2012•2013

FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN

master in de biomedische wetenschappen: klinische moleculaire wetenschappen

Masterproef

Cholesterol in hepatic inflammation: innocent bystander or still a heavily suspect?

Promotor :
dr. R. SHIRI-SVERDLOV

Nathalie Vaes

Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting klinische moleculaire wetenschappen

De transnationale Universiteit Limburg is een uniek samenwerkingsverband van twee universiteiten in twee landen: de Universiteit Hasselt en Maastricht University.

universiteit hasselt
KNOWLEDGE IN ACTION

Universiteit Hasselt | Campus Hasselt | Martelarenlaan 42 | BE-3500 Hasselt
Universiteit Hasselt | Campus Diepenbeek | Agoralaan Gebouw D | BE-3590 Diepenbeek

universiteit hasselt | Maastricht University

2012•2013
FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen: klinische moleculaire wetenschappen

Masterproef

Cholesterol in hepatic inflammation: innocent bystander or still a heavily suspect?

Promotor :
dr. R. SHIRI-SVERDLOV

Nathalie Vaes
Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting klinische moleculaire wetenschappen

universiteit hasselt | Maastricht University

Contents

Abbreviations

(2HP)-βCD:	(2-Hydroxypropyl)-β-Cyclodextrin
ABCA1:	ATP-Binding Cassette Transporter A1
ABCG1:	ATP-Binding Casette Transporter G1
AcLDL:	Acetylated Low Density Lipoprotein
BMDM:	Bone-Marrow Derived Macrophages
CD36:	Cluster of Differentiation 36
COPD:	Chronic Obstructive Pulmonary Disease
FFA:	Free Fatty Acids
HCC:	Hepatocellular Carcinoma
HDL:	High Density Lipoprotein
HFC:	High-Fat, High-Cholesterol Diet
IL:	Interleukin
KC:	Kupffer Cell
LDL(R):	Low Density Lipoprotein Receptor
LPS:	Lipopolysaccharide
LXR:	Liver X Receptor
MetS:	Metabolic Syndrome
MSR1:	Macrophage Scavenger Receptor 1
NAFLD:	Non-alcoholic Fatty Liver Disease
NASH:	Non-alcoholic Steatohepatitis
NPC:	Niemann Pick Type C
OxLDL:	Oxidized Low Density Lipoprotein
PBS:	Phosphate Buffered Saline
SR:	Scavenger Receptor
TG:	Triglycerides
TNF:	Tumor Necrosis Factor
VLDL:	Very Low Density Lipoprotein

Abstract

Introduction: Non-alcoholic steatohepatitis (NASH) is a condition characterized by liver lipid accumulation (steatosis) combined with inflammation, and is considered as the hepatic event of the metabolic syndrome. While steatosis is relatively benign and reversible, NASH can lead to progressive liver disorders and eventually, liver failure. Currently, treatment options for NASH are lacking.

Previously, we have demonstrated that hepatic inflammation is associated with cholesterol accumulation inside lysosomes of Kupffer cells (KCs), the resident macrophages of the liver. In addition, various *in vitro* studies have shown that 2-hydroxypropyl-β-cyclodextrin (2HP-βCD) can redirect lysosomal cholesterol to the cytoplasm. Relevantly, administration of 2HP-βCD to mice with Niemann-Pick Type C disease, a lysosomal cholesterol storage disorder, led to improved efflux of lysosomal cholesterol to the cytoplasm and reduced inflammation. However, the effects of 2HP-βCD have never been studied in NASH. We here **hypothesize** that by redirecting lysosomal cholesterol to the cytoplasm, 2HP-βCD can reduce the inflammatory response associated with NASH.

Materials and methods: *Ldlr*$^{-/-}$ mice were given weekly, subcutaneous injections with 2HP-βCD or PBS and were given a high-fat, high-cholesterol (HFC) diet for 12 weeks to induce NASH. In addition, bone marrow-derived macrophages of C57/Bl6 mice were cultured and stimulated with 2HP-βCD.

Results: Administration of 2HP-βCD to *Ldlr*$^{-/-}$ mice on HFC diet led to a shift in intracellular cholesterol distribution from lysosomal cholesterol accumulation towards cytoplasmic cholesterol storage. While the shift in intracellular cholesterol distribution was associated with a decrease in cholesterol crystallization, the inflammatory response was not reduced. Furthermore, cholesterol-loaded macrophages *in vitro* displayed increased levels of inflammation after treatment with 2HP-βCD.

Conclusion: In this study, we investigated for the first time the therapeutic potential of 2HP-βCD in relation to NASH. Our findings clearly demonstrate that although 2HP-βCD is able to redirect lysosomal cholesterol to the cytoplasm, inflammation and foamy KC appearance are not reduced. Furthermore, our *in vitro* data point to 2HP-βCD as being pro-inflammatory. Hence, this study provides evidence that lysosomal cholesterol accumulation is not consistently linked to hepatic inflammation and suggests that it is the type, rather than the amount of cholesterol inside lysosomes that triggers inflammation. Therefore, 2HP-βCD cannot be used as a treatment for NASH.

Significance: 2HP-βCD is being used as drug and drug carrier. However, we here show that 2HP-βCD is not able to reduce the hepatic inflammatory response and can even induce inflammation. Hence, 2HP-βCD cannot be applied as treatment strategy for NASH and one should consider side effects before using this compound in the treatment of metabolic and inflammatory diseases.

Abstract

Inleiding: Niet-alcoholische leverontsteking (NASH) is een aandoening die gekenmerkt is door de combinatie van vetopstapeling in de lever en een ontstekingsreactie. De aandoening wordt beschouwd als de lever component van het metabool syndroom. Hoewel vetopstapeling in de lever omkeerbaar is, leidt ontsteking tot verdere progressie van NASH, hetgeen uiteindelijk resulteert in leverfalen. Bovendien is er nog steeds geen behandelingsmogelijkheid voor NASH.

Voorheen hebben we aangetoond dat de leverontstekingsreactie gepaard gaat met lysosomale cholesterolstapeling in Kupffer cellen (KC), de macrofaagpopulatie van de lever. Daarnaast werd *in vitro* aangetoond dat 2-hydroxypropyl-β-cyclodextrin (2HP-βCD) lysosomaal cholesterol kan verplaatsen naar het cytoplasma. Verder is zowel de lysosomale cholesterolstapeling als de ontsteking in muizen met de lysosomale stapelingziekte Niemann-Pick Type C ziekte aanzienlijk verlaagd na behandeling met 2HP-βCD. De effecten van 2HP-βCD werden echter nog niet onderzocht in NASH. Daarom onderzoeken we hier de **hypothese** dat 2HP-βCD door het lysosomaal cholesterol te verplaatsen naar het cytoplasma, de ontstekingsreactie geassocieerd met NASH kan reduceren.

Materiaal en methoden: *Ldlr*$^{-/-}$ muizen werden gedurende een 12 weken hoog-vet, hoog-cholesterol (HFC) dieet behandeld met wekelijkse, subcutane injecties met 2HP-βCD of met een zoutoplossing. Daarnaast werden beenmergmacrofagen van C57/Bl6 muizen in kweek gebracht en behandeld met 2HP-βCD.

Resultaten: Toediening van 2HP-βCD aan *Ldlr*$^{-/-}$ muizen op HFC dieet leidde tot verplaatsing van het lysosomaal cholesterol naar het cytoplasma. Dit was geassocieerd met verminderde cholesterol kristallisatie, maar niet met een gereduceerde ontstekingsreactie. Verder induceerde 2HP-βCD ontsteking in cholesterol-beladen macrofagen *in vitro*.

Conclusie: In de huidige studie hebben we voor het eerst onderzocht of 2HP-βCD kan gebruikt worden als behandeling van NASH. Uit onze bevindingen blijkt dat 2HP-βCD lysosomaal cholesterol kan verplaatsen naar het cytoplasma, zonder de bijhorende ontstekingsreactie of de grootte van de KC te reduceren. Bovendien tonen we dat 2HP-βCD pro-inflammatoir is *in vitro*. In conclusie, lysosomale cholesterol stapeling correleert niet altijd met ontsteking (in de lever) en is het eerder het type cholesterol in de lysosomen dan de hoeveelheid die belangrijk is in de ontstekingsreactie. Als gevolg kan 2HP-βCD niet gebruikt worden om NASH te behandelen.

Significantie: 2HP-βCD wordt gebruikt als therapie en 'drugcarrier'. Echter, in dit onderzoek tonen we aan dat 2HP-βCD de ontstekingsreactie in de lever niet kan reduceren en zelfs ontsteking induceert. Daarom moet men de verscheidene effecten eerst grondig onderzoeken vooraleer 2HP-βCD te gebruiken als 'drug carrier' of anti-inflammatoire behandeling.

1. Introduction

1.1 The metabolic syndrome

The metabolic syndrome (MetS) has become one of the major public health challenges in the developed world[1, 2]. Approximately 25% of the general population is affected by the MetS, though the prevalence varies according to race, gender and age[2-4]. The MetS represents a cluster of various cardio-metabolic risk determinants including: insulin resistance, central/visceral obesity, glucose intolerance, hypertriglyceridemia, hypertension and increased very low density lipoprotein (VLDL) and decreased high density lipoprotein (HDL) cholesterol. Furthermore, a prothrombotic and a proinflammatory state are characteristic features (Figure 1.1)[2-4]. The MetS has a multi-factorial etiology, involving the interplay between environmental and genetic factors. A sedentary lifestyle, primarily characterized by physical inactivity and a 'Western' diet (high saturated fats, HDL cholesterol, high carbohydrates), combined with genetic factors that affect the specific MetS core components, increases the risk of developing the MetS[2, 4-6]. Accordingly, the growing obesity epidemic leads to an increase in the prevalence of the MetS. About 18% of the normal-weight population is affected by the MetS, rising up to 67% in obese people[2, 7]. Subjects with the MetS have a significantly higher risk for type 2 diabetes mellitus (5-fold) and cardiovascular diseases, such as atherosclerosis (2-fold)[4]. Furthermore, the liver is an important target organ in the pathogenesis of the MetS, which manifests itself in the form of metabolic fatty liver disorders[5].

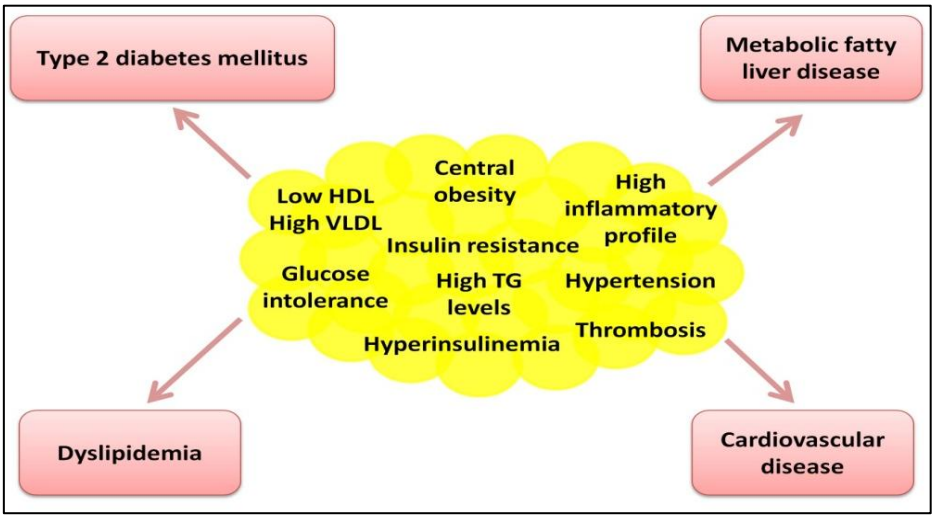


Figure 1.1 The metabolic syndrome. The cluster of metabolic risk determinants is associated with dyslipidemia, type 2 diabetes mellitus, cardiovascular disease and metabolic fatty liver disease. *HDL*, High Density Lipoprotein; *VLDL*, Very Low Density Lipoprotein; *TG*, Triglycerides.

1.2 Non-alcoholic fatty liver disease (NAFLD)

Non-alcoholic fatty liver disease (NAFLD) is defined by liver fat accumulation (steatosis), exceeding 5% of liver weight, with a minimal daily alcohol consumption (<20g/d for men; <10g/d for women)[6, 8, 9]. The development of NAFLD strongly correlates with the presence of the MetS core components. Obesity is most strongly associated with NAFLD, with a critical role for central adiposity in the pathogenesis and progression of the liver disease[8-10]. The risk of developing NAFLD is 4.5 fold higher in obese (75%) compared to in lean individuals (16.5%)[9]. In addition, approximately 90% of hyperlipidemia patients and 65% of patients with type 2 diabetes mellitus have a fatty liver with a low alcohol intake[9-11]. Furthermore, insulin resistance is present in 98% of NAFLD patients[3, 5, 9].

The liver is, except for the skin, the largest organ in the human body. It is mainly composed of hepatocytes (70–80%) and Kupffer cells, the resident macrophages of the liver (10–15%). Since the liver serves essential functions to meet the metabolic demands of the body, it has a strategic position in the circulatory system. The internal energy balance is maintained via gluconeogenesis (de novo synthesis of glucose), glycogenolysis (glycogen breakdown to glucose), and glycogenesis (glycogen synthesis from glucose for storage). In addition, the liver controls lipid metabolism by modulating cholesterol synthesis, as well as lipoprotein and TG production.

1.3 Non-alcoholic steatohepatitis, the lynchpin in the NAFLD spectrum

The umbrella term NAFLD covers a spectrum of liver disorders (Figure 1.2), ranging from steatosis, to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and hepatocellular carcinoma (HCC)[9, 12]. With a mean estimated prevalence of 26%, NAFLD is becoming the most common liver disorder in industrialized countries[13].

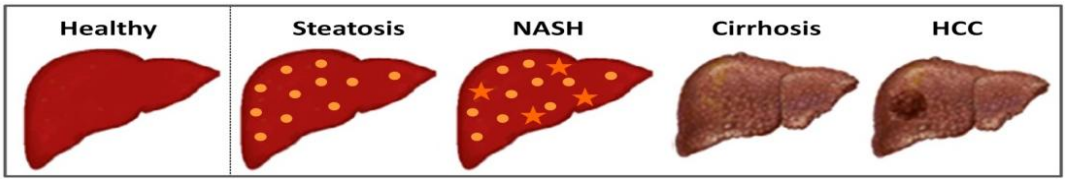


Figure 1.2 The NAFLD spectrum. A steatotic liver is characterized by fat accumulation. Progression towards NASH (steatosis and hepatic inflammation) sets the stage for the more advanced liver disorders, such as cirrhosis and HCC. *NASH, Non-Alcoholic Steatohepatitis; HCC, hepatocellular Carcinoma* (Adapted from: Galmed Pharmaceuticals and Trauner M, et al[14]).

