		**BMI**	0.262	−0.112 (−0.306, 0.085)	
Model 5			0.197	**Model 5**			0.233
CTSD levels	0.571	0.067 (−0.178, 0.320)		**CTSD activity**	0.043 *	−0.206 (−0.411, −0.007)	
Age	0.256	0.192 (−0.139, 0.513)		**Age**	0.612	0.076 (−0.219, 0.370)	
Sex	0.021 *	0.340 (0.052, 0.605)		**Sex**	0.029 *	0.299 (0.030, 0.550)	
BMI	0.506	0.121 (−0.239, 0.480)		**BMI**	0.537	0.104 (−0.226, 0.431)	
Waist circumference	0.122	−0.384 (−0.884, 0.107)		**Waist circumference**	0.114	−0.360 (−0.822, 0.090)	

Data were analysed by linear regression models: Model 1, simple regression; Model 2, Model 1 + adjustment for age; Model 3, Model 2 + adjustment for sex; Model 4, Model 3 + adjustment for BMI; Model 5, Model 4 + adjustment for waist circumference. *p<0.05, **p<0.01, ***p<0.001

Table 4.3 Plasma CTSD levels and CTSD activity are not associated with peripheral insulin sensitivity (R_d40).

Model		R_d40		Model		R_d40	
	p value	β (95% CI)	Adjusted R^2		*p* value	β (95% CI)	Adjusted R^2
Model 1			0.042	**Model 1**			−0.009
CTSD levels	0.030 *	0.231 (0.023, 0.425)		**CTSD activity**	0.666	−0.046 (−0.254, 0.163)	
Model 2			0.095	**Model 2**			0.098
CTSD levels	0.390	0.103 (−0.128, 0.324)		**CTSD activity**	0.218	−0.130 (−0.339, 0.079)	
Age	0.021 *	−0.279 (−0.495, −0.042)		**Age**	0.001 **	−0.350 (−0.552, −0.141)	
Model 3			0.162	**Model 3**			0.150
CTSD levels	0.359	0.105 (−0.117, 0.318)		**CTSD activity**	0.405	−0.086 (−0.293, 0.119)	
Age	0.582	−0.075 (−0.333, 0.188)		**Age**	0.218	−0.158 (−0.407, 0.094)	
Sex	0.007 **	0.341 (0.090, 0.561)		**Sex**	0.015 *	0.309 (0.061, 0.544)	
Model 4			0.360	**Model 4**			0.348
CTSD levels	0.583	0.055 (−0.138, 0.244)		**CTSD activity**	0.466	−0.066 (−0.247, 0.114)	
Age	0.696	−0.047 (−0.273, 0.183)		**Age**	0.374	−0.100 (−0.320, 0.122)	
Sex	0.013 *	0.279 (0.058, 0.473)		**Sex**	0.022 *	0.255 (0.037, 0.462)	
BMI	0.000 ***	−0.460 (−0.626, −0.275)		**BMI**	0.000 ***	−0.457 (−0.625, −0.277)	
Model 5			0.402	**Model 5**			0.384
CTSD levels	0.277	0.108 (−0.085, 0.293)		**CTSD activity**	0.588	−0.048 (−0.224, 0.128)	
Age	0.250	0.163 (−0.113, 0.427)		**Age**	0.569	0.075 (−0.184, 0.333)	
Sex	0.278	0.131 (−0.103, 0.353)		**Sex**	0.265	0.132 (−0.100, 0.359)	
BMI	0.365	−0.138 (−0.432, 0.161)		**BMI**	0.235	−0.175 (−0.459, 0.114)	
Waist circumference	0.012 *	−0.530 (−0.926, −0.119)		**Waist circumference**	0.019 *	−0.472 (−0.873, −0.081)	

Data were analysed by linear regression models: Model 1, simple regression; Model 2, Model 1 + adjustment for age; Model 3, Model 2 + adjustment for sex; Model 4, Model 3 + adjustment for BMI; Model 5, Model 4 + adjustment for waist circumference. *p<0.05, **p<0.01, ***p<0.001

Chapter 4

Plasma TNF-α and IL-8 concentrations are independently positively associated with plasma CTSD levels

To confirm previous studies, which indicate that plasma CTSD is associated with inflammation [16,23], we first investigated the associations between the proinflammatory cytokines TNF-α, IL-6 and IL-8 and plasma CTSD using multiple linear regression (Table 4.4). Plasma TNF-α concentrations were positively associated with plasma CTSD levels (Model 1: standardised β, 0.264 [95% CI 0.063, 0.492]; p=0.012), including after adjustment for age (Model 2: standardised β, 0.252 [95% CI 0.072, 0.458]; p=0.008) and further correction for sex (Model 3: standardised β, 0.301 [95% CI 0.107, 0.527]; p=0.004), BMI (Model 4: standardised β, 0.296 [95% CI 0.101, 0.523]; p=0.004) and waist circumference (Model 5: standardised β, 0.276 [95% CI 0.080, 0.501]; p=0.008). Adjustment for age (Model 2), age and sex (Model 3), age, sex and BMI (Model 4) and age, sex, BMI and waist circumference (Model 5) did not alter the strength and significance of the association between plasma TNF-α and plasma CTSD levels. In contrast to plasma CTSD levels, plasma CTSD activity was not associated with plasma TNF-α levels in Model 1 (standardised β, 0.102; p=0.335). Moreover, after correcting for age, sex, BMI and waist circumference as confounders in the respective linear regression models, the association between plasma CTSD activity and TNF-α remained non-significant (Table 4.4), indicating that plasma CTSD activity does not relate to plasma TNF-α levels.

In accordance with the positive association between plasma TNF-α levels and CTSD levels, plasma IL-8 levels were positively associated with plasma CTSD levels (Model 1: standardised β, 0.495 [95% CI 0.317, 0.693]; p<0.001; Table 4.5), including after adjustment for age (Model 2: standardised β, 0.353 [95% CI 0.159, 0.568]; p=0.001), and further adjustment for sex (Model 3: standardised β, 0.354 [95% CI 0.158, 0.570]; p=0.001), BMI (Model 4: standardised β, 0.349 [95% CI 0.152, 0.566]; p=0.001) and waist circumference (Model 5: standardised β, 0.333 [95% CI 0.137, 0.548]; p=0.001) (Table 4.5). Adjustment for age (Model 2), age and sex (Model 3), age, sex and BMI (Model 4) and age, sex, BMI and waist circumference (Model 5) did not influence the strength and significance of the association. In contrast to the associations with CTSD levels, although plasma IL-8 concentration was associated with CTSD activity in Model 1 (standardised β, 0.294 [95% CI 0.093, 0.492]; p=0.004) and also after adjustment for age (Model 2: standardised β, 0.234 [95% CI 0.008, 0.454]; p=0.042), plasma IL-8 levels were not associated with plasma CTSD activity after further adjustment for sex (Model 3: standardised β, 0.224 [95% CI −0.002, 0.442]; p=0.052), BMI (Model 4: standardised β, 0.226 [95% CI 0.000, 0.446]; p=0.50) and waist circumference (Model 5: standardised β, 0.220 [95% CI −0.007, 0.441]; p=0.057) (Table 4.5). Additionally, compared with the standardised β values and significance level in the association between plasma CTSD activity and plasma IL-8 levels with Model 1 and Model 2, the association between plasma CTSD levels and plasma IL-8 levels was much stronger in all models.

In contrast, no significant associations between plasma CTSD and plasma IL-6 were observed (data not shown). Altogether, our findings show that plasma CTSD levels are positively associated with plasma levels of TNF-α and IL-8, independent of age, sex, BMI and waist circumference, thereby confirming the link between plasma CTSD and inflammation.

Table 4.4 Plasma TNF-α concentration is independently positively associated with plasma CTSD levels.

Model	CTSD levels			CTSD activity		
	p value	β (95% CI)	Adjusted R^2	*p* value	β (95% CI)	Adjusted R^2
Model 1			0.059			−0.001
TNF-α	0.012*	0.264 (0.063, 0.492)		0.335	0.102 (−0.107, 0.312)	
Model 2			0.271			0.027
TNF-α	0.008**	0.252 (0.072, 0.458)		0.396	0.090 (−0.117, 0.294)	
Age	0.000***	−0.463 (−0.648, −0.281)		0.063	−0.198 (−0.402, 0.011)	
Model 3			0.275			0.035
TNF-α	0.004**	0.301 (0.107, 0.527)		0.787	0.031 (−0.192, 0.253)	
Age	0.003**	−0.371 (−0.609, −0.137)		0.024*	−0.309 (−0.568, −0.041)	
Sex	0.226	0.152 (−0.093, 0.395)		0.192	−0.185 (−0.451, −0.092)	
Model 4			0.272			0.027
TNF-α	0.004**	0.296 (0.101, 0.523)		0.747	0.037 (−0.188, 0.261)	
Age	0.003**	−0.363 (−0.602, −0.128)		0.023*	−0.314 (−0.574, −0.044)	
Sex	0.274	0.139 (−0.109, 0.384)		0.226	−0.174 (−0.444, 0.166)	
BMI	0.412	−0.079 (−0.269, 0.111)		0.588	0.059 (−0.152, 0.267)	
Model 5			0.285			0.024
TNF-α	0.008**	0.276 (0.080, 0.501)		0.836	0.024 (−0.203, 0.251)	
Age	0.001**	−0.491 (−0.779, −0.209)		0.018*	−0.398 (−0.716, −0.069)	
Sex	0.105	0.223 (−0.044, 0.488)		0.427	−0.123 (−0.416, 0.178)	
BMI	0.083	−0.287 (−0.617, 0.035)		0.686	−0.076 (−0.434, 0.287)	
Waist circumference	0.123	0.346 (−0.089, 0.791)		0.377	0.222 (−0.271, 0.708)	

Data were analysed by linear regression models: Model 1, simple regression; Model 2, Model 1 + adjustment for age; Model 3, Model 2 + adjustment for sex; Model 4, Model 3 + adjustment for BMI; Model 5, Model 4 + adjustment for waist circumference. *p<0.05, **p<0.01, ***p<0.001

Table 4.5 Plasma IL-8 concentration is independently positively associated with plasma CTSD levels

Model	CTSD levels			CTSD activity		
	p value	β (95% CI)	Adjusted R^2	*p* value	β (95% CI)	Adjusted R^2
Model 1			0.236			0.076
IL-8	0.000 ***	0.495 (0.317, 0.693)		0.004 **	0.294 (0.093, 0.492)	
Model 2			0.310			0.065
IL-8	0.001 **	0.353 (0.159, 0.568)		0.042 *	0.234 (0.008, 0.454)	
Age	0.002 **	−0.321 (−0.521, −0.123)		0.368	−0.103 (−0.325, 0.122)	
Model 3			0.302			0.076
IL-8	0.001 **	0.354 (0.158, 0.570)		0.052	0.224 (−0.002, 0.442)	
Age	0.014 *	−0.307 (−0.550, −0.065)		0.120	−0.215 (−0.480, 0.057)	
Sex	0.830	0.024 (−0.197, 0.245)		0.151	−0.183 (−0.423, 0.066)	
Model 4			0.299			0.069
IL-8	0.001 **	0.349 (0.152, 0.566)		0.050	0.226 (0.000, 0.446)	
Age	0.016 *	−0.300 (−0.544, −0.057)		0.113	−0.221 (−0.488, 0.052)	
Sex	0.907	0.013 (−0.210, 0.237)		0.176	−0.175 (−0.417, 0.077)	
BMI	0.424	−0.075 (−0.263, 0.112)		0.552	0.063 (−0.143, 0.265)	
Model 5			0.313			0.066
IL-8	0.001 **	0.333 (0.137, 0.548)		0.057	0.220 (−0.007, 0.441)	
Age	0.004 **	−0.432 (−0.722, −0.143)		0.077	−0.296 (−0.616, 0.033)	
Sex	0.391	0.109 (−0.141, 0.358)		0.390	−0.123 (−0.395, 0.156)	
BMI	0.075	−0.289 (−0.613, 0.030)		0.753	−0.057 (−0.404, 0.294)	
Waist circumference	0.106	0.355 (−0.077, 0.792)		0.414	0.199 (−0.279, 0.672)	

Data were analysed by linear regression models: Model 1, simple regression; Model 2, Model 1 + adjustment for age; Model 3, Model 2 + adjustment for sex; Model 4, Model 3 + adjustment for BMI; Model 5, Model 4 + adjustment for waist circumference. *p<0.05, **p<0.01, ***p<0.001

Chapter 4

Plasma TNF-α and IL-8 levels are not associated with hepatic insulin sensitivity

To investigate whether CTSD may be a better determinant of hepatic insulin sensitivity than systemic inflammatory factors, we next investigated the association between plasma inflammatory cytokines and hepatic insulin sensitivity. Plasma levels of the inflammatory cytokine TNF-α was only associated with hepatic insulin sensitivity in the unadjusted model (Model 1: standardised β, −0.225 [95% CI −0.463, −0.014]; *p*=0.038; Table 4.6), but after adjustment for age, sex, BMI and waist circumference, no significant associations were observed. Plasma levels of IL-8 were not significantly associated with hepatic insulin sensitivity (Table 4.6). Moreover, both inflammatory parameters did not correlate with peripheral insulin sensitivity (data not shown). Together, these findings indicate that systemic inflammatory markers have no significant predictive value for estimating hepatic insulin sensitivity.

Table 4.6 Plasma TNF-α and IL-8 concentrations are not associated with hepatic insulin sensitivity (%EGP suppression)

Model	EGP suppression (%)			Model	EGP suppression (%)		
	p value	β (95% CI)	Adjusted R^2		*p* value	β (95% CI)	Adjusted R^2
Model 1			0.039	**Model 1**			0.008
TNF-α	0.038 *	−0.225 (−0.463, −0.014)		**IL-8**	0.202	0.139 (−0.075, 0.351)	
Model 2			0.098	**Model 2**			0.055
TNF-α	0.051	−0.208 (−0.447, 0.001)		**IL-8**	0.711	0.044 (−0.191, 0.279)	
Age	0.010 *	−0.277 (−0.484, −0.068)		**Age**	0.032 *	−0.258 (−0.492, −0.023)	
Model 3			0.180	**Model 3**			0.177
TNF-α	0.421	−0.087 (−0.324, 0.137)		**IL-8**	0.571	0.063 (−0.157, 0.282)	
Age	0.757	−0.040 (−0.292, 0.213)		**Age**	0.926	0.013 (−0.253, 0.278)	
Sex	0.004 **	0.401 (0.131, 0.647)		**Sex**	0.001 **	0.445 (0.192, 0.672)	
Model 4			0.185	**Model 4**			0.180
TNF-α	0.388	−0.093 (−0.330, 0.129)		**IL-8**	0.601	0.058 (−0.161, 0.277)	
Age	0.806	−0.031 (−0.284, 0.221)		**Age**	0.883	0.020 (−0.246, 0.285)	
Sex	0.006 **	0.379 (0.108, 0.627)		**Sex**	0.001 **	0.426 (0.172, 0.655)	
BMI	0.227	−0.124 (−0.323, 0.078)		**BMI**	0.252	−0.118 (−0.317, 0.084)	
Model 5			0.200	**Model 5**			0.198
TNF-α	0.502	−0.072 (−0.307, 0.152)		**IL-8**	0.560	0.064 (−0.153, 0.281)	
Age	0.493	0.106 (−0.200, 0.412)		**Age**	0.305	0.163 (−0.151, 0.475)	
Sex	0.044 *	0.295 (0.009, 0.564)		**Sex**	0.020 *	0.327 (0.051, 0.582)	
BMI	0.590	0.094 (−0.248, 0.432)		**BMI**	0.511	0.114 (−0.226, 0.450)	
Waist circumference	0.125	−0.360 (−0.837, 0.104)		**Waist circumference**	0.100	−0.385 (−0.858, 0.076)	

Data were analysed by linear regression models: Model 1, simple regression; Model 2, Model 1 + adjustment for age; Model 3, Model 2 + adjustment for sex; Model 4, Model 3 + adjustment for BMI; Model 5, Model 4 + adjustment for waist circumference. *p<0.05, **p<0.01

Chapter 4

Discussion

Impaired liver and skeletal muscle insulin sensitivity are considered major risk factors for the development of type 2 diabetes. To enable organ-specific interventions for each (pre)diabetic individual it is, therefore, of critical importance to determine the level of hepatic and peripheral insulin sensitivity. Here, we show for the first time that plasma CTSD activity is inversely associated with hepatic insulin sensitivity, independently of age, sex, BMI and waist circumference, but not with systemic inflammation, suggesting that plasma CTSD activity may be used as a non-invasive predictive tool for hepatic insulin sensitivity.

