# Novel modulators of lipoprotein metabolism

## implications for steatohepatitis and atherosclerosis

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*This thesis is dedicated to my parents.*

谨以此论文献给我的父母.

# CONTENTS

# 1

# GENERAL INTRODUCTION

# 1. Lipids and lipoprotein metabolism

Triglycerides (TG) and cholesterol are hydrophobic lipids that are absorbed by the intestine and synthesized by the liver, and have to be transported to other tissues for biosynthetic processes (i.e. cholesterol), energy production, heat production or storage (i.e. triglycerides). Since TG and cholesterol are insoluble in a hydrophilic environment, they circulate in the blood as constituents of water-soluble particles named lipoproteins. Lipoproteins consist of a lipid-rich inner core containing TG and cholesteryl esters (CE), the storage form of cholesterol, and an amphiphilic surface containing phospholipids (PL), unesterified cholesterol and one or more apolipoproteins. According to their density, lipoproteins are subdivided into five main classes, namely (from lowest to highest density) chylomicrons (CM), very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Lipoprotein metabolism will be discussed in more detail in sections 1.1-1.3, and a schematic overview is depicted in Figure 1.

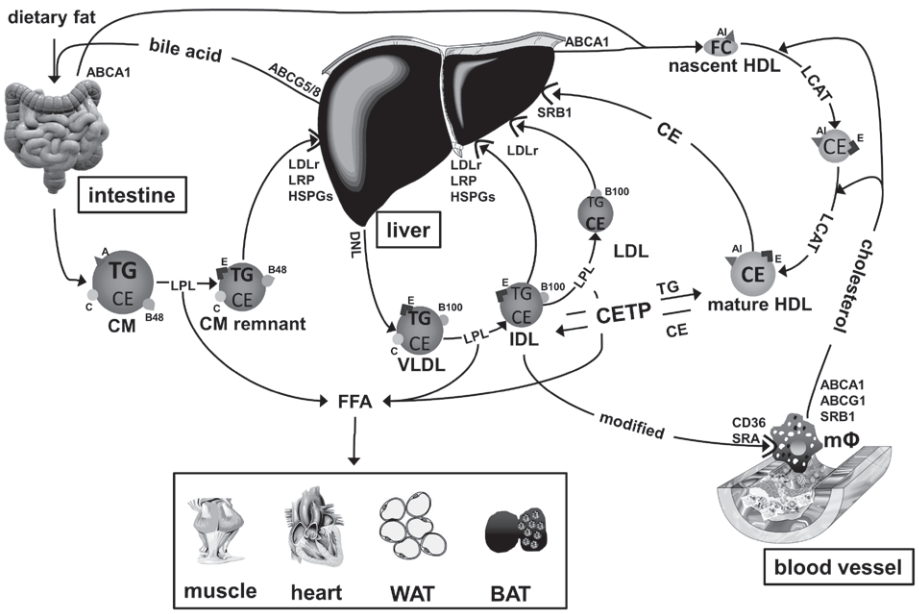


**Figure 1. Schematic overview of lipoprotein metabolism.** See text for explanation.

## 1.1 Chylomicrons

Dietary fat mainly consists of TG, but also contains phospholipids and cholesterol. In

the intestine, the digestion products of dietary TG, i.e. 2-monoacylglycerol (MG) and fatty acids (FAs) are taken up by the enterocytes. Intracellularly, TG is reconstituted from 2-MG and FA at the endoplasmic reticulum (ER) surface. TG droplets are surrounded by newly synthesized apolipoprotein B48 (apoB48), mediated by the microsomal triglyceride transfer protein (MTP), to form a prechylomicron that moves to the Golgi. There mature chylomicrons (CM) are formed by fusion with additional lipids, which are exported into the lamina propria of the basolateral membrane to enter the lymphatic system and ultimately the thoracic lymph duct [1-4]. In addition to apoB48, CMs contain other apolipoproteins including apoAI, apoAIV, apoCI, apoCII and apoCIII [5]. From the lymph system, CMs enter the blood circulation, where their TG are hydrolyzed by lipoprotein lipase (LPL) in metabolically active tissues into FAs and glycerol [6]. FAs are taken up by skeletal muscle and heart for use as energy source, by brown adipose tissue (BAT) for thermogenesis, and by white adipose tissue (WAT) for storage of excess FAs as TG. As a consequence of lipolysis, CM remnants are produced that are enriched in cholesterol and have acquired apoE, through which the remnants are taken up by the liver via the apoE-binding receptors and binding sites , such as the LDL receptor (LDLr) [7], LDLr-related protein (LRP) [7], heparan-sulphate proteoglycans (HSPGs) [8], and possibly also scavenger receptor-class B type I (SR-BI) [9].

## 1.2 VLDL, IDL and LDL

VLDL is synthesized and secreted by the liver. ApoB100 (and in rodents also apoB48 [10]) is the key structural protein of VLDL, which is also synthesized at the ER surface. Similarly to CM, the nascent apoB100 is partially lipidated to form a lipid-poor primordial VLDL particle mediated by MTP [11], and then the primordial VLDL particle fuse with TG-rich particles already present in the cytosol. The latter step is not only facilitated by MTP [12] as for chylomicrons, but also by cideB [13] (a homolog of cell death-inducing DFF45-like effector). TGs used for VLDL assembly are synthesized de novo in the ER lumen in a preventive response to FA influx as albumin-bound free FA (released from adipose tissue or taken up by the intestine via the portal vein), or as TG-derived FA (after uptake of CM or VLDL remnants) [14]. The most important genes involved in the de novo synthesis of TG are the transcription factor sterol regulatory element binding protein-1c (SREBP-1c), carbohydrate response element binding protein (ChREBP) [15], fatty acid synthase (FAS) and stearyl-Coa desaturase-1 (SCD-1)[16], while the rate-limiting enzyme for the de novo synthesis of cholesterol is 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCoA reductase).

In addition to apoB100, VLDL contains other apolipoproteins such as apoCI, apoCII, apoCIII and apoE [17, 18]. Like CM, their TGs are lipolyzed by LPL, and the VLDL remnant

(i.e. IDL) is taken up by the liver via apoE-binding receptors. In the fasted state when CM synthesis is low, LPL activity in adipose tissue is low while LPL activity in heart and skeletal muscle remains steady. Therefore, in the fasted state, VLDL-TG derived FA are mainly taken up by skeletal muscle and heart for energy use instead of by adipose tissue for storage. In addition to being taken up by the liver, IDL can be further lipolyzed, resulting in a TG-depleted lipoprotein called LDL[6, 19]. LDL has lost all apolipoproteins apart from apoB100, through which LDL can be taken up via the LDLr by the liver, but also by extrahepatic tissues that use cholesterol to maintain membrane integrity or to produce steroid hormones (e.g. adrenals, reproductive glands) [20, 21].

ApoB-containing lipoproteins (VLDL, IDL, LDL) are considered to be pro-atherogenic, as they can accumulate in the vessel wall, where they are modified and taken up by macrophages, initiating the process of atherosclerosis development.

## 1.3 HDL

Apolipoprotein AI (apoAI), the most abundant apolipoprotein of HDL, is synthesized in the liver and intestine, and is released into circulation. Subsequently, apoAI is lipidated with phospholipids (PL) via the ATP binding cassette transporter A1 (ABCA1) expressed in the liver and intestine to form a nascent discoidal HDL. This HDL particle can take up cholesterol from various peripheral tissues and cell types (e.g. from resident macrophages) via ABCA1 or from surface remnants (consisting of PL and cholesterol) upon lipolysis of TG-rich lipoproteins via phospholipid transfer protein (PLTP) [22, 23].The acquired cholesterol is then esterified by lecitin: cholesterol acyltransferase (LCAT), which results in CE accumulation in the core of the HDL particle. Under the action of LCAT, the nascent HDL becomes a mature spherical HDL containing more cholesterol and additional other apolipoproteins, such as apoAII, apoAIV, apoAV, apoCI, apoCII, apoCIII, apoE and apoM [24]. During maturation, the affinity of HDL for other cholesterol export molecules is increased, including the ATP-binding cassette transporter G1 (ABCG1) and SR-BI, which results in further cholesterol efflux from peripheral tissues and further maturation of HDL [25, 26]. CE in HDL is selectively taken up via hepatic SR-BI [27], while TG and PL in HDL can be lipolyzed by hepatic lipase (HL) and endothelial lipase (EL) [28, 29]. Alternatively, in humans and some other species, CE in HDL can be transferred to apoB-containing lipoproteins in exchange for TG via the cholesteryl ester transfer protein (CETP), and CE can subsequently be cleared by LDL receptor-mediated uptake of apoB-containing lipoproteins by the liver. Once taken up by the liver, HDL-derived cholesterol can be used for storage as CE, for assembly of VLDL, or for excretion into bile in the form of bile acids or neutral sterols.

While plasma levels of cholesterol within apoB-containing lipoproteins is positively

correlated with cardiovascular disease (CVD), the plasma HDL-cholesterol level is inversely correlated with CVD risk [30]. However, recent studies suggested that the cholesterol efflux capacity of HDL is a better predictor of CVD than the concentration of HDL-cholesterol [31, 32]. Cholesterol efflux is one of the most important steps of reverse cholesterol transport (RCT). This process describes the transport of cholesterol from the peripheral tissues like the vessel wall back to the liver, after which cholesterol is secreted via the bile into the feces. This RCT pathway is believed to be a major mechanistic basis for the protective effect of HDL on CVD. Besides mediating RCT, the antimicrobial, antioxidant, antiglycation, anti-inflammatory, nitric oxide-inducing, antithrombotic and immune-modulating properties of HDL are believed to also contribute to the anti-atherogenic actions of HDL. In addition, HDL has recently been described to beneficially affect the pathophysiology of diabetes, including glucose homeostasis and energy homeostasis [33, 34].

## 2. Atherosclerosis and non-alcoholic steatohepatitis

### 2.1 Atherosclerosis

Atherosclerosis is a multifactorial disease affecting the arteries, where lipids, connective tissue elements, smooth muscle cells (SMCs) and immune cells have accumulated, leading to a progressive narrowing of the vessel wall. With consequences such as the myocardial infarction or stroke, atherosclerosis remains the most common cause of death in the Western world [35].

Atherosclerosis development starts with the infiltration of atherogenic lipoproteins such as LDL into the vessel wall. Upon being trapped, LDL can be modified (e.g. by oxidation or aggregation), and the modified LDL then stimulates endothelial cell (EC) activation and recruitment of immune cells. Within the sub-endothelial space, monocyte-derived macrophages take up the oxidized LDL and slowly turn into the large lipid-laden "foam cells". Lesions consisting only of foam cells and other immune cells are called fatty streaks or mild lesions that mostly cause no clinical symptoms. Foam cells and activated ECs secrete inflammatory cytokines and chemokines that activate SMCs to proliferate and migrate into the atherosclerotic lesion. This causes the formation of a fibrous cap covering the fatty streak. When foam cells or SMCs die in the plaque, a necrotic core will be formed consisting of extracellular lipid and cellular debris. Depending on the composition, a plaque can be less or more vulnerable to rupture, resulting in different severity of clinical manifestations. Stable plaques usually have a thick fibrous cap and low amount of macrophages and lipid content, whereas unstable plaques have a thin fibrous cap and a relative high content of macrophages

and lipids and/or a necrotic core. Unstable plaques are prone to rupture, which leads to immediate blood clotting and the formation of a thrombus that will rapidly slow or stop blood flow, thus causing an infarction [36-38].

## 2.2 Non-alcoholic steatohepatitis

Non-alcoholic fatty liver disease (NAFLD) is currently the leading cause of chronic liver disease in the Western world and the estimated prevalence in the general population ranges between 20% and 30%, rising to as high as 90% in morbidly obese individuals [39,40]. NAFLD embraces a pathological spectrum of liver diseases, from steatosis with virtually no evidence of hepatocellular injury or liver inflammation to non-alcoholic steatohepatitis (NASH) [41]. NASH is characterized by fat accumulation (steatosis) in combination with hepatic inflammation (steatohepatitis) [42]. Although the mechanisms underlying the development of NASH are not completely established yet, the so-called "two-hit hypothesis" postulates a sequential evolution from simple steatosis to NASH, with steatosis as the first critical "hit" and a necessary prerequisite for further liver damage, such as inflammation [43, 44]. However, the "two-hit" model is challenged by findings from recent studies, which described that the development of hepatic inflammation was independent on the presence of hepatic steatosis [45, 46]. In contrast, cholesterol or its modified form, trapped inside of hepatic macrophages, is an important trigger for NASH.

Liver biopsies are currently the golden standard methods used for the diagnosis of NASH. However, there are several severe limitations to liver biopsies, such as sampling error, differences in histopathologic interpretation, as well as patient stress and discomfort, risk of bleeding and long hospitalizations. Non-invasive imaging modalities have been advocated for liver steatosis only (e.g. $^{1}$H-magnetic resonance spectroscopy; MRS), but they are insufficient to distinguish NASH from just fatty liver disease. Although some liver enzymes, such as aminotransferase (ALT) that indicate liver damage, are elevated in patients with NASH, none of them yet can replace liver biopsy. Therefore, the discovery of a blood marker with a high sensitivity and specificity for NASH is eagerly awaited [47-49] .

## 2.3 The association between atherosclerosis and NASH

The association between NASH and CVD has been studied in the last decades. Although some studies showed no significant association between NASH and markers of subclinical CVD (e.g. carotid-artery intimal medical thickness or carotid-artery calcium), most studies demonstrate that NASH is strongly associated with increased atherosclerosis [50, 51]. Historically, NASH was thought to be a causal risk factor for CVD as

patients with NASH have a higher risk of mortality than the general population, mainly due to atherosclerotic diseases [52]. However, the biological mechanisms linking NASH and CVD are still poorly understood.

Several etiologies are potentially responsible for both development of atherosclerosis and NASH. For example, insulin resistance resulting in increased lipolysis is a pathogenic factor in the development and progression of NASH, and also plays a major role in the development of CVD [53]. Also, dyslipidemia, reflected by increased plasma levels of (V)LDL-cholesterol and TG, and decreased level of HDL-cholesterol, is a major risk factors for both atherosclerotic diseases and fatty liver disease. In addition to impaired glucose and lipid metabolism, inflammation characterized by monocyte/macrophage infiltration and macrophage foam cell formation also plays a vital role in the development of both atherosclerosis and NASH [45, 54-58]. Recently, Bieghs *et al*. [59] put forward the hypothesis that NASH and atherosclerosis are actually two aspects of a shared disease with same etiology: the infiltration of macrophages.

Since atherosclerosis and NASH are strongly associated and share similar etiologies, the current treatment strategies for CVD are also tested for NASH in clinical practice, mainly aimed at improving lipid metabolism. Therapeutic options targeted to lowering plasma levels of TG and VLDL/LDL-cholesterol are established for treatment of CVD. Statins, which inhibit HMGCoA reductase thereby reducing the plasma (V)LDL-cholesterol level, are the most widely used cholesterol-lowering drugs with a significant reduction of major cardiovascular (CV) events [60, 61] up to -50% when LDL-cholesterol is reduced by 2-3 mmol/L. However, even aggressive statin therapy does not eliminate CVD; in particularly, patients with a low level of HDL-cholesterol still have a significant risk for CV events after statin therapy. Therefore, strategies that e.g. can increase the plasma HDL-cholesterol level are investigated for the treatment of CVD. Although lipid-lowering agents (e.g. statins and fibrates), anti-oxidants (e.g. vitamins C, E), and insulin-sensitizers (e.g. thiazolidinediones, metformin) are considered to have beneficial effects in NASH outcomes, none of them have as yet shown adequate and convincing benefits [62, 63]. Lifestyle modifications, such as weight loss, exercise, and restriction of nutrition intake are still the mainstays for the treatment of NASH [64]. The search for novel pharmacological strategies to treat atherosclerosis and NASH is thus still warranted.

## 3. CETP and HDL-raising strategies, implications for CVD

Since HDL-cholesterol is inversely correlated with the risk of CVD and CETP plays a vital role in HDL-cholesterol metabolism (as described in section 1.3), CETP has become one of the most important targets for the development of HDL-raising and anti-atherosclerotic strategies. Several CETP inhibitors markedly increase plasma HDL-

cholesterol, but also lipid-lowering compounds such as fibrates and niacin increase HDL-cholesterol level by affecting CETP expression and activity. However, the anti-atherogenic capacity of HDL-raising strategies is still not established.

## 3.1 CETP structure and function

Several mammalian species express CETP mRNA, including humans, monkeys, rabbits, pigs but not rats and mice [65-67]. In humans, the CETP gene is located at chromosome 16 (16q12-16q21), and consists of 16 exons and 15 introns, with the exons ranging from 32bp to 250 bp [68, 69]. The upstream flanking region of the CETP gene contains several regulatory sequences, including binding sites for SREBP, the ubiquitous nuclear factor-1, the hepatocyte nuclear factor-1 and a nuclear receptor binding site that is activated by liver X receptor (LXR) [70, 71]. In humans, CETP mRNA is expressed mainly in the liver and adipose tissue, but also to some extent in spleen, heart, small intestine, adrenal gland, and skeletal muscle [66, 67, 70, 71]. However, the relative contribution of adipose tissue and liver to total plasma CETP levels, and the cell types involved in CETP expression, are still obscure. CETP is a highly hydrophobic glycoprotein with a molecular mass ($M_r$) of 70-74 kDa [68, 69]. The crystal structure of CETP protein reveals a curved molecule with N- and C- terminal cavities and tunnel spanning the entire length of the protein [72]. CETP is secreted into plasma where it binds mainly to HDL, and promotes bidirectional transfer of CE, TG, and, to a lesser extent, PL between plasma lipoproteins. Because most of CE in plasma resides in HDL, after esterification of cholesterol by LCAT, and the majority of the TG enters the plasma as a component of CM and VLDL [known collectively as triglyceride-rich lipoproteins (TRL)], the overall effect of CETP is a net mass transfer of CE from HDL to TRLs and LDL, and in exchange for TG from TRLs to HDL [73].

## 3.2 Role of CETP in lipoprotein metabolism and atherosclerosis development

The effects of CETP on lipoprotein metabolism and atherosclerosis development in humans have been studied in subjects with genetic deficiencies of CETP. In the past two decades, at least 13 mutations in the CETP gene have been described in Japan and elsewhere [74-76]. These mutations result in decreased plasma CETP mass and activity, associated with an increase in HDL-cholesterol level. Moreover, subjects with a homozygous CETP deficiency have not only elevated plasma levels of HDL-cholesterol, apoAI and apoAII, but also decreased plasma LDL-cholesterol and apoB levels [77]. These observations suggest that inhibiting CETP in humans beneficially affects lipoprotein metabolism. However, observations on the relation between CETP deficiency and

susceptibility to develop atherosclerosis in epidemiological studies are controversial. A large meta-analysis of 92 studies involving 113.833 participants showed that subjects with CETP polymorphisms that are associated with deceased CETP activity and mass have an elevated concentration of HDL-cholesterol and a decreased risk of CVD [78]. A similar conclusion was drawn from an analysis of a cohort of 18.245 healthy women from the Women's Genome Health Study, where polymorphisms in the CETP gene that impact on HDL-cholesterol levels also impact on the future risk of myocardial infarction [79]. In contrast, other studies suggest that CETP mutations, despite of raising the plasma HDL-cholesterol level, do not lower the risk of CVD [80, 81]. In fact, the Honolulu Heart Study suggested that heterozygote CETP deficiency even increased risk of CVD [82]. Transgenic mice that express human CETP have significantly decreased plasma HDL-cholesterol level and slightly increased (V)LDL-cholesterol level [83-88]. In contrast to the clear effects on lipoprotein metabolism, the effects of CETP expression on atherosclerosis gave conflicting results in those CETP transgenic mice. CETP has been shown to be pro-atherogenic in apoE deficient, LDL receptor deficient and *APOE*3-Leiden* transgenic mice [84-86], whereas CETP was shown to be anti-atherogenic in *APOC3* and *LCAT* transgenic mice [87 88].

## 3.3 CETP inhibitors as therapeutic HDL-raising agents

Small-molecule CETP inhibitors have been developed to raise the HDL-cholesterol level and have been tested for the treatment of CVD. Torcetrapib, one of the first CETP inhibitors, at a daily dose of 60 mg, increased the plasma HDL-cholesterol and apoAI level by 70% and 25%, respectively, and decreased the plasma LDL-cholesterol and apoB level by 25% and 12.5%, respectively, in the ILLUMINATE trial [89]. Despite the beneficial effects on lipoprotein profiles, this trial was terminated early because of a statistically significant excess of deaths from both cardiovascular and noncardiovascular causes due to off-target side effects. Another CETP inhibitor, dalcetrapib, at a daily dose of 900 mg reduced CETP activity by 37%, increased the HDL-cholesterol level by 34% and reduced the LDL-cholesterol level by 7% without showing off-target adverse effects [90]. However, none of the clinical trials with dalcetrapib (dal-PLAQUE, dal-VESSEL, dal-OUTCOMES) showed beneficial effects on carotid artery wall index, endothelial function or CVD outcomes [91-93] . The REVEAL trial, evaluating the effect of another CETP inhibitor, anacetrapib, on clinical CVD outcomes is ongoing, and the outcome is expected by 2017.

## 3.4 Other CETP-modulating HDL-raising agents

Fibrates belong to a class of drugs that exert their effects by activating the peroxisome

proliferator-activated receptor (PPAR) α and, to a lesser extent PPARβ/δ and PPARγ [94]. Fibrates decrease plasma VLDL-TG by increasing VLDL-TG clearance and stimulating the oxidation of FFA, although fibrates increase VLDL-TG production in the liver [95]. In clinical trials, fibrates not only reduce plasma TG (up to -50%), but also increase plasma HDL-cholesterol (up to +25%) [96-98]. One mechanism underlying these effects of fibrates on the concentration of HDL-cholesterol could be that fibrates reduce hepatic CETP expression and plasma CETP activity [99], but it is unknown how fibrates reduce hepatic CETP expression. Fibrates were approved to treat dyslipidemia since 1993, yet there remains considerable controversy regarding their clinical efficacy for CVD [100]. Two trials of gemfibrozil demonstrated improvements in cardiovascular outcomes [101, 102], but subsequent trials of bezafibrate and fenofibrate showed no significant overall cardiovascular benefit over placebo [103-105].