Although patients with a fatty liver progress very slowly, if at all, about 20% develops NASH[6, 12, 13]. NASH is the condition characterized by liver lipid accumulation and its concurrent hepatic inflammatory response[10, 12, 13]. Whereas steatosis is a relatively benign and reversible condition, the development of hepatic inflammation can lead to irreversible liver damage. It has been estimated that nowadays 2–4%[11, 15] of the general population suffers from NASH, whereas the growing obesity epidemic results in an increasing prevalence in both obese adults (33%)[10] and children (55%)[11].

Unfortunately, it is still a challenge to identify those who have NASH. This is attributed to the fact that:(a) the primary laboratory finding of elevated serum aminotransferases (a non-invasive marker) is unreliable, and (b) a liver biopsy, is invasive, has ethical limitations and potentially (severe) complications[5, 8, 11]. Consequently, the exact prevalence is underestimated.

Currently, it is still not clear why in some (obese) individuals, but not all, a fatty liver progresses towards NASH. Nevertheless, the transition from steatosis towards NASH is crucial in the pathogenesis, because the development of inflammation sets the stage for further, irreversible liver damage[12]. Furthermore, in spite of extensive research to unravel which factors elicit the hepatic inflammatory response, the triggers remain largely unknown. Consequently, there is still no cure for NASH, thereby leading to a 6.5 fold increase in liver-related mortality[10, 11].

1.4 Macrophage cholesterol homeostasis

Cholesterol, the main sterol in mammalian tissues, is a structural component of all cell membranes and is required as precursor of bile acids and various steroid hormones. Since cholesterol is a hydrophobic molecule, it is transported in the blood packaged with proteins as lipoproteins. Low density lipoproteins (LDLs) are the main carriers of cholesterol and cholesteryl esters. Under normal circumstances, the uptake of native lipoproteins, i.e. LDLs, is tightly controlled by the intracellular cholesterol content. LDLs are taken up via LDL-receptor-mediated endocytosis and directed towards the lysosomes for degradation. Lysosomal enzymes hydrolyze lipoprotein-cholesteryl esters, releasing free cholesterol in the lysosomal compartment. Free cholesterol is then transported across the lysosomal membrane by the concerted action of the Niemann-Pick type C (NPC) proteins 1 and 2. The majority of cholesterol is mobilized to the plasma membrane, whereas excess cholesterol is stored in cytoplasmic lipid droplets or excreted during reverse cholesterol transport, mainly via the ATP-binding cassette transporters (ABCs) A1 and G1[16, 17]. As a result, the cells are provided with sufficient cholesterol for cellular functions, without overloading them[16-20].

However, macrophages are also able to ingest cholesterol via (non-)receptor-mediated pathways that are not feedback regulated by intracellular cholesterol levels. These routes primarily mediate the uptake of lipoproteins that were modified in the blood, i.e. acetylated, oxidized and aggregated LDL[17, 21, 22]. Acetylated and oxidized LDLs (acLDL and oxLDL) are mainly taken up by the scavenger receptors (SRs), macrophage scavenger receptor 1 (MSR1) and cluster of differentiation 36 (CD36), respectively (Figure 1.3)[19, 21]. Additionally, both forms can be internalized via macropinocytosis. Aggregated LDLs, on the other hand, are principally taken up via phagocytosis[22, 23]. Moreover, in the absence of the LDL receptor, cells can internalize native LDL particles via endocytosis[24]. While the unregulated uptake of (ac)LDLs leads to cytoplasmic cholesterol accumulation, the uptake of oxLDLs

disturbs intracellular cholesterol metabolism in such a way that cholesterol, i.e. both oxidized and non-oxidized cholesterol, accumulates inside lysosomes. Both will give rise to foam cells, with the latter being a specific type present in NASH and other metabolic disorders, such as atherosclerosis[25].

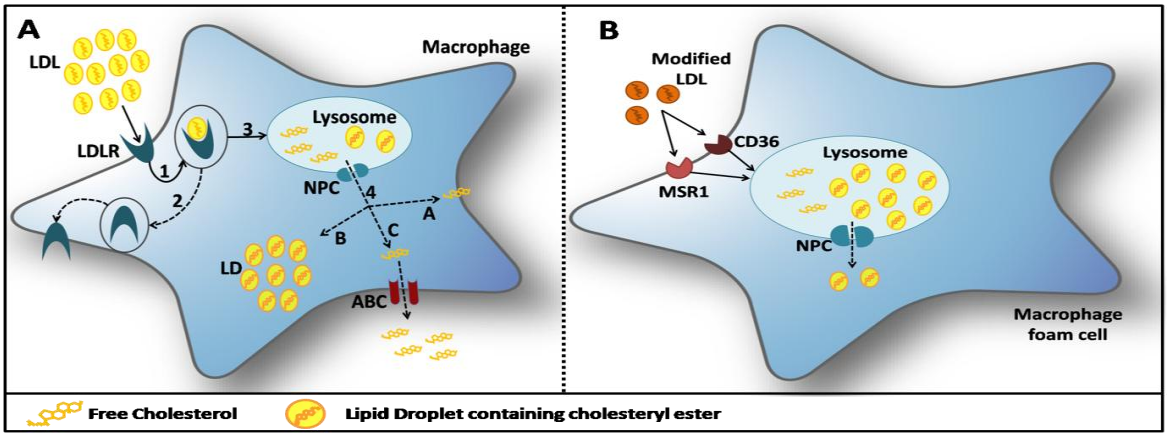


Figure 1.3 Macrophage cholesterol metabolism. A) Native LDLs bind LDL receptors (LDLR) and are taken up via receptor-mediated endocytosis (1). The LDLR is recycled (2), while LDLs are degraded in lysosomes (3). Free cholesterol is transported out of the lysosomes via NPC proteins (4) and directed to the plasma membrane (A), stored in lipid droplets (LD) (B), or excreted via ABC transporters (C). **B)** Modified LDL is mainly internalized via MSR1 and CD36, where after it becomes trapped inside lysosomes, giving rise to foamy macrophages

1.5 Lysosomal cholesterol accumulation and the MetS

Previous studies in our laboratory revealed that the onset of hepatic inflammation in hyperlipidemic, low density lipoprotein receptor knockout (*Ldlr*$^{-/-}$) mice fed a high-fat, high-cholesterol (HFC) diet is associated with foamy Kupffer cells (KCs) that contain swollen lysosomes filled with cholesterol. These foamy KC strongly resemble the foamy macrophages found in atherosclerotic lesions.[19, 26] The current view favors a model in which these foam cells are formed by the uptake of oxLDLs via the macrophage SRs and the subsequent trapping of cholesterol, i.e. oxidized and non-oxidized cholesterol, inside lysosomes[12, 19, 25, 27]. Indeed, various *in vitro* studies with macrophages showed that incubation with oxLDL leads to lysosomal cholesterol trapping[27, 28]. Furthermore, we confirmed that the uptake of oxLDL by KCs in *Ldlr*$^{-/-}$ mice fed a HFC diet leads to swollen, foamy KCS, which are characterized by lysosomal cholesterol accumulation and the presence of cholesterol crystals[12, 29, 30].

In line with our observation that lysosomal cholesterol trapping in KCs is associated with an inflammatory response, others have found evidence for this association in atherosclerosis[22, 31, 32]. Atherosclerotic lesions are characterized by the presence of foamy macrophages that contain swollen, cholesterol-engorged lysosomes, due to the uptake of oxLDLs[22, 26, 32]. As a consequence, the vascular endothelium loses its functionality, resulting in a chronic inflammatory response[33]. In addition, Niemann-Pick Type C (NPC) disease, a lysosomal storage disorder, represents another condition wherein lysosomal cholesterol accumulation and inflammation are closely related. NPC disease is caused by a mutation in either of the *NPC1* or *NPC2* genes, which encode for proteins that

transport cholesterol from the lysosome to the cytoplasm[34]. As a result, almost every cell of the body progressively accumulates cholesterol in the lysosomes, thereby disturbing total body cholesterol homeostasis and leading to inflammation in multiple organs, including the liver[34, 35].

All together, these data suggest a key role for lysosomal cholesterol accumulation in the development of inflammation in various metabolic diseases within the spectrum of the MetS, including NASH and atherosclerosis.

1.6 2-Hydroxypropyl-β-cyclodextrin (2HP-βCD), manipulator of lysosomal cholesterol content

2-Hydroxypropyl-β-cyclodextrins (2HP-βCDs) are cyclic saccharides composed of seven β(1-4)-glucopyranose units[36-38]. The rigid conical structure of 2HP-βCDs creates a hydrophobic cavity capable of solubilizing a wide variety of molecules, ranging from polar to non-polar compounds, with the highest affinity for sterols, including cholesterol[36-38]. 2HP-βCDs are modified from β-cyclodextrins (βCDs), primary degradation products of starch, via substitution of the hydroxyl groups of βCD-molecules with hydroxypropyl groups[39]. As a result, 2HP-βCDs have a 30 fold higher aqueous solubility and a higher resistance to enzymatic degradation, compared to non-modified βCD[35, 39]. In addition, because of their fairly high clearance by the kidney in almost completely intact form, the half-life of 2HP-βCDs is quite short, making them toxicologically more benign than the non-modified βCDs[40].

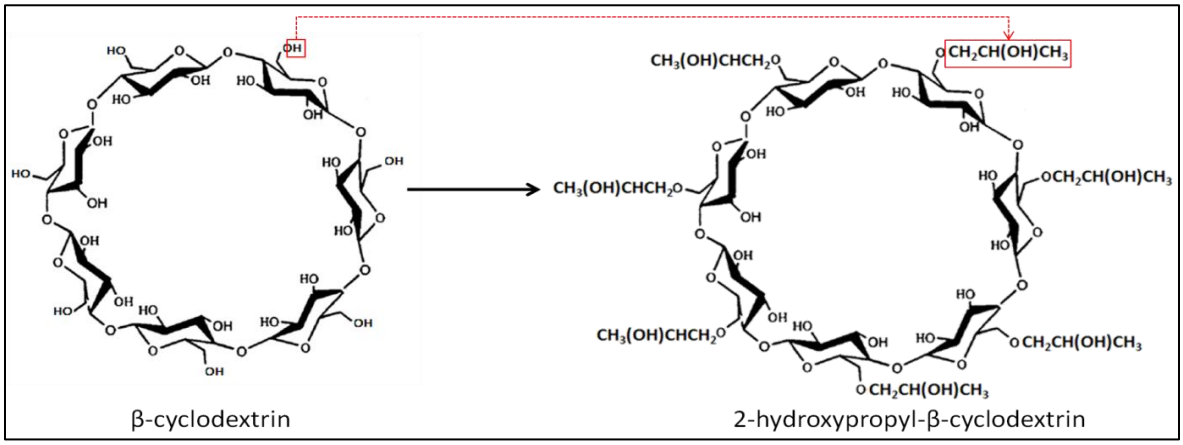


Figure 1.4 Structure of β-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin. β-cyclodextrin is a cyclic saccharide that consists of seven β(1-4)-glucopyranose units. By substituting the hydrogen of the hydroxyl group with hydroxypropyl (indicated by red boxes and the red dashed line), 2-hydroxypropyl-β-cyclodextrin is generated.

The ability of 2HP-βCD to capture sterols with a high affinity makes this compound well-suitable to manipulate the intracellular cholesterol content. In fact, various *in vitro* studies have demonstrated that at low concentrations (< 10mM), 2HP-βCD augments bidirectional flux without changing the cholesterol equilibrium between cells and lipoproteins[37, 41]. Besides, it has been shown *in vitro* that

higher concentrations of 2HP-βCD (10-100mM) enhance the rate of cholesterol efflux from a variety of cells, e.g. human and mouse fibroblasts and mouse peritoneal macrophages loaded with cholesterol, 3.5-7.5 fold compared to the physiological cholesterol acceptor HDL[37, 38, 42]. As such, 2HP-βCD serves as cholesterol shuttle if added in low concentrations, while functioning as sink (reservoir) in high concentrations[37, 42].

Furthermore, various *in vivo* studies have shown that subcutaneous administration of 2HP-βCD to *NPC1*$^{-/-}$ mice normalizes cholesterol metabolism in nearly every organ of the body[20, 34, 43-47]. 2HP-βCD is, after administration, internalized in the lysosome via bulk-phase endocytosis, where it facilitates the movement of lysosomal cholesterol into the metabolically active pool in the cytoplasm. In this way, cholesterol can take part of the normal metabolization pathway, thereby restoring the intracellular cholesterol homeostasis.

1.7 2HP-βCD and the inflammatory response

In addition to its beneficial effects on cholesterol metabolism, 2HP-βCD has been shown to have anti-inflammatory properties. Various studies in *NPC1*$^{-/-}$ mice have demonstrated that either a single subcutaneous injection or weekly subcutaneous injections with 2HP-βCD (4000 mg/kg) reduce the inflammatory response, especially in the liver and the brain of *NPC1*$^{-/-}$ mice [34, 45]. In the liver, the number of activated macrophages[34, 45] and the expression of the inflammatory marker *TNF*α[34], was significantly reduced. Furthermore, liver function was nearly normalized[34, 43]. In addition, the inflammatory response in the brain was significantly reduced, leading to decreased central nervous system degeneration[34, 45]. Moreover, intravenous infusion of 2HP-βCD to patients with NPC disease was partially effective in ameliorating hepatosplenomegaly and central nervous dysfunction[48].

Similar to the beneficial effects of 2HP-βCD on NPC disease, administration of 2HP-βCD leads to amelioration of bronchial inflammatory diseases, including chronic obstructive pulmonary disease (COPD) and asthma. In fact, these researchers revealed that male mice exposed to allergens and subjected to inhalation of 2HP-βCD, had reduced eosinophil counts, peribronchial inflammation and interleukin-13 (IL-13) expression (Patent US20120295872).

Taken all together, these data suggest that 2HP-βCD *in vivo* is able to reduce the inflammatory response.

1.8 Hypothesis, aims and objectives

Previous research in our laboratory revealed that hepatic inflammation is associated with increased lysosomal cholesterol accumulation in KCs. In addition, various studies demonstrated that 2HP-βCD can reduce the intracellular cholesterol content and the inflammatory response.