The present finding that plasma CTSD activity is associated with hepatic insulin sensitivity is in line with previous reports implicating the involvement of CTSD in mechanisms leading to the impairment of hepatic insulin sensitivity.[24] In the current study, we did not observe an association between systemic inflammatory markers and hepatic insulin sensitivity. Besides inflammation, lipid accumulation in the liver is known to impact upon hepatic insulin signalling.[24-26] For example, the intracellular lipid mediator ceramide has been extensively linked to impaired hepatic insulin sensitivity via disturbance of Akt-related pathways.[27,28] Notably, ceramide is also responsible for CTSD activation,[29] thereby highlighting ceramide as a potential mediator linking CTSD to hepatic insulin signalling. Consistently, our recently published in vivo studies also demonstrated that inhibiting CTSD activity reduces fatty liver and improves hepatic lipid metabolism.[17,18] Furthermore, *CTSD* gene knockout or mutations also lead to neuronal ceroid lipofuscinosis,[30,31] which is characterised by lipopigments and proteins accumulating in lysosomes.[32] These data, therefore, suggest that the link between CTSD activity and hepatic insulin sensitivity may be mediated by modulation of lipid metabolism. Indeed, TAG levels also associated with plasma CTSD activity in our study. Therefore, these observations urge for more in-depth investigation on how CTSD-related changes in lipid metabolism influence hepatic insulin sensitivity.

In the current study, we found an association between hepatic insulin sensitivity and plasma CTSD activity, but not with plasma CTSD levels. While CTSD levels are the major factor impacting on CTSD activity, it is clear that other factors, such as plasma pH[33] and inhibition by albumin[34] and α2-macroglobulin,[35] also influence the activity of CTSD. Additionally, the total content of CTSD includes the non-mature pro-CTSD enzyme [36]. These factors, therefore, likely explain why CTSD content did not associate with hepatic insulin sensitivity in a similar way to CTSD activity.

Additionally, sex weakens the association between plasma CTSD activity and hepatic insulin sensitivity in the current study, pointing towards the potential impact of sex on our findings. We, therefore, adjusted for sex in the linear regression models. However, even after adjusting for sex, the negative association between CTSD activity and hepatic insulin sensitivity remained significant, suggesting that plasma CTSD activity is (negatively) associated with hepatic insulin sensitivity independently of sex. The low number of women in the present

study did not, unfortunately, allow for sex-specific analyses. Future studies with larger sample sizes and sex-balanced cohorts should be considered to further validate our findings.

The liver constitutes a central position in glucose metabolism, being responsible for at least 75% of endogenous glucose output in the body.[37] Currently, the gold standard for assessing hepatic insulin sensitivity is the hyperinsulinaemic–euglycaemic clamp method.[38] However, owing to its invasive, costly and labour-intensive nature, this technique cannot be used in large populations. As an alternative, several studies have investigated the use of non-invasive plasma markers to assess insulin sensitivity.[39] However, to our knowledge, none of these markers provide specific information on hepatic insulin sensitivity. For instance, while clinical data proposed adiponectin and chemerin as potential markers for whole-body insulin sensitivity, these markers did not show specific predictive value for hepatic insulin sensitivity.[40,41] However, in the present study, we show that assessment of plasma CTSD activity, which is easier, less invasive and cost effective, holds a predictable value to evaluate hepatic insulin sensitivity. Indeed, in contrast to a required blood volume of about 150 ml, a performance duration of approximately 8 h and the high costs related to the two-step hyperinsulinaemic–euglycaemic clamp, the measurement of plasma CTSD activity requires less than 1 ml of blood (5–10µl of plasma), much less time (1.5 h) and more than 75 times lower costs. Furthermore, acquiring information on hepatic insulin sensitivity has potential in supporting the therapeutic regimen for the (pre)diabetic patients. For example, while pharmacological agents, such as metformin and glitazones, aim to specifically improve hepatic insulin resistance by inhibiting hepatic gluconeogenesis,[37,42-44] thiazolidinediones (TZDs) have extrahepatic target organs, mainly adipose tissue.[45-47] Overall, the independent correlation between plasma CTSD activity and hepatic insulin sensitivity holds value as an indication for liver-specific impairments in glucose homeostasis, which may aid in deciding on the targeted therapeutic regimen for (pre)diabetic individuals.

Despite our significant findings, the cohort that was used in our study has a relatively small sample size and an unbalanced sex population, which leads to a weak-to-moderate, although independent, association between plasma CTSD activity and hepatic insulin sensitivity. Furthermore, age-matched healthy, lean control individuals with insulin sensitivity measurements were not included in the current study. Therefore, future studies are warranted to validate our findings in larger cohorts that also include age-matched healthy normal-weight individuals.

Conclusions

The present study demonstrated that, in contrast to inflammatory markers, plasma CTSD activity was negatively associated with hepatic insulin sensitivity, independently of age, sex, BMI and waist circumference. Thus, plasma CTSD activity holds promise as a non-invasive predictive marker to assess hepatic insulin sensitivity.

Chapter 4

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Chapter 4

Supplementary data

Supplementary Table 4.1. The correlation between plasma CTSD levels/activity and other metabolic parameters related to overweight and obesity. p<0.05* is statistically significant.

		CTSD levels		CTSD activity	
	N	**P value**	**Correlation coefficients**	**P value**	**Correlation coefficients**
Hip	94	0.023*	0.244	0.350	0.100
WHR	94	0.000*	-0.417	0.531	-0.067
G0	94	0.000*	-0.456	0.066	-0.191
G120	94	0.002*	-0.324	0.996	0.001
ALT	88	0.295	-0.115	0.594	0.058
FFA	78	0.020*	-0.268	0.876	-0.018
TAG	78	0.012*	0.289	0.020*	0.263

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Chapter 5

Plasma Cathepsin D activity rather than levels correlates with metabolic parameters of type 2 diabetes in male individuals

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Chapter 5

Abstract

Objective: Type 2 diabetes mellitus is a metabolic disorder characterized by insulin resistance. Previous studies in patients demonstrated that plasma levels of cathepsin D (CTSD), which is optimally active in the acidic environment of lysosomes, correlate with insulin resistance. As plasma pH is slightly reduced in type 2 diabetic patients and we have previously shown that plasma CTSD activity is causally linked to insulin levels *in vivo,* it is likely that the activity of CTSD in plasma will be increased in type 2 diabetes compared to healthy individuals. However, so far the interaction between CTSD activity and levels to postprandial metabolic derangements in type 2 diabetes is not known.

Methods: Eighteen type 2 diabetes and 16 age-matched healthy males were given 2 consecutive standardized mixed meals, after which blood samples were collected. Plasma metabolic parameters as well as CTSD levels and activity were measured, and changes in plasma pH was assessed.

Results: In line with the elevation of plasma free fatty acids (FFA) levels in male type 2 diabetics patients, plasma pH in type 2 diabetic individuals was decreased compared to male healthy individuals. While plasma CTSD levels were similar, plasma CTSD activity was increased in male type 2 diabetic compared to male healthy individuals. Besides, plasma CTSD activity rather than levels significantly correlated with indicators of type 2 diabetes (HbA1c, HOMA-IR and glucose). Furthermore, FFA was also independently associated with plasma CTSD activity (standardized β=0.493, p=0.007).

Conclusions: Despite similar plasma CTSD levels, type 2 diabetic male individuals showed increased plasma CTSD activity compared to healthy males, which was independently linked to plasma FFA levels. Our data therefore point towards plasma CTSD as a metabolic regulator in male type 2 diabetes.

Chapter 5

Introduction

Diabetes mellitus is a global public health concern currently affecting more than 425 million people worldwide [1]. Type 2 diabetes mellitus, accounting for approximately 90% of all diabetes mellitus cases, is hallmarked by insulin resistance, a pathological phenomenon where cells fail to respond to insulin to increase glucose uptake and utilization [2,3]. However, though certain metabolic cascades have been unraveled, insight into the complete metabolic picture of insulin resistance is still lacking as it is unclear why certain patients do and others do not develop insulin resistance. In order to identify those patients at increased risk for type 2 diabetes development, it is of utmost importance to improve our understanding of the metabolic mechanisms involved with insulin resistance.

A key mechanism involved with the development of insulin resistance is lipotoxicity, an umbrella term used to identify the deleterious effects of access lipid storage in non-adipose tissue [4]. Lysosomes are essential cell organelles mediating lipid degradation, implying that under lipotoxic conditions (for example during increased cellular lipid influx) lysosomal function is compromised. Indeed, lipid-mediated damage to lysosomal membranes resulted in the unintended translocation of lysosomal enzymes into the cytosol and/or the extracellular environment [5-9]. In line with these observations, we and others previously showed that accumulation of specifically oxidized lipids in lysosomes leads to the extracellular secretion of aspartic lysosomal enzyme cathepsin D (CTSD) [10,11]. Likewise, plasma CTSD levels have been previously correlated with insulin resistance in newly diagnosed type 2 diabetes [12] and two large community cohorts without diabetes (with more than 70% of participants being healthy residents) [13] and were even implicated as non-invasive markers for different stages of NAFLD, a metabolic condition linked to type 2 diabetes [10,14]. Relevantly, the enzymatic activity of CTSD is optimally active in an acidic environment, which is maintained via pH regulation [15]. pH reductions in the interstitial fluid of diabetic patients [16] therefore imply changes in plasma CTSD activity in type 2 diabetes, which might result in metabolic changes. Furthermore, while insulin-treated diabetic rats showed increased CTSD activity in liver, kidney, heart and brain [17], inhibition of plasma CTSD activity in a steatotic rat model reduced plasma insulin levels [18]. Thus, these evidences provide support for a potential functional metabolic link between insulin resistance and plasma CTSD activity.

To validate this view, in the current study we investigated whether type 2 diabetic patients have increased plasma CTSD activity compared to healthy individuals and whether plasma CTSD levels and activity link to postprandial metabolic parameters of type 2 diabetes. For this aim, plasma pH, plasma CTSD levels and activity as well as type 2 diabetes-related plasma parameters were measured in eighteen male type 2 diabetic patients and sixteen age-matched healthy males in the postprandial state. Our study demonstrated that plasma pH decreased in male type 2 diabetic individuals as compared to male healthy individuals, which can be partly

explained by increased plasma FFA levels in type 2 diabetic patients. Additionally, compared to male healthy individuals, type 2 diabetic males demonstrated increased plasma CTSD activity. Furthermore, we observed that plasma CTSD activity, and not levels, significantly correlated with indicators of type 2 diabetes (plasma HbA1c, HOMA-IR and glucose) in males. Moreover, plasma CTSD activity also independently associated with a metabolic parameter of type 2 diabetes (plasma free fatty acids (FFA) in males). Altogether, our observations show that, despite similar plasma CTSD levels, type 2 diabetic male individuals showed increased plasma CTSD activity compared to healthy males, which was independently linked to plasma FFA levels. As FFA reduced the pH and pH was reduced in type 2 diabetic males, pH potentially played a central role in the activity of CTSD. Our data therefore point for the first time towards plasma CTSD as a metabolic regulator in male type 2 diabetes.

MATERIALS AND METHODS

Subject Characteristics

The study populations comprised of 34 Caucasian males, aged between 40 and 65 years, which were classified as age-matched healthy controls (n=16) and type 2 diabetes (n=18). All subjects were recruited by advertisement and gave written informed consent. Before and during recruitment, type 2 diabetic patients were only allowed to follow a diet or to take the glucose-lowering agents sulphonylurea and/or metformin. Exclusion criteria were excess alcohol intake (>20 units/wk), history of hepatitis and/or pancreatitis, abnormal liver and renal function tests (>2 times upper limits of normal), recent (<3 months) changes in weight (≥5%) and/or medication, history or current use of glucocorticosteroids, insulin and/or thiazolidinediones, statins or other lipid-lowering drugs. Twenty-four hours prior to examination, the subjects were refrained from heavy physical activities, and during the examination, subjects were instructed to omit their medication. The present study was approved by the ethical committee of VU University and the study protocol was in accordance with the ethical guidelines of the declaration of Helsinki (1975).

Study design

After an overnight fast, all subjects consumed 2 consecutive standardized mixed meals as breakfast and lunch (4 hours later), within 15 min. The meals were isocaloric (900 kcal), containing 75 g carbohydrates, 50 g fat (60% saturated) and 35 g protein. Subsequently, blood samples were collected from the antecubital vein 2 hours following lunch as previously described [19].

Biochemical measurements of plasma parameters

Plasma glucose concentrations were measured by a hexokinase-based technique (Roche

Diagnostics, Mannheim, Germany) and insulin concentrations by an immunoradiometric assay (Centaur, Bayer Diagnostics, Mijdrecht, The Netherlands). Plasma total cholesterol, high-density lipoproteins (HDL), triglycerides (TG), free fatty acids (FFA), gamma glutamyl transferase (GGT) and alanine transaminase (ALT) were determined by enzymatic methods (Modular, Hitachi, Japan). Low-density lipoproteins (LDL) were calculated by the Friedewald formula. Glycated hemoglobin (HbA1c) was measured by means of cation exchange chromatography (Menarini Diagnostics, Florence, Italy; reference values: 4.3-6.1%). Plasma remnants-like particle cholesterol (RLP cholesterol) was analyzed using an immuno-separation assay (Otsuka Pharmaceutical Co., Tokyo, Japan). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as fasting insulin (µU/mL) × glucose (mmol/l)/22.5 as described by Matthews *et al* [20]. Plasma apoB-48 and apoB-100 were measured using a sandwich ELISA method as previously described [21]. Plasma lactate concentration was measured via the lactate assay kit according to the protocol (Sigma-Aldrich, Netherlands). Plasma CTSD levels were determined by an enzyme-linked immunosorbent assay (USCN Life Science, Wuhan, China) and plasma CTSD activity was measured using a CTSD activity assay kit (MBL International, Woburn, USA), according to the manufacturer's protocols.

Plasma pH measurements

The impact of plasma pH on plasma CTSD activity was assessed by using pH-adjusted reaction buffer, *i.e*., pH 4.0 (mimicking lysosomal pH) and pH 7.0 (mimicking plasma pH), respectively. Further, the pH of pooled plasma derived from healthy individuals *versus* type 2 diabetes patients was determined using a Seahorse Bioscience XF96 Extracellular Flux Analyzer (Aligent, USA). While pH measurements should ideally have been performed on individual plasma samples, we performed the pH measurement on pooled plasma samples from healthy controls and type 2 diabetic patients due to the low available plasma volumes. Additionally, to investigate a change of CTSD activity within a relevant plasma pH change, we measured plasma CTSD activity after adjusting the pH from pH 7.4 to pH 7.1. Furthermore, the effect of FFA (600 µM palmitate; Sigma Aldrich, Netherlands) on plasma pH was assessed *in-vitro* using plasma derived from random healthy volunteers. The palmitate stock (1800µM, the ration of palmitic acid: bovine serum albumin (BSA)=6:1; BSA from MP Biomedicals, Netherlands) was prepared in 1.25 x MKR (Modified Krebs Ringer Buffer in MQ water) with adjusted pH of 7.5. To get 600µM FFA, 100µl FFA stock was added into 200µl plasma. Additionally, due to the influence of coagulant-EDTA, storage time and processing of blood samples (i.e., centrifuge) on plasma pH [22,23], the absolute pH value are higher than 7.4.

Statistical Analyses

Statistical analyses were performed using SPSS 24.0 (IBM, Armonk, NY, USA) and Graphpad Prism 6.0 for Microsoft Windows. All data were expressed as means ± SEM. The differences in subjects' characteristics were tested using independent sample t-test in SPSS. The differences

between two groups for the plasma pH experiment were tested using unpaired *t-test* in Graphpad Prism 6.0. Spearman's correlations determined simple correlations between plasma CTSD levels/activity and parameters related to type 2 diabetes. Subsequently, multiple linear regression analyses were performed, in which plasma CTSD levels/activity served as dependent variable in model 1 (simple regression), model 2 (model 1 + adjustment for age), model 3 (model 2 + adjustment for BMI) and model 4 (model 3 + adjustment for waist). *p*-value <0.05 was considered statistically significant.