Niacin (nicotinic acid) is the most potent HDL-cholesterol-raising drug used in clinic practice. In addition to raising HDL-cholesterol (up to +35%), niacin also decreases plasma LDL-C and TG levels (up to -25% and -50%, respectively) in humans [106]. Niacin reduces hepatic CETP expression and plasma CETP activity thereby increasing the HDL-cholesterol level in CETP Tg mice [107]. Like for fibrates, the mechanism underlying the reducing effect of niacin on hepatic CETP expression is still unclear. Although niacin has been used in clinical practice for many decades for the prevention of CVD and numerous studies demonstrated a significant reduction in CV events by the niacin intervention [108-112], the clinical efficacy of niacin for the treatment of CVD has been challenged by the latest results from the AIM-HIGH (Atherothrombosis Intervention in Metabolic Syndrome With Low HDL/High Triglycerides: Impact on Global Health Outcomes) trial [113] and HPS2-THRIVE (Heart Protection Study 2 Treatment of HDL to Reduce the Incidence of Vascular Events) trial, both of which were stopped because of failure to show clinical benefits with respect to attenuating CVD.

Overall, given the fact that CETP inhibition increases the concentration of HDL-cholesterol, CETP inhibitors and other compounds inhibiting CETP expression were thought to protect against the atherosclerosis development. However, the clinical efficacy of those compounds is challenged by the recent clinical trials (e.g. ILLUMINATE, dal-PLAQUE, dal-VESSEL, dal-OUTCOMES, AIM-HIGH, HPS2-THRIVE). Therefore, the hypothesis that raising the HDL-cholesterol level by inhibiting CETP has beneficial effects on CV events is at present not supported by clinical trials and further studies are required to investigate the role of CETP modulation in lipid metabolism and CVD.

## 4. Selected novel targets modulating lipoprotein metabolism for treatment of CVD and NASH

In addition to classical lipid-lowering drugs (e.g. statin, fibrates and niacin) used for the treatment of dyslipidemia, atherosclerosis and NASH, other novel strategies are currently under investigation, including those targeting the central and peripheral regulation of energy homeostasis and food intake (e.g. NPY and GLP-1), as well as those targeting inflammation (e.g. glucocorticoids).

### 4.1 NPY

The hypothalamus is considered as the main region of the brain regulating energy homeostasis and food intake. It contains a number of discrete neuronal populations or nuclei, one of which is the arcuate nucleus (ARC). The ARC contains two distinct groups of neurons with opposing effects on energy metabolism and food intake: one group consists of neurons with coexpression of neuropeptide Y (NPY) and agouti-related peptide (AgRP), which activates appetite; the other group consists of neurons with coexpression of pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), which inhibits appetite.

The 36-amino acid peptides NPY, peptide YY (PYY) and pancreatic polypeptide, collectively called the NPY family of peptides, affect food intake by interacting with G-protein coupled Y receptors [114, 115]. NPY is widely expressed in both the brain and the peripheral nervous system. Within the brain, NPY is highly expressed in the hypothalamus, especially in the ARC [114, 115]. NPY/AgRP-neurons can be activated by a diversity of signals, such as leptin and insulin [116]. Upon activation, NPY stimulates its Y receptors (Y1 and Y5) to activate circuits that increase food intake and fat storage. Concomitantly, by antagonizing the melanocortin 3 and 4 (MC3/4) receptors in the paraventricular nucleus (PVN), NPY/AgRP prevents the catabolic drive initiated by the melanocortin system [117].

In addition to modulation of food intake and energy expenditure, central NPY displays multiple bio-functions in experimental studies. Intracerebroventricular (ICV) injection of NPY to rats powerfully increases food intake, causes obesity [118], influences glucose metabolism [119], and increases hepatic production of VLDL-TG [120], all of which are risk factors for CVD and NASH. Although ICV administration of NPY to mice has similar effects on food intake, energy homeostasis and glucose metabolism [121, 122] as in rats, the effect of NPY administration on lipid metabolism in mice is less clear. Sympathetic nervous system-targeted NPY overexpression in mice enhances neointimal formation in response to vascular injury, indicating the direct role of central NPY in the development of vascular disease [123].

## 4.2 GLP-1

In addition to hypothalamic NPY, numerous peripheral gut hormones show an important role in regulating energy homeostasis as well, such as ghrelin [124,125], cholecystokinin [126,127], oxyntomodulin [128], peptide Y [129] and glucagon like peptide-1 (GLP-1).

GLP-1 is a cleavage production of the proglucagon molecule which is secreted by the intestinal L-cells and the brain [130,131]. It is released in response to food intake to stimulate glucose-dependent insulin production [132]. In addition, GLP-1 exerts multiple other functions, including inhibition of food intake [133], slowing the gastric emptying [134], and inhibition of glucagon secretion [135]. Moreover, administration of native GLP-1 beneficially improves glucose metabolism in type 2 diabetes mellitus (T2DM) patients [135,136], which implies GLP-1 as an ideal potential target for treatment of T2DM. GLP-1 mediates its effects via the GLP-1 receptor, a 7-transmembrane-spanning G-protein-coupled receptor that is abundantly expressed in various tissues [137], including the gastrointestinal tract, pancreatic islands, kidneys, heart and central nervous system [138].

However, therapeutic application of GLP-1 is hampered by its short circulating half-life (<2 minutes), because it is rapidly degraded by dipeptidyl peptidase 4 (DPP-4) that is widely expressed in endothelium and intestinal mucosa [139]. Therefore, pharmaceutical GLP-1 analogues that are resistant to degradation by DPP-4 have been developed with an improved pharmacokinetic profile related to a longer half-life, and with retained beneficial effects on T2DM, of which exenatide (a synthetic version of exendin-4) was approved in 2005 for the treatment of T2DM [140]. In addition to reducing body weight and improving glucose metabolism, some preliminary studies suggested that exenatide also decreases the plasma TG level in patients with T2DM [141,142], all of which are of clinical benefit for fatty liver disease. In addition, exenatide reduces hepatic lipid accumulation and reverses diet-induced hepatic steatosis in ob/ob mice [143]. Although GLP-1 receptor agonism has the potential to treat hepatic steatosis, its impact on hepatic inflammation is still uncertain. Besides the clear beneficial effects on fatty liver disease, GLP-1 receptor agonism shows controversial effects on atherosclerosis. Several studies showed that both native GLP-1 and exendin-4 inhibits atherogenesis in $ApoE^{-/-}$ mice [144,145]. However, a recent study demonstrated that taspoglutide, another long-acting GLP-1 receptor agonist, did not attenuate the development of atherosclerosis. Therefore, further studies evaluating the effects of GLP-1 receptor agonism on the development of atherosclerosis are warranted.

## 4.3 Glucocorticoids

Glucocorticoids (GCs) (cortisol in humans and corticosterone in rodents) are a class of steroid hormones secreted by the adrenals in response to a stressor, to induce

the necessary behavioral and metabolic adaptations for the individual to be able to adequately cope with the stressor [146]. GCs influence a wide variety of physiological functions, including energy homeostasis, food intake, body weight, glucose and lipid metabolism [147]. For example, GCs stimulate gluconeogenesis in the liver and inhibit glucose uptake in the muscle and adipose tissue [148]. Central administration of GCs induces hyperphagia and bodyweight gain [149]. As a consequence, patients with Cushing's syndrome (CS) who have excess of GCs display increased risk of obesity and insulin resistance [150].

In contrast to the convincing effects on the glucose metabolism, GCs have contradictory effects on lipid metabolism. GCs increase circulating level of FAs through increasing lipogenesis and VLDL secretion from the liver as well as increasing LPL activity. However, GCs appear to have both lipolytic and lipogenic effects in white adipose tissue, depending on the dose and duration of exposure [151]. Therefore, patients with excess GCs have inconsistent lipid levels. In clinical cohorts, the prevalence of hyperlipidemia in patients with CS varies from 38% to 71% [152]. Moreover, a study by Mancini *et al* [153] has shown that hyperlipidemia does not correlate to the degree of hypercortisolism in patients with CS.

In addition, the role of GCs in the development of atherosclerosis is also not yet clearly established. On one hand, GCs are shown to induce vasoconstriction [154] and endothelial dysfunction [155], both of which can induce atherosclerosis development. Human data revealed an association between increased GC levels and a risk of CVD even after long-term successful correction of GC exposure [156]. On the other hand, GCs have strong anti-inflammatory and immunosuppression properties, therefore may attenuate vascular inflammation, macrophage proliferation and differentiation [157] and thus suppressing atherosclerotic lesion formation [158]. Therefore, further studies are eagerly required to truly answer whether GCs can be used for the treatment of CVD.

## 5. Outline of this thesis

In this thesis, we aimed to gain new insights into novel modulators of lipoprotein metabolism, as well as their implications for the treatment of atherosclerosis and NASH. Firstly, we determined the role of CETP in lipid metabolism, the effect of pharmacological and dietary intervention on plasma CETP levels, as well as the cellular origin of CETP.

Since reconstituted HDL has different effects on VLDL metabolism in humans and mice, in **Chapter 2** we first evaluated the role of CETP in the effect of reconstituted HDL on VLDL metabolism by using *APOE*3-Leiden* (*E3L*) mice, an established model for human-like lipoprotein metabolism, with or without CETP expression. Previous studies in mice indicated that lipid-lowering strategies that reduce the hepatic lipid content

also reduce the hepatic CETP expression and plasma CETP concentration. To evaluate whether a reduction of the liver TG content, demonstrated to reduce hepatic CETP expression and plasma CETP levels in CETP transgenic mice, also reduces the plasma CETP concentration in humans, in **Chapter 3** we compared the effects of pioglitazone that reduces hepatic TG with that of metformin that has no effect on hepatic TG on the plasma CETP concentration. Subsequently, in **Chapter 4** we studied the effect of a lifestyle intervention (i.e. very low calorie diet), that was known to reduce hepatic TG, on plasma CETP concentration in obese patients with T2DM. To get more insight in the mechanism underlying the relation between liver lipid content and hepatic CETP expression, in **Chapter 5** we evaluated the mechanism underlying the CETP-lowering effect of niacin by using CETP transgenic mice. We showed that niacin, besides reducing the liver lipid content, also reduces the hepatic CETP expression by reducing the hepatic macrophage content. Since these data indicated that macrophages importantly contribute to hepatic CETP synthesis, we performed in **Chapter 6** more in-depth studies evaluating the contribution of various tissues and cell types to the plasma CETP level, by using both human cohorts and *E3L.CETP* transgenic mice, and were able to conclude that the plasma CETP concentration predicts hepatic macrophage content.

Then we investigated novel strategies for treatment of atherosclerosis and NASH. Since hypothalamic NPY influences energy homeostasis, food intake and lipid metabolism in rats, we set out to validate the effects of central NPY on hepatic VLDL production in wild-type mice in **Chapter 7**, to ultimately investigate whether hypothalamic NPY, by inducing dyslipidemia, affects the development of atherosclerosis. Since human studies demonstrated that GLP-1 receptor agonism decreases plasma TG, we explored the underlying mechanisms in **Chapter 8** by evaluating the effects of GLP-1 receptor analogues on hepatic VLDL production and *de novo* TG synthesis in *E3L* mice. Subsequently, in **Chapter 9** we studied the therapeutic applications of GLP-1 receptor agonist exendin-4 on the development of atherosclerosis and NASH simultaneously in *E3L.CETP* mice, and investigated the potential underlying mechanism. Because the effects of long-term glucocorticoid overexposure on the lipid metabolism and atherosclerosis development is not well established, in **Chapter 10** we investigated the effects of both transient and continuous glucocorticoid treatment on atherosclerosis development in *E3L.CETP* mice.

Finally, **Chapter 11** discusses the major findings of this thesis, and addresses the clinical implications of the results, as well as the future perspectives.

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# 2

# CETP EXPRESSION REVERSES THE RECONSTITUTED HDL-INDUCED INCREASE IN VLDL

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## ABSTRACT

Human data suggest that reconstituted HDL (rHDL) infusion can induce atherosclerosis regression. Studies in mice indicated that rHDL infusion adversely affects VLDL levels, but this effect is less apparent in humans. This discrepancy may be explained by the fact that humans, in contrast to mice, express cholesteryl ester transfer protein (CETP). The aim of this study was to investigate the role of CETP in the effects of rHDL on VLDL metabolism by using *APOE*3-Leiden* (*E3L*) mice, a well-established model for human-like lipoprotein metabolism. At 1 h after injection, rHDL increased plasma VLDL-C and TG in *E3L* mice, but not in *E3L* mice cross-bred onto a human CETP background (*E3L.CETP* mice). This initial raise in VLDL was caused by competition between rHDL and VLDL for LPL-mediated TG hydrolysis, and was thus prevented by CETP. At 24 h after injection, rHDL caused a second increase in VLDL-C and TG in *E3L* mice, whereas rHDL even decreased VLDL in *E3L.CETP* mice. This secondary raise in VLDL was due to increased hepatic VLDL-TG production. Collectively, we conclude that CETP protects against the rHDL-induced increase in VLDL. We anticipate that studies evaluating the anti-atherosclerotic efficacy of rHDL in mice that are naturally deficient for CETP should be interpreted with caution, and that treatment of atherogenic dyslipidemia by rHDL should not be combined with agents that aggressively reduce CETP activity.

# INTRODUCTION

Dyslipidemia is an important risk factor for cardiovascular disease (CVD). Current treatment mainly focuses on lowering of LDL-cholesterol (C), e.g. by statins. LDL-C lowering treatment results in a significant reduction in the morbidity and mortality of CVD, but can not prevent the majority of cardiovascular events [1, 2]. Prospective epidemiological studies have demonstrated a strong inverse correlation between HDL-C and CVD [3], and recent studies revealed that high HDL-C levels are indeed protective against plaque progression [4]. Although the exact mechanisms by which HDL protects are unclear, HDL has been shown to have antioxidant, antithrombotic and anti-inflammatory properties, and to mediate reverse cholesterol transport (RCT) via the hepatobiliary route [5]. Therefore, new strategies to raise HDL-C are currently being developed to prevent and treat CVD.

Various therapeutic strategies are currently under development to raise HDL levels, including cholesteryl ester transfer protein (CETP) inhibition, niacin, upregulation of apoAI expression and infusion of apoAI mimetics or reconstituted HDL (rHDL) [6]. Although still in early stage of development, infusion of (r)HDL seems to be a promising strategy for the treatment of CVD. Recent reviews have demonstrated that infusion of rHDL improves atherosclerotic plaque characteristics both in animal models and in humans [7-9]. For example, rHDL, composed of recombinant human apoAI$_{Milano}$ and phosphatidylcholine, rapidly mobilized tissue cholesterol and reduced the lipid and macrophage content of atherosclerotic plaques after a single injection into apoE-deficient mice [10]. Moreover, it prevented the progression of aortic atherosclerosis as well as promoted the stabilization of plaques after 6 weeks of administration [11]. Recent clinical trials assessed the effect of rHDL consisting of human apoAI and phosphatidylcholine (CSL-111) as a potential HDL-raising therapeutic strategy. Short-term infusion of CSL-111 significantly improved the plaque characterization index and coronary score on quantitative coronary angiography [12]. In addition, a single dose of rHDL led to acute changes in plaque characteristics with a reduction in lipid content, macrophage size and inflammatory mediators [13].

Albeit that rHDL thus seems to beneficially modulate atherosclerosis in mice and humans, differences have been observed with respect to modulation of VLDL levels. Infusion of rHDL into apoE-deficient mice increased (V)LDL-C in both acute and chronic studies [10, 11], whereas rHDL did not adversely affect (V)LDL-C in clinical studies [12, 13]. This discrepancy may be explained by the fact that, in contrast to mice [14], humans express CETP [15], a crucial factor involved in the metabolism of both (V)LDL and HDL by mediating the transfer of triglycerides (TG) and cholesteryl esters (CE) between these

lipoproteins. Therefore, the aim of this study was to elucidate the role of CETP in the effects of rHDL on VLDL metabolism. We used *APOE*3-Leiden* (*E3L*) transgenic mice, a unique model for human-like lipoprotein metabolism, which have been crossbred with mice expressing human CETP under control of its natural flanking regions [16], resulting in *E3L.CETP* mice. This allows distinguishing between the effect of rHDL administration on VLDL metabolism in the absence and presence of CETP-mediated lipid transfer.

## MATERIALS AND METHODS

### Animals

Hemizygous human CETP transgenic (*CETP*) mice, expressing human CETP under the control of its natural flanking regions [16], were purchased from the Jackson Laboratory (Bar Harbor, ME) and crossbred with hemizygous E3L mice [17] at our Institutional Animal Facility to obtain *E3L.CETP* mice [18]. In this study, female mice were used, housed under standard conditions in conventional cages with free access to food and water. At the age of 12 weeks, mice were fed a semi-synthetic Western-type diet, containing 1% (w/w) corn oil and 15% (w/w) cacao butter (Hope Farms, Woerden, The Netherlands) with 0.25% (w/w) cholesterol (*E3L* mice) or 0.1% (w/w) cholesterol (*E3L.CETP* mice) for three weeks, aimed at yielding comparable VLDL levels between both mouse genotypes. Upon randomization according to total plasma cholesterol (TC) and TG levels, mice received a single intravenous injection of rHDL (CSL-111; CSL Behring AG, Bern, Switzerland) (250 mg/kg in 250 µL PBS) or vehicle. Experiments were performed after 4 h of fasting at 12:00 pm with food withdrawn at 8:00 am. The institutional Ethical Committee on Animal Care and Experimentation has approved all experiments.

### Reconstituted HDL

rHDL (CSL-111) consists of apoAI isolated from human plasma and phosphatidylcholine from soybean with a molar ratio of 1:150. Before infusion, rHDL was reconstituted with 50 mL of sterile water, yielding 62.5 mL of clear, pale yellow solution, pH 7.5, and 10% (w/v) sucrose as a stabilizing agent. The final apoAI and PL concentrations were 20 and 86 mg/mL, respectively.

### Plasma lipid and lipoprotein analysis

Plasma was obtained via tail vein bleeding and assayed for TC, TG and phospholipids (PL) using the commercially available enzymatic kits 236691, 11488872 (Roche Molecular Biochemicals, Indianapolis, IN, USA) and phospholipids B (Wako Chemicals,

Neuss, Germany), respectively. The distribution of lipids over plasma lipoproteins was determined using fast protein liquid chromatography (FPLC). Plasma was pooled per group, and 50 µL of each pool was injected onto a Superose 6 PC 3.2/30 column (Äkta System, Amersham Pharmacia Biotech, Piscataway, NJ, USA) and eluted at a constant flow rate of 50 µL/min in PBS, 1 mM EDTA, pH 7.4. Fractions of 50 µL were collected and assayed for TC, TG and PL as described above.

## Plasma human apoAI concentration

Plasma human apoAI concentrations were determined using a sandwich ELISA. Goat anti-human apoAI antibody (Academy Biomedical Co., Inc., Houston, TX; 11A-G2b) was coated overnight on to Costar medium binding plate (Costar, Inc., New York, NY) (3 µg/mL) at 4°C and incubated with diluted mouse plasma (dilution, 1:100,000) for 2 h at 37°C. Subsequently, horseradish peroxidase-conjugated goat antihuman apoAI (Academy Biomedical; 11H-G1b) was added and incubated for 2 h at 37°C. Horseradish peroxidase was detected by incubation with tetramethylbenzidine (Organon Teknika, Boxtel, The Netherlands) for 15 min at room temperature. Human apoAI (Academy Biomedical; 11P-101) was used as a standard.

## In vivo clearance of VLDL-like emulsions

Glycerol tri[$^{3}$H]oleate- and [1a,2a(n)-$^{14}$C]cholesteryl oleate-double labeled VLDL-like emulsion particles (80 nm) were prepared as described by Rensen *et al*. [19]. In short, radiolabeled emulsions were obtained by adding 200 µCi of glycerol tri[$^{3}$H]oleate and 20 µCi of [$^{14}$C]cholesteryl oleate to 100 mg of emulsion lipids before sonication (isotopes obtained from GE Healthcare, Little Chalfont, U.K.). Mice were fasted for 4 h, sedated with 6.25 mg/kg acepromazine (Alfasan), 6.25 mg/kg midazolam (Roche), and 0.3125 mg/kg fentanyl (Janssen-Cilag) and injected with the radiolabeled emulsion particles (0.15 mg TG in 200 µL PBS) via the tail vein. At indicated time points after injection, blood was taken from the tail vein to determine the serum decay of glycerol tri[$^{3}$H]oleate and 20 µCi of [$^{14}$C]cholesteryl oleate.

## In vitro LPL activity assay

The effect of rHDL on LPL activity was determined essentially as described [20]. First, glycerol tri[$^{3}$H]oleate-labeled VLDL-like emulsion particles (200 µg of TG, corresponding to a final concentration of 0.5 mg/mL), prepared as described above, were added to the indicated amounts of rHDL (or vehicle containing sucrose or sodium cholate only) and heat-inactivated human serum (20 µL, corresponding to a final concentration of

5% v/v) in a total volume of 75 µL of phosphate-buffered saline. Subsequently, 0.1 M Tris.HCl (pH 8.5) was added to a total volume of 200 µL and incubation mixtures were equilibrated at 37°C. At t=0, bovine LPL (final concentration 3.5 U/mL, Sigma) in 200 µL of 120 mg/mL free fatty acid-free BSA (Sigma), corresponding with a final concentration of 60 mg/mL, was added (37°C). At t=15, 30, 60, 90 and 120 min, [$^{3}$H] oleate generated during lipolysis by LPL was extracted. Hereto, 50 µL samples were added to 1.5 mL extraction liquid ($CH_3OH$: $CHCl_3$: heptane: oleic acid (1410: 1250: 1000: 1, v/v/v/v). Samples were mixed and 0.5 mL of 0.2 M NaOH was added. Following vigorous mixing and centrifugation (10 min at 1000 g), $^{3}$H radioactivity in 0.5 mL of the aqueous phase was counted. After taking the last samples, 50 µL of the incubations were also directly counted, representing the total amount of radioactivity in the assay. Lipolysis rate (i.e. LPL activity) was calculated by linear regression between incubation time and percentage of [$^{3}$H]oleate released.