Hypothesis

We hypothesize that by redirecting lysosomal cholesterol to the cytoplasm, 2HP-βCD can reduce the inflammatory response associated with NASH.

Objective

To explore whether 2HP-βCD can be used as novel therapeutic strategy against NASH.

Approach

To investigate the therapeutic potential of 2HP-βCD in NASH, *Ldlr*$^{-/-}$ mice were given weekly, subcutaneous injections with 2HP-βCD (4000 mg/kg body weight) and placed on a HFC diet for 12 weeks to induce NASH. The control group received injections with phosphate-buffered saline (PBS). Afterwards, mice were sacrificed and livers were isolated. Lysosomal cholesterol storage and inflammatory markers were assessed by electron microscopy, histology and gene expression analysis.

Expected results

We expected that 2HP-βCD-treated mice have reduced lysosomal cholesterol accumulation and a reduced hepatic inflammatory response compared to PBS-injected mice on HFC diet.

1.9 Societal relevance

Even though NASH is recognized as a major health burden, there is currently still no cure. Since hepatic inflammation is the key event in the NAFLD spectrum towards irreversible liver damage, it is important to find therapeutic strategies to reduce the inflammatory response. With this research we aim to investigate whether 2HP-βCD is able to reduce the inflammatory response during NASH. Such knowledge can lead to a novel treatment strategy for NASH and to more insights regarding the potential of 2HP-βCD as treatment for other metabolic and inflammatory diseases.

1. Introduction

2. Materials and methods

2.1 Mice, diet and injections

Female *Ldlr*$^{-/-}$ mice on a C57/Bl6 background were housed under standard conditions and given free access to food and water. All experiments were approved by the Committee for Animal Welfare of Maastricht University and performed according to Dutch regulations.

The effects of two-hydroxypropyl-β-cyclodextrin (2HP-βCD) on NASH were investigated in *Ldlr*$^{-/-}$ mice on a high-fat, high cholesterol diet (HFC) by means of weekly, subcutaneous injections with 2HP-βCD (4000 mg/kg body weight; H5784, Sigma–Aldrich GmbH, Zwijndrecht, the Netherlands) (n=12). Phosphate-buffered saline (PBS) was used for control injections in mice receiving a regular chow (n=10) or a HFC diet for 12 weeks (n=10). The HFC diet contained 21% milk butter, 0.2% cholesterol, 46% carbohydrates and 17% casein. Blood was collected from the tail vein on the day of sacrifice (t_{12}), after a 4-hour fasting period. After 12 weeks, the mice were sacrificed by O_2/CO_2 euthanasia. Liver specimens were isolated, snap-frozen in liquid nitrogen and stored at -80°C or fixed in 4% formaldehyde/PBS.

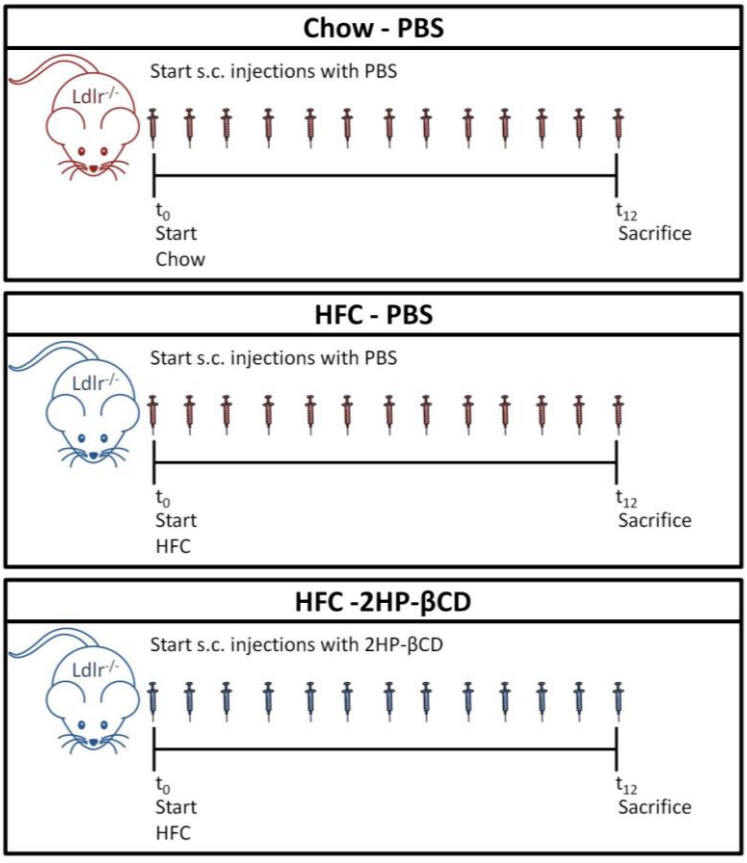


Figure 2.1 Experimental design. Female *Ldlr*$^{-/-}$ mice were put on chow or on a high-fat, high-cholesterol (HFC) diet for 12 weeks and were treated weekly, with subcutaneous (s.c.) injections of phosphate-buffered saline (PBS) (Chow-PBS and HFC-PBS, respectively). Mice in the experimental group received weekly, s.c. injections with 2-hydroxypropyl-β-cyclodextrin (2HP-βCD) during a 12-week HFC diet.

2.2 Liver lipid analysis

Approximately 50 mg of frozen liver tissue was homogenized for 30 seconds at 4800 rpm in a closed tube with 1.0 mm glass beads and 1.0 ml SET buffer (Sucrose 250 mM, EDTA 2 mM and Tris 10 mM). Cells were then completely broken down by means of two freeze-thaw cycles, one time passing through a 27-gauge syringe needle and a final freeze-thaw cycle. Protein content was determined with the bicinchoninic acid (BCA) assay method (23225; Pierce, Rockford, IL). Liver lipid content, i.e. cholesterol, triglycerides and free fatty acids, was measured with enzymatic color tests on a Bio-Rad Benchmark 550 Micro-plate reader (Bio-Rad, Hercules, Ca, USA). The cholesterol level was determined with the cholesterol CHOD-PAP method (1489232, Roche, Basel, Switzerland), TG with the GPOtrinder technique (Saint Louis, USA) and free fatty acids with the NEFAC ACS-ACOD MEHA assay method (999-75406, Wako Chemicals, Neuss, Germany). Protocols were followed according to manufacturer's instructions.

2.3 Plasma lipid analysis

Blood collected from the tail vein was centrifuged for 20 min at 3500 rpm, where after the plasma supernatant was transferred to a fresh tube. Plasma cholesterol was measured using the enzymatic color test described above, with a Benchmark 550 Microplate reader (Biorad, Hercules, Ca, USA).

2.4 Kupffer cell isolation

Liver specimens were isolated from the mice (n = 4) and digested in digestion buffer (Liberase TM, 33.3 µg/mL and 0.002% DNaseI) for 20 minutes at 37°C. Tissues were further disrupted by pushing them through a 100-µm cell strainer using wash buffer (PBS, 1% FCS, EDTA 2.5 mM). Afterwards, the cells were pelleted (1500 rpm, 10 min, 4°C) and resuspended with wash buffer. By one low-spin (300 rpm, 3 min, 4°C), the hepatocytes were removed. Supernatant was collected and centrifuged (1500 rpm, 10 min, 4°C), where after the red blood cells were lysed (lysis buffer, 3 min). The pellet that formed upon centrifugation (1500 rpm, 10 min, 4°C) was dissolved in wash buffer and incubated with the CD16/32 FcR block (1µl, 10 min, 4°C). Subsequently, wash buffer was added, followed by centrifugation (1500 rpm, 10 min, 4°C) and removal of the supernatant. To select for Kupffer cells, the samples were incubated for 20 minutes with the macrophage-specific monoclonal antibody F4/80 Allophycocyanin (1 µL/80 × 10^6 cells, 4°C; Biolegend, Breda, the Netherlands). After washing, the cells were incubated with anti-APC microbeads (200µl/100*10^6 cells; Miltenyi Biotec, Auburn, CA) for 20 minutes in the dark (4°C). Upon another centrifugation (1500 rpm, 10 min, 4°C), a pellet formed, that was washed, run into LS columns on a Quadro magnet (Miltenyi Biotec), and rinsed with wash buffer (negative fraction). Thereafter, the column was removed from the magnet and flushed again with wash buffer to capture the macrophage population (positive fraction) for further analysis.

2.5 RNA isolation

2.5.1 Liver tissue

Total RNA was isolated from mouse liver tissue specimens. Frozen liver tissues were homogenized with 1.0 ml Tri Reagent (Sigma Aldrich, Saint Louis, USA) and 1.0 mm glass beads in a closed tube for 30 seconds at 4800 rpm. To ensure complete dissociation of nucleoprotein complexes, the samples were incubated in the Tri Reagent for 5 minutes (RT). Afterwards, the samples were mixed with 200 µl chloroform and incubated for 10 minutes at RT. By a centrifugation step (12000g, 15 min, 4°C), an aqueous phase formed, that was transferred to a fresh eppendorf tube. Upon addition of 0.5 ml isopropanol and another centrifugation (12000g, 5 min, 4°C), the RNA precipitated. After a wash step (1.0 ml 70% ethanol), centrifugation (12000g, 5 min, 4°C) and removal of the supernatant, the pellet was dissolved in an appropriate volume DEPC sterile H_2O. All materials used throughout the procedure were RNAse free and the samples were placed on ice at all times. The RNA concentration and quality were determined on a Nanodrop ND-1000 spectrophotometer. When the RNA quality was too low, the samples were cleaned with the High Pure RNA isolation Kit (11828665001, Roche, Manheim, Germany) according to the manufacturer's guidelines.

2.5.2 Kupffer cells

Total RNA was isolated from both the negative and positive fraction of the Kupffer cell isolation (n=4 for both fractions) with the same protocol as described for the liver tissue, except the homogenizing step.

2.6 Quantitative PCR

2.6.1 Liver tissue

The isolated RNA from each individual mouse (500ng) was converted into first-strand complementary DNA (cDNA) with the iScript cDNA synthesis kit (170-8891**,** Bio-Rad, Hercules, CA, USA). To determine changes in gene expression, a quantitative polymerase chain reaction (qPCR) of 10ng cDNA was performed either on a Bio-Rad MyIQ™ with the Bio-Rad iQ™5 v2 software (Bio-Rad, Hercules, CA, USA) using the IQ™ Sensimix SYBR with fluorescein (QT615-05, Bioline, Hercules, CA, USA) or on the Applied Biosystems 7900HT using the SensiMix™ SYBR (Quantace, London, UK). For each gene, a standard curve was generated using a serial dilution of a cDNA pool, and Cyclophilin A was used as reference gene in order to standardize for the amount of cDNA. Primer sets (Table 2.1) were developed with Primer Express version 2.0 (Applied Biosystems, Foster city, CA, USA) using default settings. Data generated with the qPCR were analyzed with the comparative Ct method.

2.6.2 Kupffer cells

RNA isolated from the negative and positive fraction of the Kupffer cell isolation (400ng) was first converted into cDNA, where after changes in gene expression were determined with qPCR as described above. To standardize for the amound of cDNA, *CD68* was used as reference gene. *CD68* is a transmembrane glyprotein that is highly expressed by monocytes and macrophages. Hence, using this gene allows to correct for the amount of KCs present in the samples. Primer set development (Table 2.1) and qPCR data analysis were performed as described above.

Table2.1. Primer sequences for qPCR

Gene	Forward primer	Reverse primer
Cyclophilin A	TTCCTCCTTTCACAGAATTATTCCA	CCGCCAGTGCCATTATGG
CD68	TGACCTGCTCTCTCTAAGGCTACA	TCACGGTTGCAAGAGAAACATG
ABCA1	CCCAGAGCAAAAAGCGACTC	GGTCATCATCACTTTGGTCCTTG
ABCG1	TCGGACGCTGTGCGTTTT	CCCACAAATGTCGCAACCT
CD36	GCCAAGCTATTGCGACATGA	AAAAGAATCTCAATGTCCGAGACTTT
MSR-1	CATACAGAAACACTGCATGTCAGAGT	TTCTGCTGATACTTTGTACACACGTT
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
ICAM	CTACCATCACCGTGTATTCGTTTC	CGGTGCTCCACCATCCA
MCP1	GCTGGAGAGCTACAAGAGGATCA	ACAGACCTCTCTCTTGAGCTTGGT

2.6 Liver histology

7-µm thick frozen liver sections were fixed in acetone and stained with a marker for infiltrated macrophages and neutrophils (Mac-1; 1:100), CD68 Kupffer cells (FA11; 1:100) and T cells (KT3; 1:5). After fixation, the slides were blocked with 30% H_2O_2 (0.1%, 5 min) to minimize background coloring. Prior to incubation with the 1st antibody, slides were incubated with 4% fetal calf serum (FCS)/1x PBS + 1:5 Avidin D Block solution (amplification, Red ABC kit, Vector Laboratories, USA). The primary antibodies were diluted in 4% FCS/1x PBS + 1:5 Biotin Block solution (amplification, Red ABC kit, Vector Laboratories, USA). After incubation with the first antibody (60 minutes), the slides were incubated another 60 minutes with the secondary antibody solution containing 4%FCS/2% normal mouse serum (NMS)/1:300 α-Rat-Bio/1x PBS. Then, the slides were incubated in a 1x PBS solution with 1:50 Avidin D solution + 1:50 Biotin solution (amplification, Green ABC kit, Vector Laboratories, USA), followed by a staining with 13-Amino-9-ethylcarbozale (AEC) as color substrate using the AEC kit (2% buffer, 3% AEC and 2% H_2O_2 in demi water). Nuclear counterstaining was done by incubation with Haematoxylin. Slides were enclosed with Faramount aqueous mounting medium.

To stain the frozen liver sections with a marker for neutrophils (NIMP; 1:100), the slides were manipulated according to an analogous procedure without amplification steps and using α-Rat-PO (1:100) as secondary antibody.

Pictures of all frozen liver sections were taken with a Nikon DMX1200 digital camera and the ACT-1 v2.603 software from Nikon Corporation. Immune cells were counted in six microscopical views with a 200x magnification and are marked as cells/mm². For the CD68 staining, pictures of each microscopical view (6 pictures, 200x magnification) were analyzed with Adope Photoshop CS2 v.9.0. Liver sections were quantified by percentage of CD68 positive area.