Results

General characteristics of healthy controls and type 2 diabetic subjects

Thirty-four males were enrolled in the study, consisting of 16 healthy controls and 18 type 2 diabetes with a mean age of 56.8 and 55.1 years, respectively. General characteristics (Table 5.1) show that BMI, waist, systolic (SBP) and diastolic blood pressure (DBP) were significantly higher in male patients with type 2 diabetes compared to healthy males. Plasma levels of glucose, HbA1c and insulin as well as HOMA-IR were significantly higher in male type 2 diabetic patients compared to male healthy controls, pointing towards disturbed glucose metabolism in male type 2 diabetic patients. Plasma lactate in type 2 diabetic male patients was also significantly higher than healthy males. No significant differences were found in postprandial plasma TG, RLP-cholesterol, ApoB48 and ApoB100 between the male healthy controls and type 2 diabetes. Plasma FFA levels significantly increased, while plasma HDL significantly decreased in type 2 diabetes compared to healthy individuals, indicating disturbed lipid metabolism in male type 2 diabetic patients. In line, plasma levels of ALT, GGT and hs-CRP were significantly higher in male type 2 diabetic patients than healthy males.

Reduced plasma pH in male type 2 diabetic patients due to elevated FFA levels.

To confirm whether type 2 diabetes have lower plasma pH compared with healthy individuals, we measured the pH of pooled plasma from male healthy individuals *versus* male type 2 diabetes. Our data showed a significant reduction in plasma pH of male type 2 diabetes compared to healthy males (Fig.5-1A; pH reduction=-0.19, p<0.001), though the pH values were outside of the physiological range. Next, *ex-vivo* settings were tested using plasma derived from healthy volunteers to investigate whether the slight decline in plasma pH in type 2 diabetic patients is related to increased levels of FFA, which is known to play a key role in the pathology of type 2 diabetes [24]. As expected, treatment with FFA (600 µM) resulted in a significant reduction in plasma pH (Fig. 5-1B). Altogether, these data show the potential of FFA to significantly impact the plasma pH, which might influence plasma CTSD activity in type 2 diabetes.

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Table 5.1. General characteristics of healthy controls and type 2 diabetic subjects.

	Parameters	Control	T2DM	*p*
Basic factors	**Number, N**	16	18	
	Age (year)	56.8 ± 1.9	55.1 ± 1.3	0.477
	BMI (kg/m^2)	26.9 ± 0.7	32.9 ± 1.1	0.000*
	Waist (cm)	98.3 ± 2.2	113.9 ± 2.8	0.000*
	SBP (mmHg)	122.9 ± 2.1	136.0 ± 3.3	0.003*
	DBP (mmHg)	76.3 ± 1.7	84.1 ± 1.2	0.001*
Glucose-related parameters	**Glucose (mmol/L)**	6.01 ± 0.19	8.75 ± 0.51	0.000*
	HbA1c (%)	5.53 ± 0.08	6.74 ± 0.16	0.000*
Insulin-related parameters	**Insulin (pmol/L)**	147.04 ± 18.18	295.58 ± 33.46	0.001*
	HOMA-IR	0.99 ± 0.14	4.06 ± 0.56	0.000*
	Lactate (mmol/L)	0.51 ± 0.03	0.70 ± 0.05	0.008*
Lipid-related parameters	**TG (mmol/L)**	2.36 ± 0.33	3.01 ± 0.40	0.221
	FFA (mmol/L)	0.24 ± 0.01	0.32 ± 0.02	0.002*
	HDL (mmol/L)	1.26 ± 0.08	0.90 ± 0.04	0.007*
	RLP-Chol (mg/mL)	13.21 ± 1.56	13.65 ± 1.72	0.850
	ApoB100 (mg/dL)	110.29 ± 6.89	124.87 ± 6.01	0.121
	ApoB48 (µg/mL)	15.38 ± 1.78	13.87 ± 1.27	0.493
Liver indicators	**ALT (U/L)**	20.00 ± 2.25	32.29 ± 3.55	0.007*
	GGT (U/L)	21.31 ± 2.78	33.00 ± 3.08	0.010*
Inflammation	**Hs-CRP (mg/L)**	0.85 ± 0.16	1.92 ± 0.31	0.006*

Data are mean ± SEM. T2DM, type 2 diabetes mellitus; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, hemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance; TG, triglyceride; FFA, free fat acids; HDL, high density lipid protein; RLP-chol, remnants like particle cholesterol; GGT, gamma-glutamyl transpeptidase; ALT, alanine transaminase; Hs-CRP, high sensitive C-reaction protein; Note: p: Control vs T2DM. *p<0.05 is statistically significant.

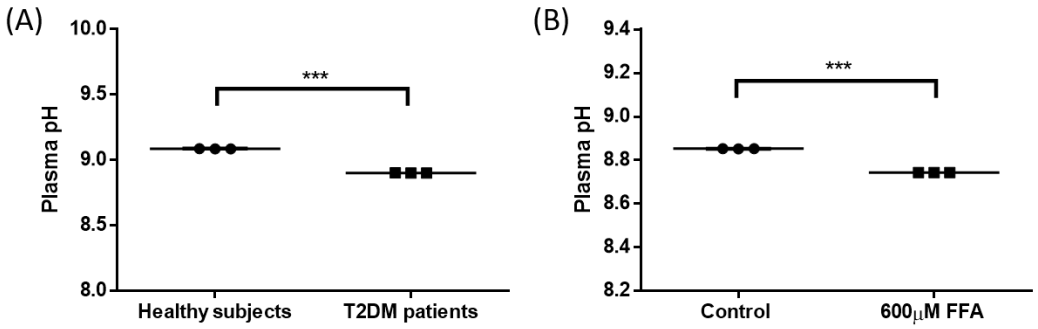

Figure 5-1. Reduced plasma pH in male type 2 diabetes due to elevated FFA levels. (A) Plasma pH in T2DM male individuals compared to male healthy subjects, (B) the effect of high concentrations of FFA (600 µM) on plasma pH. Data is mean ± SEM. ****p*<0.001. T2DM, type 2 diabetes mellitus.

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Plasma CTSD activity is superior to levels in distinguishing type 2 diabetes from healthy controls in male individuals

To confirm that plasma CTSD indeed maintains reduced activity outside of the lysosomes, the activity of plasma CTSD was measured at pH 7 (physiological pH) and at pH 4 (lysosomal pH). Our data demonstrated that plasma CTSD maintained ~50% of its proteolytic activity at neutral pH compared to acidic pH (Fig. 5S1). Next, based on the above observations, plasma CTSD activity and levels were measured to compare the difference between type 2 diabetes and healthy subjects in males. No significant differences in plasma CTSD levels were observed between male type 2 diabetes and healthy individuals (Fig. 5-2A). In line with the reduced plasma pH, plasma CTSD activity was significantly higher in male type 2 diabetic patients compared to male healthy individuals (Fig. 5-2B, $p<0.001$), suggesting that plasma CTSD activity is linked to type 2 diabetes progression. Likewise, as shown in Fig. S2, we observed that plasma CTSD activity at pH 7.1 increased compared to pH 7.4, supporting a change of CTSD activity within a relevant plasma pH change. Additionally, while lactate levels were significantly higher in type 2 diabetic males, these levels did not correlate with plasma CTSD activity, suggesting that lactate likely did not influence plasma CTSD activity (data not shown).

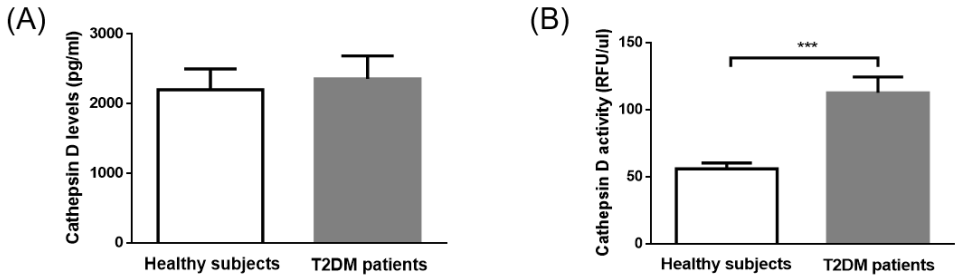

Figure 5-2. **In contrast to CTSD level, CTSD activity distinguishes between healthy controls and T2DM in male individuals**. (A) CTSD levels, (B) CTSD activity. Data is mean ± SEM. ***p<0.001. T2DM, type 2 diabetes mellitus.

Plasma CTSD activity rather than levels significantly correlates with type 2 diabetes-indicators in male individuals

To investigate whether plasma CTSD activity is correlated with type 2 diabetes-indicators (*ie.,* HbA1c (%), HOMA-IR and glucose) compared with plasma CTSD levels, Spearman's correlations were performed. In line with the finding that plasma CTSD activity was significantly higher in male type 2 diabetes compared with healthy males, we found that plasma CTSD activity positively correlated with HbA1c (%) (Fig. 5-3B; $r=0.577$, $p=0.002$), HOMA-IR (Fig. 5-3D; $r=0.398$, $p=0.027$) and plasma glucose (Fig. 5-3F; $r=0.465$, $p=0.015$). In contrast, levels of plasma CTSD did not correlate with indicators of type 2 diabetes, including HbA1c (%), HOMA-IR and glucose (Fig. 5-3A, C, E). Additionally, linear regression analyses were performed to evaluate whether the correlation between plasma CTSD activity and type 2 diabetic indicators (HbA1c (%), HOMA-IR and glucose) were dependent or independent of age, BMI and waist. As shown in table 5S1, no significant associations were observed between

plasma CTSD activity and HbA1c (%), HOMA-IR as well as glucose after adjustment of age, BMI and waist, indicating dependency of these parameters on the link between plasma CTSD activity and HbA1c (%)/HOMA-IR/glucose. Likewise, HbA1c (%), HOMA-IR and glucose were also not independently associated with plasma CTSD levels (data not shown). Taken together, our data indicate that plasma CTSD activity rather than levels correlates with type 2 diabetic indicators (plasma HbA1c, HOMA-IR and glucose), but their associations are dependent of age, BMI and waist.

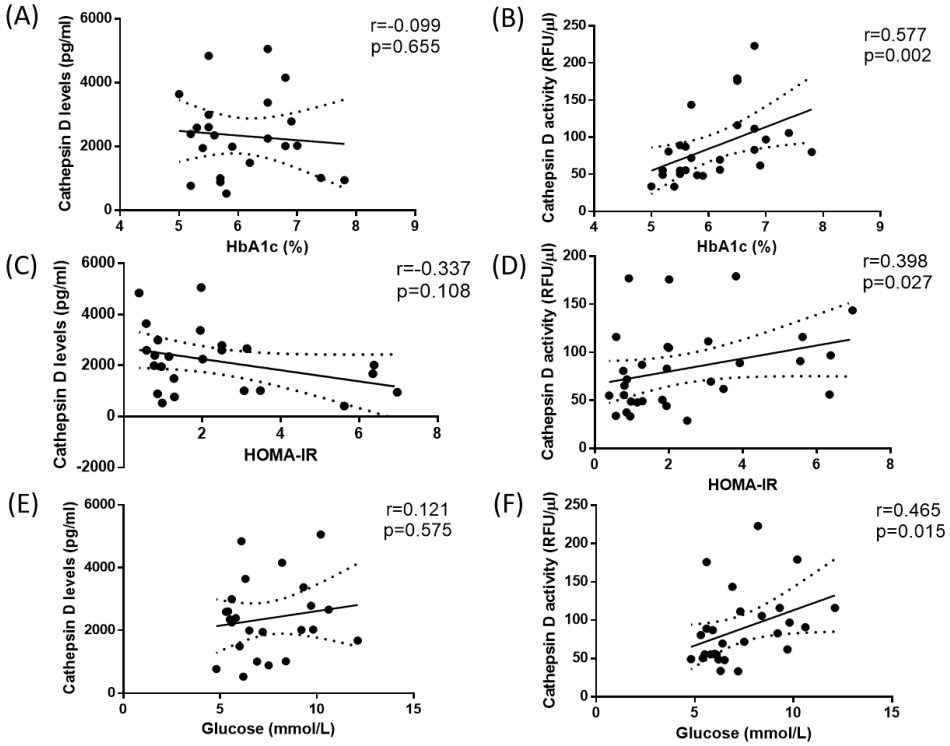

Figure 5-3. Spearman correlations between CTSD levels and activity versus type 2 diabetes-indicators in male individuals. CTSD levels (A, C, E) or activity (B, D, F) *vs.* HbA1c (%), HOMA-IR and glucose (mmol/L), respectively. Spearman's correlations were performed. $p<0.05$ is considered statistically significant.

Metabolic parameter related to type 2 diabetes (FFA) is independently associated with plasma CTSD activity in male individuals

Insulin and FFA play a central role in metabolic disturbances related to insulin resistance and type 2 diabetes. For this reason, we investigated whether plasma FFA and insulin are associated with plasma CTSD levels and activity. Firstly, Spearman's correlation was performed to analyze the simple correlation between plasma CTSD levels/activity and metabolic parameters of type 2 diabetes (plasma FFA and insulin). As shown in Fig. 5-4A and B, plasma CTSD activity, but not levels, correlated with plasma FFA ($r=0.424$, $p=0.022$). Both plasma CTSD levels and activity were not correlated with insulin (Fig. 5-4C and D). To further evaluate whether these correlations were dependent or independent of age, BMI and waist, linear regression analyses were performed. As displayed in table 5.2, plasma FFA positively associated with CTSD activity (standardized $\beta=0.494$, $p=0.006$), even after adjustment for age

(Model 2: standardized ß=0.492, p=0.008), BMI (Model 3: standardized ß=0.471, p=0.008) and further correction for waist (Model 4: standardized ß=0.493, p=0.007). However, plasma insulin levels were not positively associated with CTSD activity even adjustment of age, BMI and waist (as shown in table 5S1). Altogether, these data demonstrate that the metabolic parameter of type 2 diabetes (plasma FFA) is independently associated with plasma CTSD activity, linking plasma CTSD activity to insulin resistance via changes in FFA metabolism.

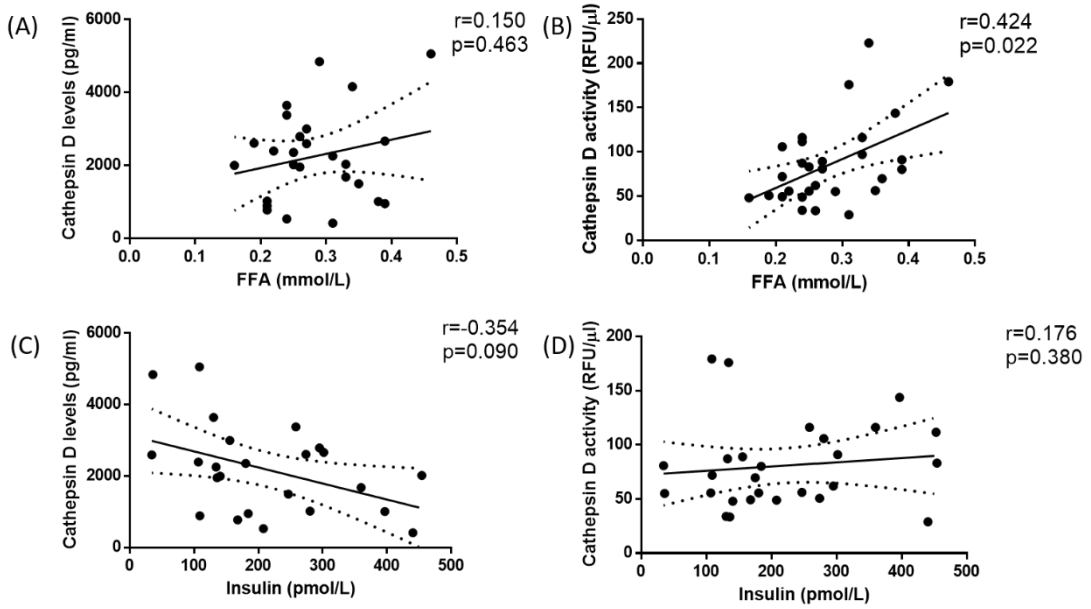

Figure 5-4. Plasma CTSD activity rather than levels significantly correlates with metabolic parameter of type 2 diabetes (FFA) in male individuals. CTSD levels (A, C) or activity (B, D) *vs.* FFA and insulin, respectively. Spearman's correlations were performed. $p<0.05$ is considered statistically significant.