## Hepatic VLDL-TG and VLDL-apoB production

Mice were fasted for 4 h, with food withdrawn at 8:00 a.m., prior to the start of the experiment. During the experiment, mice were sedated as described above. At t=0 min blood was taken via tail bleeding and mice were i.v. injected with 100 µL PBS containing 100 µCi Trans$^{35}$S label to measure de novo total apoB synthesis. After 30 min, the animals received 500 mg of tyloxapol (Triton WR-1339, Sigma-Aldrich) per kg body weight as a 10% (w/w) solution in sterile saline, to prevent systemic lipolysis of newly secreted hepatic VLDL-TG [21]. Additional blood samples were taken at 15, 30, 60, and 90 min after tyloxapol injection and used for determination of plasma TG concentration. At 120 min, the animals were sacrificed and blood was collected by orbital puncture for isolation of VLDL by density gradient ultracentrifugation. $^{35}$S-apoB was measured in the VLDL fraction and VLDL-apoB production rate was shown as dpm.h$^{-1}$ [22].

## Statistical analysis

All data are presented as means ± SD. Data were analyzed using the unpaired Student's *t* test. *P*-values less than 0.05 were considered statistically significant.

# RESULTS

## Infusion of rHDL transiently increases plasma apoAI and phospholipid levels in both *E3L* and *E3L.CETP* mice

To investigate the role of CETP in the effects of rHDL infusion on VLDL metabolism, female *E3L* mice with or without human CETP expression received a single intravenous injection of rHDL. To assess the kinetics of rHDL that consists of humans apoAI and PL, plasma levels of human apoAI and phospholipid was determined over time (Fig. 1) rHDL caused a transient increase in plasma human apoAI and PL levels in both *E3L* mice (Fig. 1A, C) and *E3L.CETP* mice (Fig. 1B, D). Human apoAI and PL were cleared at a similar rate, and were disappeared from plasma after approximately 24 h. At 1 h after injection, lipoproteins in plasma were separated and the distribution of human apoAI and PL were determined (Fig. 2). rHDL appeared to integrate into the endogenous HDL pool in both *E3L* and *E3L.CETP* mice, since both human apoAI (Fig. 2A, B) and PL (Fig. 2C, D) eluted in fractions representing HDL. In addition, PL derived from rHDL selectively integrated into (V)LDL fraction (Fig. 2C, D). The presence of rHDL-PL in (V)LDL is not due the presence of large rHDL aggregates that would elute in the void volume, since apoAI is not detected in the void volume (Fig. 3A), but is explained by a time-dependent transfer of PL to endogenous VLDL as evident from incubation of rHDL with plasma from *E3L* mice (Fig. 3B) and *E3L.CETP* mice (Fig. 3C) in vitro.

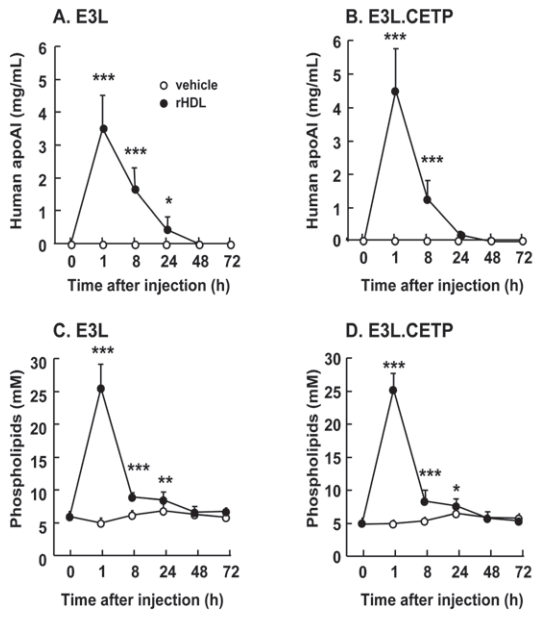


**Figure 1. Effect of rHDL on plasma human apoAI and phospholipid in E3L and E3L.CETP mice.** *E3L* (A, C) and *E3L.CETP* (B, D) mice were fed a Western-type diet for 3 weeks. Subsequently, they received a single intravenous injection of rHDL (250 mg/kg in 250 µL PBS) or vehicle. Blood was drawn at the indicated time points and plasma was assayed for human apoAI (A, B) and phospholipid (C, D). Values are means ± SD (n=8-10); **P*<0.05, ***P*<0.01, ****P*<0.001 as compared to the control group.

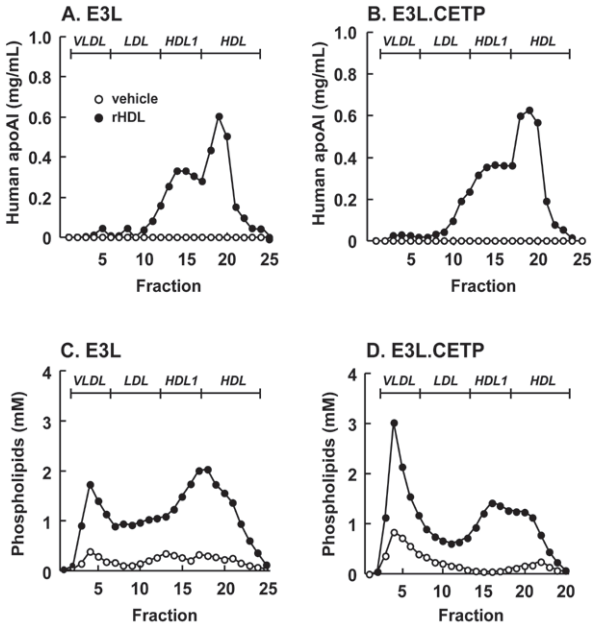


**Figure 2. Effect of rHDL on the lipoprotein distribution of human apoAI and phospholipid at 1 h after injection in *E3L* and *E3L.CETP* mice.** *E3L* (A, C) and *E3L.CETP* (B, D) mice were fed a Western-type diet for 3 weeks. Subsequently, they received a single intravenous injection of rHDL (250 mg/kg in 250 µL PBS) or vehicle. After 1 h, blood was drawn and plasma was pooled per group (n=8-10). Pooled plasma was fractionated using FPLC on a Superose 6 column and the individual fractions were assayed for human apoAI (A, B) and phospholipid (C, D).

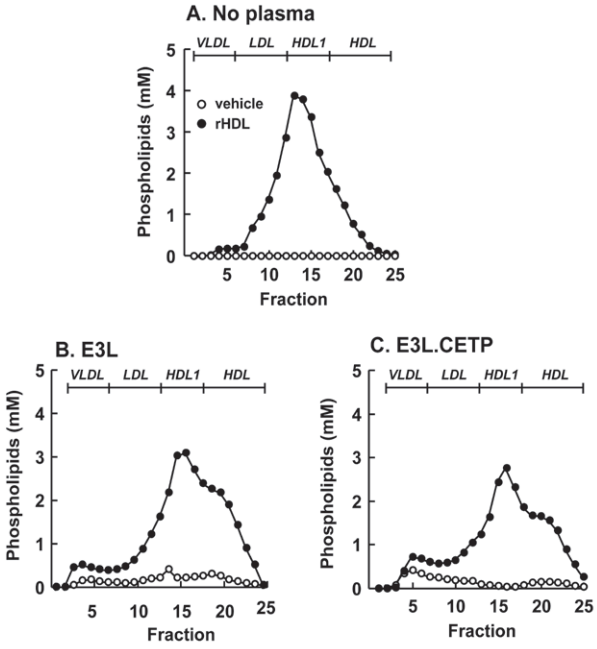


**Figure 3. Effect of in vitro incubation of rHDL with *E3L* and *E3L.CETP* mouse plasma on phospholipid distribution.** *E3L* and *E3L.CETP* mice were fed a Western-type diet for 3 weeks, and fresh plasma was collected. rHDL was incubated (1 h at 37°C) without mouse plasma (A) or with plasma of *E3L* mice (B) or *E3L.CETP* mice (C). Samples were pooled per group (n=8-10) and fractionated using FPLC on a Superose 6 column, and the individual fractions were assayed for phospholipid.

## Infusion of rHDL affects plasma levels of endogenous lipids differentially in *E3L* and *E3L.CETP* mice

Albeit rHDL was cleared at a similar rate in *E3L* and *E3L.CETP* mice, its effects on endogenous plasma levels of cholesterol and TG were clearly different in both mouse types (Fig. 4). At 1 h after injection, rHDL significantly increased plasma cholesterol (C) in both *E3L* mice (+63%; P<0.001) (Fig. 4A) and *E3L.CETP* mice (+28%; P<0.01) (Fig. 4B). However, at 24 h after injection, rHDL still significantly increased plasma cholesterol in *E3L* mice (+26%, P<0.01) (Fig. 4A) but actually decreased plasma cholesterol in *E3L.CETP* mice (-22%, P<0.01) (Fig. 4B). In addition, whereas rHDL caused a significant increase in plasma TG levels in *E3L* mice at both 1 h (+89%; P<0.01) and 24 h after injection (+67%; P<0.01) (Fig. 4C), rHDL did not significantly increase plasma TG at any time point in *E3L.CETP* mice (Fig. 4D).

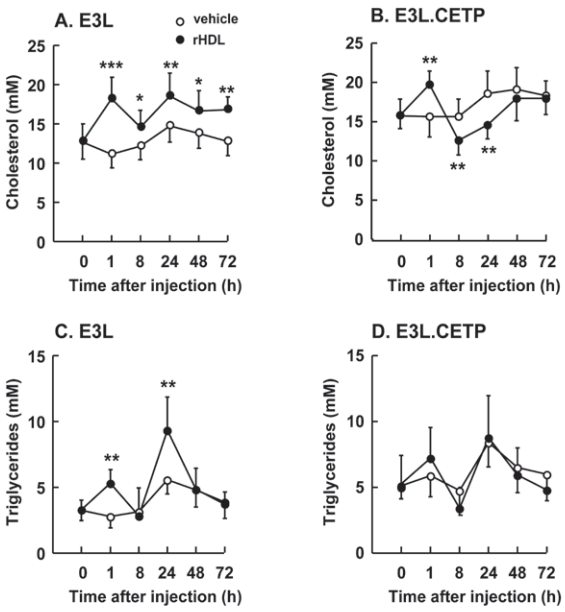


**Figure 4. Effect of rHDL on plasma cholesterol and triglycerides in *E3L* and *E3L.CETP* mice.** *E3L* (A, C) and *E3L.CETP* (B, D) mice were fed a Western-type diet for 3 weeks. Subsequently, they received a single intravenous injection of rHDL (250 mg/kg in 250 µL PBS) or vehicle. Blood was drawn at the indicated time points and plasma was assayed for total cholesterol (A, B) and triglycerides (C, D). Values are means ± SD (n=8-10); **P*<0.05, ***P*<0.01, ****P*<0.001 as compared to the control group.

## At short term, rHDL raises HDL-C in *E3L* and *E3L.CETP* mice, and increases VLDL mainly in *E3L* mice

To investigate the mechanism underlying the early effects of rHDL infusion on plasma lipids, plasma was obtained at 1 h after injection and lipoproteins were fractionated by FPLC (Fig. 5). rHDL increased HDL-C in both *E3L* mice (Fig. 5A) and *E3L.CETP* mice (Fig. 5B), indicating that rHDL induces a rapid cholesterol efflux from peripheral tissues into

plasma. In addition, rHDL markedly increased VLDL-C (Fig. 5A) and VLDL-TG (Fig. 5C) in *E3L* mice, while its VLDL-increasing effect was only modest in *E3L.CETP* mice (Fig. 5B, D). To investigate whether the raise in VLDL was due to competition between rHDL and VLDL for binding and subsequent TG hydrolysis by LPL, we assessed the effect of rHDL on the plasma kinetics of intravenously injected glycerol tri[$^{3}$H]oleate [$^{14}$C]cholesteryl oleate double-labeled VLDL-like emulsion particles (Fig. 6). Indeed, rHDL decreased the plasma clearance of the VLDL-like emulsion particles, including glycerol tri[$^{3}$H]oleate and [$^{14}$C]cholesteryl oleate, in both *E3L* mice (Fig. 6A, C) and *E3L.CETP* mice (Fig. 6B, D). An in vitro LPL activity assay confirmed that rHDL dose-dependently decreases LPL-mediated lipolysis of VLDL-like emulsion particles (Fig. 7), whereas sucrose and sodium cholate at amounts present at the various rHDL concentrations did not (not shown). These data thus indicate that rHDL competes for the binding of VLDL-like emulsion particles with LPL in both *E3L* and *E3L.CETP* mice, resulting in delayed clearance of TG-derived fatty acids (i.e. $^{3}$H-activity) as well as the resulting core remnants (i.e. $^{14}$C-activity). The fact that rHDL does not substantially raise VLDL levels in *E3L.CETP* mice is thus probably related to rapid remodeling of VLDL by CETP.

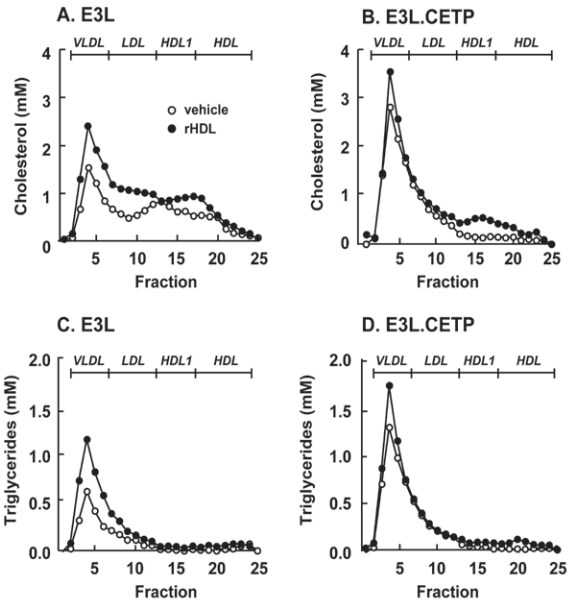


**Figure 5. Effect of rHDL on lipoprotein distribution of cholesterol and triglycerides at 1 h after injection in *E3L* and *E3L.CETP* mice.** *E3L* (A, C) and *E3L.CETP* (B, D) mice were fed a Western-type diet for 3 weeks. Subsequently, they received a single intravenous injection of rHDL (250 mg/kg in 250 µL PBS) or vehicle. Blood was drawn and plasma was pooled per group (n=8-10). Pooled plasma was fractionated using FPLC on a Superose 6 column and the individual fractions were assayed for total cholesterol (A, B) and triglycerides (C, D).

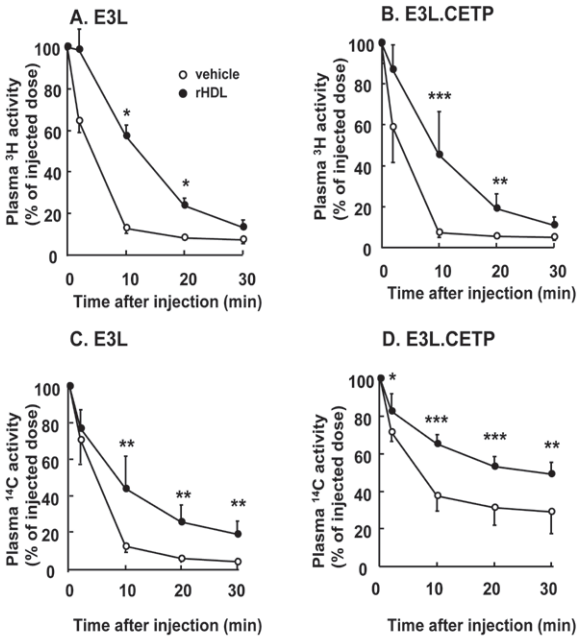


**Figure 6. Effect of rHDL on the plasma clearance of VLDL-like emulsion particles in *E3L* and *E3L.CETP* mice.** *E3L* (A, C) and *E3L.CETP* (B, D) mice were fed a Western-type diet for 3 weeks, and they received a single intravenous injection of rHDL (250 mg/kg in 200 µL PBS) or vehicle. After 1 min, mice were intravenously injected with glycerol tri[$^{3}$H]oleate- and [$^{14}$C]cholesteryl oleate-double labeled VLDL-like emulsion particles (0.15 mg TG in 200 µL PBS). Blood was drawn at the indicated time points and $^{3}$H and $^{14}$C-activity was determined. Values are means ± SD (n=8); **P*<0.05, ***P*<0.01, ****P*<0.001 as compared to the control group.

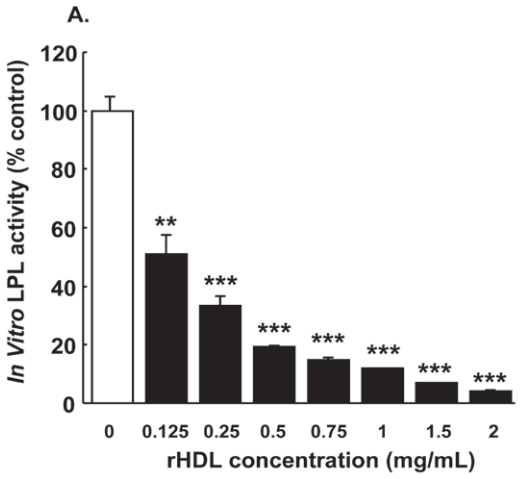


**Figure 7. Effect of rHDL on in vitro LPL activity** Glycerol tri[$^{3}$H]oleate-labeled VLDL-like emulsion particles were incubated at 37 °C with bovine LPL (3.5 U/mL) in 0.1 M Tris.HCl (pH 8.5) in the presence of heat-inactivated human serum (5%, v/v) and free fatty acid-free BSA (60 mg/mL). [$^{3}$H]oleate generated during lipolysis was extracted after 15, 30, 60, 90 and 120 min of incubation. The lipolysis rate (i.e. LPL activity) was calculated by the linear regression between incubation time and percentage of [$^{3}$H]oleate generated. Values are means ± SD (n=3); ***P*<0.01, ****P*<0.001 as compared to control incubations containing vehicle.

## At long term, rHDL raises VLDL in *E3L* mice and decreases VLDL in *E3L.CETP* mice

To determine the mechanism underlying the divergent long-term effects of rHDL infusion on plasma was also obtained at 24 h after administration and lipoproteins

were fractionated by FPLC (Fig. 8). In both *E3L* and *E3L.CETP* mice, the effect of rHDL on increasing HDL-C levels had disappeared (Fig. 8A, B). However, whereas rHDL still significantly raised VLDL-C (+60%) (Fig. 8A) and VLDL-TG (+86%) (Fig. 8C) in *E3L* mice, rHDL actually decreased VLDL-C (-25%) in *E3L.CETP* mice (Fig. 8B). Since it has been shown that increasing the flux of HDL to the liver can increase the availability of substrate for hepatic VLDL synthesis and subsequently VLDL-TG secretion [23], we speculated that rHDL may have increased the VLDL production. Therefore, the effect of rHDL on VLDL production was evaluated after injection of Triton WR1339 (tyloxapol) to block LPL-mediated lipolysis (Fig. 9). Indeed, at 24 h after administration of rHDL, the VLDL-TG production rate was increased in *E3L* mice (+36%; P<0.01) (Fig. 9A). ApoB production was not affected (Fig. 9C), indicating that rHDL increases lipidation of VLDL particles rather than increasing the VLDL particle secretion rate. Likewise, rHDL tended to increase the VLDL-TG production rate (Fig. 9B) without affecting the apoB production rate (Fig. 9D) in *E3L.CETP* mice.

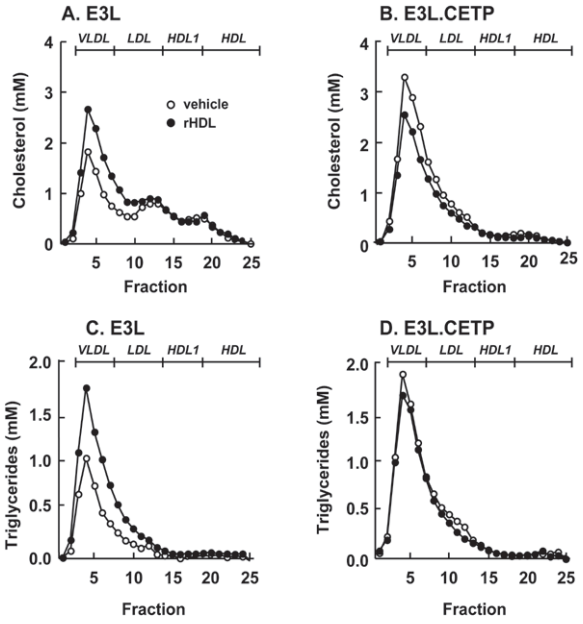


**Figure 8. Effect of rHDL on lipoprotein distribution of cholesterol and triglycerides at 24 h after injection in *E3L* and *E3L.CETP* mice.** *E3L* (A, C) and *E3L.CETP* (B, D) mice were fed a Western-type diet for 3 weeks. Subsequently, they received a single intravenous injection of rHDL (250 mg/kg in 250 µL PBS) or vehicle. Blood was drawn and plasma was pooled per group (n=8-10). Pooled plasma was fractionated using FPLC on a Superose 6 column and the individual fractions were assayed for total cholesterol (A, B) and triglycerides (C, D).