2.7 Electron microscopy

Electron microscopy was performed to explore the intracellular cholesterol distribution in Kupffer cells. Freshly isolated livers were perfused and fixed by syringe injections with 2.5% glutaraldehyde and postfixed in 2% gluraraldehyde/0.1 M cacodylate buffer (10min). Afterwards, the liver biopsies were kept in 0.1 M cacodylate buffer/7.5% sucrose at 4°C until further processing. To stain the lysosomes of the Kupffer cells, acid phosphatase chemistry was performed. The samples were frozen for 1 hour at -30°C and then cut into 50-µm-thick cryosections. These sections were incubated according to the cerium-based method of Robinson and Karnovsky for the localization of acid phosphatase[49]. After incubation, the sections were washed two times in 0.1 M cacodylatebuffer/5% sucrose and refixed in 3% glutaraldehyde/cacodylate buffer for 1 hour. Subsequently, the slices were rinsed overnight (4°C) in veronal acetate buffer (pH 7.4) and postfixed for 30 min in 2% osmium tetroxide/veronal buffer/4% sucrose. After dehydration in 70%-100% ethanol, the liver sections were embedded in epoxy resin and cut for electron microscopy. Electron microscopy pictures were taken with a Philips CM100 TEM. The KCs were scored from 0 to 6 for their lysosomal cholesterol content, with a score of 0 indicating no lysosomal cholesterol and a score of 6 indicating extreme cholesterol accumulation inside the lysosomes. The same scoring index was used for cytoplasmic cholesterol accumulation and cholesterols crystals, with a score of 0 indicating no cytoplasmic cholesterol or crystals and score of 6 indicating excessive cytoplasmic cholesterol or crystals.

2.8 Cell culture

Bone-marrow cells were isolated from the femurs and tibiae of C57/Bl6 mice and cultured in RPMI-1640 medium (GIBCO Invitrogen, Breda, the Netherlands), supplemented with 10% heat-inactivated fetal calf serum (Bodinco B.V., Alkmaar, the Netherlands), 100U/ml penicillin, 100 µg/ml streptomycin, 2mM L-glutamine (All GIBCO Invitrogen, Breda, the Netherlands), and 20% L929-conditioned medium (LCM), for 8 days to generate bone marrow-derived macrophages as described previously[50] Cells were treated for 24h with 25µg/ml oxLDL (Intracel, Frederick, USA), incubated with or without 2µM 2HP-βCD for 4h and then stimulated with lipopolysaccharide (LPS) for an additional 4h. Then the cells were collected, where after RNA was isolated and gene expression analysis was performed as described above.

2.9 Statistical analysis

Data were analyzed by two-tailed, unpaired, t tests using GraphPad Prism, version 5.0 for Windows for comparing both treatment groups with the control injected group. Data are represented as mean ± standard error of mean (SEM) and considered significant at $P < 0.05$ (* $P < 0.05$; ** $P<0.01$ and *** $P<0.001$, respectively).

3. Results

Decreased lysosomal cholesterol accumulation in 2HP-βCD-injected mice compared to PBS-injected mice on HFC diet

Electron microscopy revealed that administration of 2HP-βCD to *Ldlr*$^{-/-}$ mice fed an HFC diet resulted in a strong decrease in the level of lysosomal cholesterol inside KCs, which was not observed in the PBS-injected mice on HFC diet. Correspondingly, KCs of 2HP-βCD-injected mice accumulated more cytoplasmic cholesterol, compared to PBS-injected mice on HFC diet (Figure 3.1). Thus, these data confirm that our treatment with 2HP-βCD was effective.

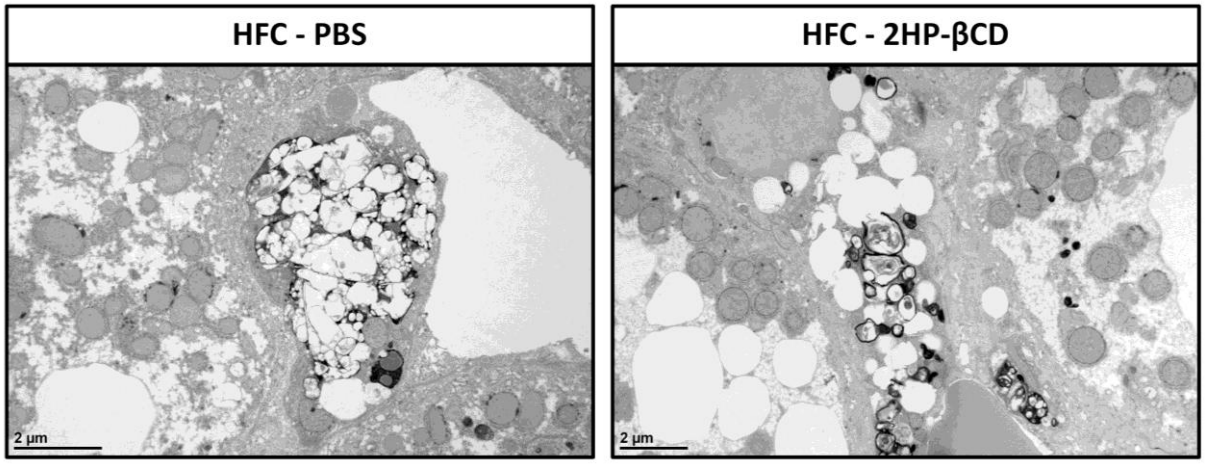


Figure 3.1 Intracellular cholesterol distribution. A) Representative electron microscopy pictures of KCs from PBS- and 2HP-βCD-injected *Ldlr*$^{-/-}$ mice on a HFC diet. Lysosomes are indicated in black by acid phosphatase staining.

Reduced relative weight gain in 2HP-βCD-injected mice compared to PBS-injected mice on HFC diet

To determine the effect of 2HP-βCD on the global health condition, total body weight and relative weight gain, as well as liver and spleen weights were measured. Total body weight was not different between the three groups (Figure 3.2A). However, PBS-injected mice on an HFC diet had a higher percentage of relative weight gain compared to mice fed a regular chow. Treatment with 2HP-βCD, on the other hand, resulted in a significantly reduced relative weight gain compared to PBS-injected mice on an HFC diet (Figure 3.2B). Both the liver and spleen weight were calculated relative to the total body weight. After a 12-week HFC diet, the liver and spleen weight were both significantly increased. Treatment with 2HP-βCD had no effect on the relative liver weight, nor on the relative spleen weight (Figure 3.2C-D). Thus, these data demonstrate that mice on HFC diet have a reduced relative weight gain after treatment with 2HP-βCD

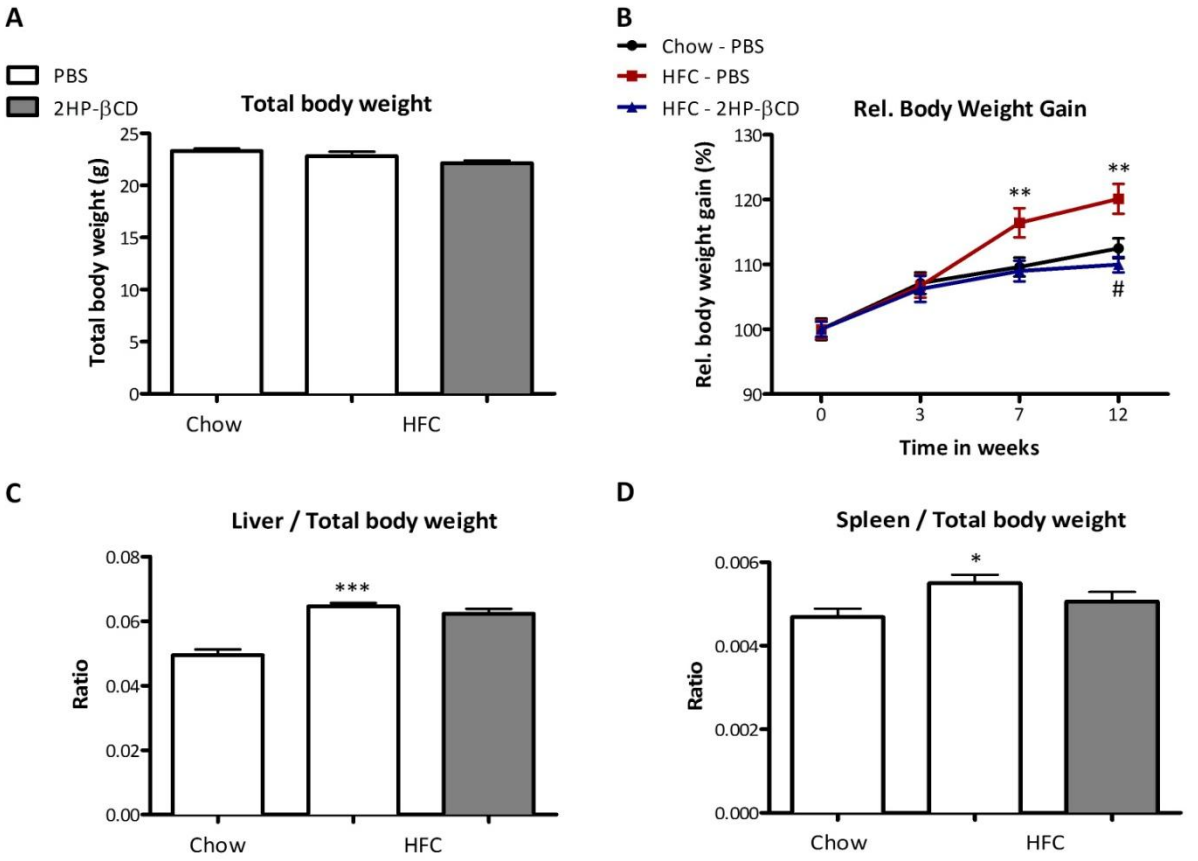


Figure 3.2 Total body weight, relative weight gain, liver and spleen weight. A) Total body weight, **B)** relative weight gain, **C)** liver weight and **D)** spleen weight of *Ldlr*$^{-/-}$ mice on regular chow, PBS-injected and 2HP-βCD-injected mice on HFC diet. Relative weight gain data are shown relative to *Ldlr*$^{-/-}$ mice on chow. Asterisks indicate significant difference from *Ldlr*$^{-/-}$ mice on chow. *Indicates $P < 0.05$ and ** $P < 0.01$. # Indicates $P < 0.001$ compared to control mice on HFC diet.

No difference in lipid levels between 2HP-βCD–injected mice and PBS-injected mice on HFC diet

To identify the effect of 2HP-βCD on liver lipid levels in hyperlipidemic mice, biochemical measurements of liver cholesterol, triglycerides (TG) and free fatty acids (FFA) were carried out (Figure 3.3A). After a 12-week HFC diet, all liver lipid levels were significantly increased compared to chow-fed mice. Liver lipid levels did not differ between 2HP-βCD-injected and PBS-injected mice on a HFC diet. In addition, the effect of 2HP-βCD on plasma cholesterol was established by measuring the level of cholesterol in the plasma (Figure3.3B). PBS-injected mice on the HFC diet had significantly higher plasma cholesterol levels compared to mice on a regular chow. Plasma cholesterol in *Ldlr*$^{-/-}$ mice fed a HFC diet was not altered by injections with 2HP-βCD. All together, these data show that 2HP-βCD injections do not affect plasma cholesterol, nor the severity of steatosis.

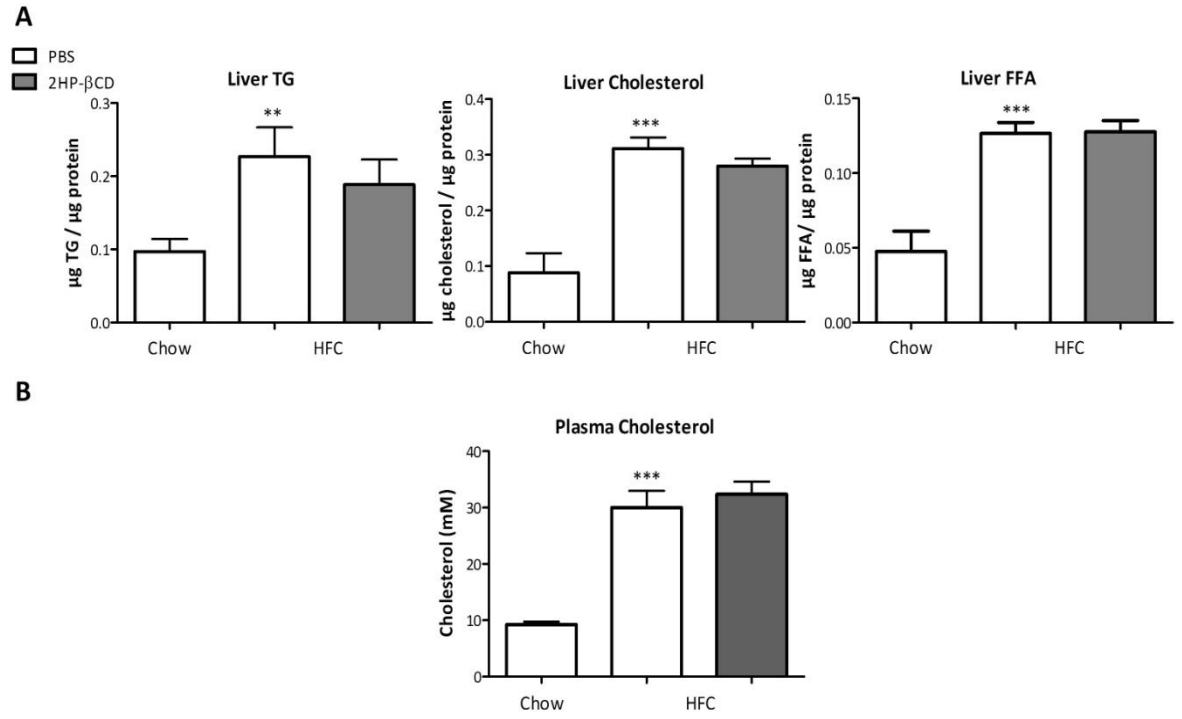
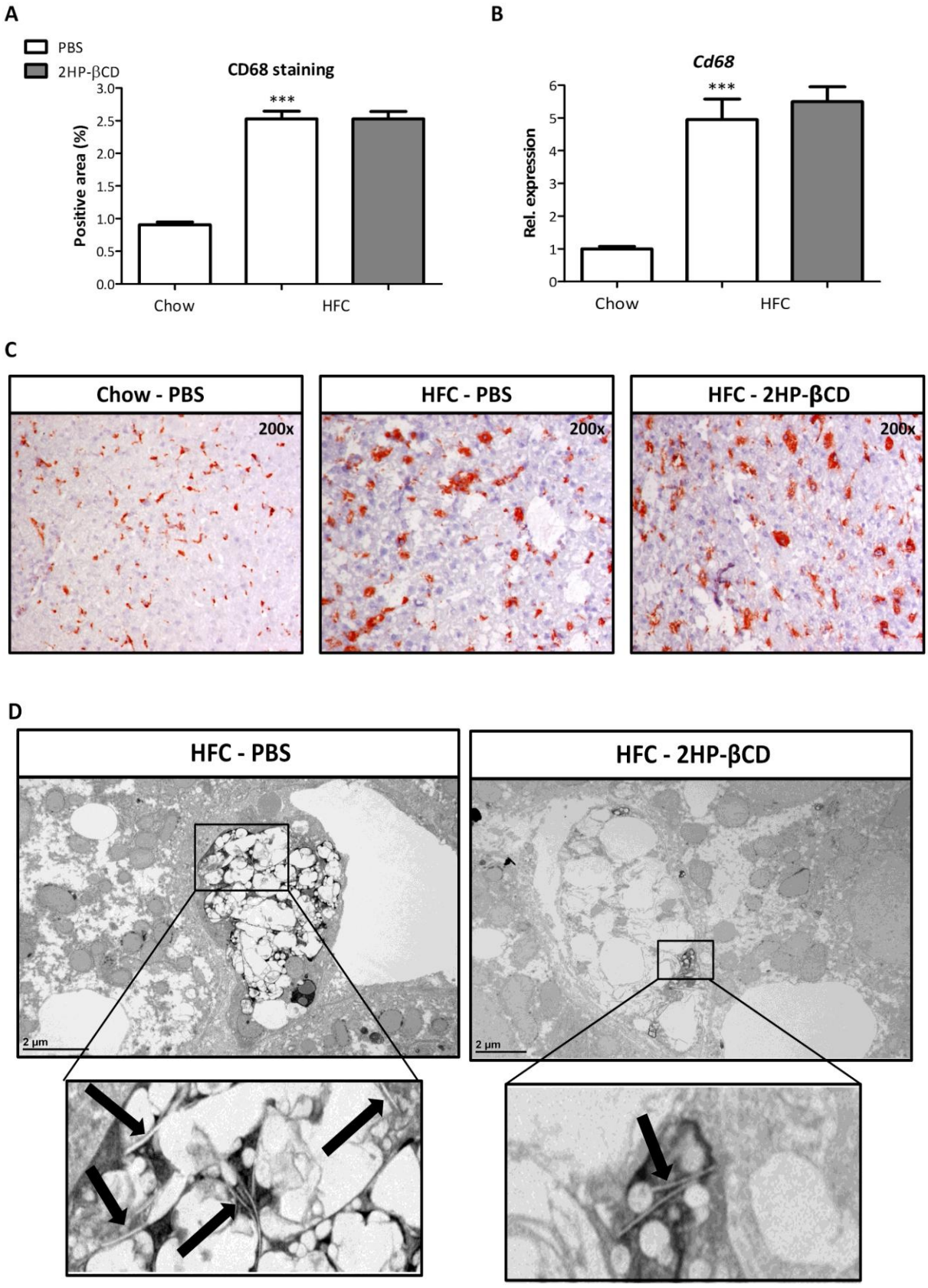