Table 5.2 Plasma FFA is independently (positively) associated with plasma CTSD activity in male individuals

Dependent variable: CTSD activity			
Independent Variables		**FFA**	
Models	***p* value**	**Standardized coefficients β**	**R^2**
Model 1	0.006	0.494	0.244
Model 2	0.008	0.492	0.250
Model 3	0.008	0.471	0.329
Model 4	0.007	0.493	0.349

Data were analyzed by linear regression models: Model 1, simple regression; Model 2, model 1 + adjustment for age; Model 3, model 2 + adjustment for BMI; Model 4, model 3 + adjustment for waist. $p < 0.05$ is statistically significant.

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Discussion

In the current study, we show that a metabolically-induced reduction of plasma pH in male type 2 diabetes patients correlates with increased plasma CTSD activity, which on its turn is linked to elevated plasma lipid and glucose levels. Our data therefore point towards plasma CTSD as a novel metabolic regulator in type 2 diabetes. These findings are in line with our previous studies that demonstrated a role for plasma CTSD activity in lipid metabolism and insulin resistance [18,25,26].

Under healthy conditions, CTSD has been demonstrated to play important roles in maintaining numerous physiological functions, including degradation of intracellular proteins, activation of hormones, growth factors and enzymatic precursors, hydrolysis of LDL cholesterol, and regulating programmed cell death [15]. Besides physiology, CTSD has also been reported to be involved with the pathogenesis of a whole array of disorders such as in cancer, Alzheimer and metabolic syndrome related diseases [14,27,28]. Indeed, emerging studies currently have focused on the role of CTSD in metabolic syndrome, linking CTSD to NAFLD [14] and type 2 diabetes [26]. More specifically, increasing reports link CTSD activity to disturbances in lipid metabolism [18,25], a finding which we could also confirm in here in our study. Therefore, with lipotoxicity as one of the key drivers for type 2 diabetes, CTSD might be an auspicious new player in the complex network contributing to insulin resistance by mechanisms that need to be further explored.

Our finding that plasma pH is influenced by changes in plasma FFA suggests an essential role for pH in regulating physiological processes related to metabolism. Indeed, enzyme activity is known to be highly dependent on the pH, a phenomenon that has been best described within the intralysosomal environment [29]. Building further on this modulatory role of pH on enzyme activity, in this study, our findings imply that pH also influences enzyme activity in the plasma, suggesting the impact of pH also beyond the intralysosomal environment. However, as our pH measurements were outside of the physiological range, the link between FFA, pH and enzyme activity should be considered with caution at this stage as more research is necessary to confirm this claim. Nevertheless, a reduction of 0.05 units from the normal blood pH (being 7.40) in diabetic patients indeed results in acidosis [30], a condition which accelerates the progression of pathological features of diabetes by hampering the activity of metabolic enzymes such as phosphofructokinase [31]. Furthermore, subtle reductions in pH also increase cathepsin activity in-vivo or ex-vivo in the context of cancer [32-35]. Taken this modulatory function of pH on enzyme activity into account, it is possible that the plasma pH reduction in diabetic patients [16,36,37] also influenced CTSD activity in our study. Regardless, plasma pH has an essential modulatory function on metabolic processes by influencing enzyme activity and for this reason future studies should be done to clarify this role of pH on metabolic processes in more detail.

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The observation that metabolic parameter (plasma FFA) related to type 2 diabetes associated with plasma CTSD activity (as dependent variable) in males implies an influence of this metabolic parameter on plasma CTSD activity. Indeed, considering pH as a key indicator for maintenance of enzyme activity, we here describe that plasma FFA changes plasma pH likely via the acidic characteristics associated with FFA [38], thereby influencing plasma CTSD activity. These findings raise the question whether this pH-mediated metabolic influence on plasma CTSD activity has functional consequences. Our findings that (1) type 2 diabetic patients have higher plasma CTSD activity compared to healthy controls and (2) plasma CTSD activity positively correlates with indicators of type 2 diabetes (HbA1c, HOMA-IR and glucose) point towards the link between plasma CTSD activity and disturbed glucose metabolism (or insulin resistance) in type 2 diabetes. Moreover, we recently found that inhibition of extracellular CTSD activity reduced plasma insulin levels and hepatic lipids in rats with hepatic steatosis [18]. Likewise, our group also previously observed that inhibiting CTSD activity via pepstatin A (an aspartic lysosomal enzymes inhibitor) reduces the gene expression of CD36 (a transporter of FFA)[25] that also mediates the suppression of FFA on insulin signaling [39]. This data thereby suggests that CTSD activity is likely involved with insulin signaling via regulating FFA metabolic pathways (i.e., CD36 transporter). Furthermore, previous reports have proven the ability of CTSD to proteolysis and influence the bioavailability of insulin-like growth factors (IGFs), factors that have been extensively linked to insulin resistance [40,41]**.** These observations place plasma CTSD activity at the center of metabolic programming as in this way, CTSD receives "signals" from metabolic factors such as FFA on one hand and regulates glucose metabolism via insulin or IGFs on the other hand. Therefore, CTSD should be further investigated as a central metabolic regulator in the context of type 2 diabetes and potentially other metabolic diseases.

While in contrast to a previous study [12], we did not observe changes in plasma CTSD levels in type 2 diabetes patients, we observed that type 2 diabetes showed increased plasma CTSD activity, which also correlated metabolic parameters of type 2 diabetes. As starvation influences the secretion of lysosomal enzymes, one potential explanation for the lack of correlation with CTSD levels might be related to the fact that subjects in our study did not undergo overnight fasting prior to blood sampling [42,43], and as such the differences in plasma CTSD levels were below detection levels. Another explanation for the lack of correlation between plasma CTSD levels and type 2 diabetes in the current study could be related to the known effect of estrogen in increasing CTSD expression [44] as the previous report included both males and females [12], whereas all the subjects in our study are males. It is also noteworthy to mention that in our cohort, it cannot be completely excluded that the increase in CTSD activity is due to the presence of other diseases that coincide with type 2 diabetes (such as NAFLD). As plasma ALT levels, however, did not correlate with plasma CTSD activity (data not shown), it seems unlikely that the presence of NAFLD influenced our results.

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In the current study, the identification of CTSD as a metabolic regulator might have implications for improving type 2 diabetes treatment. While a variety of drugs have currently been developed to treat type 2 diabetes [45-47], they are often featured by high costs and/or serious sides effects [45]. Considering its functions as a metabolic regulator, CTSD-related strategies to treat type 2 diabetes may provide a relevant alternative for these existing anti-diabetic agents. A previous study by our group demonstrated that inhibiting circulating CTSD activity reduces plasma insulin levels in steatotic rats [18], confirming the potential therapeutic value of directly targeting CTSD to improve insulin sensitivity. However, to fully disclose CTSD and related pathways as a new therapeutic alternative to treat type 2 diabetes, additional and larger cohorts are essential to verify these results, and more in depth investigation into the mechanisms of CTSD are necessary. Nevertheless, our observations implying the functional role of CTSD as a metabolic regulator holds clinical value as it can lead to new ways to treat type 2 diabetes.

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Chapter 5

Supplementary data

Supplementary Table 5S1. Plasma HbA1c (%), HOMA-IR, glucose and insulin are not independently associated with plasma CTSD activity.

Dependent variable: plasma CTSD activity						
Independent Variables	**HbA1c (%)**			**HOMA-IR**		
Models	***p* value**	**Standardized coefficients β**	**R^2**	***p value***	**Standardized coefficients β**	**R^2**
Model 1	0.017	0.463	0.214	0.221	0.268	0.049
Model 2	0.022	0.457	0.217	0.219	0.282	0.050
Model 3	0.176	0.337	0.239	0.962	-0.014	0.098
Model 4	0.108	0.409	0.295	0.969	-0.012	0.098

Dependent variable: plasma CTSD activity						
Independent Variables	**Glucose**			**Insulin**		
Models	***p* value**	**Standardized coefficients β**	**R^2**	***p value***	**Standardized coefficients β**	**R^2**
Model 1	0.050	0.381	0.145	0.552	0.120	0.014
Model 2	0.066	0.377	0.145	0.572	0.117	0.016
Model 3	0.234	0.251	0.231	0.548	-0.161	0.112
Model 4	0.118	0.344	0.295	0.558	-0.165	0.112

Data were analyzed by linear regression models: Model 1, simple regression; Model 2, model 1 + adjustment for age; Model 3, model 2 + adjustment for BMI; Model 4, model 3 + adjustment for waist. $p < 0.05$ is statistically significant.

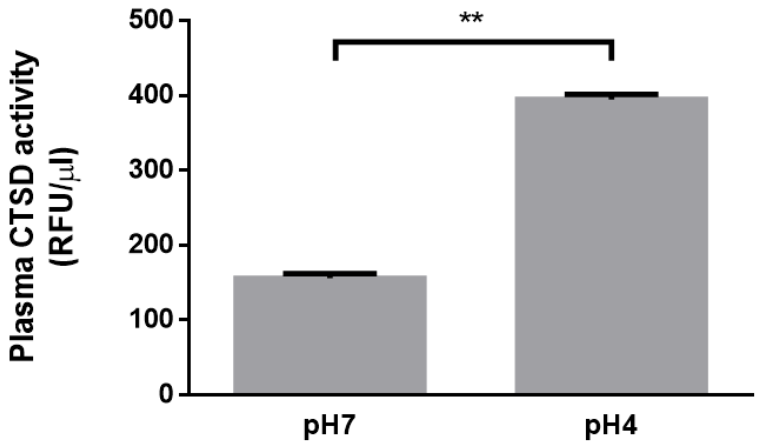

Figure. 5S1. Plasma CTSD maintains reduced enzymatic activity at neutral pH. Data is mean ± SEM. **p<0.01.

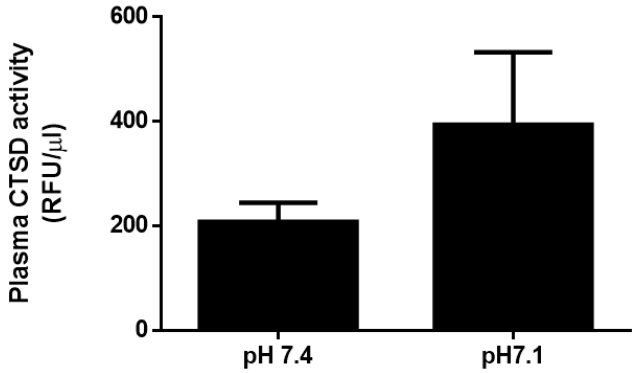

Figure. 5S2. A change of CTSD activity within a relevant plasma pH range

Chapter 6

Insulin resistance is positively associated with plasma cathepsin D activity in NAFLD patients

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Under review

Chapter 6

Abstract

Previous studies associated plasma cathepsin D (CTSD) activity with hepatic insulin resistance in overweight and obese humans. Insulin resistance is a major feature of non-alcoholic fatty liver disease (NAFLD) and is one of the multiple hits determining the progression towards non-alcoholic steatohepatitis (NASH). In line, we have previously demonstrated that plasma CTSD levels are elevated in patients with NASH. However, it is not known whether insulin resistance associates with plasma CTSD activity in NAFLD. To increase our understanding regarding the mechanisms by which insulin resistance mediates NAFLD progression, fifty-five liver biopsy or MRI-proven NAFLD patients (BMI>25kg/m^2) were included to investigate the link between plasma CTSD activity to insulin resistance in NAFLD. We concluded that HOMA-IR and plasma insulin levels are independently associated with plasma CTSD activity in NAFLD patients (standardized coefficient β: 0.412, 95% CI: 0.142~0.679, p=0.004 and standardized coefficient β: 0.495, 95% CI: 0.236~0.758, p=0.000, respectively). Together with previous studies, these data suggest that insulin resistance may contribute to NAFLD progression via elevation of CTSD activity in plasma. As such, these data pave the way for testing CTSD inhibitors as a pharmacological treatment of NAFLD/NASH.

Chapter 6

Introduction

Non-alcoholic fatty liver disease (NAFLD) covers a disease spectrum ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) (steatosis and inflammation with or without fibrosis), liver cirrhosis and hepatocellular carcinoma [1]. While the pathogenesis of NAFLD has been extensively studied, the mechanisms underlying NAFLD progression are not completely understood, resulting in the lack of well-defined effective ways to prevent NAFLD development and progression.

One of the key factors that contributes to the transition from NAFLD progression to NASH is insulin resistance [2]. Relevantly, previous research in our group showed that plasma activity of the lysosomal enzyme cathepsin D (CTSD) is associated with hepatic insulin sensitivity in overweight and obese individuals [3]. Moreover, we have previously demonstrated that plasma CTSD levels are elevated in NASH patients [4,5], thereby linking CTSD to a progressed NAFLD state. The positive correlation between plasma CTSD and hepatic inflammation on one hand, and the inverse correlation between hepatic insulin sensitivity and the plasma CTSD activity in overweight and obese humans on the other hand suggest that insulin resistance is positively associated with plasma CTSD activity in NAFLD patients. However, it is not yet known whether insulin resistance associates with plasma CTSD activity in NAFLD. Investigating the link between plasma CTSD activity to insulin resistance in NAFLD patients will increase our understanding regarding the mechanisms by which insulin resistance mediates NAFLD progression.

In the current paper, plasma CTSD activity and insulin resistance-related parameters were determined in fifty-five NAFLD patients. The associations between parameters of insulin resistance and plasma CTSD activity were analyzed with adjustment for age, sex, BMI and waist.

Materials and methods

Subjects characteristics

Fifty-five subjects were included in the present study including 29 men and 26 women, with an age ranging from 20 to 65 years and BMI ≥25 kg/m^2. All participants were proven NAFLD via liver biopsy (according to the criteria of NAFLD activity score) or chemical shift magnetic resonance imaging (MRI). Metabolic syndrome was diagnosed based on the International Diabetes Federation (IDF) definition [6]. Exclusion criteria were excessive ethanol consumption (male ≥ 14 and female ≥ 7 standard beverages per week), causes for secondary hepatic fat accumulation (medication, Wilson's disease, viral infections, starvation or parenteral nutrition and microvesicular steatosis on liver biopsy), pregnancy and breastfeeding, a history of bariatric surgery, liver cirrhosis and/or hepatocellular carcinoma, extrahepatic malignancy(s) within the last 5 years, subjects who use insulin and individuals about to undergo or are recovering from a surgical or otherwise medical procedure. All subjects were recruited from

Maastricht University Medical Centre (MUMC+) and CO-EUR (a second line eating disorder clinic) between September 2015 and October 2018. These participants gave written informed consent before involvement in this study. The study was approved by the Medical-Ethical Committee of Maastricht University (ClinicalTrials.gov Identifier: NCT02422238), and was performed in accordance with the principles of the Declaration of Helsinki, as revised in 2008.

Biochemical analyses

After overnight fasting, venous blood was collected into pre-chilled tubes at Maastricht University Medical Center (MUMC+). After centrifuging (1000 x g; 10 min; 4°C), plasma was snap-frozen in liquid nitrogen and then stored at -80°C until analyses. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (GGT), bilirubin, Alkaline Phosphatase (AP), total cholesterol, low density lipoprotein cholesterol (LDL-cholesterol), high density lipoprotein cholesterol (HDL-cholesterol), triglyceride, fasting plasma glucose (FPG), hemoglobin A1c (HbA1C) and plasma insulin concentrations were determined using routine analyses at the clinical chemistry department of the Maastricht UMC+ hospital. Insulin resistance was estimated by means of the homeostasis model of assessment-insulin resistance (HOMA-IR) with the following formula HOMA-IR = (fasting plasma insulin (mU/L) × fasting plasma glucose (mmol/L)) / 22.5 [7]. Plasma CTSD activity was measured by a CTSD activity assay kit according to the manufacturer's protocol (MBL International, Woburn, USA).