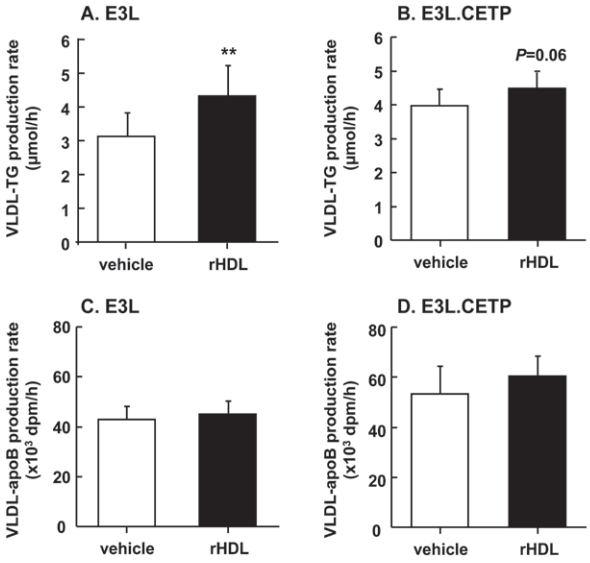


**Figure 9. Effect of rHDL on the hepatic VLDL-TG production at 24 h after injection in *E3L* and *E3L.CETP* mice.** *E3L* (A, C) and *E3L.CETP* (B, D) mice were fed a Western-type diet for 3 weeks, and they received a single intravenous injection of rHDL (250 mg/kg in 250 µL PBS) or vehicle. At 24 h after rHDL or vehicle injection, mice were injected with Trans$^{35}$S label and tyloxapol to block VLDL-TG clearance. Blood was drawn at the indicated time points and plasma TG concentrations were determined. VLDL-TG production rate was calculated from the slopes of the TG-time curves from the individual mice (A, B). At 120 after tyloxapol injection, mice were exsanguinated and VLDL was isolated by ultracentrifugation. $^{35}$S-activity was determined and VLDL-apoB production rate was calculated as dpm.h$^{-1}$ (C, D). Values are means ± SD (n=7-11); ***P*<0.01 as compared to the control group.

# DISCUSSION

In this study, we investigated the role of CETP in the effects of rHDL on VLDL metabolism by using *E3L* mice with or without human CETP expression. In both *E3L* and *E3L.CETP* mice, rHDL caused a similar transient increase in plasma human apoAI and PL levels and induced a transient increase in the endogenous HDL-C pool. However, rHDL caused an increase in VLDL in *E3L* mice, at both 1 h and 24 h after injection, which was prevented by CETP expression in *E3L.CETP* mice.

We observed that rHDL caused a rapid increase in VLDL-TC and VLDL-TG in *E3L* mice within 1 h after administration. This is in line with previous observations showing that rHDL, composed of human apoAI$_{Milano}$ and PL, also increased the VLDL-TC pool in apoE-deficient mice at 1 h after injection [10]. These effects can not be simply explained by transfer of lipid compounds from rHDL to VLDL, since rHDL does not contain cholesterol or triglycerides. Rather, we speculated that infusion of a substantial amount of rHDL may interfere with endogenous VLDL catabolism. Indeed, rHDL decreased the plasma clearance of VLDL-like emulsion particles, including the clearance of both glycerol

tri[$^{3}$H]oleate and [$^{14}$C]cholesteryl oleate. Apparently, rHDL competes with endogenous VLDL for binding to triacylglycerol hydrolases, resulting in impaired hydrolysis of TG within VLDL. Indeed, rHDL dose-dependently inhibited LPL activity in an in vitro assay. At an rHDL concentration of 0.625 mg/mL, resulting in an rHDL: TG ratio similar to the in vivo situation, rHDL inhibited LPL activity by as much as 80%. Sucrose and sodium cholate, both present in rHDL, did not inhibit LPL activity in vitro and are thus unlikely to inhibit LPL in vivo. As a consequence of LPL inhibition by rHDL in vivo, the clearance of core remnants is attenuated and plasma VLDL levels are increased. Our finding that normalization of elevated PL levels at 8 h after injection also normalized plasma TG levels in *E3L* mice is consistent with such a mechanism.

In addition to increasing VLDL at 1 h after injection, rHDL caused a second raise in VLDL-TC and VLDL-TG in *E3L* mice at 24 h after administration. Since competition of rHDL for VLDL clearance mechanisms can be excluded at this time point, as rHDL has been cleared from the circulation, we hypothesized that rHDL may have caused an increase in hepatic VLDL production. Indeed, we observed that rHDL increased the production rate of VLDL-TG without affecting the production rate of VLDL-apoB. Since each VLDL particle contains a single molecule of apoB, this indicates that rHDL infusion increases the lipidation of hepatic apoB rather than increasing the number of VLDL particles produced. Interestingly, it has previously been observed that increasing the flux of HDL to the liver by hepatic overexpression of SR-BI also increases the VLDL-TG production rate, and that HDL-derived cholesterol can be re-secreted from the liver within VLDL particles [23]. Therefore, we postulate that, in a similar manner, infusion of rHDL causes an increased net flux of lipids to the liver. We did show that rHDL transiently enhanced HDL-C, which may at least partly be attributed by efflux of cholesterol from peripheral tissues. This increased HDL-C may subsequently be taken up by the liver via SR-BI, which could then be re-secreted within VLDL. However, it is even more likely that a high flux of rHDL-associated PL to the liver enhances the amount of hepatic TG available for secretion as VLDL-TG. Indeed, it has been demonstrated that HDL-associated phosphatidylcholine that has been taken up by hepatocytes is converted to TG after phosphatidylcholine-phospholipase C-mediated hydrolysis of phosphatidylcholine resulting in diacylglycerol that is subsequently converted into TG by DGAT2 [24].

Although rHDL infusion caused a clear hyperlipidemic side-effect in *E3L* mice, there was no increase in VLDL in *E3L.CETP* mice at any time point, albeit rHDL induced a significant decrease of VLDL clearance at short term and tended to increase VLDL production at long term in *E3L.CETP* mice similarly to *E3L* mice. Expression of CETP thus clearly prevents the adverse effects of rHDL on VLDL levels. These data are in line with various human studies in which infusion of rHDL was used as an experimental

treatment of CVD [12], diabetes [25] and inflammation [26]. In these clinical studies, no specific adverse VLDL increasing effects have been reported, which is in line with our data that human CETP may protect against rHDL-induced elevation of VLDL.

It is interesting to speculate on the mechanism(s) underlying the protective effect of CETP on the rHDL-induced raise in VLDL. It is well-known that CETP mediates the transfer of CE from HDL particles to LDL and VLDL particles in exchange for TG, and that this reciprocal neutral lipid transfer approaches equilibrium under physiological conditions. However, under conditions of increased VLDL levels as observed at 1 and 24 h after administration of rHDL to *E3L* mice, the increase in VLDL results in elevated acceptor activity for CETP, which would result in an increased net rate of TG transfer from VLDL particles to both HDL and LDL particles [27]. Both TG-enriched HDL and LDL particles are avidly bound to hepatic lipase (HL) that effectively hydrolyzes TG (as well as PL) to form small dense LDL and HDL, respectively [28], thereby effectively eliminating TG from plasma.

Based on our data and literature studies, we propose the following mechanism by which CETP has a protective effect on rHDL-induced increase in VLDL. Infusion of rHDL initially decreases VLDL clearance via blocking of LPL-mediated lipolysis and, at a later stage, increases VLDL production via HDL-mediated delivery of lipids to the liver, both of which processes increase plasma VLDL levels. The transient accumulation of VLDL particles leads to an increase of the CETP activity, resulting in an accelerated transfer of TG from VLDL to HDL and LDL, in which TG is hydrolyzed quickly through the action of HL.

Since our primary research question was to examine the effect of CETP on the rHDL-induced increase on VLDL metabolism, we used saline as a control for rHDL treatment similarly as applied in clinical trials. It should be noted that such a study set-up does not allow evaluating the individual contributions of apoAI versus lipids. As the cholesterol-efflux properties of rHDL could largely depend on the apoAI moiety, it would be interesting to investigate the effect of rHDL as compared to apoAI-free lipid micelles on cholesterol mobilization into plasma as well as VLDL metabolism in future studies.

In addition to rHDL infusion therapy, other strategies to raise HDL-C are currently being developed to prevent and treat CVD, alone or combined with LDL-lowering drugs, among which CETP inhibitors. The first CETP inhibitor torcetrapib increased HDL-C levels by approximately 60% [29], but failed to demonstrate any effect on the primary atherosclerotic burden as assessed by carotid intima-media thickness and coronary intravascular ultrasound imaging [30, 31] and even increased cardiovascular events and mortality, accompanied by off-target effects [32]. However, other CETP inhibitors such as dalcetrapib [33] and anacetrapib [34] have now progressed into phase III clinical trails

without showing any off-target toxicity. Additionally, niacin effectively increases HDL by 25-30% [35] and improves carotid intima-media thickness [36]. We recently showed that the HDL-raising effect of niacin is caused by reducing the hepatic CETP expression and plasma CETP protein [37]. Since the HDL-raising effects of CETP inhibitors and niacin thus both depend on reducing CETP activity in plasma, whereas CETP activity now appears crucial to prevent the raise in VLDL as induced by rHDL infusion, combining rHDL with these HDL-raising agents could reveal adverse VLDL effects, and as a consequence counteract the potentially protective effect of rHDL in CVD.

In conclusion, our results show that rHDL infusion induces an increase in VLDL levels, which is prevented by CETP expression. Therefore, we anticipate that studies evaluating the anti-atherosclerotic efficacy of rHDL in mouse models that are naturally deficient for CETP should be interpreted with caution, and that treatment of atherogenic dyslipidemia as a risk factor for CVD by rHDL should not be combined with agents that aggressively reduce CETP activity.

## ACKNOWLEDGEMENTS

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# 3

# PIOGLITAZONE DECREASES PLASMA CHOLESTERYL ESTER TRANSFER PROTEIN MASS, ASSOCIATED WITH A DECREASE IN HEPATIC TRIGLYCERIDE CONTENT, IN PATIENTS WITH TYPE 2 DIABETES

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## ABSTRACT

Thiazolidinediones reduce hepatic steatosis and increase HDL cholesterol levels. In mice with human-like lipoprotein metabolism (*APOE*3-Leiden.CETP* transgenic mice), a decrease in hepatic triglyceride (TG) content is associated with a decrease in plasma cholesteryl ester transfer protein (CETP) mass and an increase in high-density lipoprotein (HDL)-cholesterol levels. Therefore, the aim of the present study was to assess the effects of pioglitazone on CETP mass in patients with type 2 diabetes mellitus (T2DM). We included 78 men with T2DM (age 56.5 ± 0.6 years; HbA1c 7.1 ± 0.1%) who were randomized to treatment with pioglitazone (30 mg/day) or metformin (2000 mg/day) and matching placebos, in addition to glimepiride. At baseline and after 24 weeks of treatment plasma HDL cholesterol levels and CETP mass were measured, and hepatic TG content was assessed by proton magnetic resonance spectroscopy. Pioglitazone decreased hepatic TG content (5.9 [interquartile range 2.6-17.4] versus 4.1 [1.9-12.3]%, $P<0.05$), decreased plasma CETP mass (2.33±0.10 vs. 2.06±0.10 µg/ml, $P<0.05$) and increased plasma HDL-cholesterol levels (1.22±0.05 vs. 1.34±0.05 mmol/l, $P<0.05$). Metformin did not significantly change any of these parameters. In conclusion, a decrease in hepatic TG content by pioglitazone is accompanied by a decrease in plasma CETP mass and associated with an increase in HDL-cholesterol levels. These results in patients with T2DM fully confirm recent findings in mice.

# INTRODUCTION

Hepatic steatosis is a prevalent condition in patients with type 2 diabetes mellitus (T2DM) and is associated with an increased cardiovascular risk [1, 2]. Furthermore, many patients with T2DM display dyslipidemia characterized by high plasma levels of apolipoprotein (apo) B-lipoproteins and triglycerides (TG) and low plasma levels of high-density lipoprotein (HDL) cholesterol. Recently, Toledo *et al.* [3] showed that hepatic steatosis is associated with more severe hyperlipidemia in T2DM, which might contribute to the increased risk of cardiovascular disease.

To reduce this increased cardiovascular risk in T2DM, regular treatment algorithms include lipid lowering drugs. Our previous studies in *APOE*3-Leiden.CETP* transgenic mice, a well-established model for human-like lipoprotein metabolism, showed that treatment with either statins [4], fibrates [5] or niacin [6] resulted in a reduction in plasma apoB-lipoprotein and TG levels and an increase in HDL cholesterol. Moreover, these treatments reduced hepatic lipid content (i.e., both TG and cholesterol) as well as the hepatic expression and plasma levels of cholesteryl ester transfer protein (CETP) [4-6]. CETP is a protein that mediates the heteroexchange of cholesteryl esters from HDL to (V)LDL with a simultaneous exchange of triglycerides from (V)LDL to HDL. These studies thus suggest that lowering of hepatic TG content in *APOE*3-Leiden.CETP* mice increased HDL cholesterol levels by reduction of plasma CETP mass.

Because the correlation between hepatic TG content and plasma CETP mass has not been studied in humans, the aim of this study was to evaluate whether the relationship between lowering of hepatic TG content and decreased plasma CETP mass also exists in humans. Hepatic TG content can be lowered by thiazolidinediones, including pioglitazone [7]. Indeed, in a previous study, we reported that both antidiabetic drugs pioglitazone and metformin improved insulin sensitivity in men with T2DM, whereas only pioglitazone reduced hepatic TG content [8]. Therefore, we used pioglitazone treatment as a model to study the effects of a change in hepatic TG content on CETP mass in patients with T2DM and used metformin treatment as a negative control.

# MATERIALS AND METHODS

## Study design

This study used the data from the Pioglitazone Influence on tRiglyceride Accumulation in the Myocardium in Diabetes (PIRAMID) study. This was a prospective, randomized, double-blind, intervention study, which compared the effects of pioglitazone and

metformin on cardiac function and metabolism. The results were reported previously [8]. The study design will be summarized here. The study included male patients with T2DM without cardiovascular disease or diabetes related complications. Inclusion criteria were body mass index (BMI) 25-32 kg/m$^2$, age between 45-65 years, and diabetes well controlled with metformin, a sulfonylurea or both (HbA1c 6.5-8.5%). Patients were excluded on the following criteria: uncontrolled hypertension (blood pressure > 150/85 mmHg), medical history of diabetes-related complications, liver disease, cardiovascular disease, or use of thiazolidinediones or insulin before the study. This study was executed at two hospitals in the Netherlands (Leiden University Medical Center, Leiden and VU University Medical Center, Amsterdam), and both local ethics committees gave their approval. All participants signed informed consent.

If patients met the inclusion criteria, their glucose-lowering medication was switched to glimepiride monotherapy, untill a stable dose was reached 2 weeks before the start of the intervention. At baseline, patients were randomized to metformin (500 mg twice daily, titrated to 1000 mg twice daily) or pioglitazone (15 mg once daily, titrated to 30 mg once daily after 2 weeks) in addition to glimepiride. In both the metformin and pioglitazone groups, 39 patients were included. In the pioglitazone group 34 and in the metformin group 37 patients completed the study.

Patients were studied at baseline and after 24 weeks treatment. Blood sampling and magnetic resonance spectroscopy were performed after an overnight fast.

## Hepatic triglyceride content

Hepatic TG content was measured by proton ($^1$H) magnetic resonance spectroscopy on a 1.5 Tesla whole-body magnetic resonance scanner (Gyroscan ACS/NT15; Philips, Best, the Netherlands). The technical details were described previously [8]. The voxel was placed in the hepatic parenchyma, carefully preventing placement of the voxel in vascular structures. Java Magnetic Resonance User interface software (jMRUI version 2.2, Leuven, Belgium) was used for fitting of the spectra. Technical details of spectra acquisition and spectra quantification were formerly described [8, 9]. Spectra with and without water suppression were acquired to calculate hepatic TG content relative to water (signal amplitude of triglyceride/ signal amplitude water x 100%).

## Plasma cholesterol and CETP mass analysis

All plasma samples were obtained in the postabsorptive state on the day of randomization and at 24 weeks and analyzed in one laboratory (Leiden, the Netherlands). To ascertain that we could make adequate correlations, these measurements were performed in the same blood samples. Plasma triglyceride and cholesterol concentrations were

determined using a commercially available enzymatic kit (1488872 and 236691; Roche Molecular Biochemicals, Indianapolis, IN, USA). This cholesterol assay was also used for determination of plasma HDL cholesterol after precipitation of apoB-lipoproteins from 20 µl of plasma by adding 10 µl of heparin (500 unites/ml; LEO Pharma, The Netherlands) and 10 µl of 0.2 mol/l $MnCl_2$ [6]. Plasma apoB100 was determined with a Human ApoB ELISA kit (3715-1H; Mabtech, Nacka Strand, Sweden). The plasma CETP mass was quantified using a CETP ELISA Daiichi kit (Daiichi Pure Chemicals, Tokyo, Japan).

### Statistical analysis

Data are expressed as means ± standard error of the mean (SEM) or as median (interquartile range) because hepatic TG content and plasma TG were not normal distributed. Paired *t* tests or Wilcoxon signed-ranks tests were used for within-group differences. We used ANOVA to assess between-group differences. For correlation analysis, Spearman correlation analyses were used. We used SPSS software (version 16.0, SPSS, Chicago, Illinois, USA) for statistical analyses. $P < 0.05$ was considered statistically significant.

## RESULTS

At baseline, patients in both treatment groups were well-matched for age (pioglitazone 56.8 ± 1.0 years and metformin 56.4 ± 0.9 years, between group $P > 0.05$), duration of diabetes (pioglitazone 4 [3-6] years and metformin 3 [1-5] years, $P > 0.05$), and body mass index (pioglitazone 28.2 ± 0.5 kg/m$^2$ and metformin 29.3 ± 0.6 kg/m$^2$, between group $P > 0.05$) [8].

**Table 1. Hepatic triglyceride content, plasma CETP mass, cholesterol, triglycerides, and apoB100 at baseline and after 24 weeks of treatment**

| | Pioglitazone | | Metformin | | *P* value between groups |
|---|---|---|---|---|---|
| | Baseline | 24weeks | Baseline | 24weeks | |
| Hepatic TG content (%) # | 5.9 (2.6-17.4) | 4.1 (1.90-12.3)* | 7.7 (3.7-23.9) | 10.7 (5.1-22.0) | < 0.01 |
| CETP mass (µg/ml) | 2.33 ± 0.10 | 2.06 ± 0.10* | 2.44 ± 0.08 | 2.24 ± 0.12 | NS |
| HDL cholesterol (mmol/l) | 1.22 ± 0.05 | 1.34 ± 0.05* | 1.22 ± 0.06 | 1.23 ± 0.06 | 0.02 |
| Total cholesterol (mmol/l) | 4.90 ± 0.16 | 5.19 ± 0.22* | 5.15 ± 0.18 | 4.99 ± 0.18 | < 0.01 |
| Triglycerides (mmol/l) | 1.74(0.97-3.09) | 1.37(0.88-1.80) | 2.08(1.08-2.74) | 1.23(0.68-1.92) | NS |
| ApoB100 (mg/dl) | 84.88 ± 5.08 | 75.41 ± 4.03* | 89.72 ± 5.01 | 74.58 ± 4.22* | NS |

Data are means ± SEM or median (interquartile range). # Data from van der Meer et al [8].

*Within group $P < 0.05$; NS = not significant

### Treatment effect

The effects of treatment on hepatic TG content and plasma lipid profiles are presented in Table 1. As we showed before [8], treatment with pioglitazone for 24 weeks decreased hepatic TG content [5.9 (2.6-17.4) vs. 4.1 (1.9-12.3)%, $P< 0.05$)]. This was accompanied by a decrease in plasma CETP mass (2.33±0.10 vs. 2.06±0.10 µg/ml, $P< 0.05$) and an increase in plasma HDL cholesterol levels (1.22 ± 0.05 vs. 1.34 ± 0.05 mmol/l, $P< 0.05$). Treatment with metformin did not significantly affect either of these parameters. Treatment with pioglitazone and metformin both decreased plasma TG and apoB100 significantly; however, there was no difference in the reduction between the groups (Table 1).

## Correlations

Changes in plasma CETP mass after 24 weeks in the pioglitazone-treated patients correlated with changes in hepatic TG content ($r= 0.34$, $P <0.05$), although this association was not present in the metformin group.

## Effect of statin use in the pioglitazone-treated group

In the pioglitazone group, 19 of the 39 patients used a statin at start of the study. Table 2 compares the changes in lipid levels and hepatic TG content between statin users versus non-statin users in the patients treated with pioglitazone. In nonstatin users, pioglitazone decreased liver TG content [6.4 (2.5-18.9) vs. 4.9 (1.9-14.7)%, $P< 0.05$], increased HDL cholesterol levels (1.14 ± 0.06 vs. 1.32 ± 0.06 mmol/l, $P< 0.05$), and decreased CETP mass (2.64 ± 0.14 vs. 2.16 ± 0.12 µg/ml, $P< 0.05$). However, remarkably, in statin users, pioglitazone also reduced liver TG content [8.0 (2.8-16.5) vs. 3.7 (1.9-10.9)%, $P< 0.05$], but did not affect either HDL cholesterol levels or CETP mass. ApoB100 decreased significantly only in the statin users (83.07 ± 8.29 vs. 68.77 ± 5.34 mg/dl, $P< 0.05$)

**Table 2. Hepatic triglyceride content, plasma CETP mass, cholesterol, triglycerides, and apoB100 in patients treated with pioglitazone, selected on the use of statins at baseline**

| | No statin use | | Statin use | | *P* value between groups |
|---|---|---|---|---|---|
| | Baseline | 24weeks | Baseline | 24weeks | |
| Hepatic TG content (%) | 6.4 (2.5-18.9) | 4.9 (1.90-14.7)* | 8.0 (2.8-16.5) | 3.7 (1.9-10.9)* | NS |
| CETP mass (µg/ml) | 2.64 ± 0.14 | 2.16 ± 0.12* | 1.98 ± 0.09 | 1.96 ± 0.15 | 0.03 |
| HDL cholesterol (mmol/l) | 1.14 ± 0.06 | 1.32 ± 0.06* | 1.30 ± 0.06 | 1.36 ± 0.08 | NS |
| Total cholesterol (mmol/l) | 5.32 ± 0.22 | 5.87 ± 0.35 | 4.44 ± 0.19 | 4.55 ± 0.19 | NS |
| Triglycerides (mmol/l) | 1.74(0.82-3.11) | 1.32(0.80-3.02) | 1.61(1.02-2.41) | 1.46(0.99-1.69) | NS |
| ApoB100 (mg/dl) | 86.59 ± 6.22 | 82.06 ± 5.77 | 83.07±8.29 | 68.77 ± 5.34* | NS |

Data are means ± SEM or median (interquartile range).