Figure 3.3 Lipid levels of *Ldlr*$^{-/-}$ mice on a regular chow and of PBS-and 2HP-βCD-injected mice on a HFC diet. A) Liver cholesterol, FFA and TG. Data are shown as the ratio to the total amount of proteins in the liver. **B)** Plasma cholesterol levels. Data are shown relative to *Ldlr*$^{-/-}$ mice on chow. Asterisks indicate significant difference from *Ldlr*$^{-/-}$ mice on chow. ** Indicates $P < 0.01$ and ** $P < 0.001$.

Shift in intracellular cholesterol distribution after 2HP-βCD injections does not alter foamy KC appearance

Immunohistochemistry for CD68, a macrophage marker, was performed to examine the size of the KCs. The percentage of the CD68 positive area in the liver was increased in PBS-injected mice on HFC diet, compared to chow-fed mice (Figure 3.4A). In line with these date, gene expression of *CD68* was significantly higher in control mice on HFC diet compared to chow-fed mice (Figure 3.4B). Representative histological pictures of the CD68 staining confirmed that livers of HFC-fed mice harbor larger foamy KCs compared to mice on chow (Figure 3.4C). On the other hand, no difference in CD68 positivity, nor in the level of *CD68* gene expression was observed in HFC-fed mice after treatment with 2HP-βCD. Electron microscopy confirmed that the size of the KCs did not differ between 2HP-βCD-injected mice and PBS-injected mice on HFC diet (Figure 3.4D). However, there was a shift in the intracellular cholesterol distribution. While KCs of PBS-injected mice accumulated excessive amounts of lysosomal fat after a 12-week HFC diet, KCs of 2HP-βCD-injected mice stored more fat inside the cytoplasm. Accordingly, less cholesterol crystals were found in the KCs of 2HP-βCD-injected mice compared to control mice (Figure 3.4E), as demonstrated by representative electron microscopy pictures (Figure 3.4D). Thus, the shift in intracellular cholesterol distribution after treatment with 2HP-βCD is accompanied by reduced cholesterol crystallization, but not with reduced foamy KC appearance.


A
PBS
2HP-βCD
CD68 staining
***
Positive area (%)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Chow
HFC
B
Cd68
***
Rel. expression
6
5
4
3
2
1
0
Chow
HFC
C
Chow - PBS
200x
HFC - PBS
200x
HFC - 2HP-βCD
200x
D
HFC - PBS
2 µm
HFC - 2HP-βCD
2 µm

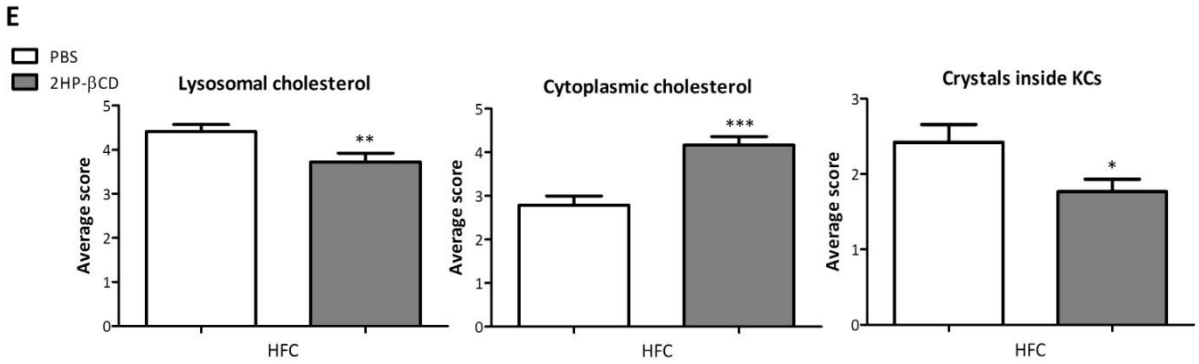


Figure 3.4 Foamy KC appearance. A) Liver sections were stained for CD68 and quantified for the percentage CD68 positive area. **B)** Gene expression analysis of *CD68*. Data are shown relative to *Ldlr*$^{-/-}$ mice on chow. Asterisks indicate significant difference from *Ldlr*$^{-/-}$ mice on chow. *** Indicates P < 0.001. **C)** Representative histological images of CD68 stained liver sections of *Ldlr*$^{-/-}$ mice on chow, PBS-injected and 2HP-βCD-injected mice on HFC diet. **D)** Representative electron microscopy pictures of foamy KCs. Acid phosphatase staining indicates the lysosomes of KCs. Cholesterol crystals are indicated by black arrows. **E)** Scoring of lysosomal and cytoplasmic cholesterol in PBS-injected and 2HP-βCD-injected mice on HFC diet, with a score of 0 indicating no lysosomal or cytoplasmic cholesterol, and a score of 6 indicating extreme cholesterol accumulation in either the lysosomes or the cytoplasm of KCs. The same scoring index was used for the amount of cholesterol crystals. ** indicates P < 0.01 and *** P < 0.001

To explore whether the shift in intracellular cholesterol distribution can certainly be attributed to the administration of 2HP-βCD, differences in cholesterol uptake and in cholesterol efflux were investigated. Differences in cholesterol uptake were investigated by means of gene expression of *Msr1* and *CD36*, two scavenger receptors which are important for the uptake of modified cholesterol. Livers of PBS-injected mice on HFC diet expressed higher levels of *Msr1* and *CD36*, although not significant for *CD36*, compared to chow-fed mice (Figure 3.5A). Furthermore, the expression of *CD36* was significantly increased in the KCs from PBS-injected mice on HFC diet compared to mice on chow (Figure 3.5B). Thus, upon HFC feeding, the uptake of modified cholesterol is increased. Analogue to the observation that there was no difference in the size of foamy KCs between 2HP-βCD-injected mice and PBS-injected mice, *Msr1* and *CD36* expression in the liver and *CD36* expression in KCs, was not changed by 2HP-βCD-injections (Figure 3.5A-B).

To examine cholesterol efflux, gene expression of the efflux transporters, *Abca1* and *Abcg1,* was measured in the liver and in the KCs. HFC feeding induced, a 2-fold and a 4-fold increase in the hepatic expression of *Abca1* and *Abcg1*, respectively (Figure 3.5A). KCs of mice on the HFC diet also expressed significantly higher levels of both genes (Figure 3.5B). Administration of 2HP-βCD to *Ldlr*$^{-/-}$ mice fed a HFC diet had no influence on the total liver or KC-specific expression of both cholesterol transporters. Taken together, these data demonstrate that there is no difference in the uptake or efflux of modified cholesterol between PBS- and 2HP-βCD-injected mice on HFC diet.

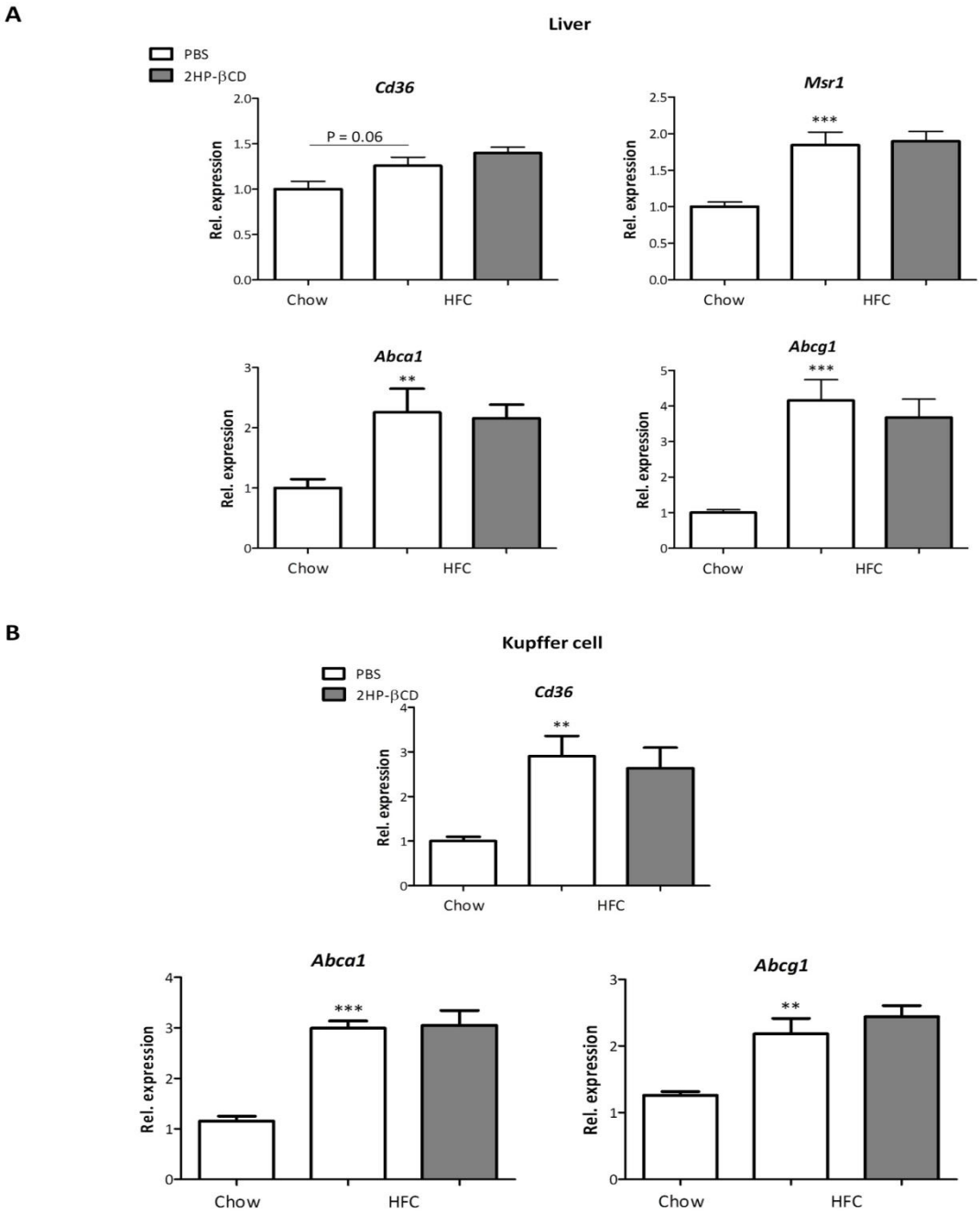


Figure 3.5 Gene expression analysis of genes involved in cholesterol uptake and efflux. A) Total liver expression of *CD36* and *Msr1*, two genes important in cholesterol uptake, and of *Abca1* and *Abcg1*, two genes involved in cholesterol efflux of *Ldlr*$^{-/-}$ mice on regular chow, PBS- and 2HP-βCD-injected mice on HFC diet. **B)** KC-specific expression of *CD36, Abca1* and *Abcg1.* Data are shown relative to *Ldlr*$^{-/-}$ mice on chow. Asterisks indicate significant difference from *Ldlr*$^{-/-}$ mice on chow. ** Indicates P < 0.01 and *** P < 0.001.