Magnetic Resonance Imaging

The hepatic fat fraction was measured with chemical shift gradient echo MRI. On three MRI sections, four circular region of interest (ROIs) of 5 cm^2 were drawn in the liver. Artefact, vascular and biliary structures were avoided and the ROI was copied from the in-phase (IP) image to the opposed-phase (OP) image. The average ROI signal intensity (SI) for the IP and OP image were calculated. The mean signal intensity loss of all 12 ROIs was calculated with the following formula: (SI IP- SI OP)/ 2*SI IP*100%[8].

Data statistics

Statistical analyses were performed using SPSS 25.0 (IBM, Armonk, NY, USA) for Microsoft Windows. All data were expressed as means ± SEM. A bivariate correlation was used to analyze the simple correlations between plasma CTSD activity and other parameters. Then multiple linear regression analyses were performed for the association between plasma CTSD activity and insulin/ HOMA-IR, in which plasma CTSD activity was added as dependent variable and either HOMA-IR or insulin as independent variables resulting in model 1 (simple regression), model 2 (model 1 + adjustment for age), model 3 (model 2 + adjustment for sex), model 4 (model 3 + adjustment for BMI) and model 5 (model 4 + adjustment for waist). *p*-value <0.05 was considered statistically significant. Interactions between co-variates (age, sex,

BMI and waist) in the multiple regression analyses was also tested but no significant interactions were found (data not shown).

Results

Anthropometric and clinical characteristics of the study participants

The baseline characteristics of the population are summarized in Table 6.1. Fifty-five NAFLD patients were included in our study, of which 29 males and 26 females with a mean age of 51.4 years old and mean BMI of 32.3 Kg/m^2. The hepatic fat fraction (%) ranged from 5.2 to 45.5%. Furthermore, plasma CTSD activity ranged from 93.9 RFU/µL to 324.3 RFU/µL, with a mean of 179.6 ± 8.1 RFU/µL.

Table 6.1. Baseline clinical characteristics of the study participants.

	Mean ± SEM	Ranges
Age, yrs	51.4 ± 1.6	20~65
Sex (M/F)	29/26	-
BMI, kg/m^2	32.3 ± 0.7	24.6~46.3
Waist, cm	106.8 ± 1.6	82.0~146.9
Hip, cm	112.4 ± 1.6	96.0~154.3
SBP (mmHg)	135.0 ± 2.0	104~177
DBP (mmHg)	81.1 ± 1.3	64~113
Heart rate (bpm)	67.6 ± 1.5	49~100
MetS (Yes/No)	31/24	-
MetS (points)	3.0 ± 0.2	0~5
HbA1c (%)	5.8 ± 0.1	4.6~8.8
Fasting glucose, mmol/L	6.2 ± 0.2	4.6~11.7
Insulin, pmol/L	99.3 ± 8.9	17.7~364.0
HOMA-IR	4.2 ± 0.5	0.6~23.8
Total Cholesterol (mmol/L)	5.2 ± 0.1	3.3~7.7
Triglycerides (mmol/L)	2.1 ± 0.3	0.7~15.6
HDL Cholesterol (mmol/L)	1.3 ± 0.1	0.5~2.2
LDL Cholesterol (mmol/L)	3.2 ± 0.1	0.9~5.4
GGT (U/L)	54.6 ± 6.2	12~256
ALT (U/L)	51.2 ± 6.0	7~272
AST (U/L)	33.9 ± 2.6	11~118
Bilirubin (µmol/L)	9.3 ± 0.7	3.7~28.7
Alkaline phosphatase (IU/L)	95.0 ± 4.0	52~186
Hepatic steatosis (%)	19.1 ± 1.4	5.2~45.5
CTSD activity, RFU/µL	179.6 ± 8.1	93.9~324.3

Data are mean ± SEM. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, hemoglobin; MetS: metabolic syndrome; HOMA-IR, homeostasis model assessment of insulin resistance; HDL, high density lipid protein; LDL, low density lipid protein; GGT, gamma-glutamyl transpeptidase; ALT, alanine transaminase; AST, Aspartate transaminase.

Chapter 6

HOMA-IR and plasma insulin levels are positively associated with plasma CTSD activity

To investigate whether insulin resistance-related parameters correlate with plasma CTSD activity in NAFLD patients, multiple linear regression analyses were performed. As shown in Table 6.2, HOMA-IR was positively associated with plasma CTSD activity (Model 1: standardized β=0.413, 95% Cl: 0.156~0.667, p=0.002), even after adjustment for age (Model 2: standardized β=0.417, 95%Cl: 0.162~0.669, p=0.002), sex (Model 3: standardized β=0.394, 95%Cl: 0.129~0.656, p=0.004), BMI (Model 4: standardized β=0.410, 95%Cl: 0.143~0.674, p=0.003) and waist (Model 5: standardized β=0.412, 95%Cl: 0.142~0.679, p=0.004). Furthermore, we found that plasma insulin levels were also positively associated with plasma CTSD activity (Model 1: standardized β=0.472, 95%Cl: 0.225~0.723, p=0.000), independent of age (Model 2: standardized β=0.487, 95%Cl: 0.243~0.735, p=0.000), sex (Model 3: standardized β=0.468, 95%Cl:0.214~0.726, p=0.001), BMI (Model 4: standardized β=0.493, 95%Cl: 0.237~0.753, p=0.000) and waist (Model 5: standardized β=0.495, 95%Cl: 0.236~0.758, p=0.000). Our data therefore demonstrate that insulin resistance is independently associated with plasma CTSD activity in the context of NAFLD.

Table 6.2. HOMA-IR and plasma insulin levels are positively associated with plasma CTSD activity independent of age, sex, BMI and waist.

Dependent variable: Plasma CTSD activity							
HOMA-IR				**Plasma insulin levels (Insulin)**			
Models	**Adjusted R square**	**Standardized coefficient β (95% CI)**	***p* value**	**Models**	**Adjusted R square**	**Standardized coefficient β (95%CI)**	***p* value**
Model 1	0.154			**Model 1**	0.208		
HOMA-IR		0.413 (0.156~0.667)	0.002	**Insulin**		0.472 (0.225~0.773)	0.000
Model 2	0.167			**Model 2**	0.231		
HOMA-IR		0.417 (0.162~0.669)	0.002	**Insulin**		0.487 (0.243~0.735)	0.000
Age		0.169 (-0.084~0.418)	0.188	**Age**		0.196 (-0.049~0.435)	0.115
Model 3	0.158			**Model 3**	0.221		
HOMA-IR		0.394 (0.129~0.656)	0.004	**Insulin**		0.468 (0.214~0.726)	0.001
Age		0.193 (-0.071~0.452)	0.149	**Age**		0.213 (-0.041~0.462)	0.099
Sex		-0.095 (-0.369~0.178)	0.485	**Sex**		-0.074 (-0.338~0.139)	0.572
Model 4	0.159			**Model 4**	0.230		
HOMA-IR		0.410 (0.143~0.674)	0.003	**Insulin**		0.493 (0.237~0.753)	0.000
Age		0.181 (-0.084~0.441)	0.177	**Age**		0.200 (-0.053~0.468)	0.120
Sex		-0.064 (-0.345~0.216)	0.646	**Sex**		-0.036 (-0.305~0.234)	0.791
BMI		-0.132 (-0.388~0.129)	0.319	**BMI**		-0.158 (-0.404~0.094)	0.276
Model 5	0.141			**Model 5**	0.214		
HOMA-IR		0.412 (0.142~0.679)	0.004	**Insulin**		0.495 (0.236~0.758)	0.000
Age		0.190 (-0.093~0.468)	0.184	**Age**		0.211 (-0.060~0.476)	0.125
Sex		-0.086 (-0.448~0.275)	0.632	**Sex**		-0.062 (-0.408~0.284)	0.720
BMI		-0.086 (-0.608~0.438)	0.745	**BMI**		-0.104 (-0.602~0.398)	0.684
Waist		-0.053 (-0.608~0.499)	0.844	**Waist**		-0.063 (-0.594~0.464)	0.805

Data were analyzed by linear regression models: Model 1, simple regression; Model 2, model 1 + adjustment for age; Model 3, model 2 + adjustment for sex; Model 4, model 3 + adjustment for BMI; Model 5, model 4 + adjustment for waist. $p<0.05$ is statistically significant.

Chapter 6

Discussion

We here show that plasma insulin levels and HOMA-IR are independently associated with plasma CTSD activity in NAFLD patients. Together with previous studies, our finding raise the possiability that insulin resistance may contribute to NAFLD progression via elevation of plasma CTSD activity.

While insulin resistance is mostly associated with type 2 diabetes, its pathophysiology is known as an important mediator towards more severe states of NAFLD progression such as non-alcoholic steatohepatitis (NASH) [9]. Our current observation that plasma CTSD activity associates with insulin resistance in this NAFLD cohort therefore implies that plasma CTSD may contribute to NASH progression by regulating insulin resistance. This view is in line with previous studies by us and others that demonstrate that plasma CTSD mediates insulin signaling [3,10,11], hepatic inflammation [5,10,12] and lipotoxicity [10,12], all aspects that are involved with insulin sensitivity [13,14]. However, we like to clarify that our findings only provide initial evidences for a link between insulin resistance, plasma CTSD activity and NAFLD progression and require further in-depth investigation to validate our current observations. Nevertheless, these data support the approach to test CTSD inhibitors as a pharmacological treatment of NAFLD/NASH. A limitation of the current study is that the cohort is relatively small. Future studies that include larger patient cohorts are therefore warranted to validate our findings.

Chapter 6

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Chapter 6

Chapter 7

General discussion

Chapter 7

While emerging studies have demonstrated that CTSD is involved in metabolism and inflammation, its link to different aspects of the MetS has not been completely understood. In the present thesis, we aimed to provide clinical insights into the link between plasma CTSD and MetS-related diseases (NAFLD and T2DM). Here the key findings are discussed in the context of these metabolic diseases along with a prospective view on their clinical implications.

Plasma CTSD is linked to metabolic dysfunctions in MetS-related disorders

Under healthy conditions, metabolism is in a balanced equilibrium between catabolism and anabolism, with lipid metabolism and glucose metabolism being two major sources of cellular energy needs. During the process of metabolism, nutrients (for instance lipids and glycogen) are degraded by enzymes (i.e., lysosomal enzymes) for the use of energy and inversely the body can also synthesize lipids and glycogen for the storage of energy accordingly. These processes thus maintain the balance between catabolism and anabolism in healthy conditions. However, under MetS conditions, the balance between catabolism and anabolism is disturbed, leading to disturbances of lipid and glucose metabolism. Consequently, it results in dyslipidemia and/or hyperglycemia in MetS patients. Relevantly, it has been demonstrated that dysfunction of lysosomal enzymes (such as CTSD) in white adipose tissue and liver is involved with obesity-related pathology (i.e., the disturbed lipid and glucose metabolism).[1] However, while it has been demonstrated that extracellular CTSD is elevated in MetS-related conditions, it remains unclear what associations extracellular CTSD has with other parameters related to MetS. This current thesis has shown that increased plasma CTSD is associated with disturbed lipid and glucose metabolism in NAFLD and T2DM patients **(Chapter 3, 5 & 6)**, suggesting that extracellular CTSD may play an important regulatory role in different aspects of MetS.

Studies have shown that CTSD regulates ABCA1 (a type of lipid transporter)-mediated lipid efflux,[2] pointing towards the possibility that CTSD likely influences lipid metabolism via ABCA1 transporter. Furthermore, previous studies from our group have shown that inhibiting CTSD activity reduced systemic and hepatic lipids, thus improving lipid metabolism in in-vivo hyperlipidemic animal models.[3,4] In line, we have observed that hepatic steatosis and myosteatosis were independently associated with plasma CTSD levels in NAFLD patients (**Chapter 3**). Together, these evidences suggest that plasma CTSD may influence or be an indicator of altered lipid metabolism. As previously mentioned, in addition to disturbed lipid metabolism, CTSD dysfunction has also been associated with disturbances in glucose metabolism. For instance, previous studies demonstrated that increased plasma CTSD correlated with insulin resistance in newly-diagnosed T2DM patients.[5] Similarly, this thesis also links plasma CTSD to glucose metabolism (**Chapter 4, 5 & 6**). As demonstrated in **Chapter 5**, we observed increased plasma CTSD activity in T2DM patients compared to healthy individuals while plasma CTSD activity was also associated with metabolic parameters of T2DM (i.e.,

glucose, HbA1c (%) and HOMA-IR). Moreover, given the fact that insulin is an important regulator for metabolism,[6] the current thesis demonstrated that plasma insulin is independently associated with plasma CTSD activity in NAFLD patients **(Chapter 6)**. In line, a previous study has shown that inhibiting extracellular CTSD activity reduces the plasma insulin level in steatotic rats,[4] thus pointing towards a role for plasma CTSD in regulating insulin.
Additionally, it has been shown that a small reduction (i.e., 0.05 units) of normal blood pH causes acidosis in diabetes. Acidosis then further aggravates the pathological condition of diabetes via influencing the activity of metabolic enzymes,[7,8] suggesting a functional role of pH in the metabolic process. As diabetic patients have a lower pH in their blood than non-diabetic individuals,[8] it implies that plasma pH reduction in diabetic patients might influence plasma CTSD activity (enzyme activity is highly dependent on pH). In line, we indeed observed a lower plasma pH in T2DM patients compared to healthy controls, which was likely induced by elevated plasma FFA levels. This pH reduction indeed resulted in the increased plasma CTSD activity in T2DM patients **(Chapter 5)**. Given the fact that increased plasma CTSD activity also correlates with metabolic parameters of T2DM discussed in **Chapter 5**, it therefore suggests that plasma CTSD might be involved in the metabolic disturbances in T2DM that are mediated by reduced plasma pH. Furthermore, we observed that plasma FFA is independently associated with plasma CTSD activity in T2DM patients (**Chapter 5**). Consistently, studies have demonstrated that inhibition of CTSD via Pepstatin A decreased gene expression of Cluster of differentiation 36 (CD 36, a scavenger receptor for uptake of long chain fatty acids[9]), that also mediates the suppression of FFA on insulin signaling.[3,10] Thus, these data suggest that plasma CTSD is likely involved in glucose metabolism through influence on FFA pathways. Altogether, because plasma CTSD has a role in regulating insulin as well as being regulated by metabolic parameters such as FFA, it highlights the central role of plasma CTSD in the metabolic process.

Another regulator of metabolism is the circadian rhythm.[11] The circadian rhythm regulates the secretion of hormones (i.e., cortisol that regulates glycogenolysis, lipolysis, and proteolysis and melatonin that potentiates central and peripheral insulin action via regulating GLUT4 expression or triggering the insulin-signaling pathway),[12,13] thereby maintaining metabolic homeostasis. Relevantly, cortisol and melatonin have been shown to disturb CTSD activity in in-vivo studies,[14-16] suggesting that the proposed modulatory function of plasma CTSD on metabolism could also be related to changes in circadian rhythm.

In conclusion, the findings in this thesis suggest that plasma CTSD has strong ties to metabolic dysfunction, which holds the potential to be used as a metabolic marker in the context of MetS. To ascertain this, future studies focusing on prognostic analyses and determining cut-off values for plasma CTSD measurements should be performed in addition to further investigating the underlying mechanism and the potential role of plasma CTSD in the context of MetS as these knowledges can provide novel insights for the development of diagnostic and therapeutic tools for MetS related diseases.

Chapter 7

The muscle as a metabolic organ linked to plasma CTSD levels

The muscle is a metabolic organ that accounts for around 40% of total human body weight in healthy individuals [17] and plays an important role in systemic metabolism homeostasis.[18,19] Indeed, the muscle has been suggested to play a vital role in maintaining blood glucose and energy balance. For instance, during starvation the muscle is able to utilize both glucose and fatty acids as fuel and also serves as a source of amino acids for energy usage by other tissues and organs.[20] However, excessive lipids (i.e., triglycerides and free fatty acids), glucose as well as physical inactivity, disturb metabolism in skeletal muscle.[19] Of note, ectopic fat infiltration in skeletal muscle which includes both intramyocellular fat (fat infiltration within myocytes) and intermuscular fat (visible fat within the fascia surrounding skeletal muscle) induces myosteatosis. It has been demonstrated that myosteatosis is strongly associated with obesity, MetS, diabetes and NAFLD,[21,22] highlighting the link between myosteatosis and MetS. As mentioned earlier, plasma CTSD is a metabolic regulator, which suggests that plasma CTSD might be linked with myosteatosis. Indeed, in the current thesis we identified the association between myosteatosis and plasma CTSD levels in NAFLD patients (**Chapter 3**), demonstrating the significant role of the muscle as a metabolic organ in MetS.