*Within group $P <0.05$; NS = not significant

## Discussion

In this study, we assessed the associations among changes in hepatic TG content, plasma CETP mass, and lipid profiles in patients with T2DM. The results show that pioglitazone decreased hepatic TG content, associated with decreased plasma CETP mass and increased HDL cholesterol levels. These findings are in full concordance with our recent studies in *APOE*3-Leiden.CETP* mice, which showed that classical lipid-lowering drugs concurrently lowered hepatic lipid content and decreased hepatic CETP mRNA expression, resulting in decreased plasma CETP mass [4-6].

In this study, plasma TG and plasma apoB100 decreased equivalently in both the pioglitazone and metformin group. Accordingly, other studies have shown that pioglitazone and metformin both decrease plasma TG [10-13]. Given the generally observed inverse relationship between plasma TG and HDL cholesterol found in epidemiological studies, a decrease in plasma TG may induce an increase in HDL cholesterol only. However, in the present study the decrease in plasma TG was not different between groups and plasma HDL cholesterol only increased in the pioglitazone group, indicating that another mechanism may be responsible for eliciting this difference, i.e., the reduction in CETP.

It is intriguing to speculate on the mechanism underlying the correlation between hepatic lipid content and plasma CETP mass. It is known that cholesterol derivatives are agonists for the nuclear receptor liver X receptor α (LXRα). Activation of LXRα results in increased transcription of CETP and sterol regulatory element-binding proteins-1c (SREBP-1c) [14]. Thus, our studies in *APOE*3-Leiden.CETP* mice suggest that a decrease in the hepatic cholesterol content, associated with a decrease in cholesterol derivatives, such as oxysterols, reduces LXRα activation, thereby further downregulating CETP mRNA transcription. It is therefore likely that in our study pioglitazone decreased hepatic cholesterol content in addition to TG content, thereby downregulating LXRα-activity. Although it is not possible at this time to noninvasively measure hepatic cholesterol content in humans, studies in *APOE*3-Leiden.CETP* mice with a human-like protein profile generally demonstrate a strong relation between hepatic cholesterol and hepatic TG content.

Notably, thiazolidinediones such as pioglitazone, can also upregulate LXRα expression via activation of peroxisome proliferator-activated receptor-γ, which is most dominantly expressed in adipose tissue [15]. Moreover, CETP expression is prominent in adipose tissue. Radeau *et al.* [16] have shown that CETP mRNA expression in adipose tissue correlates with plasma CETP concentrations. The increased subcutaneous fat mass as induced by pioglitazone treatment may contribute to increased plasma CETP

levels, thereby potentially counteracting the decrease in plasma CETP levels due to the decrease in hepatic lipid content by pioglitazone. Therefore, the observed decrease in plasma CETP mass due to the decrease in hepatic TG content may, in fact, be larger.

To our knowledge only one other study evaluated the effect of thiazolidinediones on CETP expression, but the hepatic TG content was not studied. Chappuis *et al.* [17] performed a cross-over study comparing pioglitazone versus rosiglitazone in 17 patients with T2DM. They found that rosiglitazone increased total cholesterol levels and decreased plasma CETP activity in accordance with our study. However, pioglitazone did not have any effect on these parameters. The differences in their results on the effects of pioglitazone compared with our study may be due to the shorter treatment duration of 12 weeks and/or to the smaller sample size in the previous study.

Finally, we found that pioglitazone did not further decrease CETP mass in patients who already used a statin. Experimental studies in *APOE*3-Leiden* mice have demonstrated that statins decrease plasma CETP mass by decreasing hepatic mRNA expression, again related to decreased hepatic cholesterol content [4]. Apparently the effect of statins is dominant over the effect of pioglitazone. We hypothesize that statins specifically decrease hepatic cholesterol content and downregulate CETP mRNA expression. Therefore, additional lowering of hepatic TG content will not result in an additional decrease in CETP expression. However, the current study was not designed to assess the effects of statins on these parameters.

A limitation of this study is that only men were included. Kinoshita *et al.* [18] showed that men have lower CETP activity than women; thus, these data may not be readily extrapolated to women with T2DM.

In summary, this study shows that in male patients with T2DM a decrease in hepatic TG content by pioglitazone is associated with a decrease in CETP mass and an increase in HDL cholesterol levels. Furthermore, we confirmed that use of statins is associated with a decreased plasma CETP mass. Both findings are in full agreement with our recent findings in *APOE*3-Leiden.CETP* mice and support the validity of these mice as a model for human-like lipoprotein metabolism.

## ACKNOWLEDGEMENTS

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# 4

# PROLONGED CALORIC RESTRICTION IN OBESE PATIENTS WITH TYPE 2 DIABETES MELLITUS DECREASES PLASMA CETP AND INCREASES APOLIPOPROTEIN AI LEVELS WITHOUT IMPROVING THE CHOLESTEROL EFFLUX PROPERTIES OF HDL

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## ABSTRACT

Using a mouse model for human-like lipoprotein metabolism we previously observed that reduction of the hepatic triglyceride (TG) content resulted in a decrease in plasma cholesteryl ester transfer protein (CETP) and an increase in HDL levels. The aim of the present study was to investigate the effects of prolonged caloric restriction in obese patients with type 2 diabetes mellitus, resulting in a major reduction in hepatic TG content, on plasma CETP and HDL levels. We studied 27 obese (BMI: 37.2±0.9 kg/m$^2$) insulin-dependent patients with type 2 diabetes mellitus (14 men, 13 women, age 55±2 years) who received a 16-week very low calorie diet (VLCD). At baseline and after a 16-week VLCD, plasma lipids, lipoproteins and CETP were measured. Furthermore, functionality of HDL with respect to inducing cholesterol efflux from human monocyte cells (THP-1) was determined. A 16-week VLCD markedly decreased plasma CETP concentration (-18%, $P<0.01$) and increased plasma apoAI levels (+16%, $P<0.05$), without significantly affecting plasma HDL-cholesterol and HDL-phospholipids. Although a VLCD results in HDL that is less lipidated, the functionality of HDL with respect to inducing cholesterol efflux in vitro was unchanged. In conclusion, the marked decrease in hepatic TG content induced by a 16-week VLCD is accompanied by a decrease in plasma CETP concentration and an increase in ApoAI levels, without improving the cholesterol efflux properties of HDL in vitro.

# INTRODUCTION

Patients with type 2 diabetes mellitus display a typical atherogenic dyslipidemia marked by increased plasma triglycerides (TG) and very low density lipoprotein (VLDL)-cholesterol concentrations, and decreased high density lipoprotein (HDL)-cholesterol levels. Furthermore, hepatic steatosis, which is also strongly associated with cardiovascular disease (CVD) risk [1, 2] is frequently observed in patients with type 2 diabetes mellitus [3-5].



Previously, we demonstrated that the HDL-raising effect of various classical lipid-lowering drugs was caused by a reduction in plasma cholesteryl ester transfer protein (CETP) that mediates the net transfer of cholesteryl esters from HDL to (V)LDL. In *APOE*3-Leiden.CETP* mice, a well-established animal model for human-like lipoprotein metabolism, statins [6], fibrates [7], and niacin [8] decrease the hepatic lipid content (i.e. both TG and cholesterol) resulting in a decreased hepatic CETP expression accompanied by decreased plasma CETP levels, and a consequently increased plasma HDL. Recently, we showed that a similar mechanism may account for the HDL-raising effect of pioglitazone in humans. In patients with type 2 diabetes mellitus, pioglitazone decreased hepatic TG content [9], accompanied by a decrease in plasma CETP concentration and increase in HDL level [10]. In contrast, metformin did not affect either hepatic TG, plasma CETP or HDL levels [10].

Lifestyle interventions such as diet-induced weight reduction and exercise are very important in the treatment of obese patients with type 2 diabetes mellitus. Recently, we reported that a 16-week very low calorie diet (VLCD) in obese patients with type 2 diabetes mellitus significantly decreased plasma total cholesterol and TG levels and markedly reduced hepatic TG content [11], but the potential beneficial effect of a VLCD on plasma CETP and HDL levels has not been studied. Therefore, using plasma samples from that study [11], we now investigated whether prolonged caloric restriction reduces CETP concentration and thereby increases HDL levels in obese patients with type 2 diabetes mellitus.

# METHODS

## Patients

The study protocol has previously been described in detail [11]. Twenty-seven obese patients with insulin-dependent type 2 diabetes mellitus (14 men and 13 women) were included (mean ± standard error of the mean: age: 55±2 years, BMI: 37.2±0.9 kg/m$^2$.

HbA1c: 7.8±0.2%). At baseline patients used 82±11 units of insulin per day with or without concomitant use of metformin and/or sulfonyl ureum derivates. Exclusion criteria were: smoking, unstable weight during 3 months before inclusion or any other chronic disease. In the previously published paper [11], we described only 12 out of the 27 patients, from whom $^{1}$H-MRS scans could be obtained. The local ethics committee approved this protocol. All patients gave written informed consent and the study was performed in accordance with the Declaration of Helsinki.



## Study design

Patients were studied before start and after completion of the 16-week VLCD. Three weeks before start of the VLCD all oral blood glucose lowering medication was stopped and insulin therapy was intensified. The day before the start of the VLCD intervention, only short-acting insulin was prescribed. Patients did not use any blood glucose-lowering medication, including insulin, during the 16-week VLCD. The VLCD consisted of 3 liquid food shakes (Modifast Intensive; kindly provided by Nutrition & Santé, Antwerp, Belgium) containing a total of 450 kcal/day and all essential micro- and macronutrients. Thirteen of the 27 subjects simultaneously followed an exercise program in addition to the VLCD. Since exercise had no effect on outcome parameters (Supplemental Table 1), data of all subjects were pooled for the present analyses.

**Supplemental Table 1. Changes in plasma CETP and (apo)lipoprotein values induced by 16 weeks of VLCD in obese patients with type 2 diabetes mellitus without or with an exercise program.**

| Plasma parameters | Non-exercise (n=14) | Exercise (n=13) | *P* value |
|---|---|---|---|
| Δ CETP (μg/mL) | -0.41 ± 0.14 | -0.49 ± 0.23 | 0.7651 |
| Δ Total cholesterol (mM) | -0.67 ± 0.34 | -0.86 ± 0.20 | 0.6356 |
| Δ Triglycerides (mM) | -0.87 ± 0.19 | -1.32 ± 0.50 | 0.4128 |
| Δ Phospholipids (mM) | -0.37 ± 0.08 | -0.45 ± 0.12 | 0.5822 |
| Δ LDL-C (mM) | -0.64 ± 0.30 | -0.63 ± 0.24 | 0.9866 |
| Δ ApoB100 (mg/dL) | -11.3 ± 7.4 | -26.8 ± 7.3 | 0.1477 |
| Δ HDL-C (mM) | 0.00 ± 0.09 | 0.15 ± 0.06 | 0.1896 |
| Δ HDL-PL (mM) | -0.03 ± 0.08 | 0.05 ± 0.05 | 0.4477 |
| Δ ApoAI (mg/dL) | 34.5 ± 12.2 | 8.2 ± 11.3 | 0.1270 |

Changes (Δ) are calculated by subtracting values obtained after VLCD from those obtained at baseline. Data are presented as means ± SEM. P-values are calculated using Unpaired Student's t test. CETP, cholesteryl ester transfer protein; VLCD, very low calorie diet; PL, phospholipids; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; HDL-PL, high density lipoprotein-phospholipids.

## Hepatic triglyceride content

Hepatic TG content was measured in supine position using $^{1}$H-MRS on a 1.5 Tesla whole-body MR scanner (Gyroscan ACS/NT15; Philips, Best, The Netherlands), exactly as previously described [11].

## Plasma (apo)lipoprotein and CETP analyses



All plasma samples were obtained after an overnight fast before the start (i.e. after stopping all glucose-lowering medication including insulin) and after the 16-week VLCD protocol, stored in aliquots at -80°C, and analyzed after thawing once in a single laboratory (Leiden, The Netherlands). To ascertain that we could make adequate correlations, all analyses were performed within the same blood samples in the same assay runs. Plasma cholesterol and TG concentrations were determined using enzymatic kits (no. 236691 and 11488872, respectively, Roche Molecular Biochemicals, Indianapolis, IN, USA). Plasma phospholipids were determined using the phospholipids B kit (Wako Chemicals, Neuss, Germany). Plasma CETP concentration was quantified using kit 'CETP ELISA Daiichi' (Daiichi Pure Chemicals Co, Ltd, Tokyo, Japan). HDL fractions were obtained after precipitation of ApoB-lipoproteins from 50 µL plasma by adding 25 µL 36% polyethylene glycol 6000 (PEG6000, no.81260, Sigma Aldrich, Inc, USA.) The HDL-cholesterol and phospholipids were determined as described above. Plasma ApoAI and ApoB100 levels were determined with the 'Human ApoAI ELISA kit' (no. 3710-1H, Mabtech AB, Sweden) and 'Human ApoB ELISA kit' (no. 3715-1H; Mabtech AB, Sweden), respectively.

## Cholesterol efflux study

Cholesterol efflux to total plasma and ApoB-depleted human plasma were determined using the human monocyte cell line THP-1 as cholesterol donor. THP-1 cells were obtained from European Collection of Cell Cultures (ECACC), and maintained in medium A (RPMI 1640 with 25 mM HEPES Buffer, supplemented with 10% fetal bovine serum, 1% L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin) at 37°C in 5% $CO_2$. Before the experiment, THP-1 cells were seeded into 24 well plates at density of $5x10^5$ cells per well and differentiated into macrophages with 0.1 µM phorbol 12-myristate-13-acetate (PMA; no. P1585, Sigma Aldrich, Inc, USA) within 3 days. Macrophages were washed three times with PBS and incubated in medium B (RPMI 1640 with 25 mM HEPES buffer, supplemented with 2% fetal bovine serum, 1% L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µg/mL acetyl-LDL and 10 µCi/ml [1α,2α(n)-$^{3}$H]-cholesterol (no.NET139001MC, Perkin Elmer, Netherlands) for 1 day at 37°C in 5% $CO_2$. After incubation, cells were washed 3 times with PBS and the efflux assay was started

by adding total human plasma or ApoB-depleted human plasma diluted to 1% in medium C (RPMI 1640 with 25 mM HEPES buffer, supplemented with 1% L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.5 mg/ml BSA). The whole assay was carried out in triplicate. To be able to normalize results between series of experiments and to correct for plate-to-plate variation, efflux to a standard preparation of HDL (50 µg protein/mL) was determined in triplicate. After 4 hours incubation, medium was collected and centrifuged. Subsequently, [$^3$H]cholesterol was quantified by liquid scintillation counting. Total cellular $^3$H-cholesterol was determined after extraction of the cells with 0.1 M NaOH. Cholesterol efflux rate was calculated by dividing the $^3$H-activity in the medium by the sum of the $^3$H-activity in the medium and the cell extract. Background values (the efflux in the absence of plasma) were subtracted.

### Statistical analysis

Data are expressed as means ± SEM. Paired *t*-tests were used for the statistical comparisons between measurements at baseline and after 16 weeks of caloric restriction. For correlation analysis, Pearson's correlation analysis was used. A *P*-value < 0.05 was considered statistically significant.

## RESULTS

In line with our previous observations in 12 patients [11], a subset of the 27 patients who were included in the present study, the VLCD profoundly reduced bodyweight from 113.1±3.7 to 87.7±2.9 kg ($P<0.05$) and decreased BMI from 37.2±0.9 to 28.9±0.8 kg/m$^2$ ($P<0.05$). In addition, in the 12 patients from whom $^1$H-MRS scans could be obtained, hepatic TG content considerably reduced from 21.2±4.2 to 3.0±0.9% (n =12, $P<0.001$) as reported previously [11].

### Plasma CETP and (apo)lipoproteins

Compared to baseline, VLCD decreased plasma CETP concentration (-18.2%, $P<0.01$). In addition, VLCD reduced plasma levels of total cholesterol (-13.1%, $P<0.001$), TG (-45.1%, $P<0.001$), phospholipid (-15.2%, $P<0.0001$), LDL-cholesterol (-15.8%, $P<0.01$) and ApoB100 (-13.9%, $P<0.01$). VLCD did not alter plasma HDL-cholesterol and HDL-phospholipids, but increased ApoAI (+16.2%; P<0.05) (Table 1). The change in bodyweight after 16 weeks of VLCD did not correlate with the change in either plasma triglycerides ($R^2$=0.0000; $P$=0.9952), total cholesterol ($R^2$=0.0471; $P$=0.2769), phospholipid ($R^2$=0.0305; $P$=0.3837), LDL-cholesterol ($R^2$=0.0320; $P$=0.3717), HDL-cholesterol ($R^2$=0.0086; $P$=0.6460) or HDL-phospholipid ($R^2$=0.0013; $P$=0.8570).

**Table 1. Plasma CETP and (apo)lipoprotein levels in obese patients with type 2 diabetes mellitus and hepatic steatosis in response to 16 weeks of VLCD.**

| Plasma parameters | Baseline | After VLCD | Delta (%) | *P* value |
|---|---|---|---|---|
| CETP (µg/mL) | 2.48 ± 0.15 | 2.03 ± 0.14 | -18.2 | 0.0021 |
| Total cholesterol (mM) | 5.76 ± 0.30 | 5.00 ± 0.22 | -13.1 | 0.0007 |
| Triglycerides (mM) | 2.41 ± 0.28 | 1.32 ± 0.10 | -45.1 | 0.0003 |
| Phospholipids (mM) | 2.69 ± 0.11 | 2.28 ± 0.07 | -15.2 | 0.0000 |
| LDL-C (mM) | 3.99 ± 0.27 | 3.36 ± 0.20 | -15.8 | 0.0028 |
| ApoB100 (mg/dL) | 130 ± 6 | 111 ± 5 | -13.9 | 0.0016 |
| HDL-C (mM) | 0.84 ± 0.04 | 0.91 ± 0.06 | - | NS |
| HDL-PL (mM) | 0.98 ± 0.03 | 0.98 ± 0.05 | - | NS |
| ApoAI (mg/dL) | 135 ± 10 | 156 ± 11 | +16.2 | 0.0174 |

Delta-values are calculated by comparing values obtained after VLCD to those obtained at baseline from obese patients with T2DM. Data are presented as means ± SEM (n=27). *P*-values are calculated using Paired Student's *t* test. CETP, cholesteryl ester transfer protein; VLCD, very low calorie diet; PL, phospholipids; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; HDL-PL, high density lipoprotein-phospholipids; NS, not significant.

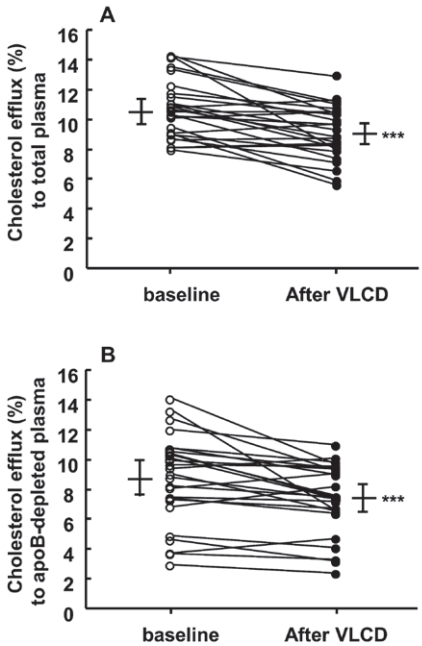
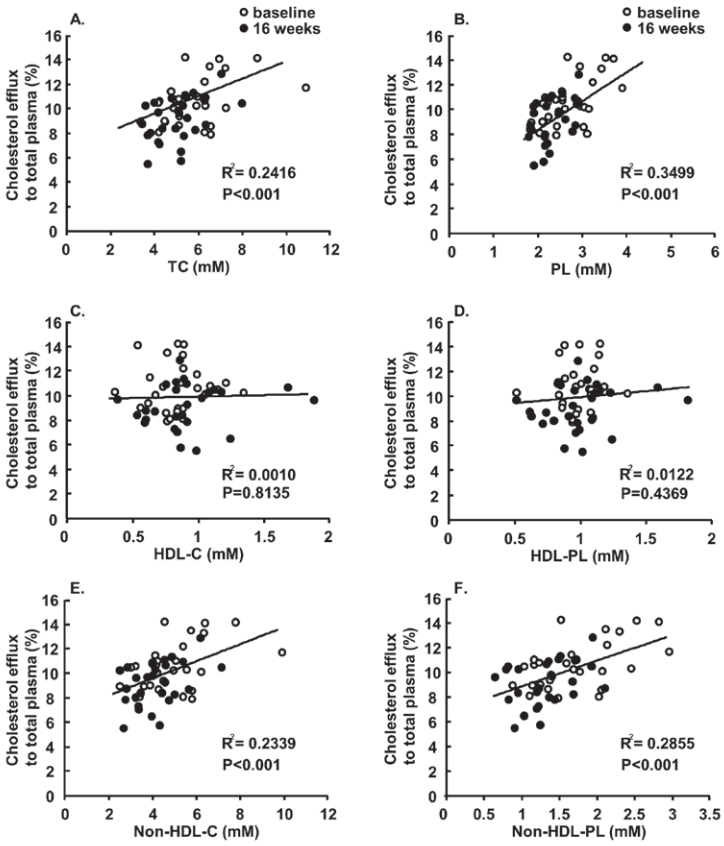



**Figure 1. Cholesterol efflux to total plasma and apoB-depleted plasma.** THP-1 cells were loaded with [3H]cholesterol and incubated for 4 h at 37°C with total plasma (1% v/v) (A) or ApoB-depleted plasma (1% v/v) (B), obtained before and after VLCD from 27 obese patients with T2DM. Cholesterol efflux rate is calculated by dividing $^{3}$H-activity in the medium by the sum of the $^{3}$H-radioactivity in the medium and cell extract. Data are means ± SEM. *P*-values are calculated using Paired Student's *t* test. ***$P<0.001$ as compared to baseline. ApoB, Apolipoprotein B; HDL, high density lipoprotein; VLCD, very low calorie diet.