No difference in inflammation between 2HP-βCD-injected and PBS-injected mice on an HFC diet

To determine whether treatment with 2HP-βCD affects the inflammatory response in *Ldlr*$^{-/-}$ mice, liver sections were stained for the inflammatory markers: Mac-1 (infiltrated macrophages and neutrophils), NIMP (neutrophils) and CD3 (T cells). The number of infiltrated macrophages and neutrophils, but not T cells, was significantly increased after a 12-week HFC diet, compared to mice

on regular chow. Administration of 2HP-βCD had no effect on the number of immune cells in the liver of mice fed a HFC diet (Figure 3.6A). In Figure 3.6B, representative histological pictures of the Mac-1 staining for all three groups are shown.

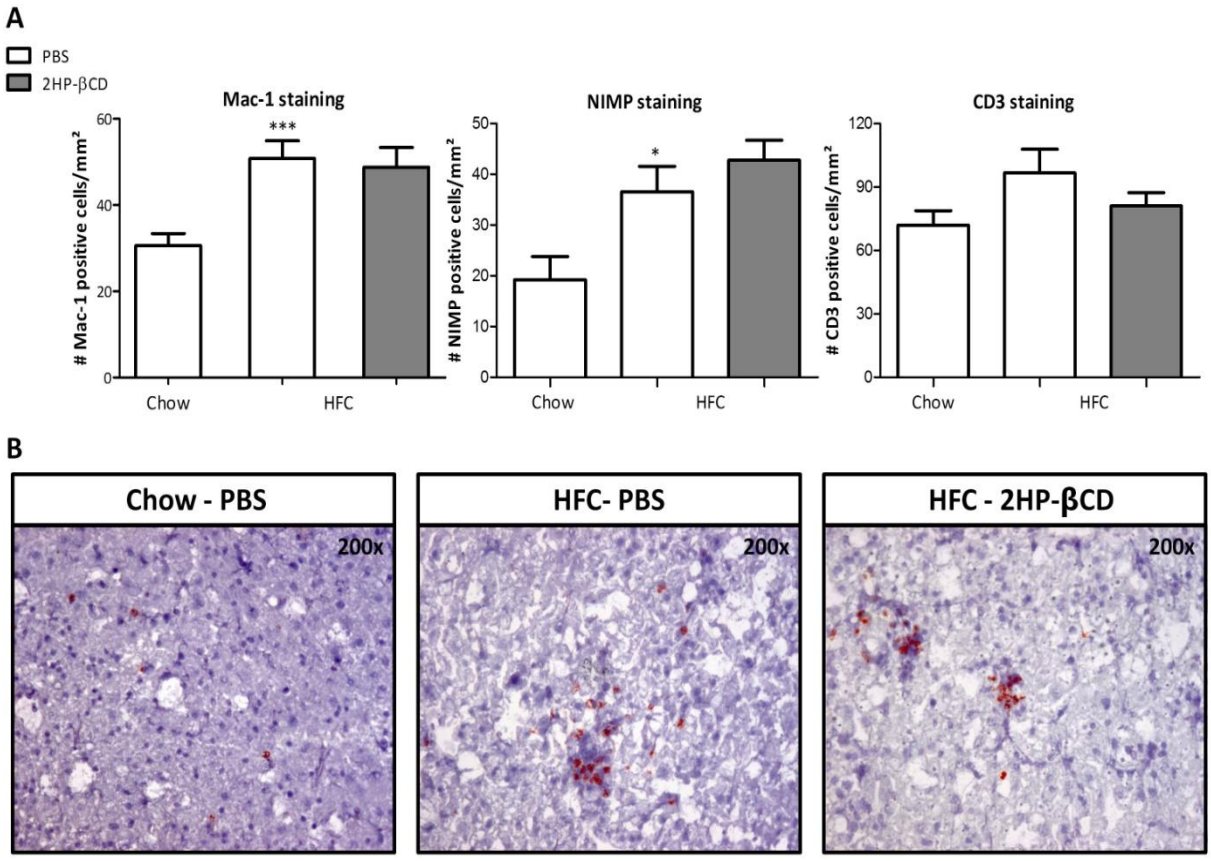


Figure 3.6 Livers sections were stained for inflammatory markers. A) Liver sections were stained for infiltrated macrophages (Mac-1), neutrophils (NIMP) and T cells (CD3), and positive stained immune cells were counted. **B)** Representative histological pictures of the Mac-1 staining for *Ldlr*$^{-/-}$ mice on chow, PBS-injected and 2HP-βCD-injected mice on HFC diet, respectively, at a 200x magnification. Asterisks indicate significant difference from *Ldlr*$^{-/-}$ mice on chow. * Indicates $P < 0.05$ and ***Indicates $P < 0.001$.

Gene expression analysis of various markers, which are known to be involved in the hepatic inflammatory process, confirmed our histological observations. The expression of tumor necrosis factor (*Tnf*), monocyte chemoattractant protein 1 (*Mcp1*) and intercellular adhesion molecule (*Icam*) was significantly higher in the livers of PBS-injected mice on HFC diet compared to mice on chow. Further, the livers of mice that were injected with 2HP-βCD expressed similar mRNA levels of these inflammatory markers as PBS-injected, HFC-fed mice (Figure 3.7A).

To further investigate the inflammatory response, the contribution of the KCs to the inflammatory response was examined. First, it was confirmed that the positive fraction of the KC isolation effectively contained KCs. As shown in Figure 3.7B, the positive fraction contained approximately 2 to 3 fold more KCs compared to the negative fraction, wherein still some KCs are found, in addition to

other immune cells that are not *F4/80* positive. Similar to what was observed in the liver, gene expression of both *Tnf* and *Icam* was significantly higher in KCs of *Ldlr*$^{-/-}$ mice after HFC feeding, whereas injections with 2HP-βCD did not alter the expression of *Tnf* and *Icam* in mice fed a HFC diet (Figure 3.7C). Thus, these data demonstrate that 2HP-βCD-injections did not reduce the hepatic inflammatory response induced by HFC diet.

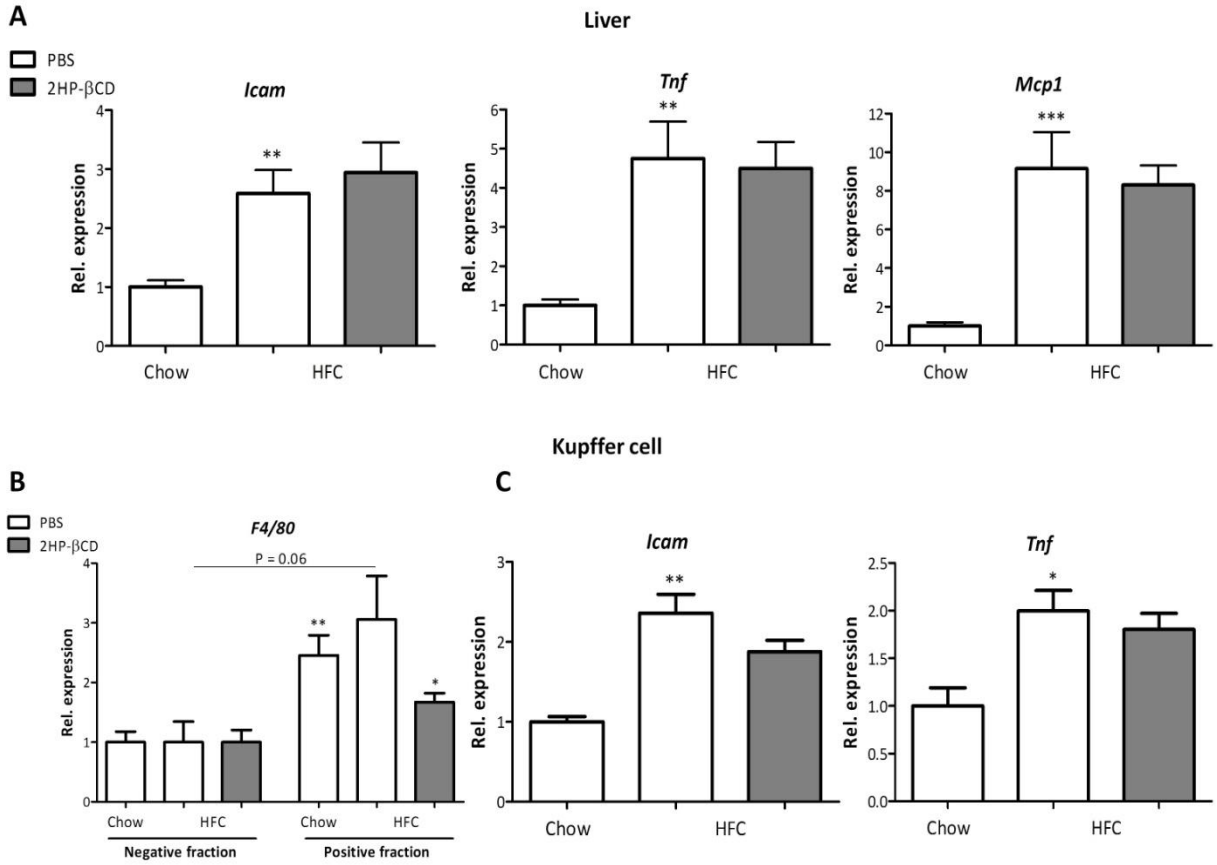


Figure 3.7 Gene expression of inflammatory markers from *Ldlr*$^{-/-}$ mice on chow, PBS-injected mice and 2HP-βCD-injected mice on HFC diet. A) Gene expression of *Icam*, *Tnf* and *Mcp1* in total liver. **B)** Gene expression of F4/80 in the negative (not F4/80 positive cells) and positive fraction (F4/80 positive cells) of the KC isolation. *F4/80* data of the positive fraction are shown relative to the same group in the negative fraction. Asterisks indicate significantly different from the same group in the negative fraction. **C)** Gene expression of *Icam* and *Tnf* in the isolated KCs. Gene expression data are shown relative to *Ldlr*$^{-/-}$ mice on chow. * Indicates $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

2HP-βCD induces inflammation in bone-marrow derived macrophages

To confirm our *in vivo* data, the effects of 2HP-βCD were also investigated in macrophage foam cells *in vitro*. To induce foam cells, bone-marrow derived macrophages (BMDM) were incubated with oxLDL and LPS. A subsequent 4h treatment with 2HP-βCD led to an increase in the expression of the pro-inflammatory marker *Tnf*, and a decrease in the expression of the anti-inflammatory marker *IL-10*.

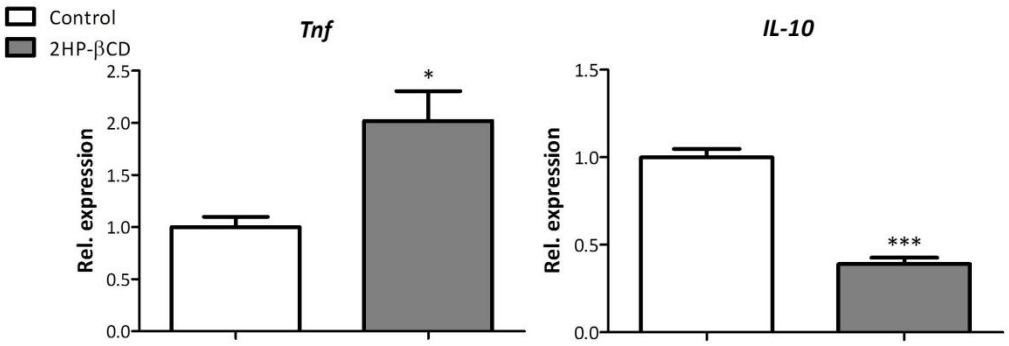


Figure 3.8 Gene expression of Tnf and IL-10 in BMDM. BMDM were incubated with oxLDL (25µg/ml;24h) and LPS (4h) to stimulate foam cell formation. The cells were then treated with 2HP-βCD (2µM; 4h), and collected for gene expression analysis. Data are shown relative to control BMDM. * indicates $P < 0.05$ and *** $P < 0.001$.

4. Discussion

Due to the fact that NASH is becoming a major health burden, many studies have been carried out to unravel the mechanisms that trigger the development of hepatic inflammation. In the present study we show for the first time that 2HP-βCD, a compound that redirects cholesterol from the lysosomes to the cytoplasm, cannot be used as treatment for NASH. This is probably attributed to the fact that it is the lysosomal accumulation of oxidized cholesterol rather than normal cholesterol, that triggers the inflammatory response. In addition, we demonstrate that the size of the KCs is not altered with the shift in intracellular cholesterol distribution and that foam cells are not directly correlated with the inflammatory state. Finally, we reveal the pro-inflammatory effects of 2HP-βCD *in vitro*, indicating that one should consider pro-inflammatory effects of 2HP-βCD before/when using this compound in the treatment of metabolic and inflammatory diseases.

2HP-βCD redirects cholesterol from the lysosome to the cytoplasm, without reducing foamy KC appearance

It has been almost 20 years since the first article was published that 2HP-βCD is able to manipulate intracellular cholesterol content. While low concentrations of 2HP-βCD enhance bidirectional flux of cholesterol, without changing cholesterol homeostasis[37], high concentrations efficiently remove cholesterol from macrophage foam cells *in vitro*[37, 42]. Nevertheless, it was only recently published that the lysosomal cholesterol storage in mutant *NPC1*$^{-/-}$ mice was significantly reduced after administration of 2HP-βCD[34, 45]. This is attributed to the fact that 2HP-βCD is internalized in the lysosome where it facilitates movement of cholesterol into the metabolically active pool in the cytoplasm[20]. In line with these observations, we here demonstrate that 2HP-βCD can redirect lysosomal cholesterol to the cytoplasm, with a resultant decrease in lysosomal cholesterol storage and an increase in cholesterol accumulation in the cytoplasm of KCs. However, whereas this leads to a normalization of the cholesterol metabolism in almost every organ in *NPC1*$^{-/-}$ mice[34, 45], the cholesterol metabolism inside KCs during NASH is not improved. In line with these observations, foamy KC appearance was not reduced in NASH after treatment with 2HP-βCD, which is in contrast to observations in *NPC1*$^{-/-}$ mice[34, 45]. In fact, genes involved in the regulation of cholesterol homeostasis, i.e. liver X receptor (LXR) and its downstream targets, such as *Abca1* and *Cyp7a1*, were activated in mutant NPC mice after treatment with 2HP-βCD, while in NASH, gene expression was unaltered. However, these differences can be attributed to the fact that the NPC*1*$^{-/-}$ mice are much younger compared to the *Ldlr*$^{-/-}$ mice used in the present study (49 days and 168 days, respectively). Since difference in age already lead to differences in basal cholesterol metabolism, it is likely that the effects of 2HP-βCD are less pronounced in older compared to younger mice[51].