Myosteatosis has been shown to induce insulin resistance, disturbed lipid metabolism and inflammation.[23] Relevantly, lipid accumulation (i.e., oxidized LDL) has been shown to stimulate the secretion of CTSD into the circulation.[24,25] The increased secretion of CTSD induced by lipid overload can be explained by lysosomal exocytosis, a process which is regulated by intracellular Ca^{2+} channels.[26] Lipid accumulation in cells is known to induce chronic intracellular Ca^{2+} overload, subsequently stimulating lysosomes to fuse with the plasma membrane to release lysosomal enzymes (i.e., CTSD) into the circulation via exocytosis.[27,28] In line, we demonstrated that myosteatosis is independently associated with plasma CTSD levels in NAFLD patients (**Chapter 3**). As such, these data suggest that the muscle might contribute to plasma CTSD levels via lysosomal exocytosis induced by lipid overload in the muscle. Alternatively, intracellular lipid overload could also disturb CTSD trafficking pathway, leading to the extracellular secretion of CTSD rather than being targeted to lysosomes (i.e., pro-CTSD).[24,29-31] In contrast, our observed positive association between myosteatosis and plasma CTSD levels in NAFLD patients also raises the question whether elevated plasma CTSD levels could lead to myosteatosis which is a possibility considering the function of CTSD as a mediator in the development of ectopic lipid accumulation.[3] However, whether myosteatosis leads to increased plasma CTSD levels, vice versa, or both is a matter of future debate. Nevertheless, our data show a link between myosteatosis and plasma CTSD in NAFLD patients, implying an involvement of the skeletal muscle in MetS-associated disorders. Additionally, while hepatic steatosis independently associates with plasma CTSD levels, in this thesis, the association between myosteatosis and plasma CTSD levels in NAFLD patients was found to be independent of sex, age, BMI, waist and hepatic steatosis (**Chapter 3**), suggesting that the muscle

independently contributes to plasma CTSD levels without the influence of the liver. In line, recent studies indeed have indicated that myosteatosis is not correlated with hepatic steatosis in NAFLD patients.[32] Altogether, the results in this thesis suggest the muscle as a metabolic organ contributing to plasma CTSD levels.

Plasma CTSD as a valuable component to determine organ-specific (hepatic) insulin resistance

Insulin resistance is a hallmark of T2DM,[33] which can be manifested as whole-body or organ-specific insulin resistance. Examples of organ-specific insulin resistance are peripheral and hepatic insulin resistance. Notably, peripheral insulin resistance decreases the ability of insulin to stimulate the glucose uptake into peripheral tissue (mainly skeletal muscle), while hepatic insulin resistance reduces insulin-mediated suppression of glucose production by the liver.[34] Of note, whole body insulin resistance is the sum of peripheral and hepatic insulin resistance,[35] among which the former can be divided into muscle insulin resistance and adipose tissue insulin resistance. It has been shown that the muscle, adipose tissue and liver account for about 60–70%, 10% and 30% of whole-body insulin mediated glucose disposal, respectively.[36] While insulin resistance develops simultaneously in multiple organs in many individuals, the severity of insulin resistance is different among the various tissues,[37] suggesting organ-specific interventions might be an effective way to improve insulin sensitivity. From clinical observations, it appears that some (pre)diabetic patients that mainly suffer from hepatic insulin resistance, mostly respond to interventions that improve hepatic insulin sensitivity (i.e., metformin), whereas others who have peripheral insulin resistance, will benefit from interventions that aim to improve peripheral insulin sensitivity (i.e., peroxisome proliferator-activated receptor-γ agonist and exercise).[38-40] Currently, the gold standard used in the clinic to determine insulin resistance (whole-body insulin resistance) is via the hyperinsulinemic-euglycemic clamp.[41] When combined with a radiolabeled glucose tracer, the two-step hyperinsulinemic–euglycemic clamp is considered as the golden standard to quantify organ-specific insulin resistance .[42] However, the clamp method cannot be used in a large population in clinical practice as it is an invasive, costly, time-consuming and labor-intensive procedure.[37] Alternatively, the new indexes for insulin resistance have been developed based on the measurement of plasma glucose and insulin concentrations from the oral glucose tolerance test in the clinic, for instance, homeostasis model assessment index for insulin resistance (HOMR-IR).[35,43] Although these indexes provide a simple procedure to assess whole-body insulin resistance, they cannot give information for the severity and magnitude of organ-specific insulin resistance. Therefore, to achieve higher intervention efficacy, new intervention methods based on an organ-specific approach to determine organ specific insulin resistance are warranted. Currently, clinical studies have shown to create linear regression models to predict skeletal muscle insulin sensitivity (Muscle-ISI) based on routine clinical and biochemical measurements and nuclear magnetic resonance (NMR)-metabolomics against the

golden standard measurement.[44] While these studies suggest Muscle-ISI model could be used as easy, less time consuming and inexpensive tools for determining muscle insulin sensitivity in large clinical cohort studies, it still needs to measure a variety of fasting serum metabolic parameters, such as glucose, insulin, triglycerides and FFA. Additionally, given the fact that there is no existing consensus on how to quantify adipose tissue insulin resistance so far,[45] Søndergaard *et al.* compared three methods: adipose tissue insulin resistance index (Adipo-IR that is calculated from a single measurement of post absorptive concentrations of FFA and insulin), the single step insulin clamp (using a palmitate tracer) and the multistep pancreatic clamp (using somatostatin to inhibit endogenous insulin secretion and calculating the insulin concentration required for a 50% suppression of lipolysis).[46-49] Their study found that both adipo-IR and the one-step hyperinsulinemic-euglycemic clamp technique are good predictors and can be used in a large population for measuring adipose tissue insulin sensitivity, whereas the multistep pancreatic clamp technique is a more effective tool to be used for mechanistic studies of adipose tissue insulin action though it cannot be used in large clinical studies. Although current methods are able to determine organ-specific insulin resistance, there are still some limitations, which make them unable to be widely used in clinical studies. In this thesis, we suggest that plasma CTSD activity could potentially be utilized to determine hepatic insulin sensitivity in overweight and obese individuals (**Chapter 4**), which provides a non-invasive, less time-consuming and easier-performed approach to determine hepatic insulin resistance. However, in order to claim plasma CTSD as a potential marker, prognostic analyses need to be performed and cut-off values should be determined in other cohorts. Additionally, the value of plasma CTSD as biomarkers should also be investigated in combination with other (organ-specific) markers.[50]

Studies have shown that obesity-induced ectopic lipid accumulation in the liver associated with abnormalities of lysosomal protease expression/function, which also contributes to inflammation in adipocytes.[1] Additionally, ectopic hepatic lipid accumulation is one of the major triggers leading to impaired hepatic insulin sensitivity.[51] In **Chapter 4**, we indeed observed that TAG levels correlate with plasma CTSD activity and systemic inflammatory factors (IL-8 and TNF α) are also independently associated with plasma CTSD levels in overweight and obese humans. While previous research demonstrated that inhibiting extracellular CTSD reduces fatty liver in a steatotic rat model,[4] in this thesis, we demonstrated for the first time that plasma CTSD activity is inversely associated with hepatic insulin sensitivity in overweight and obese humans (**Chapter 4**). Collectively, these data suggest that the probable involvement of plasma CTSD activity in impairing hepatic insulin sensitivity is likely a consequence of disturbed lipid metabolism.

In addition, while it has been shown that inflammation is a trigger to induce hepatic insulin resistance,[52] systemic inflammation was not associated with peripheral/hepatic insulin sensitivity in our study (**Chapter 4**). This is consistent with studies showing that obesity-

associated inflammation is beneficial to maintain insulin sensitivity via regulation of energy metabolism which also explains the failure of anti-inflammatory therapy in the treatment of insulin resistance.[53] Previous studies from our group have demonstrated that plasma CTSD levels are associated with hepatic inflammation.[25,50] Likewise, other studies demonstrated that CTSD is up-regulated in inflammatory bowel disease.[54] In line, the current thesis also observed that plasma CTSD levels are positively associated with systemic inflammation (IL-8 and TNF α) in overweight and obese humans **(Chapter 4)**, suggesting that plasma CTSD is associated with metabolic inflammation in general. In contrast to the previously mentioned studies suggesting systemic inflammation to be beneficial for maintenance of insulin sensitivity, others have shown that inflammation is involved with the development of insulin resistance.[55,56] For instance, TNF-α can result in the decreased expression of the insulin receptor, insulin receptor substrate 1 (IRS1) and Glut4 genes, as well as a reduction of insulin stimulated glucose uptake.[57] These evidences suggest that the association of plasma CTSD with hepatic insulin resistance could be mediated by metabolic inflammation. Further research into these conflicting findings could further aid in determining the potential value of plasma CTSD in diagnosing metabolic syndrome-related diseases.

Overall, the findings in this thesis provide a new approach on determining organ-specific insulin resistance and might also present a new view on the mechanism of insulin resistance via the involvement of plasma CTSD activity and inflammation. These findings could be utilized to aid in the process of deciding on organ-specific therapeutic agents for pre(diabetic) individuals.

Insulin resistance contributes to NAFLD via elevation of CTSD activity

Insulin resistance is known as an important trigger for the development and progression of NAFLD.[58,59] Indeed, studies have shown that insulin resistance contributes to the evolution of NAFLD by increasing *de novo* lipogenesis and FFA flux to the liver via reduced inhibition of lipolysis,[60] thus leading to hepatic lipo-toxicity. As described in **Chapter 2**, the harmful lipid intermediates (i.e., diacylglycerols and ceramides)[61] from lipo-toxicity products in the liver can promote the release of ROS, leading to oxidative stress and thereby progression from steatosis to NASH.[62,63] Similarly, it has been shown that insulin resistance also accelerates the entire pathologic spectrum of NASH partly via the upregulation of genes for lipogenesis, inflammation and fibrogenesis in animal models,[64] implying a pivotal role of insulin resistance in the pathogenesis of NAFLD progression.

In the current thesis, we demonstrated that insulin resistance and plasma insulin levels are independently associated with plasma CTSD activity in NAFLD patients (**Chapter 6**). Together with our previous studies[3,4,65] which highlight the effect of plasma CTSD in the context of the NAFLD pathology, these findings allow us to speculate that insulin resistance might contribute

to NAFLD progression via elevation of plasma CTSD activity. Indeed, in our previous studies we have observed elevated levels of plasma CTSD in NASH patients, which is linked with hepatic inflammation [25,50] and hepatic steatosis (**Chapter 3**). Inhibiting CTSD activity via a specific extracellular CTSD inhibitor reduced hepatic steatosis and plasma insulin levels in steatotic rats, thus improving lipid metabolism and insulin resistance.[4] Likewise, another study from our group demonstrates that inhibiting CTSD via Pepstatin A (an inhibitor of aspartyl proteases) leads to the reduction of hepatic inflammation and the improvement of lipid metabolism in murine steatohepatitis model.[3] Importantly, this thesis also shows that plasma CTSD activity is linked to hepatic insulin resistance and T2DM (**Chapter 4 and Chapter 5**). As such, these evidences show a link between increased plasma CTSD activity, hepatic inflammation, insulin resistance and lipo-toxicity (three pathological factors that closely contribute to NAFLD progression). Relevantly, studies have shown that CTSD can cleave active insulin into inactive insulin intermediates.[66] Additionally, CTSD can also cleave the insulin growth factor binding proteins (IGFBPs) that combines with IGF/insulin to regulate glucose metabolism.[67,68] Of note, IGFBPs are synthesized in large amounts by liver, which have a high degree of homology with the insulin receptor.[69,70] With the insulin receptor having a liver-specific isoform[71] accompanied by the high degree of similarity between IGFBPs and insulin receptor means that CTSD could potentially also be able to cleave liver insulin receptor. Therefore, based on these proteolytic properties of CTSD we speculate that the enzymatic activity of CTSD might impair insulin signalling in the liver leading to insulin resistance and further contributes to NAFLD progression through this mechanism (as shown in **Figure 7-1**). Additionally, insulin resistance is known to induce impaired glucose tolerance that has been shown to result in metabolic acidosis (a reduction of blood pH).[72,73] As previously mentioned in **Chapter 5**, a reduction in blood pH can increase the activity of plasma CTSD. This is therefore in line with our observation that insulin resistance is independently and positively associated with plasma CTSD activity in NAFLD patients. Overall, our findings in this thesis, together with our earlier studies, raise the possibility that the contribution of elevated plasma CTSD activity to the progression of NAFLD is potentially mediated by its enhancing effect on insulin resistance.

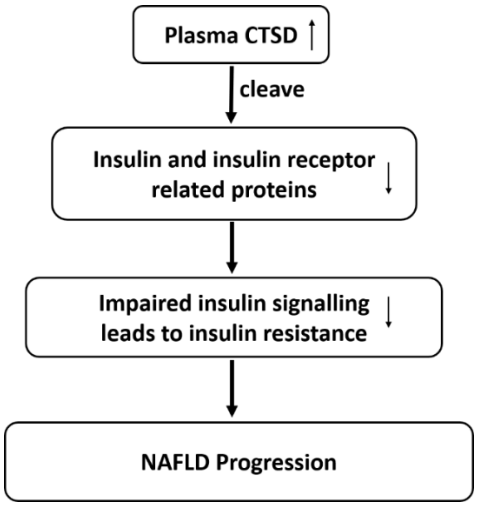

Figure 7-1. Potential mechanism of plasma CTSD activity contributing to NAFLD progression.

Chapter 7

Clinical implications of the findings

Diagnosis

As mentioned earlier, HOMA-IR or other indexes calculated from oral glucose tolerance test is more commonly used in clinic practice to assess insulin resistance. However, this approach cannot provide information for organ-specific insulin resistance. Alternatively, the two-step hyperinsulinemic–euglycemic clamp combined with a glucose tracer is considered as the gold standard for assessing peripheral and hepatic insulin sensitivity currently in the clinic.[74,75] However, this technique is an invasive, costly and labor-intensive procedure,[76] which is not able to be used in large population. Therefore, developing novel non-invasive, cost-effective and easy-to-measure markers to determine peripheral and hepatic insulin sensitivity for enabling organ-specific intervention is highly desirable for (pre)diabetic individuals.

While there are several studies that have investigated alternative non-invasive plasma markers to determine whole body and organ-specific insulin sensitivity,[77-79] none of them are specific for peripheral or hepatic insulin sensitivity. For instance, previous studies have demonstrated that increased liver enzymes γ-glutamyltransferase (GGT) and alanine aminotransferase (ALT) can serve as markers for both systemic and hepatic insulin sensitivity in healthy individuals.[77] However, GGT and ALT which are effectively used to diagnose steatosis,[80] didn't show distinctively predictive value for either systemic or hepatic insulin sensitivity. In addition, other studies also showed that inflammatory factors (i.e., interleukin-1 receptor antagonist (IL-1RA) and high-sensitivity C-reactive protein (hs-CRP)) can also be used for predicting insulin sensitivity,[81] whereas these markers did not hold predictive value for organ-specific insulin sensitivity. As such, there is a need for markers that are suitable to determine organ-specific insulin sensitivity. In the present thesis, we have shown that plasma CTSD activity might potentially be useful as a non-invasive, easier and cost-effective marker to assess hepatic insulin sensitivity in overweight and obese individuals **(Chapter 4)**. This suggested potential is based on the observation that plasma CTSD activity is inversely associated with hepatic insulin sensitivity independent of age, sex, BMI and waist circumference **(Chapter 4)**. In line, previous studies have also indicated that plasma CTSD is associated with insulin resistance in newly-diagnosed T2DM patients,[5] further confirming our observations. Based on these data it is reasonable to envision that hepatic insulin sensitivity could potentially be determined by measuring levels of plasma CTSD activity. However, in order to claim plasma CTSD as a potential marker, prognostic analyses need to be performed and cut-off values should be determined in other cohorts. Additionally, the value of plasma CTSD as biomarkers should also be investigated in combination with other (organ-specific) markers.