## Cholesterol efflux

VLCD decreased cholesterol efflux from THP-1 cells to total plasma obtained from patients compared to plasma from baseline (-14.5%, $P<0.001$) (Fig. 1A). Similarly, the capacity of apoB-depleted plasma obtained after VLCD to promote cholesterol efflux was lower than that of apoB-depleted plasma obtained at baseline (-14.9%; $P<0.001$) (Fig. 1B).



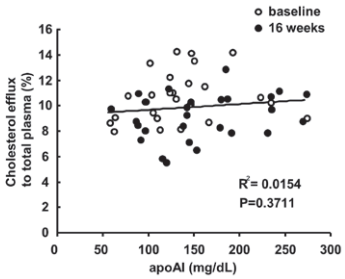



**Supplemental Figure 1. Correlation between cholesterol efflux and plasma lipids.** THP-1 cells were loaded with [$^3$H]cholesterol and incubated for 4 h at 37°C with total plasma (1% v/v) obtained at baseline and after VLCD from 27 obese patients with T2DM. Cholesterol efflux rate was calculated by dividing $^3$H-activity in the medium by the sum of the $^3$H-radioactivity in the medium and cell extract. Cholesterol efflux was plotted against total cholesterol (TC, A), phospholipid (PL, B), HDL-C (C), HDL-PL (D), Non-HDL-C (E), and Non-HDL-PL (F), and correlations were calculated.

Correlation analysis showed that cholesterol efflux to total plasma positively correlated with plasma total cholesterol ($R^2$=0.2416; $P<0.001$) and plasma total

phospholipid ($R^2$=0.3499; $P$<0.001). Cholesterol efflux to total plasma positively correlated with non-HDL-cholesterol ($R^2$=0.2339; $P$<0.001) and non-HDL-phospholipid ($R^2$=0.2855; $P$<0.001) rather than HDL-cholesterol or HDL-phospholipid (both $P$>0.05) (Supplemental Fig. 1). Moreover, no significant correlation was observed between cholesterol efflux to plasma and apoAI (Supplemental Fig. 2).






**Supplemental Figure 2. Correlation between cholesterol efflux and plasma apoAI.** THP-1 cells were loaded with [$^3$H]cholesterol and incubated for 4 h at 37°C with total plasma (1% v/v) obtained at baseline and after VLCD from 27 obese patients with T2DM. Cholesterol efflux rate was calculated by dividing $^3$H-activity in the medium by the sum of the 3H-radioactivity in the medium and cell extract. Cholesterol efflux was plotted against apoAI, and correlations were calculated.

## DISCUSSION

A main finding from the present study is that prolonged caloric restriction by a 16-week VLCD in obese patients with type 2 diabetes mellitus and hepatic steatosis, which considerably reduces hepatic TG content (-85%) [11], also markedly decreases plasma CETP concentration (-18.2%). This observation corroborates our recent finding that a reduction of the hepatic lipid content (-30.5%), as induced by pioglitazone, also associates with a reduction in plasma CETP concentration (-11.6%) in patients with type 2 diabetes mellitus [10]. However, the potency of prolonged caloric restriction to reduce hepatic lipid content and plasma CETP concentration exceeds that of pioglitazone treatment considerably.

These data are in full accordance with our previous observations that lowering hepatic lipids (i.e. TG as well as cholesterol) in *APOE*3-Leiden.CETP* mice by classical lipid-lowering drugs decreased hepatic CETP mRNA expression, resulting in decreased plasma CETP concentration [6-8]. Since CETP expression is regulated by liver X receptor alpha (LXRα) for which oxysterols are natural ligands [12] and the liver cholesterol level determines LXRα activation [13], we concluded from those studies that a decrease in hepatic cholesterol content, associated with a decrease in cholesterol derivatives,

reduces hepatic LXRα activation, thereby downregulating CETP mRNA transcription. Although it is unknown whether hepatic TG levels reflect levels of hepatic cholesterol and oxysterols in the present study, as we cannot assess hepatic (oxy)sterols noninvasively in humans, hepatic TG and cholesterol levels were highly correlated ($r$=0.867) in 33 Chinese subjects (Dr. P. Parini, personal communication). Therefore, it is likely that the reduction in plasma CETP concentration induced by VLCD also reduces hepatic LXRα-activated CETP mRNA transcription thereby reducing plasma CETP. Our data corroborate those of Laimer *et al.* [14] who showed that substantial weight loss in morbidly obese women induced by laparoscopic gastric banding surgery also decreased plasma CETP mass (-8.3%) at 1 year after surgery.

The effect of caloric restriction on HDL levels is still under debate. Although a single study reported that an average of 6 years of caloric restriction in 18 subjects increased HDL-cholesterol [15], other studies demonstrated that both 6 months and 2 years of caloric restriction in 8 subjects in fact decreased plasma HDL-cholesterol [16, 17]. Moreover, caloric restriction had conflicting effects on HDL-cholesterol among different diet groups even in one study: HDL-cholesterol was either increased or unaffected [18]. The current study is the first to show that caloric restriction by a VLCD increased the plasma level of the main HDL protein constituent ApoAI (+16.2%), which is supposed to be a good (negative) predictor of CVD risk [19]. However, the VLCD did not increase HDL-cholesterol levels. Our previous studies in mice [6-8] and in patients with type 2 diabetes mellitus [10] showed that reduction of hepatic lipids and plasma CETP, as induced by drugs, were in fact related to increased plasma HDL-cholesterol levels. In fact, treatment of only 20 patients with type 2 diabetes mellitus with pioglitazone resulted in a significant increase in HDL-cholesterol despite less pronounced decreases in hepatic lipid and plasma CETP [10], suggesting that our present study including 27 patients would not be underpowered to detect an potential effect on HDL-C. It is known that the LXRα target ATP binding cassette A1 (ABCA1) plays a crucial role in HDL maturation by mediating the lipidation of plasma ApoAI [20]. Furthermore, hepatic ABCA1 is the main contributor to the loading of HDL with cholesterol as evidenced by studies in mice that selectively lack ABCA1 from the liver [21]. Collectively, it is thus conceivable that the dramatic reduction in hepatic lipids largely downregulates the hepatic expression of ABCA1, resulting in reduced lipidation of plasma ApoAI with liver-derived cholesterol, thereby counteracting the expected rise in HDL-cholesterol due to the reduction in CETP.

The reduced ratio of HDL-cholesterol over apoAI as induced by VLCD may be expected to result in an increased ability of total plasma and ApoB-depleted plasma to induce cholesterol efflux from cholesterol-laden macrophages. However, we observed that the VLCD actually reduced the capacity of total plasma and ApoB-depleted plasma

to induce cholesterol efflux from THP-1 cells, even after discarding the three subjects showing the largest decrease in cholesterol efflux from the analysis. Correlation analysis revealed that HDL constituents (cholesterol, phospholipid and apoAI) did not correlate with cholesterol efflux to plasma. Instead, the decrease in cholesterol efflux to plasma was mainly related to the decrease in total phospholipid levels in plasma. Collectively, these data indicate that the total lipoprotein surface area in plasma, rather than the HDL level, determines the capacity of plasma to induce cholesterol efflux.



Our findings are in line with those of a recent study showing that the cholesterol efflux capacity of plasma was independent of total HDL-C or ApoAI levels [22]. In fact, independently of HDL-cholesterol, sera with high efflux capacity had a significant increase in ABCA1-mediated efflux due to the presence of preß-1 HDL. Likewise, another study showed that, although both pioglitazone and statins increase HDL-cholesterol, pioglitazone but not statins increases the cholesterol efflux capacity of plasma, and no correlation was noted between the change in HDL-cholesterol and the change in cholesterol efflux capacity [23]. Overall, the cholesterol efflux capacity of plasma is thus not simply related to HDL-cholesterol or apoAI, although the capacity of HDL to mediate cholesterol efflux from macrophages has recently been established to strongly inversely associate with carotid intima-media thickness and likelihood of angiographic coronary artery disease [23]. The fact that we were unable to detect a correlation between cholesterol efflux and HDL-cholesterol or apoAI thus probably indicates that VLCD does not improve the functionality of HDL with respect to mediating cholesterol efflux.

A limitation of the current study may be the small study group. Although we initially included 27 patients for the VLCD intervention, hepatic TG quantification by MRS was possible in only 12 patients, mainly related to limitations for maximum weight and circumference of the MRI scanner. Secondly, the design of the current human study does not permit to assess causal relationships between hepatic lipid content and plasma CETP or ApoAI level. Nonetheless, the results are in accordance with data obtained from mechanistic studies in a mouse model relevant for human lipoprotein metabolism [6-8].

In conclusion, this study indicates that prolonged caloric restriction, which considerably reduces hepatic TG content, also markedly decreases plasma CETP concentration. This is in full accordance with our previous findings in *APOE*3-Leiden.CETP* mice, in which we showed that classical lipid-lowering drugs concurrently lowered hepatic lipids resulting in decreased plasma CETP concentration. Furthermore, the VLCD increased plasma apoAI levels without improving functionality of HDL with respect to cholesterol efflux from macrophages.

## ACKNOWLEDGMENTS

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# 5

# NIACIN REDUCES PLASMA CETP LEVELS BY DIMINISHING LIVER MACROPHAGE CONTENT IN CETP TRANSGENIC MICE

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## ABSTRACT

The anti-dyslipidemic drug niacin has recently been shown to reduce the hepatic expression and plasma levels of CETP. Since liver macrophages contribute to hepatic CETP expression, we investigated the role of macrophages in the CETP-lowering effect of niacin in mice. In vitro studies showed that niacin does not directly attenuate CETP expression in macrophages. Treatment of normolipidemic human CETP transgenic mice, fed a Western-type diet with niacin for 4 weeks, significantly reduced the hepatic cholesterol concentration (-20%), hepatic CETP gene expression (-20%), and plasma CETP mass (-30%). Concomitantly, niacin decreased the hepatic expression of CD68 (-44%) and ABCG1 (-32%), both of which are specific markers for the hepatic macrophage content. The decrease in hepatic CETP expression was significantly correlated with the reduction of hepatic macrophage markers. Furthermore, niacin attenuated atherogenic diet-induced inflammation in liver, as evident from decreased expression of TNF-alpha (-43%). Niacin similarly decreased the macrophage markers and absolute macrophage content in hyperlipidemic *APOE*3-Leiden.CETP* transgenic mice on a Western-type diet. In conclusion, niacin decreases hepatic CETP expression and plasma CETP mass by attenuating liver inflammation and macrophage content in response to its primary lipid-lowering effect, rather than by attenuating the macrophage CETP expression level.

# INTRODUCTION

The anti-dyslipidemic drug niacin, also known as nicotinic acid, lowers plasma levels of pro-atherogenic lipids/lipoproteins, including very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) as well as triglycerides (TG). The lipid-lowering effect of niacin has been widely recognized as an action on adipose tissue, where it reduces the mobilization and flux of free fatty acids from adipocytes into the plasma by inhibiting intracellular lipolysis [1, 2]. In addition to lowering pro-atherogenic lipoprotein levels, niacin increases the level of anti-atherogenic high-density lipoprotein (HDL) in normolipidemic as well as hypercholesterolemic subjects [3]. Several clinical trials have shown that niacin reduces cardiovascular disease and myocardial infarction incidence, providing an emerging rationale for the use of niacin in the treatment of atherosclerosis [4, 5].

Previously, we have shown that niacin increases HDL by reducing the hepatic expression and plasma levels of the pro-atherogenic cholesteryl ester transfer protein (CETP) in *APOE*3-Leiden.CETP* transgenic mice that exhibit a human-like lipoprotein profile [6]. Importantly, a similar effect of niacin treatment on plasma CETP has also been detected in the human clinical setting (25-30% decrease; Chapman *et al.*, unpublished data). CETP, as a lipid transfer protein, has an established role in cholesterol metabolism [7]. It modifies the arterial intima cholesterol content via altering the concentration and function of plasma lipoproteins. Human population investigations favor low CETP as atheroprotective; this is supported by animal models where overexpression of CETP increased concentration of apoB-lipoprotein-cholesterol and atherosclerosis [8]. Since CETP expression is driven by liver X receptor (LXR) activation, the reduction in hepatic CETP expression may be secondary to reduced liver lipid levels. However, the exact mechanism behind the hepatic CETP-lowering effect of niacin is still unresolved.

The liver consists of several different types of cells, including hepatocytes and non-parenchymal cells such as resident macrophages, also known as Kupffer cells. Kupffer cells reside in the sinusoidal space of the liver and represent approximately 80-90% of the body's resident macrophages [9, 10]. Kupffer cells are derived from monocytes that arise from bone marrow progenitors and migrate from the circulation [11]. Interestingly, Van Eck *et al.* [12] have shown a 47-fold higher expression of CETP mRNA in liver Kupffer cells than in hepatocytes of CETP transgenic mice. Furthermore, immunolocalization studies by Pape *et al.* [13] have also suggested that non-parenchymal cells are the primary site of CETP expression in livers from cynomolgus monkeys. Combined, these studies indicate that bone marrow-derived CETP is an important contributor to hepatic CETP expression and plasma CETP mass. Since the niacin receptor GPR109A is expressed in macrophages [14, 15] and niacin has been shown to exhibit potent anti-inflammatory

activities independent of its lipid lowering action [16-18], it is important to determine whether there is a direct action of niacin on liver macrophages. The aim of the current study was therefore to investigate whether macrophages are involved in the hepatic CETP-lowering effect of niacin, by using CETP transgenic mice on a wild-type and *APOE*3-Leiden* transgenic background.

## MATERIALS AND METHODS



### Animals

Twelve to fourteen week old female CETP transgenic mice expressing the human CETP transgene under the control of its natural flanking regions (*CETP* Tg; strain 5203; C57BL/6J N10) [19] were used. The animals were fed a semi-synthetic Western-type diet containing 15% (w/w) fat and 0.25% (w/w) cholesterol (Diet W, Special Diet Services, Witham, UK) for 3 weeks (run-in), after which the diet for the treatment group was supplemented with 2% niacin (Sigma-Aldrich) for 4 weeks. Given the ~7-fold higher total metabolic rate [20, 21] and ~6-fold higher glomerular filtration rate [22] in mice as compared to humans, the 2% dose of niacin given to the mice corresponds to approximately 18 g/day for an average 70 kg human subject. Although this dosage is higher than the therapeutic dose of niacin used in the clinical setting [23], in pharmaceutical literature a relatively high dose of dietary niacin is commonly used dose to study the biological effect of niacin in mice [24-26]. After an overnight fast, mice were euthanized, bled via orbital exsanguination, and perfused in situ through the left cardiac ventricle with ice-cold PBS (pH 7.4) for 20 minutes. Tissues were dissected and snap-frozen in liquid nitrogen. One lobe of the liver was dissected free of fat and stored in 3.7% neutral-buffered formalin (Formal-fixx, Shandon Scientific Ltd., UK) for histological analysis. Animal care and procedures were performed in accordance with the national guidelines for animal experimentation. All protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

Some of the data reported here are derived from post-hoc analyses on samples from a previous study executed in *APOE3*Leiden.CETP* mice [6]. In the indicated study, female *APOE3*Leiden.CETP* mice were fed a semi-synthetic cholesterol-rich diet for 3 weeks to obtain similar total cholesterol levels. After matching, mice received a Western-type diet without or with 1% niacin (Sigma) for 3 weeks.

### Culture of bone marrow-derived macrophages

Bone marrow from female CETP Tg mice was harvested by flushing the femurs and

tibias with PBS (pH 7.4). Single-cell suspensions were prepared by passing the cells through a 70 µm cell strainer (BD, Breda, The Netherlands). Cell concentration was adjusted to 8 x $10^6$ cells/mL, and cells were placed on a non-tissue culture treated Petri dish in RPMI1640 (PAA Laboratories) containing 20% (v/v) fetal calf serum (FCS), 2 mM/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 1% (v/v) non-essential amino acids, 1% (v/v) pyruvate, and 30% (v/v) L929-conditioned media for 7 days to specifically induce macrophage differentiation. Optimal differentiation was confirmed microscopically by visual examination of cell morphology (i.e. shape) and using routine blood cell analysis (Sysmex XT-2000iV Veterinary Hematology analyzer; Sysmex Corporation). Macrophages were harvested and cultured on 12-well plate in DMEM (PAA Laboratories) containing 10% (v/v) FCS, 2 mM/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at a density of 0.5 x $10^6$ cells/mL. After 24 hours, non-adherent cells were removed, and macrophages were incubated in the absence or presence of niacin (Sigma-Aldrich) at a concentration of 0.1 µM, 1 µM, 10 µM, and 100 µM for 24 hours.



## Tissue lipid analysis

Lipids were extracted from liver using the Folch method. Briefly, 100 mg of tissue was homogenized with chloroform/methanol (1:2). The homogenate was centrifuged to recover the upper phase, which was further washed with chloroform-0.9% NaCl (1:1, pH 1.0). After centrifugation, the lower chloroform phase containing lipids was evaporated and the retained lipids were solubilized in 2% Triton X-100 by sonication. Protein content of the tissue homogenates was analyzed by BCA assay (Pierce Biotechnology, Thermo Fisher Scientific BV, IL, USA). Total cholesterol and triglyceride contents of the lipid extract were determined using enzymatic colorimetric assays (Roche Diagnostics, Mannheim, Germany). Data were expressed relative to the protein content.

## RNA isolation and gene expression analysis

Total RNA was isolated using acid guanidinium thiocyanate (GTC)-phenol-chloroform extraction. Briefly, 500 µL of GTC solution (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% N-lauroylsarcosine; Sigma-Aldrich) was added to each sample, followed by acid phenol:chloroform extraction. The RNA in aqueous phase was precipitated with isopropanol. The quantity and purity of the isolated RNA were examined using ND-1000 Spectrophotometer (Nanodrop, Wilmington, DE, USA). One microgram of the isolated RNA from each sample was converted into cDNA by reverse transcription with RevertAid™ M-MuLV Reverse Transcriptase (Promega, Madison, WI, USA). Negative controls without addition of reverse transcriptase were prepared for each sample.

Quantitative real-time PCR was carried out using ABI Prism 7700 Sequence Detection system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions using the primers in Table 1. 36B4, Beta-actin, and GAPDH were used as internal housekeeping genes. Amplification curves were analyzed using 7500 Fast System SDS software V1.4 (Applied Biosystems, Foster City, CA, USA). Transcripts that showed Ct>35 were considered not detectable. The relative expression of each gene was expressed as comparative numerical fold changes 2−(ΔΔCT). Standard error of the mean (SEM) and statistical significance were calculated using ΔΔCt formula.



**Table 1: Primers used for quantitative real-time PCR**

| Gene | Accession | Forward Primer | Reverse Primer |
|---|---|---|---|
| 36B4 | NM007475 | GGACCCGAGAAGACCTCCTT | GCACATCACTCAGAATTTCAATGG |
| ABCA1 | NM013454 | GGTTTGGAGATGGTTATACAATAGTTGT | TTCCCGGAAACGCAAGTC |
| ABCG1 | NM009593 | AGGTCTCAGCCTTCTAAAGTTCCTC | TCTCTCGAAGTGAATGAAATTTATCG |
| APOA1 | NM009692 | ACTCTGGGTTCAACCGTTAGTCA | TCCCAGAAGTCCCGAGTCA |
| ATGL | NM025802 | TGCCCTCAGGACAGCTCC | TTGAACTGGATGCTGGTGTTG |
| Beta-actin | X03672 | AACCGTGAAAAGATGACCCAGAT | CACAGCCTGGATGGCTACGTA |
| CD68 | NM009853 | CCTCCACCCTCGCCTAGTC | TTGGGTATAGGATTCGGATTTGA |
| CETP | NM000078 | CAGATCAGCCACTTGTCCAT | CAGCTGTGTGTTGATCTGGA |
| CHOP | MMCHOP10 | CTCTTGACCCTGCGTCCCTAG | TGGGATGTGCGTGTGACCT |
| FAS | NM007988 | GGCGCGGCACCTATGGCGAGG | CTCCAGCAGTGTGCGGTGGTC |
| GAPDH | NM008084 | TCCATGACAACTTTGGCATTG | TCACGCCACAGCTTTCCA |
| HSL | NM001039507 | CTGACAATAAAGGACTTGAGCAACTC | AGGCCGCAGAAAAAAGTTGAC |
| LPL | NM008509 | CCAGCAACATTATCCAGTGCTAG | CAGTTGATGAATCTGGCCACA |
| SREBP-1C | NM011480 | GGAGCCATGGATTGCACATT | CCTGTCTCACCCCCAGCATA |
| TNF-alpha | X02611 | GCC AGC CGA TGG GTT GTA | AGGTTGACTTTCTCCTGGTATGAGA |

## CETP mass determination in plasma

Plasma CETP mass was determined by ELISA, using a commercially available immunoturbidimetry kit (Daiichi Pure Chemicals, Tokyo, Japan) according to the manufacturer's instructions. Endogenous CETP activity was determined by a fluorescent method using donor liposomes enriched with nitrobenzoxadiazole-labeled cholesteryl esters (RB-CETP; Roar Biomedical, New York, NY) as described [27].