Furthermore, with increasing age, 2HP-βCD is cleared much faster from the bloodstream[43], whereby less 2HP-βCD will reach the site of action and the response will be less effective. Indeed, our data are in line with the observation that foamy KC appearance in 160 day old mutant NPC mice is not reduced[45]. The fact that 2HP-βCD is cleared much faster from the bloodstream in older mice, could have been circumvented by injecting 2HP-βCD two-daily instead of weekly. However, injecting more frequently would have given rise to more foamy macrophages in the liver[40], which in turn would have led to a disrupted, more inflammatory phenotype.

Taken all together, these data provide evidence that 2HP-βCD is able to redirect cholesterol from the lysosome to the cytoplasm, without reducing foamy KC appearance.

2HP-βCD does not affect lipid levels but reduces relative weight gain

Even though earlier studies have shown that only long term treatment or treatment with a high dose of βCD, but not 2HP-βCD, led to a reduction in body weight gain[52], we here show for the first time that treatment of *Ldlr*$^{-/-}$ mice with 2HP-βCD leads to a decrease in body weight gain. A possible explanation for this finding could be that complexation of 2HP-βCD with cholesterol reduces cholesterol absorption[53] and increases the excretion of cholesterol from the liver as bile acid[43, 53]. However, this is very unlikely since treatment with 2HP-βCD does not reduce plasma cholesterol, liver cholesterol or liver expression of *Abca1* en *Abcg1*. A second possibility could be the presence of increased cellular damage or inflammation. This is most likely not the case as the relative spleen weight, the first indication of increased cellular damage, is not elevated after 2HP-βCD treatment. Finally, this decrease could be explained by decreased food intake or reduced energy expenditure after treatment with 2HP-βCD[52]. However, as the mice were not in metabolic cages, this cannot be said with certainty.

Thus, from the data described above, we cannot draw a clear conclusion. Further research is needed, e.g. determine bile acid composition and intestinal analysis, to explore what caused the reduction in body weight gain.

2HP-βCD administration does not reduce hepatic inflammation

Several lines of evidence have indicated that lysosomal cholesterol accumulation and inflammation are closely related. Our previous studies have shown a clear link between lysosomal cholesterol accumulation in KCs and hepatic inflammation. In addition, others have found this association in both atherosclerosis[32] and NPC disease[34]; where cholesterol trapping inside lysosomes, due to a defective cholesterol efflux from the lysosome to the cytoplasm, is associated with increased inflammation. In fact, we have demonstrated that prevention of the uptake of modified LDL particles

(i.e. oxLDL) by deletion of *CD36* and *Msr1*[19, 21], the two main scavenger receptors, or by immunization with heat-inactivated pneumoccoci[29], was associated with a significant reduction in lysosomal cholesterol accumulation and hepatic inflammation. Relevantly, recent studies have revealed that treatment of *NPC1*$^{-/-}$ mice with 2HP-βCD dramatically reduced lysosomal cholesterol storage and the concomitant inflammatory response[34, 45]. Due to its ability to reduce lysosomal cholesterol accumulation and inflammation, 2HP-βCD seems to be a very promising strategy to treat NASH.

In contradiction to our hypothesis, we here show that 2HP-βCD is not able to reduce the inflammatory response during NASH, despite its efficacy to redirect lysosomal cholesterol to the cytoplasm and to reduce cholesterol crystallization. Moreover, this observation is in contrast to our previous findings where a reduction in lysosomal cholesterol storage was associated with decreased cholesterol crystal formation and inflammation[54]. A possible explanation for the different effect on inflammation could be that administration of 2HP-βCD to *NPC1*$^{-/-}$ mice leads to a decrease in total cholesterol levels, whereas total cholesterol level and the expression of *CD36* and *Msr1* is not reduced in *Ldlr*$^{-/-}$ mice on HFC diet after treatment with 2HP-βCD. As such, in the presence or absence of 2HP-βCD, a similar amount of cholesterol in the plasma will become oxidized and then trapped inside lysosomes. Oxidized cholesterol has been shown to be able to immediately damage the lysosomal membrane, thereby leading to inflammation[55] however, the direct link between non-oxidized cholesterol and inflammation is not yet clear. Our data therefore clearly suggest that disturbances in cholesterol metabolism and trafficking are not the main trigger for inflammation, but rather the actual oxidations that occurs when lipids levels are high.

Therapeutic potential of 2HP-βCD in metabolic and inflammatory diseases

The hydrophobic cavity of the three-dimensional structure of the 2HP-βCD molecule allows not only the encapsulation of sterols but also of various (anti-inflammatory) drugs[36-38]. Consequently, 2HP-βCD is being used extensively in various pharmaceutical applications to improve bioavailability of the drugs[39]. Various approved and market drugs in Europe and in the United States contain 2HP-βCD. For instance, Mitozytrex, composed of mitomycin and 2HP-βCD, is an anti-tumor drug for the treatment of gastro-intestinal cancers and breast cancer. Another example is Sporanox, itraconazole mixed with 2HP-βCD, a drug used in the treatment of patients with fungal and systemic infections[39]. In addition, 2HP-βCD is now being used in clinical trials as therapy for NPC disease and has been shown to ameliorate the inflammatory state[48]. However, in this study we demonstrate for the first time that 2HP-βCD is not able to reduce the inflammatory response during NASH. Further, we demonstrate that 2HP-βCD even has pro-inflammatory effects *in vitro.* Thus, this study suggests that the application of 2HP-βCD in pharmaceuticals should be re-evaluated and that more *in vitro* and *in*

vivo models of investigations as well as clinical investigations are required to improve and expand our current knowledge of the diverse effects of 2HP-βCD.

In conclusion, our results clearly demonstrate that 2HP-βCD cannot be used as a treatment for NASH. Moreover, despite its ability to transfer lysosomal cholesterol to the cytoplasm, 2HP-βCD cannot reduce foamy KC appearance nor the inflammatory response. Hence, our data provide evidence that inflammation during NASH is not always associated with lysosomal cholesterol accumulation, nor with foam cell formation. Furthermore, our data suggest that it is not the amount of cholesterol inside lysosomes of KCs, but rather the type of cholesterol, i.e. oxidized cholesterol, which triggers the inflammatory response in NASH. In addition, we reveal pro-inflammatory effects of 2HP-βCD *in vitro* and suggest that one should consider these effects and thus re-evaluate the use of 2HP-βCD as drug or drug carrier.

5. Conclusion and synthesis

Non-alcoholic steatohepatitis (NASH) is a condition characterized by liver lipid accumulation (steatosis) combined with inflammation. While steatosis is relatively benign and reversible, inflammation leads to irreversible liver damage and eventually, hepatocellular carcinoma and liver failure. Though, it is currently still unclear what mechanisms trigger the development of inflammation.

In the current study we showed that lysosomal cholesterol accumulation is not consistently linked with hepatic inflammation. Further, we established that 2HP-βCD has no potential as treatment for NASH. Even though lysosomal cholesterol storage is reduced upon treatment with 2HP-βCD, this effect was not accompanied by a concomitant decrease in hepatic inflammation. Previous studies from our group and others in combination with the data in this study suggest that 2HP-βCD is only able to redirect lysosomal, non-oxidized cholesterol to the cytoplasm, whereby the oxidized fraction remains in the lysosome. Taken together, these data propose that not the amount, but the type of cholesterol inside lysosomes is important in the development of inflammation.

Moreover, we revealed that although 2HP-βCD could not affect inflammation during NASH, it induces pro-inflammatory response in macrophages *in vitro*. Since 2HP-βCD is being used extensively in the clinic either as drug or as drug carrier, our results point out that one should be careful with this compound in the treatment of metabolic and inflammatory diseases.

To further investigate why 2HP-βCD is not able to reduce hepatic inflammation during NASH, additional experiments should be performed. A staining for oxidized cholesterol can be used to explore which type of cholesterol accumulates inside lysosomes during hepatic inflammation. Further, the expression of various lysosomal enzymes: i.e. lysosomal acid lipase and cathepsin D, can be determined to examine the role of lysosomal damage during inflammation.

Additionally, the differential effects of 2HP-βCD need further attention in *in vitro*, *in vivo* and clinical investigations, with emphasis on possible mechanisms whereby 2HP-βCD exerts its diverse effects. In this way, it would expand our current knowledge, thereby creating opportunities for better treatment options for metabolic and inflammatory diseases, such as NASH.

6. Acknowledgements

The first paragraph is normally dedicated to thank the supervisor. However, I would like to thank Tom Houben first, because if it wasn't for him, I would have never met my supervisor: Dr. Ronit Shiri-Sverdlov. So, many thanks Tom for introducing me as a motivated student from Hasselt University.

Now, I would like to thank Dr. Ronit Shiri-Sverdlov for given me the opportunity to show that I was indeed the motivated student from Hasselt she was looking for. Further, I would like to thank her for her unconditional help: assigning me to a new project, comments, suggestions, advice…, at all times: day or night!

Next, I would like to thank Tim Hendrikx for teaching and helping me with the whole arsenal of new lab techniques. Besides, I want to thank him for helping me in the writing process: from proposal(s), to thesis, poster and presentations. At last, many thanks for showing me the rich world of cholesterol crystals and helping me in the transition towards my new project.

In that regard, I also want to thank Sofie Walenbergh, who took me under her wings so that I had a new project for my thesis. Many thanks for all the support, the help and the funny/tiring hours of scoring intracellular cholesterol in KCs.

Further, I would like to thank Mike Jeurissen for his advice, help and support and. I also want to thank Patrick van Gorp for all the help in the lab and for teaching me the 'A-Z of qPCR'.

Finally, many thanks to the other colleagues for their support, help and advice, and to all the other students for the numerous funny moments, help with 'Toshiba-computer-problems' and Muffin-moments!

6. Acknowledgements

7. References

1. Alberti KG, Zimmet P, Shaw J. The metabolic syndrome--a new worldwide definition. Lancet. 2005;366(9491):1059-62. Epub 2005/09/27.
2. Bruce KD, Hanson MA. The developmental origins, mechanisms, and implications of metabolic syndrome. The Journal of nutrition. 2010;140(3):648-52. Epub 2010/01/29.
3. Duvnjak L, Duvnjak M. The metabolic syndrome - an ongoing story. Journal of physiology and pharmacology : an official journal of the Polish Physiological Society. 2009;60 Suppl 7:19-24. Epub 2010/04/24.
4. Grundy SM. Metabolic syndrome pandemic. Arteriosclerosis, thrombosis, and vascular biology. 2008;28(4):629-36. Epub 2008/01/05.
5. Loria P, Lonardo A, Carulli L, Verrone AM, Ricchi M, Lombardini S, et al. Review article: the metabolic syndrome and non-alcoholic fatty liver disease. Alimentary pharmacology & therapeutics. 2005;22 Suppl 2:31-6. Epub 2005/10/18.
6. Farrell GC, Larter CZ. Nonalcoholic fatty liver disease: from steatosis to cirrhosis. Hepatology. 2006;43(2 Suppl 1):S99-S112. Epub 2006/02/01.
7. Marchesini G, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini R, et al. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. Hepatology. 2003;37(4):917-23. Epub 2003/04/02.
8. Kotronen A, Yki-Jarvinen H. Fatty liver: a novel component of the metabolic syndrome. Arteriosclerosis, thrombosis, and vascular biology. 2008;28(1):27-38. Epub 2007/08/11.
9. Paschos P, Paletas K. Non alcoholic fatty liver disease and metabolic syndrome. Hippokratia. 2009;13(1):9-19. Epub 2009/02/26.
10. Mishra A, Younossi ZM. Epidemiology and Natural History of Non-alcoholic Fatty Liver Disease. Journal of Clinical and Experimental Hepatology. 2012;2(2):135-44.
11. Bellentani S, Scaglioni F, Marino M, Bedogni G. Epidemiology of non-alcoholic fatty liver disease. Dig Dis. 2010;28(1):155-61. Epub 2010/05/13.
12. Wouters K, van Gorp PJ, Bieghs V, Gijbels MJ, Duimel H, Lutjohann D, et al. Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis. Hepatology. 2008;48(2):474-86. Epub 2008/07/31.
13. Armstrong MJ, Houlihan DD, Bentham L, Shaw JC, Cramb R, Olliff S, et al. Presence and severity of non-alcoholic fatty liver disease in a large prospective primary care cohort. Journal of hepatology. 2012;56(1):234-40. Epub 2011/06/28.
14. Trauner M, Arrese M, Wagner M. Fatty liver and lipotoxicity. Biochimica et biophysica acta. 2010;1801(3):299-310. Epub 2009/10/28.
15. Agrawal S, Duseja AK. Non-alcoholic Fatty Liver Disease: East Versus West. Journal of Clinical and Experimental Hepatology. 2012;2(2):122-34.
16. Cuchel M, Rader DJ. Macrophage reverse cholesterol transport: key to the regression of atherosclerosis? Circulation. 2006;113(21):2548-55. Epub 2006/06/01.
17. Tang C, Oram JF. The cell cholesterol exporter ABCA1 as a protector from cardiovascular disease and diabetes. Biochimica et biophysica acta. 2009;1791(7):563-72. Epub 2009/04/07.
18. Soccio RE, Breslow JL. Intracellular cholesterol transport. Arteriosclerosis, thrombosis, and vascular biology. 2004;24(7):1150-60. Epub 2004/05/08.
19. Bieghs V, Verheyen F, van Gorp PJ, Hendrikx T, Wouters K, Lutjohann D, et al. Internalization of modified lipids by CD36 and SR-A leads to hepatic inflammation and lysosomal cholesterol storage in Kupffer cells. PloS one. 2012;7(3):e34378. Epub 2012/04/04.
20. Vance JE, Peake KB. Function of the Niemann-Pick type C proteins and their bypass by cyclodextrin. Current opinion in lipidology. 2011;22(3):204-9. Epub 2011/03/18.
21. Bieghs V, Wouters K, van Gorp PJ, Gijbels MJ, de Winther MP, Binder CJ, et al. Role of scavenger receptor A and CD36 in diet-induced nonalcoholic steatohepatitis in hyperlipidemic mice. Gastroenterology. 2010;138(7):2477-86, 86 e1-3. Epub 2010/03/09.