Overall, the data in this thesis suggests that plasma CTSD activity could be used to determine hepatic insulin sensitivity, which is beneficial for achieving organ-specific intervention for pre(diabetes) individuals. Besides CTSD, studies have also indicated that cathepsin S, K and L

are also linked to insulin resistance.[82-84] Future studies should be performed to further confirm whether the predicative value of plasma CTSD activity and other potential lysosomal enzymes are worthwhile to be utilized as non-invasive markers for T2DM.

Therapy

Due to the lack of knowledge regarding the underlying mechanism for the progression of NAFLD (i.e., the development of hepatic inflammation), there are no effective pharmacotherapies approved for NAFLD yet, especially for NASH.[3,85] Given the fact that NAFLD has become the most prevalent chronic liver disease worldwide,[86] there is an urgent need to develop an effective pharmaceutical intervention for the treatment of NAFLD.

Insulin resistance is pivotal for the progression of NAFLD,[87] which has been demonstrated to promote simple fatty liver to NASH.[64] Additionally, NAFLD and T2DM often coexist and act synergistically to drive adverse clinical outcomes suggesting that insulin resistance is an important contributor to NAFLD progression.[88,89] As such, therapeutic interventions aimed at improving insulin resistance might be an effective way for preventing NAFLD progression and also a novel therapeutic tool for NAFLD.

In the current thesis, we have demonstrated that insulin resistance might be involved in the progression of NAFLD via elevation of plasma CTSD activity **(Chapter 6)**. Therefore, therapies aimed at targeting CTSD activity might lead to improved clinical outcomes for NAFLD patients. As described in **chapter 2**, multiple therapies that target insulin and glucose metabolism have been investigated (i.e., GLP1R agonists and DPP4 inhibitor that have been administrated to diabetic patients) for the treatment of NAFLD.[90-92] However, none of them is currently approved yet due to side effects or ongoing clinical trials.[93,94] In this case, it will be worthwhile to investigate whether targeting extracellular CTSD activity has an influence on NAFLD development as targeting extracellular CTSD activity has less side effects.

Relevantly, the previous studies from our group have demonstrated that CTSD plays a key role in the development of hepatic inflammation and regulation of lipid metabolism.[3] Indeed, in these studies we have observed that inhibiting plasma CTSD activity reduces plasma insulin levels and hepatic steatosis in a steatotic rat model and also decreases hepatic inflammation in murine steatohepatitis.[3,4] These findings suggest that targeting CTSD for the treatment of NAFLD might exert effects via three distinct mechanisms, including the improvement of insulin resistance and lipid metabolism as well as the reduction of hepatic inflammation (as shown in **Figure 7-2**). Therefore, additional researches via inhibiting circulating CTSD activity in preclinic and clinic studies are warranted to further confirm whether targeting plasma CTSD activity is an effective pharmacotherapy for NAFLD.

Chapter 7

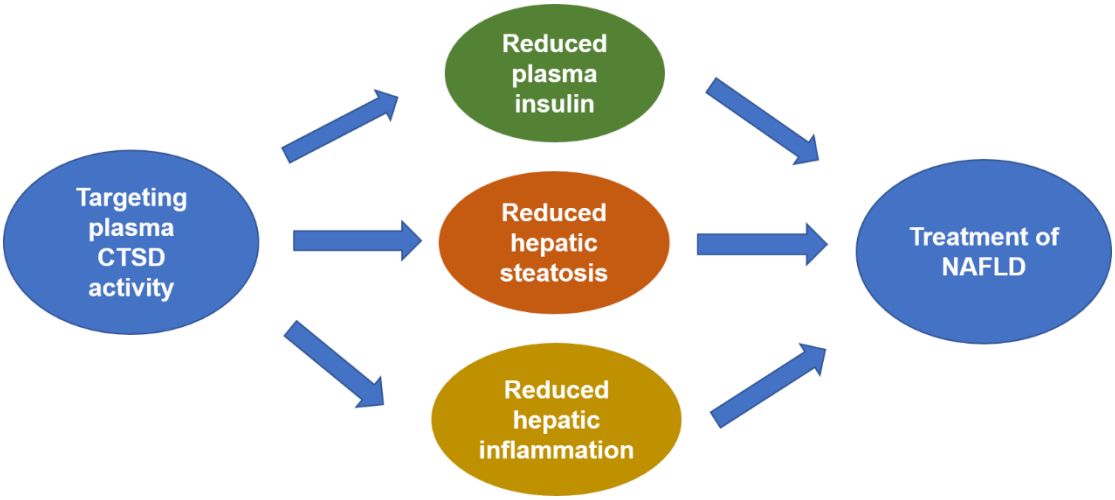

Figure 7-1. Targeting plasma CTSD activity is a potential therapeutic tool for the treatment of NAFLD.

Next to NAFLD, the identification of plasma CTSD serving as a metabolic regulator in T2DM **(Chapter 5)** also implies a potential for targeting plasma CTSD as a treatment for T2DM. Indeed, we observed that plasma CTSD activity is increased in T2DM patients compared with healthy individuals **(Chapter 5)**, suggesting a possible role of plasma CTSD activity in the pathogenesis of T2DM. Though multiple drugs have been developed and approved for the treatment of T2DM,[95] the high cost and side effects are currently existing disadvantages.[96,97] Therefore, plasma CTSD might be a promising and potentially less toxic (considering its extracellular nature) therapeutic target for T2DM treatment. Apart from CTSD, dysfunction of other plasma lysosomal enzymes has also been linked to T2DM.[82,83] Similarly, those plasma lysosomal enzymes might also be worthwhile to be investigated as targets for T2DM therapy.

Altogether, in the present thesis, our data suggests a potential therapeutic value of targeting plasma CTSD activity for the treatment of NAFLD and T2DM. As dysfunction of lysosomes and lysosomal enzymes play important roles in metabolic diseases,[1] future studies should therefore be conducted in great details to assess the viability of targeting of plasma CTSD and other plasma lysosomal enzymes as novel less toxic therapeutic tools for metabolic syndrome related diseases.

Chapter 7

Novel findings of this thesis

In the current thesis, we aimed to provide insight into the link between plasma CTSD and different aspects of metabolic syndrome in general. The novel findings from this thesis are summarized below (as shown in table 7.1).

1. Myosteatosis in NAFLD patients correlates with plasma CTSD levels (**Chapter 3**)
2. Plasma CTSD activity is (independently) inversely associated with hepatic insulin sensitivity (**Chapter 4**)
3. Plasma pH reduction increases plasma CTSD activity (**Chapter 5**)
4. Type 2 diabetic male individuals showed increased plasma CTSD activity compared to healthy males (**Chapter 5**)
5. Plasma CTSD is linked to metabolic dysfunctions in MetS-related diseases (**Chapter 3, 4 and 5**)
6. Insulin resistance is independently associated with plasma CTSD activity in NAFLD patients (**Chapter 6**)

Possible non-invasive marker for assessing hepatic insulin sensitivity

1. Plasma CTSD activity
2. other lysosomal enzymes in the plasma

Possible therapeutic options for NAFLD/T2DM

1. Targeting plasma CTSD
2. Modulating lysosomal function
3. Blocking the activity of other lysosomal enzymes in the plasma

Table 7.1 The main findings, limitations and applications of experimental chapters of the thesis

Chapters	Main findings	Limitations	Applications
Chapter 3	-Myosteatosis in NAFLD patients correlates with plasma CTSD levels	Sample size is relatively small.	Mechanistic Knowledge to better understand the extra-hepatic characteristics in NAFLD
Chapter 4	-Plasma CTSD activity is (independently) inversely associated with hepatic insulin sensitivity	The cohort that was used in our study has a relatively small sample size and an unbalanced sex population.	Plasma CTSD activity holds promise as a non-invasive predictive marker to assess hepatic insulin sensitivity
Chapter 5	-Plasma pH reduction increases plasma CTSD activity -Type 2 diabetic male individuals showed increased plasma CTSD activity compared to healthy males, which was independently linked to plasma FFA levels.	Plasma samples were used from long term storage, which could affect the absolute pH value.	Plasma CTSD as target for intervention in insulin resistance of T2DM
Chapter 6	-Insulin resistance is independently associated with plasma CTSD activity in NAFLD patients	The sample size is relatively small	Plasma CTSD could be used as an intervention target for the treatment of NAFLD

Chapter 7

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Appendices

Appendices

Summary

Chapter 1 provides a global overview of MetS, T2DM and NAFLD. After introducing the formation and location of lysosomes, lysosomal enzymes as well as lysosomal function, the critical importance of lysosomal enzymes in metabolism in the context of MetS was interpreted. Furthermore, the formation of CTSD as well as its role in metabolism, inflammation and MetS were introduced in more details. Finally, the thesis aim and outline are summarized.

Chapter 2 gives an overview of recent knowledge regarding NAFLD, including current epidemiological data and its related clinical and economic costs, an overview of pathophysiological hepatic processes in NAFLD as well as the currently investigated therapeutic approaches for NAFLD.

Chapter 3 investigates whether, besides the liver, also the muscle associates with plasma CTSD levels. The findings demonstrate a positive and independent association of myosteatosis with plasma CTSD levels in NAFLD patients, identifying a link between plasma CTSD levels and the muscle. This observed link supports the concept of a significant role of the skeletal muscle in metabolic disturbances in metabolic syndrome-related disorders.

Chapter 4 explores the links between plasma CTSD (levels/activity) and peripheral/hepatic insulin sensitivity as well as systemic inflammation in overweight and obese humans. The data demonstrates that plasma CTSD activity, but not systemic inflammation, inversely associates to hepatic insulin sensitivity, suggesting that plasma CTSD activity holds the potentiality to determine hepatic insulin sensitivity in (pre)diabetic individuals.

Chapter 5 assesses the link between plasma CTSD (activity/levels) and metabolic parameters of T2DM. In this chapter, we observe that despite similar levels of plasma CTSD between T2DM patients and healthy individuals, a metabolically-induced reduction of the plasma pH likely results in increased plasma CTSD activity in T2DM patients. Besides, increased plasma CTSD activity was independently linked to plasma FFA levels. Our data therefore point toward plasma CTSD as a metabolic regulator in male type 2 diabetes.

Chapter 6 mainly focuses on the association between plasma CTSD activity and insulin resistance in NAFLD patients. The findings demonstrate that insulin resistance is independently associated with plasma CTSD activity in NAFLD patients. Together with our previous studies, it raises the possibility that insulin resistance might involve in the progression of NAFLD via elevation of plasma CTSD activity.

Chapter 7 discusses the overall conclusions of this thesis in the context of these metabolic diseases along with a perspective view on their clinical implications.

Appendices

Samenvatting

Hoofdstuk 1 geeft een globaal overzicht van MetS, T2DM en NAFLD. Na het introduceren van de vorming en locatie van lysosomen, lysosomale enzymen en lysosomale functie, werd het cruciale belang van lysosomale enzymen in het metabolisme in de context van MetS geïnterpreteerd. Bovendien werden de vorming van CTSD en de rol ervan in metabolisme, ontsteking en MetS in meer details geïntroduceerd. Ten slotte worden het doel en de opzet van het proefschrift samengevat.

Hoofdstuk 2 geeft een overzicht van recente kennis betreffende NAFLD, inclusief huidige epidemiologische gegevens en de gerelateerde klinische en economische kosten, een overzicht van pathofysiologische leverprocessen in NAFLD en de momenteel onderzochte therapeutische benaderingen voor NAFLD.

Hoofdstuk 3 onderzoekt of, naast de lever, ook de spier associeert met plasma CTSD levels. De bevindingen tonen een positieve en onafhankelijke associatie aan van myosteatosis met plasma CTSD-levels bij NAFLD-patiënten, waarbij een verband wordt geïdentificeerd tussen plasma-CTSD-levels en de spier. Deze waargenomen link ondersteunt het concept van een belangrijke rol van de skeletspier bij metabole stoornissen bij metabool syndroom-gerelateerde aandoeningen.

Hoofdstuk 4 onderzoekt de verbanden tussen plasma CTSD (levels / activiteit) en perifere / hepatische insulinegevoeligheid, evenals systemische inflammatie bij mensen met overgewicht en obesitas. De gegevens tonen aan dat plasma CTSD activiteit, maar niet systemische ontsteking, omgekeerd evenredig is met hepatische insulinegevoeligheid, wat suggereert dat plasma CTSD-activiteit heeft de potentie om de hepatische insulinegevoeligheid bij (pre) diabetische personen te bepalen.

Hoofdstuk 5 onderzoekt het verband tussen plasma CTSD (activiteit / levels) en metabole parameters van T2DM. In dit hoofdstuk zien we dat ondanks vergelijkbare levels van plasma CTSD tussen T2DM patiënten en gezonde individuen, een metabolisch geïnduceerde verlaging van de plasma pH waarschijnlijk resulteert in verhoogde plasma CTSD activiteit bij T2DM patiënten. Bovendien was verhoogde plasma CTSD activiteit onafhankelijk gekoppeld aan plasma FFA levels. Onze gegevens wijzen daarom op plasma CTSD als een metabolische regulator van diabetes type 2 in mannen.

Hoofdstuk 6 richt zich voornamelijk op de associatie tussen plasma CTSD activiteit en insulineresistentie bij NAFLD patiënten. De bevindingen tonen aan dat insulineresistentie onafhankelijk geassocieerd is met plasma CTSD activiteit bij NAFLD patiënten. Samen met onze eerdere studies, verhoogt het de mogelijkheid dat insulineresistentie kan leiden tot de

progressie van NAFLD via verhoging van plasma-CTSD-activiteit.

Hoofdstuk 7 bespreekt de algemene conclusies van dit proefschrift in de context van deze metabole ziekten, samen met een perspectief op de klinische implicaties.

Appendices

总结

第一章对代谢性紊乱综合征，2 型糖尿病和非酒精性脂肪肝进行了全面介绍。然后在介绍了溶酶体，溶酶体酶以及溶酶体功能的形成和位置之后，还解释了溶酶体酶在代谢性紊乱综合征中对代谢的重要性影响。此外，在这一章节中还更加详细地介绍了组织蛋白酶 D 的形成及其在新陈代谢，炎症和代谢性紊乱综合征中的作用。最后总结了论文的目的和基本框架。

第二章概述了有关非酒精性脂肪肝的最新知识，包括当前的流行病学数据及其相关的临床和经济负荷，同时也概述了非酒精性脂肪肝的病理发病机制以及当前研究的对非酒精性脂肪肝的治疗方法。

第三章，我们主要研究了除肝脏外，肌肉是否也与血浆组织蛋白酶 D 水平有关。我们的发现表明 NAFLD 患者的肌肉脂肪含量与血浆组织蛋白酶 D 水平呈独立的正相关，从而确定了血浆组织蛋白酶 D 水平与肌肉之间的联系。我们所观察到这种联系表明骨骼肌在代谢综合征相关疾病的代谢紊乱中起重要作用。

第四章探讨了超重和肥胖人群血浆组织蛋白酶 D（水平/活性）与外周/肝胰岛素敏感性以及全身性炎症之间的联系。我们的数据表明血浆组织蛋白酶 D 的活性而非全身性炎症与肝胰岛素敏感性呈负相关，这表明血浆组织蛋白酶 D 活性有潜在的可能性可作为确定（糖尿病前期）个体肝胰岛素敏感性的标志物。

第五章评估了血浆组织蛋白酶 D（活性/水平）与 2 型糖尿病代谢参数之间的联系。在本章中，我们观察到，尽管在男性 2 型糖尿病患者和男性健康个体之间血浆组织蛋白酶 D 的水平相似，但是代谢诱导的血浆 pH 降低可能会导致 2 型糖尿病患者的血浆组织蛋白酶 D 活性增加。此外，血浆组织蛋白酶 D 活性的增加与血浆游离脂肪酸水平独立相关。因此，我们的数据表明血浆组织蛋白酶 D 可作为男性 2 型糖尿病的代谢调节剂。

第六章主要研究了非酒精性脂肪肝患者血浆组织蛋白酶 D 活性与胰岛素抵抗之间的关系。研究结果表明，非酒精性脂肪肝患者的胰岛素抵抗与血浆组织蛋白酶 D 活性独立相关。结合我们以前的研究结果，它很可能表明胰岛素抵抗可能通过血浆组织蛋白酶 D 活性的升高而参与了非酒精性脂肪肝的发展。

第七章在这些代谢性疾病的背景下，讨论了本论文的主要结论，并对它们的临床意义进行了展望。

Appendices

Impact paragraph

Social-economical and clinical relevance

With the global increase of obesity, MetS has become a global and escalating public health threat.[1] Currently, it is estimated that 12–37% of the Asian population, 12–26% of the European population and more than 30% of Northern American population suffers from MetS.[2,3] Moreover, individuals suffering from MetS have high risks to develop NAFLD, T2DM and other metabolic diseases,[4,5] emphasizing the public health threat that MetS brings to the world.