## Immunohistochemistry

Macrophage content in livers of *APOE*3-Leiden.CETP* mice treated with or without niacin [6] was analyzed by immunohistochemistry staining. The liver was embedded in O.C.T™ Compound (Tissue-Tek, Sakura finetek, Tokyo, Japan), and subsequently sectioned using a Leica CM 3050S cryostat at 8 µm intervals. After incubation with blocking solution (5% goat serum), macrophages were detected using a rat anti-murine F4/80 antibody (AbD Serotec, Oxford, UK). A rabbit anti-rat IgG/HRP was used as second antibody (Dako, Heverlee, Belgium). Sections were developed using NovaRED Peroxidase Substrate Kit (Vector Laboratories, Peterborough, UK) according to the manufacturer's instructions. Slides were counterstained with hematoxylin (Sigma-Aldrich). Apoptotic cells were detected by terminal deoxynucleotidyl transferase–mediated dUTPbiotin nick-end labeling (TUNEL) with an in situ cell death detection kit (Roche). Nuclei were counterstained with 0.3% Methylene Green.

### Statistical analysis

Statistical analyses were performed by the unpaired Student's *t*-test for independent samples (Instat GraphPad software, San Diego, USA). Statistical significance was defined as $P<0.05$. Data are expressed as means±SEM.

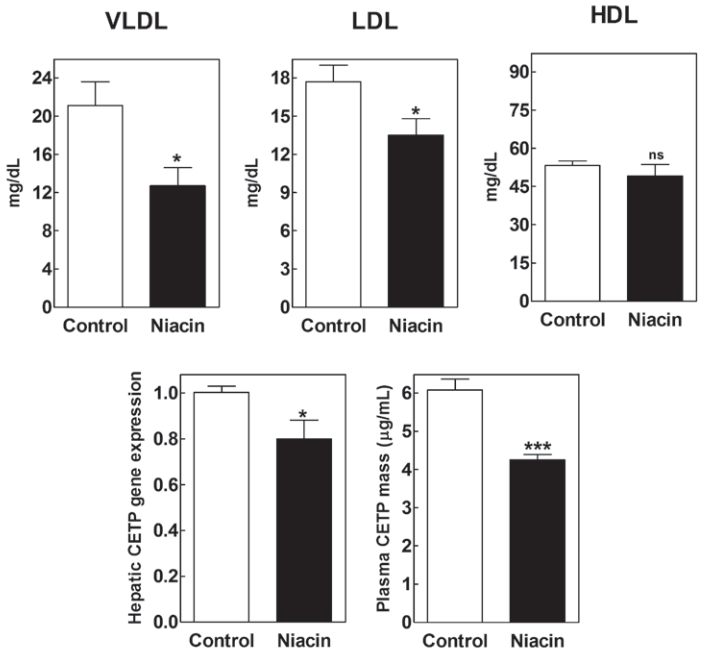



**Figure 1. Effect of niacin on plasma lipoprotein concentrations, hepatic CETP expression, and plasma CETP mass in *CETP* Tg mice**. *CETP* Tg mice were fed a Western-type diet with or without supplementation of niacin (w/w) for 3 weeks before analysis. Values are means±SEM. ns, not significant. *$P<0.05$; ***$P<0.001$.

## RESULTS

### Niacin lowers VLDL/LDL levels and reduces plasma CETP mass in *CETP* Tg mice

In agreement with its established lipid lowering capacity in the human situation, four weeks of niacin treatment induced a significant decrease in plasma levels of pro-atherogenic apoB-containing lipoproteins VLDL (-40%; $P<0.05$) and LDL (-24%; $P<0.05$) in Western-type diet fed *CETP* Tg mice (Fig. 1). Although a CETP-dependent increase in plasma HDL levels has previously been noted upon niacin treatment in our *APOE3*Leiden* mouse model [6], we did not observe a significant change in plasma HDL-

cholesterol levels in *CETP* Tg mice upon feeding the diet supplemented with niacin (Fig. 1), probably because of low (V)LDL levels as acceptor of CETP-mediated HDL-CE transfer. In line with our previous data from *APOE*3-Leiden.CETP* transgenic mice [6], niacin treatment did result in a significant reduction in hepatic CETP gene expression (-20%: P<0.05) and plasma CETP mass (-30%; P<0.001) in *CETP* Tg mice (Fig. 1). Probably due to the low amount of substrate available for CETP action, i.e. relatively low plasma VLDL/LDL levels, and associated low CETP activity already under basal (non-niacin) conditions we did not observe a concomitant decrease in the endogenous plasma CETP activity upon niacin treatment in our *CETP* Tg mouse model (0.35±0.03 mmol/mL/h for niacin vs 0.37±0.03 mmol/mL/h for controls; *P*>0.05).



Gene expression analysis on livers revealed that the relative mRNA expression level of the primary protein moiety of HDL, apolipoprotein A1 (APOA1), was as expected [28] markedly stimulated (+84%; *P*<0.05) by niacin treatment (Fig. 2). Li *et al.* [26] recently showed that activation of the niacin receptor GPR109A diminishes the hepatic expression of ABCA1 and impairs the efflux of cholesterol from hepatocytes to APOA1. In accordance, we also detected a significant decrease in hepatic ABCA1 expression (-45%; *P*<0.05) in mice subjected to niacin treatment (Fig. 2).

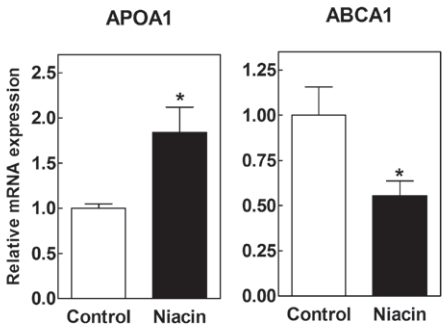



**Figure 2. Effect of niacin on HDL-associated gene expression in liver of *CETP* Tg mice.** Relative expression levels as fold compared to control of apolipoprotein A1 (APOA1) and ATP-binding cassette transport A1 (ABCA1). Values are means±SEM. *$P$<0.05.

## Niacin treatment does not affect LXR activity in livers of *CETP* Tg mice

As evident from the Oil red O stainings depicted in Fig. 3A, Western-type high cholesterol/high fat diet feeding was associated with the appearance of neutral lipid stores within hepatocytes of control mice. In contrast, livers of niacin-treated mice showed virtually no lipid droplets (Fig. 3A). Quantification of intra-hepatic lipid levels revealed that the effect on neutral lipids stores upon niacin treatment coincided with a

reduction in hepatic total cholesterol levels (-20%; $P<0.01$; Fig. 3B).

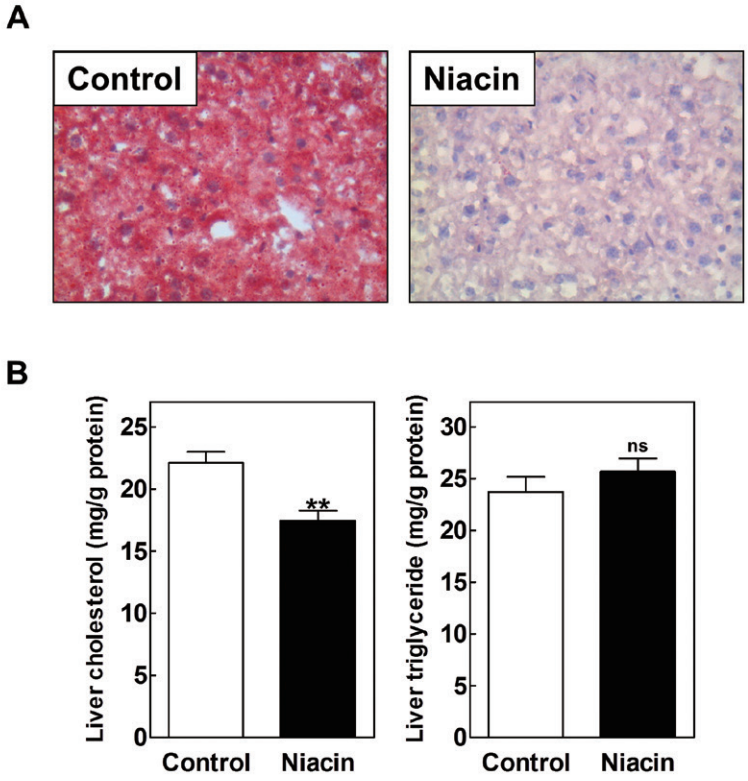



**Figure 3. Effect of niacin on hepatic neutral lipid stores in *CETP* Tg mice.** (A) Neutral lipid content was visualized by Oil red O staining. (B) Liver cholesterol and triglyceride concentrations corrected for cellular protein content. Values are means±SEM. ns, not significant. **$P<0.01$.

The cholesterol sensor liver X receptor (LXR) is able to directly stimulate CETP and ABCA1 transcription through specific LXR responsive elements in their promoter regions [29, 30]. To evaluate whether niacin decreased hepatic CETP expression and ABCA1 by attenuating LXR activation, we measured the effect of niacin on the expression of the other established LXR target genes SREBP-1C, APOE, and LPL. The hepatic expression of these three genes remained unchanged after niacin treatment (data not shown), indicating that the reduction of hepatic CETP expression was not due to a change in LXR activation upon niacin treatment.

## Niacin does not change macrophage CETP expression in vitro

Previous studies using cultured peritoneal macrophages have indicated that niacin at a concentration of 100 μM can directly affect macrophage function by altering their calcium flux [31] or gene expression profile [32] to a similar extent as observed in vivo in mice treated with doses of 0.3-1% niacin. To assess whether niacin directly attenuates CETP expression in macrophages, bone marrow-derived macrophages from *CETP* Tg mice were exposed to various concentrations of niacin (0.1 μM, 1 μM, 10 μM, and 100 μM) for 24 hours. Niacin treatment did not significantly alter CETP expression. Furthermore, niacin did not affect relative mRNA expression levels of the LXR-regulated targets SREBP-1C or APOE, or the cholesterol metabolism-related genes ABCA1, ABCG1, SR-B1, CD36 in cultured bone marrow-derived macrophages (data not shown).



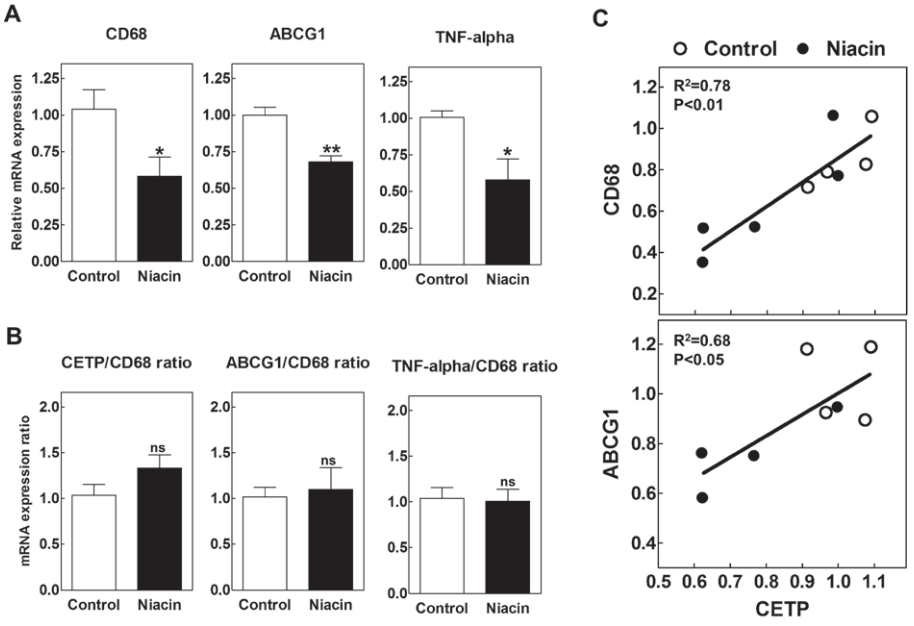



**Figure 4. Effect of niacin on hepatic macrophage gene expression in *CETP* Tg mice.** (A) Relative expression levels as fold compared to control of macrophage marker CD68, the ATP binding cassette transporter G1 (ABCG1), and tumor necrosis factor-alpha (TNF-alpha). (B) Ratios between the expression level of CETP and CD68, ABCG1 and CD68, TNF-alpha and CD68 were calculated. (C) Correlation between hepatic CETP and CD68/ABCG1 expression was linearly plotted. Values are means±SEM. ns, not significant. **P*<0.05; ***P*<0.01.

## Niacin reduces the liver macrophage content in *CETP* Tg mice

Since these data indicate that niacin may reduce hepatic CETP expression by reducing

the liver macrophage content, we evaluated the effect of niacin on the established macrophage markers CD68 and ABCG1 [33-35]. Niacin treatment significantly decreased hepatic expression of CD68 by 44% ($P<0.05$) and ABCG1 by 32% ($P<0.01$) (Fig. 4A). However, niacin did not affect the CETP/ABCG1 and ABCG1/CD68 expression ratios (Fig. 4B). Combined, these findings suggest that niacin - in line with our in vitro data - does not directly reduce the expression of CETP on macrophages, but in fact reduces hepatic CETP expression by diminishing the liver content of (CETP-expressing) macrophages.

In accordance with a decreased liver macrophage content, gene expression of the pro-inflammatory M1 macrophage subtype marker TNF-alpha decreased by 43% ($P<0.05$) after niacin treatment (Fig. 4A). Since the TNF-alpha/CD68 ratio did not change after niacin treatment (Fig. 4B) and the anti-inflammatory M2 macrophage marker interleukin-10 (IL-10) could not be detected in either treatment group (Ct>35; data not shown), it seems that treatment of CETP Tg mice with niacin did not affect the in vivo macrophage phenotype.



The comparable reductions of hepatic CETP, liver macrophage markers, and liver inflammation markers suggest that the decrease of hepatic CETP expression is caused by a reduced amount of inflammatory macrophages in liver. Indeed, as evident from Fig. 4C linear regression showed a significant and strong positive correlation between hepatic CETP and CD68 expression ($P<0.01$; $R^2=0.78$), as well as between hepatic CETP and ABCG1 expression ($P<0.05$, $R^2=0.68$).

Consistent with these results, post-hoc analysis on livers of *APOE*3-Leiden.CETP* mice treated with niacin, from our previous study, in which the CETP-lowering effect of niacin was first observed [6], revealed similar significant reductions in hepatic gene expression of the macrophage markers CD68 (-51%; $P<0.01$) and ABCG1 (-45%; $P<0.01$) (Fig. 5A). In addition, there were also significant correlations between hepatic CETP and CD68 ($P<0.001$; $R^2=0.75$) or ABCG1 ($P<0.001$; $R^2=0.85$) expression (Fig. 5A). The reduction of hepatic macrophage content was further visualized by staining of F4/80-positive cells, where niacin significantly reduced the number of macrophages in the liver by 28% ($P<0.01$) (Fig. 5B).

In agreement with a prominent contribution of the liver macrophage-derived CETP to total plasma CETP levels, we observed a significant positive correlation between the plasma CETP level and hepatic CD68 mRNA expression in *CETP* Tg mice treated with niacin ($P<0.05$; Supplemental Fig. 1A) and between the plasma CETP level and the number of macrophages in livers of *APOE*3-Leiden.CETP* mice treated with niacin ($P<0.05$; Supplemental Fig. 1B).

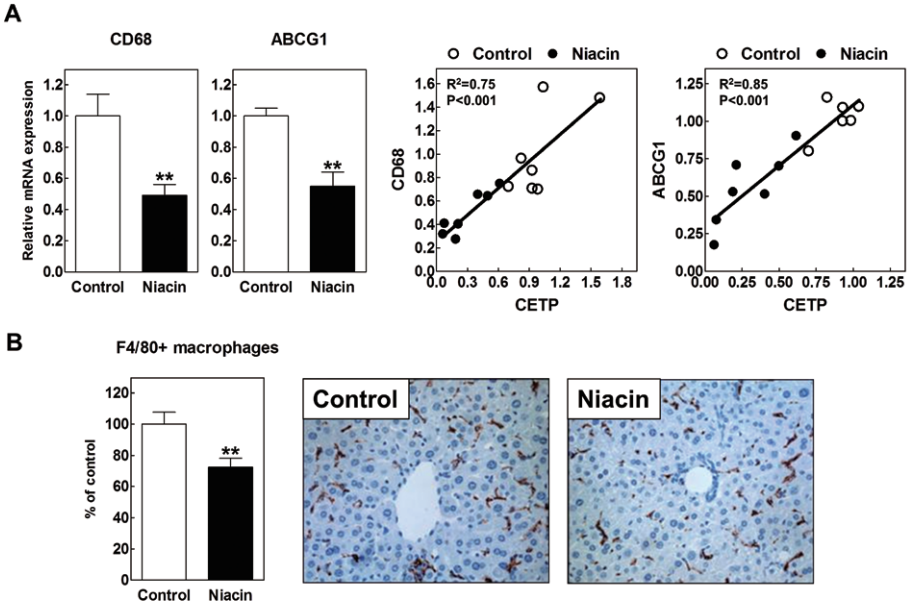
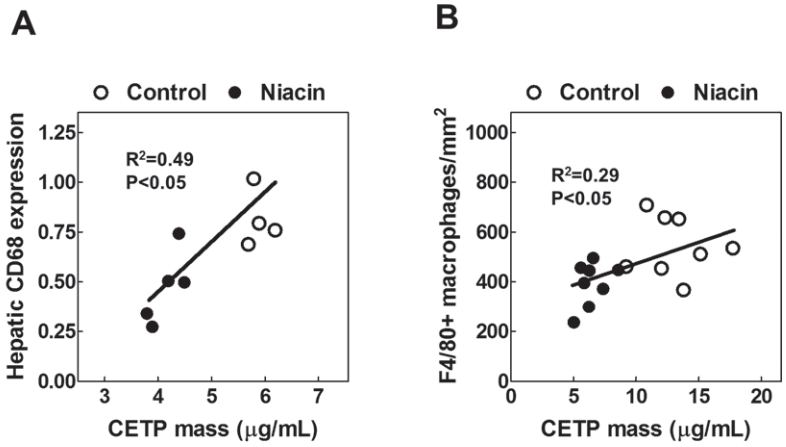



**Figure 5. Effect of niacin on hepatic macrophage gene expression and number of macrophages in *APOE*3-Leiden.CETP* mice.** (A) Relative expression levels as fold compared to control of macrophage marker CD68 and the ATP binding cassette transporter G1 (ABCG1). Correlation between hepatic cholesteryl ester transfer protein (CETP) and CD68/ABCG1 expression was linearly plotted. (B) Macrophage content in the liver was visualized via immunohistochemistry staining with F4/80 antibody, and the number of positive cells were counted and expressed as percentage of control group. Representative pictures are shown. Values are means±SEM. ***P*<0.01.




**Supplemental Figure 1. Correlations between the plasma CETP level and hepatic CD68 mRNA expression in *CETP* Tg mice treated with 2% niacin (A) and the plasma CETP level and the number of macrophages in livers of *APOE*3-Leiden.CETP* mice treated with 1% niacin (B).**

## Niacin does not induce apparent liver toxicity in *CETP* Tg mice

Mild hepatic toxicity is a known side-effect of high dose niacin treatment in humans [36]. In addition, a case of severe liver toxicity, i.e. fulminant hepatic failure, upon niacin treatment has been reported [37]. We therefore evaluated possible hepatotoxic effects of niacin treatment in the current study. The liver structure of *CETP* Tg mice fed the diet without or with niacin appeared normal. In addition, no TUNEL-positive apoptotic cells were noted in livers of either treatment group (Fig. 6A). In fact, niacin decreased the hepatic mRNA expression level of the pro-apoptotic molecule C/EBP homologous protein (CHOP; -66%; $P<0.01$; Fig. 6B) that is highly sensitive to endoplasmatic reticulum stress [38]. Combined, these findings suggest that niacin may actually diminish hepatotoxicity. In line with an overall lower hepatic stress level upon niacin exposure, plasma aspartate aminotransferase (AST/GOT) and alanine aminotransferase (ALT/GPT) levels both tended to decrease ($P=0.08$ for both) in *APOE*3-Leiden.CETP* mice subjected to niacin treatment (Fig. 6C).



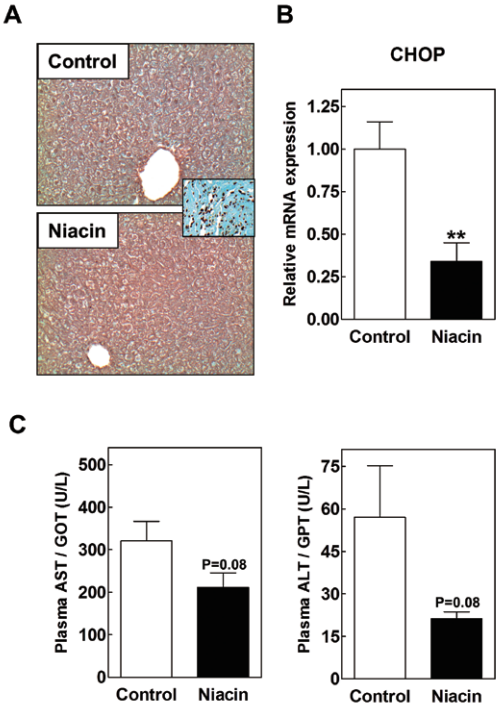



**Figure 6. Effect of niacin on liver toxicity in *CETP* Tg mice and *APOE*3-Leiden.CETP* mice.** (A) No TUNEL-positive apoptotic cells could be detected in livers *CETP* Tg mice treated with or without niacin, while our positive control (parallel stained vain graft material) did show TUNEL-positive staining (black nuclei; inset). (B) Relative expression levels as fold compared to control of C/EBP homologous protein (CHOP) in *CETP* Tg mice treated with niacin. (C) Plasma aspartate aminotransferase (AST/GOT) and alanine aminotransferase (ALT/GPT) in *APOE*3-Leiden.CETP* mice treated with niacin. Values are means±SEM. **$P<0.01$.