7. References

22. Griffin EE, Ullery JC, Cox BE, Jerome WG. Aggregated LDL and lipid dispersions induce lysosomal cholesteryl ester accumulation in macrophage foam cells. Journal of lipid research. 2005;46(10):2052-60. Epub 2005/07/19.
23. Ghosh S. Macrophage cholesterol homeostasis and metabolic diseases: critical role of cholesteryl ester mobilization. Expert review of cardiovascular therapy. 2011;9(3):329-40. Epub 2011/03/29.
24. Kruth HS, Huang W, Ishii I, Zhang WY. Macrophage foam cell formation with native low density lipoprotein. The Journal of biological chemistry. 2002;277(37):34573-80. Epub 2002/07/16.
25. Bieghs V, Rensen PC, Hofker MH, Shiri-Sverdlov R. NASH and atherosclerosis are two aspects of a shared disease: central role for macrophages. Atherosclerosis. 2012;220(2):287-93. Epub 2011/09/21.
26. Jerome WG, Yancey PG. The role of microscopy in understanding atherosclerotic lysosomal lipid metabolism. Microscopy and microanalysis : the official journal of Microscopy Society of America, Microbeam Analysis Society, Microscopical Society of Canada. 2003;9(1):54-67. Epub 2003/02/25.
27. Maor I, Aviram M. Oxidized low density lipoprotein leads to macrophage accumulation of unesterified cholesterol as a result of lysosomal trapping of the lipoprotein hydrolyzed cholesteryl ester. Journal of lipid research. 1994;35(5):803-19. Epub 1994/05/01.
28. Yancey PG, Jerome WG. Lysosomal cholesterol derived from mildly oxidized low density lipoprotein is resistant to efflux. Journal of lipid research. 2001;42(3):317-27. Epub 2001/03/20.
29. Bieghs V, van Gorp PJ, Walenbergh SM, Gijbels MJ, Verheyen F, Buurman WA, et al. Specific immunization strategies against oxidized low-density lipoprotein: a novel way to reduce nonalcoholic steatohepatitis in mice. Hepatology. 2012;56(3):894-903. Epub 2012/02/16.
30. Walenbergh SM, Koek GH, Bieghs V, Shiri-Sverdlov R. Non-alcoholic steatohepatitis: The role of oxidized low-density lipoproteins. Journal of hepatology. 2013;58(4):801-10. Epub 2012/11/28.
31. Moore KJ, Freeman MW. Scavenger receptors in atherosclerosis: beyond lipid uptake. Arteriosclerosis, thrombosis, and vascular biology. 2006;26(8):1702-11. Epub 2006/05/27.
32. Itabe H, Takano T. Oxidized low density lipoprotein: the occurrence and metabolism in circulation and in foam cells. Journal of atherosclerosis and thrombosis. 2000;7(3):123-31. Epub 2001/08/02.
33. Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction: a marker of atherosclerotic risk. Arteriosclerosis, thrombosis, and vascular biology. 2003;23(2):168-75. Epub 2003/02/18.
34. Liu B, Turley SD, Burns DK, Miller AM, Repa JJ, Dietschy JM. Reversal of defective lysosomal transport in NPC disease ameliorates liver dysfunction and neurodegeneration in the npc1-/- mouse. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(7):2377-82. Epub 2009/01/28.
35. Davidson CD, Ali NF, Micsenyi MC, Stephney G, Renault S, Dobrenis K, et al. Chronic cyclodextrin treatment of murine Niemann-Pick C disease ameliorates neuronal cholesterol and glycosphingolipid storage and disease progression. PloS one. 2009;4(9):e6951. Epub 2009/09/15.
36. Lopez CA, de Vries AH, Marrink SJ. Molecular mechanism of cyclodextrin mediated cholesterol extraction. PLoS computational biology. 2011;7(3):e1002020. Epub 2011/04/02.
37. Yancey PG, Rodrigueza WV, Kilsdonk EP, Stoudt GW, Johnson WJ, Phillips MC, et al. Cellular cholesterol efflux mediated by cyclodextrins. Demonstration Of kinetic pools and mechanism of efflux. The Journal of biological chemistry. 1996;271(27):16026-34. Epub 1996/07/05.
38. Christian AE, Haynes MP, Phillips MC, Rothblat GH. Use of cyclodextrins for manipulating cellular cholesterol content. Journal of lipid research. 1997;38(11):2264-72. Epub 1997/12/10.

39. Davis ME, Brewster ME. Cyclodextrin-based pharmaceutics: past, present and future. Nature reviews Drug discovery. 2004;3(12):1023-35. Epub 2004/12/02.
40. Stella VJ, He Q. Cyclodextrins. Toxicologic pathology. 2008;36(1):30-42. Epub 2008/03/14.
41. Kritharides L, Kus M, Brown AJ, Jessup W, Dean RT. Hydroxypropyl-beta-cyclodextrin-mediated efflux of 7-ketocholesterol from macrophage foam cells. The Journal of biological chemistry. 1996;271(44):27450-5. Epub 1996/11/01.
42. Atger VM, de la Llera Moya M, Stoudt GW, Rodrigueza WV, Phillips MC, Rothblat GH. Cyclodextrins as catalysts for the removal of cholesterol from macrophage foam cells. The Journal of clinical investigation. 1997;99(4):773-80. Epub 1997/02/15.
43. Liu B, Ramirez CM, Miller AM, Repa JJ, Turley SD, Dietschy JM. Cyclodextrin overcomes the transport defect in nearly every organ of NPC1 mice leading to excretion of sequestered cholesterol as bile acid. Journal of lipid research. 2010;51(5):933-44. Epub 2009/12/08.
44. Abi-Mosleh L, Infante RE, Radhakrishnan A, Goldstein JL, Brown MS. Cyclodextrin overcomes deficient lysosome-to-endoplasmic reticulum transport of cholesterol in Niemann-Pick type C cells. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(46):19316-21. Epub 2009/11/04.
45. Ramirez CM, Liu B, Taylor AM, Repa JJ, Burns DK, Weinberg AG, et al. Weekly cyclodextrin administration normalizes cholesterol metabolism in nearly every organ of the Niemann-Pick type C1 mouse and markedly prolongs life. Pediatric research. 2010;68(4):309-15. Epub 2010/06/29.
46. Aqul A, Liu B, Ramirez CM, Pieper AA, Estill SJ, Burns DK, et al. Unesterified cholesterol accumulation in late endosomes/lysosomes causes neurodegeneration and is prevented by driving cholesterol export from this compartment. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2011;31(25):9404-13. Epub 2011/06/24.
47. Camargo F, Erickson RP, Garver WS, Hossain GS, Carbone PN, Heidenreich RA, et al. Cyclodextrins in the treatment of a mouse model of Niemann-Pick C disease. Life sciences. 2001;70(2):131-42. Epub 2002/01/15.
48. Matsuo M, Togawa M, Hirabaru K, Mochinaga S, Narita A, Adachi M, et al. Effects of cyclodextrin in two patients with Niemann-Pick Type C disease. Molecular genetics and metabolism. 2013;108(1):76-81. Epub 2012/12/12.
49. Ikura Y, Ohsawa M, Suekane T, Fukushima H, Itabe H, Jomura H, et al. Localization of oxidized phosphatidylcholine in nonalcoholic fatty liver disease: impact on disease progression. Hepatology. 2006;43(3):506-14. Epub 2006/02/24.
50. Kanters E, Pasparakis M, Gijbels MJ, Vergouwe MN, Partouns-Hendriks I, Fijneman RJ, et al. Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. The Journal of clinical investigation. 2003;112(8):1176-85. Epub 2003/10/17.
51. Dietschy JM, Turley SD. Control of cholesterol turnover in the mouse. The Journal of biological chemistry. 2002;277(6):3801-4. Epub 2001/12/26.
52. Gould S, Scott RC. 2-Hydroxypropyl-beta-cyclodextrin (HP-beta-CD): a toxicology review. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2005;43(10):1451-9. Epub 2005/07/16.
53. Ferezou J, Riottot M, Serougne C, Cohen-Solal C, Catala I, Alquier C, et al. Hypocholesterolemic action of beta-cyclodextrin and its effects on cholesterol metabolism in pigs fed a cholesterol-enriched diet. Journal of lipid research. 1997;38(1):86-100. Epub 1997/01/01.
54. Bieghs V, Hendrikx T, van Gorp PJ, Verheyen F, Guichot YD, Walenbergh SM, et al. The cholesterol derivative 27-hydroxycholesterol reduces steatohepatitis in mice. Gastroenterology. 2013;144(1):167-78 e1. Epub 2012/10/09.
55. Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG, et al. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature. 2010;464(7293):1357-61. Epub 2010/04/30.

7. References

8. Supplementary information

8.1 Explanation letter: Experimental change

Dear Sir/Madame,

Within the following two pages, I want to explain why the initially planned experiment changed into a new topic in my thesis.

Background Non-alcoholic steatohepatitis (NASH), defined by hepatic lipid accumulation combined with inflammation, is considered the hepatic event of the metabolic syndrome. The development of inflammation represents the key step in the pathogenesis, as it sets the stage for irreversible liver damage, i.e. liver cirrhosis and liver cancer. However, the risk factors that drive progression towards hepatic inflammation are still unclear. Consequently, therapeutic options for NASH are lacking. Hence, it is of critical importance to unravel which mechanisms trigger hepatic inflammation.

Plan A
In our previous research, we have shown that hepatic inflammation is associated with increased cholesterol accumulation inside lysosomes of Kupffer cells. Lysosomes are critical players in autophagy, the process by which cells degrade (damaged) intracellular components and lipids. Thus, lysosomal dysfunction disturbs the autophagy process, an important feature of lysosomal storage disorders. In addition, recent studies have revealed that inhibition of autophagy activates the inflammasome, thereby triggering the maturation and the release of pro-inflammatory cytokines. Taken together, these data suggest an important role for autophagy during the development of hepatic inflammation. Therefore, we **hypothesize** that disturbed autophagy in KCs, leads to increased hepatic inflammation during NASH

Experimental setup To investigate whether disturbed autophagy in KCs leads to increased hepatic inflammation during NASH, *Ldlr*$^{-/-}$ mice were transplanted with bone marrow from mice with or without a macrophage-specific Atg7 knockout (Atg7-floxed or Atg7-wild-type mice). The role of autophagy in the hepatic inflammatory response was planned to be investigated by analyzing the level of autophagy, the degree of hepatic inflammation and steatosis, together with plasma and liver lipid content, and cholesterol distribution in Kupffer cells. According to our hypothesis, we anticipated that *Ldlr*$^{-/-}$ mice with a macrophage-specific Atg7 knockout will have increased hepatic inflammation, compared to their wild type counterparts.

What went wrong About one and a half year ago, the mice experiment started after a genotyping analysis. Unfortunately, there was a mistake in the genotyping analysis leaving only Atg7-wild-type mice and no Atg7-floxed mice carrying the macrophage-specific Atg7 deletion.

8. Supplementary information

Plan B

Recently, our group has shown that cholesterol distribution is critical in the development of hepatic inflammation, i.e. hepatic inflammation is associated with the accumulation of cholesterol inside lysomes of Kupffer cells, whereas cytoplasmic cholesterol is related with a lower inflammatory response. Further, both *in vitro* and *in vivo* studies have demonstrated that cellular cholesterol content and the transfer of cholesterol out of the lysosome into the cytoplasm can be mediated via cyclodextrin. Cyclodextrin is a cyclic oligosaccharide with a hydrophilic exterior and an hydrophobic interior that has a very high affinity for sterols, i.e. cholesterol. Interestingly, chronic cyclodextrin treatment of murine Niemann-Pick C disease, a lysosomal storage disorder, results in reduced lysosomal cholesterol storage in brain and liver, normalized autophagy levels and amelioration of the disease. Together these data suggest that cyclodextrin has the potential to decrease the inflammatory response. Therefore, we **hypothesize** that cyclodextrin will reduce lysosomal cholesterol storage and inflammation during NASH.

Experimental setup To investigate to what extent cyclodextrin is able to interfere with the accumulation of cholesterol in lysosomes and with the inflammatory state, *Ldlr*$^{-/-}$ mice were injected with either PBS or cyclodextrin. To determine whether cyclodextrin can be used as therapeutical option, the degree of hepatic inflammation, steatosis and autophagy, together with plasma and liver lipid content, and cholesterol distribution in Kupffer cells will be analyzed. According to our hypothesis, we anticipate that *Ldlr*$^{-/-}$ mice receiving cyclodextrin will have a reduced inflammatory state compared with non-treated *Ldlr*$^{-/-}$ mice.

Feasibility The mice are already sacrificed and I already started some analyses. I have stained cryo-liver sections for Mac-1 (macrophages), CD3 (T-cells), and NIMP (neutrophils). Further, I have determined plasma lipid content, and I have cutted paraffine liver and spleen sections. In addition, analysis of a small portion of the electron microscopy pictures has been done, and further analysis is currently in process. In the coming weeks I will isolate RNA from total liver and KCs for gene-expression analysis and I will determine liver lipid content. In addition, I will perform some other staining both on cryo- and paraffin sections, i.e. CD68, Oil Red O and H&E. Thus, even though the analysis of the electron microscopy might not yet be complete, I will still have sufficient data to write a complete thesis.

With best regards,

Nathalie Vaes

Auteursrechtelijke overeenkomst

Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:
Cholesterol in hepatic inflammation: innocent bystander or still a heavily suspect?

Richting: **master in de biomedische wetenschappen-klinische en moleculaire wetenschappen**
Jaar: **2013**

in alle mogelijke mediaformaten, - bestaande en in de toekomst te ontwikkelen - , aan de Universiteit Hasselt.

Niet tegenstaand deze toekenning van het auteursrecht aan de Universiteit Hasselt behoud ik als auteur het recht om de eindverhandeling, - in zijn geheel of gedeeltelijk -, vrij te reproduceren, (her)publiceren of distribueren zonder de toelating te moeten verkrijgen van de Universiteit Hasselt.

Ik bevestig dat de eindverhandeling mijn origineel werk is, en dat ik het recht heb om de rechten te verlenen die in deze overeenkomst worden beschreven. Ik verklaar tevens dat de eindverhandeling, naar mijn weten, het auteursrecht van anderen niet overtreedt.

Ik verklaar tevens dat ik voor het materiaal in de eindverhandeling dat beschermd wordt door het auteursrecht, de nodige toelatingen heb verkregen zodat ik deze ook aan de Universiteit Hasselt kan overdragen en dat dit duidelijk in de tekst en inhoud van de eindverhandeling werd genotificeerd.

Universiteit Hasselt zal mij als auteur(s) van de eindverhandeling identificeren en zal geen wijzigingen aanbrengen aan de eindverhandeling, uitgezonderd deze toegelaten door deze overeenkomst.

Voor akkoord,

Vaes, Nathalie

Datum: **10/06/2013**