Being the hepatic event of MetS, NAFLD has become a common cause of chronic liver disease in the world.[6] The prevalence of NAFLD is currently estimated to be 25% in the world, among which NASH makes up 5% of the world population. Similarly, diabetes is one of the largest global public health concerns, whose prevalence has been rising in recent decades. According to the recent report of the International Diabetes Federation (IDF), 451 million adults live with diabetes in the world in 2017, which is expected to increase to 693 million by 2045 if no effective prevention is conducted.[7,8] Among the prevalence of diabetes, T2DM accounts for about 90% of all cases. In line with the high prevalence of NAFLD and T2DM, the economic burden of NAFLD and T2DM are also huge. For instance, the United States spent approximately 103 billion dollars per year on NAFLD-related costs[9] and the healthcare costs of diabetes accounts for 10% of global health care expenditure (USD 760 billion).[10] Besides the economic burden, NAFLD and T2DM also bring huge social and clinical burdens to the world. For instance, more social or medical workers are needed to take care of NAFLD and T2DM patients. As there is no available non-invasive biomarker to diagnosis NASH and no effective pharmaceutic therapy for the treatment of NAFLD due to the largely unknown underlying mechanism of NAFLD progression (i.e., hepatic inflammation), clinicians also face a major challenge in diagnosing and treating patients with NAFLD. Likewise, the treatment of T2DM in the clinic also has some limitations due to the high price and side effects of antidiabetic reagents. Therefore, there is an enormous demand for developing novel diagnostic markers and effective therapeutics for NAFLD and T2DM as well as for MetS in general.

In the current thesis, we propose that insulin resistance might contribute to NAFLD progression via elevation of plasma CTSD activity, suggesting that plasma CTSD activity might be a promising target for the prevention and treatment of NAFLD. This finding is valuable from a clinical point of view as it might lead to the development of new effective pharmaceutic therapies for NAFLD, thus reducing social and economic burden. Additionally, as previously discussed, determining organ-specific insulin resistance rather than whole-body insulin resistance is beneficial to achieve higher intervention efficacy via organ-specific interventions. The current thesis indeed demonstrated that plasma CTSD activity might be useful as a marker

to determine hepatic insulin sensitivity in pre(diabetic) individuals, thereby providing support for clinicians to prevent the disease progression and apply the organ-specific therapy for T2DM patients. Furthermore, this thesis also indicated that plasma CTSD activity that is increased in T2DM patients compared to healthy controls, also associates with metabolic parameters of T2DM, suggesting that plasma CTSD activity is likely involved in the pathogenesis of T2DM. Therefore, from a therapeutic point of view, these findings imply that plasma CTSD activity is a promising and potential target for the treatment of T2DM. Moreover, the findings of this thesis might also be interesting for other related metabolic diseases, such as, atherosclerosis and lysosomal storage disorders as these diseases have similarities in the perspective of pathology (i.e., lipid accumulation and lysosomal dysfunction).[11-13] For instance, in the context of atherosclerosis, studies have shown that CTSD is upregulated in atherosclerotic lesions.[14] Besides CTSD, other cathepsins including cathepsin B and X have also been found to play important roles in atherosclerosis, where these cathepsins can participate in the modification and accumulation of LDL cholesterol, cellular targeting of inflammatory cells, and extracellular matrix (ECM) remodelling, thereby contributing to the pathogenesis and progression of atherosclerosis.[15] This confirms the previous suggestion that CTSD and other cathepsins could be potential targets of intervention for the prevention of atherosclerosis. Likewise, CTSD has also been found to be involved in lysosomal storage disorders such as neurodegenerative disorders (i.e., Alzheimer's disease).[16,17] Indeed, current studies have demonstrated that CTSD is closely associated with the mechanisms of neurodegeneration by playing a role in the processing of Alzheimer's disease pathogenic proteins and autophagy.[18-20] Given this fact, researchers suggest CTSD as a therapeutic target for the treatment of Alzheimer's disease.[21] In addition to CTSD, other lysosomal enzymes, such as cathepsin B and cathepsin S are also reported to be involved in Alzheimer's disease [22-24] likely via initiating apoptotic cell death and activating inflammatory processes[25,26]. As such, our findings in this thesis could be extrapolated to other metabolic diseases where the clinical relevance of targeting other lysosomal enzymes in the context of those metabolic-related diseases should be further considered for investigation. Altogether, the successful translation of our findings from this thesis into the clinic in the future would probably improve the life quality of NAFLD, T2DM and MetS patients in general and alleviate the social, economic and clinical burdens that these diseases bring to the world.

Novelty of the concept

In this thesis, we investigated the link between plasma CTSD to different aspects of MetS (i.e., NAFLD and T2DM) in clinical cohorts. Here, we demonstrated for the first time that except for the liver, myosteatosis also links to plasma CTSD levels in NAFLD patients, further confirming the important role of the muscle in the context of MetS. Additionally, while studies have shown that insulin resistance contributes to the pathogenesis of NAFLD, the underlying mechanism is still not yet fully understood. Together with our previous findings, this thesis

proposes that insulin resistance might contribute to the progression of NAFLD via elevation of plasma CTSD activity, thereby elucidating part of a potential mechanism for the progression of NAFLD. Moreover, as previously discussed, assessing organ-specific insulin resistance is urgent for achieving organ-specific intervention for (pre)diabetic individuals. While currently there is no available non-invasive tool to determine organ-specific insulin resistance, this thesis suggests that plasma CTSD activity might be a potential non-invasive marker to determine hepatic insulin sensitivity, which is a non-invasive, easier-performed and faster approach. Thus, our finding provides a novel concept for a non-invasive tool to identify organ-specific insulin resistance. Finally, this thesis also demonstrated that plasma CTSD activity is likely involved in the pathogenesis of T2DM, thereby adding new mechanistical insight into T2DM progression and suggesting that targeting plasma CTSD activity might be of therapeutic value for T2DM patients.

Future plan

While the findings in this thesis provide novel diagnostic and therapeutic options for T2DM and NAFLD as well as MetS in general, more efforts have to be put to achieve the clinical translation of our findings to patients and public health. In order to implement the use of plasma CTSD activity as a marker to assess hepatic insulin sensitivity in the clinic, prognostic analyses need to be performed and cut-off values should be determined in other cohorts. Additionally, the value of plasma CTSD as biomarkers should also be investigated in combination with other (organ-specific) markers. Additionally, while there are commercial CTSD inhibitors available, so far, they are not capable of targeting circulating CTSD activity specifically. Hence, further research to develop specific inhibitors for targeting plasma CTSD activity which will have less side effects is particularly attractive for the treatment of NAFLD and T2DM. Apart from these two aspects, another one of our focuses is to perform validation studies in additional models and cohorts also in the context of other metabolic diseases. This is already in progress as this project is supported by a competitive grant from the TKI (a collaborative project between national and international academic researchers together with companies and patient organizations). For instance, *in-viv*o experiments in insulin resistance mouse models (i.e., *ob/ob* mice) will be performed with specific CTSD inhibitors to validate the finding that plasma CTSD plays an important role in insulin resistance. In addition to animal models, our group also has access to plasma samples of a hepatocellular carcinoma (HCC) cohort and alcoholic liver disease (ALD) cohort which we will use to validate our findings with respect to plasma CTSD in lipid metabolism, inflammation and insulin resistance. Besides the role of CTSD in metabolic diseases, investigating the role of other lysosomal enzymes in the context of metabolic diseases is another valuable direction for future research. As mentioned earlier, other lysosomal enzymes also play important roles in metabolic diseases. For instance, cathepsin S has been suggested as a potential biomarker and therapeutic target in inflammatory bowel diseases (IBD) as it has been shown that cathepsin S is a key driver of

intestinal inflammation[27]. Therefore, investigating the role of cathepsin S in other metabolic diseases in the context of inflammation would be very promising. Finally, given the fact that CTSD has dual mechanisms of action either through ligand (CTSD levels) interactions or protease activity (CTSD activity),[28] figuring out the difference between CTSD levels and activity, especially from the perspective of underlying mechanisms (i.e., molecular signalling pathway) is also one of our points of interest for further research. Based on our finding that plasma CTSD activity is always associated with insulin resistance in different cohorts, it would be interesting to first investigate the effect of CTSD enzymatic activity on insulin signalling pathway via utilizing specific CTSD activity inhibitors. Additionally, LDL receptor-related protein-1 (LRP1) receptor interactions with CTSD is a good start to explore the effect of CTSD as a ligand in terms of lipid metabolism and inflammation signalling pathways[29,30]. Overall, our findings from this thesis provide multiple ideas for future research which can be built upon to better understand the link between lysosomal enzymes and metabolic diseases.

Appendices

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Curriculum Vitae

Lingling Ding was born on March 7th, 1990 in Henan province, China. After finishing her high school in her home town, she started her Bachelor education in Pharmacy at Xinxiang medical university (Xinxiang, Henan province, China) in September 2009. During the four years of college (2009-2013), she got National Encouragement Scholarship twice, Major Award of Xinxiang Medical University and Outstanding student of Xinxiang medical University. After finishing an internship at the Institute of Medicinal Plant Development of Chinese Academy of Medical Science (Beijing, China), she acquired her Bachelor in Medicine in July 2013.

Subsequently, she participated in the Master 'Pharmaceutics' at the Tianjin University of Traditional Chinese Medicine and Tianjin International Joint Academy of Biotechnology and Medicine in September 2013. With excellent study achievements, she got National Scholarship, Outstanding Student in Tianjin University of Traditional Chinese Medicine, Excellent graduate in Tianjin University of Traditional Chinese Medicine and Second-class scholarship in Tianjin International Joint Academy of Biotechnology and Medicine during her master period (2013-2016). For this degree, her research project was about "targeted nanoparticle drug delivery system in breast cancer". After finishing her master thesis in this project, she got her master degree of Medicine in June 2016.

In July 2016, she worked as a researcher in the institute of pharmaceutical research in Beijing Kawin technology company until August 2017. Then in the same year, she started her PhD program at the department of Molecular Genetics within the School of Nutrition and Translational Research in Metabolism (NUTRIM). Her PhD was supervised by Prof. dr. Ronit Shiri-Sverdlov, dr. Tom Houben and dr. Yvonne Oligschlaeger. The topic of her research was to identify the functional role of lysosomal enzyme cathepsin D in metabolic syndrome. During this period, she was supported by the Chinese Scholarship Council (2017.09-2021.09).

Appendices

List of publications

1. **Lingling Ding**, Tom Houben, Yvonne Oligschlaeger, Albert V. Bitorina, Bart Verwer, Maarten E. Tushuizen & Ronit Shiri-Sverdlov. (2020) Frontiers in Endocrinology. https://doi.org/10.3389/fendo.2020.575070 (2019 impact factor: 3.644)
2. **Lingling Ding**, Yvonne Oligschlaeger, Ronit Shiri-Sverdlov, Tom Houben. (2020) Nonalcoholic Fatty Liver Disease. Handbook of Experimental Pharmacology. Springer, Berlin, Heidelberg (2019 impact factor 15.550)
3. **Lingling Ding**[#], Gijs H. Goossens[#], Yvonne Oligschlaeger, Tom Houben, Ellen E. Blaak* & Ronit Shiri Sverdlov*. Plasma cathepsin D activity is negatively associated with hepatic insulin sensitivity in overweight and obese humans. Diabetologia 63, 374–384 (2020) (2019 impact factor:7.518).
4. **Lingling Ding**, Toon. J. I. De Munck, Yvonne Oligschlaeger, Jef Verbeek, Ger. H. Koek, Tom Houben, Ronit Shiri-Sverdlov. Insulin resistance is positively associated with plasma cathepsin D activity in NAFLD patients. (Under review)
5. **Lingling Ding,** Toon. J. I. De Munck, Yvonne Oligschlaeger, Inês Magro dos Reis, Jef Verbeek, Ger. H. Koek, Tom Houben, Ronit Shiri-Sverdlov. Myosteatosis in NAFLD patients correlates with plasma Cathepsin D. (Under review)
6. Albert V. Bitorina[#], Yvonne Oligschlaeger[#], **Lingling Ding**, Tulasi Yadati, Tom Houben, Rianne D.W.Vaes, Steven W.M. Olde Damink, Jan Theys, Ronit Shiri-Sverdlov. OxLDL as an inducer of a metabolic shift in cancer cells. (Under revision)
7. Kati Mokkala, Johanna Gustafsson, Tero Vahlberg, Anita C.E. Vreugdenhil, **Lingling Ding**, Ronit Shiri-Sverdlov, Jogchum Plat, Kirsi Laitinen. Serum Cathepsin D in overweight and obese women during pregnancy: Exploring the relation with metabolic and inflammatory markers and evaluating the effects of fish oils and probiotics in a randomized intervention trial. (Under review)
8. **Lingling Ding**, Jiawei Li, Rui Huang, Zhidong Liu, Chunhua Li, Shaozi Yao, Jinyan Wang, Dongli Qi, Nan Li, Jiaxin Pi. Salvianolic acid B protects against myocardial damage caused by nanocarrier TiO2; and synergistic anti-breast carcinoma effect with curcumin via codelivery system of folic acid-targeted and polyethylene glycol-modified TiO2 nanoparticles, International Journal of Nanomedicine, 2016:11 5709–5727 (2019 impact factor: 5.115)
9. **Lingling Ding**, Zhidong Liu, Mike Okweesi Aggrey, Chunhua Li, Jing Chen and Ling Tong. Nanotoxicity: The Toxicity Research Progress of Metal and Metal-Containing Nanoparticles, Mini-Reviews in Medicinal Chemistry, 2015, 15, 529-542. (2019 Impact factor:2.733)
10. Zhidong Liu[#], Qian Zhang[#], **Lingling Ding**, Chunhua Li, Zhongpeng Yin, Guoqiang Yan, Jiaxin Pi, Jiawei Li, Nan Li, Dongli Qi. Preparation procedure and pharmacokinetic study of water-in-oil nanoemulsion of Panax notoginseng saponins for improving the oral bioavailability, Current Drug Delivery, 2015, June 7. (2019 impact factor:1.582)

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Oral presentations

- June, 2018: Genetic & Cell biology Seminar meeting, Maastricht, the Netherlands
  Title: Cathepsin D in health and disease

- September, 2018: NUTRIM Research Line 2 lunch meeting, Maastricht, the Netherlands
  Title: Correlation of Plasma Cathepsin D level and activity with Insulin resistance and systemic inflammation

- April, 2019: NUTRIM Research Line 2 lunch meeting, Maastricht, the Netherlands
  Title: Plasma Cathepsin D activity rather than levels correlates with T2DM

- April, 2019: Student visit day (Bachelor and Master Students from University of Freiburg, Germany)
  Title: Cathepsin D in nonalcoholic steatohepatitis.

- May, 2020: NUTRIM Research Line 2 lunch meeting, Maastricht, the Netherlands
  Title: CTSD activity and circadian clock in metabolic syndrome patients

Poster presentations

- November, 2018: Annual NUTRIM Symposium, Maastricht, the Netherlands
  Title: Plasma Cathepsin D activity is influenced by food consumption

- November, 2019: Annual NUTRIM Symposium, Maastricht, the Netherlands
  Title: Plasma cathepsin D activity rather than levels correlates with metabolic parameters of T2DM

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On September 17th 2017, I took my first international flight to Dusseldorf (German), then my boyfriend picked me up in the Dusseldorf airport. After one day's short break in Aachen, I went to Maastricht, a totally unfamiliar city for me, for my PhD study. At the beginning, I was full of curiosity and a little fear for this city and Maastricht University. After four years' studying and living in Maastricht, I already fall in love with this city and the people I meet here. Now, I would say that Maastricht is like my second home, where I am so lucky to meet so many nice people and also have so many unforgettable memories. Hereby, I would like to express my gratitude and thanks to my family, friends and colleagues.

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