## Niacin modulates white adipose tissue gene expression and lipids in *CETP* Tg mice

Niacin executes its primary lipid lowering action in adipocytes within white adipose tissue, where it via GPR109a-mediated modulation of intracellular signalling pathways inhibits lipolysis by decreasing the activity of adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) [39]. We did not detect a change in the relative mRNA expression level of ATGL and HSL in abdominal white adipose tissue of niacin-treated mice, excluding a direct transcriptional effect of niacin on the ATGL-HSL axis (Fig. 7A). In contrast to what one would expect in response to the diminished lipolytic activity, a marked decrease in the white adipose tissue triglyceride content (-70%; $P<0.01$; Fig. 7B) was noted upon niacin treatment. However, in agreement with similar observations in patients with impaired glucose tolerance treated with extended release niacin [40], a significant decrease (-38%; $P<0.01$) in the white adipose tissue expression of fatty acid synthase (FAS) was also observed upon niacin exposure (Fig. 7A). Since we subjected the mice to an overnight fast, both groups of mice contained low levels of abdominal white adipose tissue at sacrifice. As a result, we did not see an apparent change in the body weight of niacin-treated mice (data not shown).

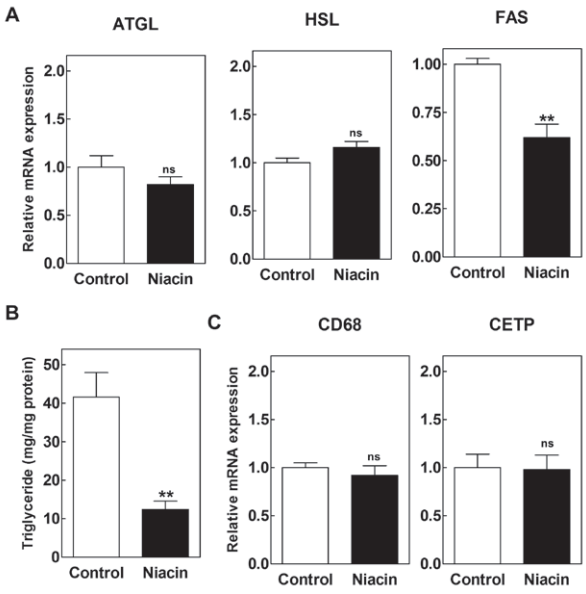



**Figure 7. Effect of niacin on abdominal white adipose tissue triglyceride content and gene expression in *CETP* Tg mice.** (A) Relative expression levels as fold compared to control of adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and fatty acid synthase (FAS). (B) White adipose tissue triglyceride concentration corrected for cellular protein content. (C) Relative expression levels as fold compared to control of macrophage marker CD68 and CETP. Values are means±SEM. ns, not significant. $^{**}P<0.01$.

The relative expression levels of CD68 and CETP in abdominal white adipose tissue were unaffected by niacin treatment (Fig. 7C). This suggests that the effect of niacin treatment on plasma CETP levels can be attributed to attenuation of the macrophage-derived CETP expression specifically in the liver and argues against a niacin-induced general cytotoxic (i.e. apoptotic/necrotic) effect on macrophages in vivo.

## DISCUSSION



Niacin lowers plasma CETP levels both in mice [6] as well as in the human clinical setting (unpublished data; Chapman et al.). However, the mechanism behind the niacin-induced decrease in CETP levels has thus far not been delineated.

To explain the CETP-lowering effect of niacin, we set out to investigate the effect of niacin on macrophages. Our observations in vitro showed that niacin at various concentrations did not reduce CETP expression in cultured macrophages derived from *CETP* Tg mice. Neither did niacin alter cholesterol metabolism-related genes in macrophages, such as ABCA1, ABCG1, and SR-B1. We thus conclude that niacin does not directly regulate expression of CETP or other lipid-related genes in macrophages.

Luo *et al.* [29] have previously demonstrated that CETP is trans-activated by nuclear receptor LXR, suggesting its role in regulating CETP expression in vivo. Therefore, we proposed in our previous study that niacin may decrease the hepatic CETP mRNA expression via LXR responsive element in the CETP promoter following decreased hepatic cholesterol content [6]. However, our current showed that niacin did not directly regulate expression of LXR-regulated target genes, such as ABCA1, in cultured macrophages in vitro. Our in vivo data further confirmed that niacin did not regulate the hepatic expression of classical LXR targets such as SREBP-1C, APOE, or LPL. In addition, although niacin treatment reduced the gene expression of ABCG1 in liver, it did not affect the ABCG1/CD68 expression ratio, indicating that niacin does not reduce the relative expression level of ABCG1 per macrophage. The reduction in ABCG1 in vivo is thus probably not simply the consequence of reduced LXR activation. Therefore, it is suggested that either direct or indirect regulation of LXRs in the liver is not the main mechanism by which niacin reduces CETP expression.

The liver is a unique immunological site responding to inflammation. Antigen-rich blood from the gastrointestinal tract and the peripheral circulation enters the hepatic parenchyma, passes through a network of liver sinusoids and is scanned by immune cells including macrophages and lymphocytes [41]. Thus, liver macrophages have profound implications in many aspects of the hepatic inflammatory response [42]. Plasma pro-atherogenic lipoproteins, mainly (V)LDLs, are important determinants of

liver inflammation. Recent evidence has indicated an increased hepatic inflammation and macrophage content upon high-fat diet-induced hyperlipidemia. In C57Bl/6J mice fed a high fat diet, upregulation of hepatic expression of CD68 was found associated with increased hepatic lipid content [43]. Another study showed that in the LDL receptor knockout mice fed a high fat diet containing cholesterol, an increase of CD68 expression in the liver was correlated with increased plasma VLDL cholesterol levels. Omitting cholesterol from the diet rapidly reduced plasma triglyceride and VLDL-cholesterol accumulation, associated with significantly lowered CD68 expression in liver together with other inflammatory genes [44]. In humans, a similar correlation between increased presence of CD68-positive Kupffer cells and the histological severity of human hepatic lipid content in fatty liver has been reported [45]. Such correlations between altered macrophage content and circulatory inflammatory factors define macrophage infiltration as a common response against hepatic and circulatory inflammation.



In the current study, niacin treatment reduced cholesterol content in the liver. In line with this attenuated liver fat accumulation, we further observed a significant reduction of the pro-inflammatory M1 macrophage marker TNF-alpha in liver. TNF-alpha is critically involved in the pathophysiology of liver steatosis, and this cytokine is primarily secreted by Kupffer cells and liver-infiltrating macrophages [46]. Taken together, the results suggested an attenuated liver inflammation after niacin treatment.

In line with the attenuated diet-induced inflammation in the liver, the hepatic gene expression of CD68 and ABCG1 were also reduced upon niacin treatment. CD68 has been defined as a reliable macrophage marker and widely used for quantification of macrophage content in numerous studies [47-49]. ABCG1 has also been shown to be a reliable marker to assess Kupffer cell content in the liver, since ABCG1 is not expressed in hepatocytes [50, 51]. In the current study, a reduction in the hepatic TNF-alpha expression coincided with decreased CD68 and ABCG1 gene expression in liver, and also a reduced number of macrophages in liver, indicating an attenuated macrophage infiltration into the liver and/or an increased macrophage efflux/emigration from the liver and thus an overall decreased liver macrophage content. More importantly, the significant positive correlation between hepatic CETP and both CD68 and ABCG1 expression observed in both the current study and in the present post-hoc analysis of our previous study [6] suggests that the liver macrophage is a primary contributor to hepatic and total plasma CETP mass, and that the hepatic CETP reduction induced by niacin treatment is a direct consequence of a reduced macrophage content of the liver.

Fig. 8 illustrates the proposed mechanism underlying the action of niacin on hepatic CETP expression. We propose that the primarily reduced hepatic cholesterol accumulation via the lipid-lowering effect of niacin leads to attenuated hepatic

inflammation, and thus less macrophage infiltration into and/or increased macrophage efflux/emigration out of the liver. The decreased amount of hepatic macrophages leads to an overall reduction in hepatic CETP expression and a lower plasma CETP level.

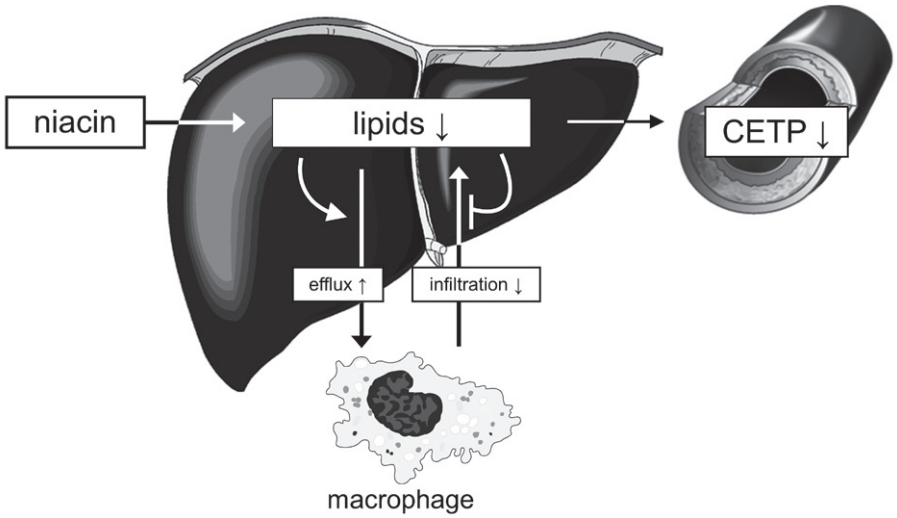



**Figure 8. Proposed mechanism underlying the action of niacin on hepatic CETP expression and plasma CETP mass.** We propose that the primarily reduced hepatic cholesterol accumulation via the lipid-lowering effect of niacin leads to attenuated hepatic inflammation, and thus less macrophage infiltration into and/or increased macrophage emigration out of the liver. The decreased amount of hepatic macrophages, which are significant contributors of CETP, leads to an overall reduction in hepatic CETP expression and a lower plasma CETP level.

In conclusion, our study sheds new light on the mechanism underlying the CETP-lowering effect of niacin. We have shown that niacin does not directly alter macrophage CETP expression, but attenuates liver inflammation and the macrophage content in response to its primary lipid-lowering effect, which leads to a decrease in hepatic CETP expression and plasma CETP mass. These findings further substantiate our working hypothesis that CETP in plasma is primarily derived from bone marrow-derived cells, i.e. macrophages.

## ACKNOWLEDGEMENTS

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# 6

# PLASMA CHOLESTERYL ESTER TRANSFER PROTEIN: A BIOMARKER FOR HEPATIC MACROPHAGES

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## ABSTRACT

Cholesteryl ester transfer protein (CETP) reduces HDL-cholesterol and is a pharmaceutical target for treating dyslipidemia. However, the cellular origin of plasma CETP in humans is uncertain. We aimed to elucidate the relative contribution of adipose tissue and liver to plasma CETP pools and the cellular origin of plasma CETP. To assess the association between adipose tissue mass and CETP, waist circumference and plasma CETP were measured in 1434 subjects from a general population. To further characterize the source of CETP, biopsies of liver and adipose tissue from 94 subjects were analysed for gene and protein expression, and these were correlated with plasma CETP and lipid parameters. Detailed mechanistic studies on the cellular origin of CETP and mode of action of lipid-lowering drugs that lower plasma CETP were performed using *APOE*3-Leiden.CETP* (*E3L.CETP*) transgenic mice, a well-established model for human-like lipoprotein metabolism. Plasma CETP did not correlate with waist circumference, suggesting that central adipose tissue does not contribute to plasma CETP. Microarray analysis of liver and adipose tissue biopsies showed that CETP expression was highest in liver. Immunohistochemistry revealed that hepatic CETP is primarily expressed by macrophages. CETP expression in liver, but not adipose tissue, positively correlated with plasma CETP, and inversely correlated with plasma HDL-cholesterol. Selective elimination of macrophages from liver versus adipose tissue in *E3L.CETP* mice virtually abolished hepatic CETP expression, accompanied by largely reduced plasma CETP and increased plasma HDL-cholesterol. Lipid-lowering drugs that are known to reduce plasma CETP and to increase HDL in humans, reduced the hepatic macrophage content, simultaneously reducing plasma CETP and increasing HDL-cholesterol in *E3L.CETP* mice. In conclusion, plasma CETP is predominantly derived from hepatic macrophages, and may serve as a plasma biomarker for hepatic macrophage content in non-alcoholic steatohepatitis.

# INTRODUCTION

The metabolic syndrome (MetS) is a complex disorder defined by interrelated risk factors for type 2 diabetes and cardiovascular disease, including central obesity, hyperglycemia, hypertension and dyslipidemia. In particular, many patients with the clinical diagnosis of MetS show a pro-atherogenic lipid profile with increased very low density lipoprotein (VLDL)-triglycerides (TG) and decreased high density lipoprotein (HDL)-cholesterol (C) [1]. Cholesteryl ester (CE) transfer protein (CETP) plays a pivotal role in the metabolism of VLDL and HDL by mediating the transfer of TG from VLDL to HDL in exchange for CE, resulting in increased (V)LDL-C and decreased HDL-C [2]. Genetic deficiency for CETP increases HDL-C and lipid-lowering compounds that reduce plasma CETP induce a beneficial lipoprotein profile including reduced VLDL-TG and raised HDL-C [3,4]. Therefore, CETP inhibition is a current target for the treatment of low HDL and the pro-atherogenic lipid profile associated with MetS to reduce cardiovascular disease risk [5].



Previous studies have demonstrated that liver and adipose tissue are the two major sources of circulating CETP in several mammalian species. Other tissues, such as spleen, heart, small intestine, adrenal gland and skeletal muscle express CETP mRNA to only a minor extent [6-10]. However, the relative contribution of liver and adipose tissue to total plasma CETP, and the cell types involved in CETP synthesis, remain to be unambiguously determined. Few studies have suggested that hepatocytes may be responsible for the expression and secretion of CETP from the liver [6]. However, other studies suggested that nonparenchymal cells including Kupffer cells are the principal source of CETP in the liver [6,11]. Although adipose tissue CETP expression was shown to be associated with plasma CETP in a small cohort of 13 men [12], an association between plasma CETP and adiposity has not been conducted in large general populations as yet.

To design novel CETP-target strategies and to understand their ability to reduce cardiovascular disease risk, it is crucial to understand the cellular origin of plasma CETP. Therefore, in this study we aimed to elucidate the cellular origin of CETP in the *APOE*3-Leiden.CETP* mouse model and in a translational setting using human cohorts. These mice express the human CETP gene under control of its natural regulatory flanking regions, ensuring a similar CETP expression pattern as in humans [13-16]. Our data show that the liver, rather than adipose tissue, predominantly contributes to plasma CETP, and that hepatic CETP is exclusively produced by macrophages. Moreover, specific lipid-lowering drugs decrease the hepatic macrophage content and thus increase HDL-C via reduction of hepatic CETP production. We therefore conclude that plasma CETP is a biomarker for the macrophage content of the liver.

## MATERIALS AND METHODS

### Design of human studies

Two independent populations were selected. The first cohort was obtained from the general population in Rijswijk, The Netherlands, consisting of 1434 non-diabetic subjects between 40-70 years of age (654 males, 780 females). Exclusion criteria included diagnosed diabetes, known terminal disease, and a history of psychiatric disorder or substance abuse. Waist circumference was measured and venous blood samples were taken after overnight fasting (approx. 12 h) for measurement of the plasma CETP concentration. The Rijswijk study was approved by the review board of South West Holland and performed in accordance with the Declaration of Helsinki.



The second study consisted of 93 severely obese subjects (BMI 30-74 kg/m$^2$) who underwent elective bariatric surgery from 2006 to 2009 at the Dept. of General Surgery, Maastricht University Medical Center, Maastricht, The Netherlands, as described [17]. Subjects using anti-inflammatory drugs or having acute or chronic inflammatory diseases, degenerative diseases, and subjects reporting alcoholic intake >10 g/day, were excluded. During surgery, biopsies from liver, subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) were taken for mRNA isolation and hybridization. Venous blood samples were obtained after overnight fasting (approx. 8 h) on the morning of surgery for analysis of the plasma CETP concentration and lipid parameters. This study was approved by the Medical Ethics Board of Maastricht University Medical Centre, in line with the Declaration of Helsinki. All participants provided informed written consent.

Details of all parameters measured in both population cohorts are provided in the Methods section in the Supplementary Appendix.

### Design of mouse studies

Female *APOE*3-Leiden.CETP* (*E3L.CETP*) transgenic mice expressing the human CETP gene under the control of its natural flanking regions [18] were used, and housed under standard conditions with a 12 h light/dark cycle with free access to food and water unless indicated otherwise. Mice were fed a semi-synthetic Western-type diet (WTD), containing 0.1% (w/w) cholesterol, 1% (w/w) corn oil and 15% (w/w) cocoa butter (Hope Farms, Woerden, The Netherlands).

In a first experiment, mice were fed WTD for 4 weeks, randomized according to body weight, plasma total cholesterol (TC) and triglyceride (TG) levels, and received two intraperitoneal injections of liposomal clodronate (4 ml/kg bodyweight; purchased from Dr. N. van Rooijen, VUmc, Amsterdam) at a 3-day interval to deplete macrophages from the liver [19, 20], and were terminated 3 days after the second injection. In a second

experiment, mice were fed WTD, without (control) and with 0.04% (w/w) fenofibrate or 1% (w/w) niacin (both from Sigma, St. Louis, MO, USA) for 4 additional weeks before sacrificing. Experiments were performed after 4 h of fasting at 12:00 pm with food withdrawn at 8:00 am. The institutional Ethical Committee on Animal Care and Experimentation from the Leiden University Medical Center, Leiden, The Netherlands had approved all animal experiments.

In both experiments, blood was obtained via tail vein bleeding into heparin-coated capillary tubes. The tubes were placed on ice and centrifuged, and the obtained plasma was snap-frozen in liquid nitrogen and stored at -20°C until further analysis. Plasma was assayed for CETP and lipid concentrations and lipoprotein profiles (see the Methods section in the Supplementary Appendix). After mice had been sacrificed, liver and gonadal adipose tissue samples were collected to measure the expression of selected genes by quantitative real-time PCR and proteins by immunohistochemistry (see the Methods section in the Supplementary Appendix).



### Statistical Analysis

Categorical variables are presented as frequencies and percentages, and continuous variables as means and standard deviations, or medians and interquartile ranges for variables with skewed distributions. Pearson correlation was used to estimate the association between waist circumference and plasma CETP in Rijswijk study. In the bariatric surgery cohort, Spearman correlation was used to determine the correlation between expression of *CETP* and *MARCO* in the liver, SAT, VAT; the association between *CETP* expression in liver, SAT, VAT and plasma CETP level; as well as the association between *CETP* expression in liver, SAT, VAT and plasma HDL-C level, respectively. Statistical differences between groups were assessed with the nonparametric Mann-Whitney U test for two independent groups or two-way ANOVA with Tukey's post-hoc test for multiple comparisons. All reported *P* values are two-tailed, and *P* values of less than 0.05 were considered statistically significant.

## RESULTS

### Waist circumference is not associated with plasma CETP concentration in humans

The characteristics of 1434 subjects (654 males and 780 females) from a non-diabetic population in Rijswijk are shown in Supplementary Table 1. Mean (±SD) waist circumference was 99 ± 11 cm for males (n=654) and 89 ± 12 cm for females (n=780). The

median value for plasma CETP concentration was significantly lower in males [2.31 (1.90-2.72) µg/ml] compared to females [2.44 (2.02-2.86) µg/ml] ($P$<0.001). However, plasma CETP did not differ between quintiles of waist circumference in either males (Fig. 1A; $P$=0.328) or females (Fig. 1B; $P$=0.571), and no correlation between waist circumference and plasma CETP concentration was observed in either males (Fig. 1C; $r$=0.007, $P$=0.857) or females (Fig. 1D; $r$=-0.024, $P$=0.509). Excluding subjects who received lipid-lowering medication (statins) (82 males and 61 females) did not change the results for males ($r$=0.023, $P$=0.583) and females ($r$=-0.003, $P$=0.930). Our findings suggest that central obesity measured as waist circumference does not correlate with the plasma CETP in a general population.



**Supplementary Table 1 Characteristics of the non-diabetic population cohort in Rijswijk study. ***

| Characteristic | Male (N=654) | Female (N=780) |
|---|---|---|
| Age — yr | 54.0±8.2 | 53.7±8.3 |
| Current smoking — no. (%) | 189 (28.9) | 202 (25.9) |
| Metabolic Syndrome † — no. (%) | 293 (45) | 245 (32) |
| Medication — no. (%) | | |
| Statin | 82 (12.5) | 61 (7.8) |
| Blood pressure lowering medication | 128 (19.6) | 132 (16.9) |
| Blood pressure — mmHg | | |
| Systolic blood pressure | 136±19 | 127±20 |
| Diastolic blood pressure | 84±11 | 81±11 |
| Waist circumference — cm | 99±11 | 89±12 |
| Total cholesterol — mmol/liter | | |
| Median | 5.58 | 5.67 |
| Interquartile range | 4.84-6.27 | 4.99-6.38 |
| HDL-cholesterol — mmol /liter | | |
| Median | 1.13 | 1.44 |
| Interquartile range | 0.96-1.33 | 1.23-1.70 |
| Triglycerides — mmol /liter | | |
| Median | 1.32 | 1.05 |
| Interquartile range | 0.93-1.90 | 0.81-1.45 |
| Glucose – mmol/liter | 5.44±1.16 | 5.13±0.77 |
| CETP — µg/ milliliter | | |
| Median | 2.31 | 2.44 |
| Interquartile range | 1.90-2.72 | 2.02-2.86 |

* Plus-minus values are means±SD. To convert values for cholesterol to milligrams per deciliter, divide by 0.02586. To convert values for triglycerides to milligrams per deciliter, divide by 0.01129. HDL denotes high-density lipoprotein; CETP denotes cholesteryl ester transfer protein.

† The diagnosis of metabolic syndrome is based on the IDF criteria.[36] Therefore two or more of the following criteria in addition to an increased waist circumference (male ≥ 94 cm, female ≥ 80 cm), had to be present: triglycerides ≥ 1.7 mmol/l (≥ 150mg/dl), HDL-cholesterol < 1.03 mmol/l (<40 mg/dl) in male and < 1.29 mmol/l (<50 mg/dl) in female, fasting glucose ≥ 5.6 mmol/l (150 mg/dl), blood pressure ≥ 130/85 mmHg or the use of blood pressure lowering medication.

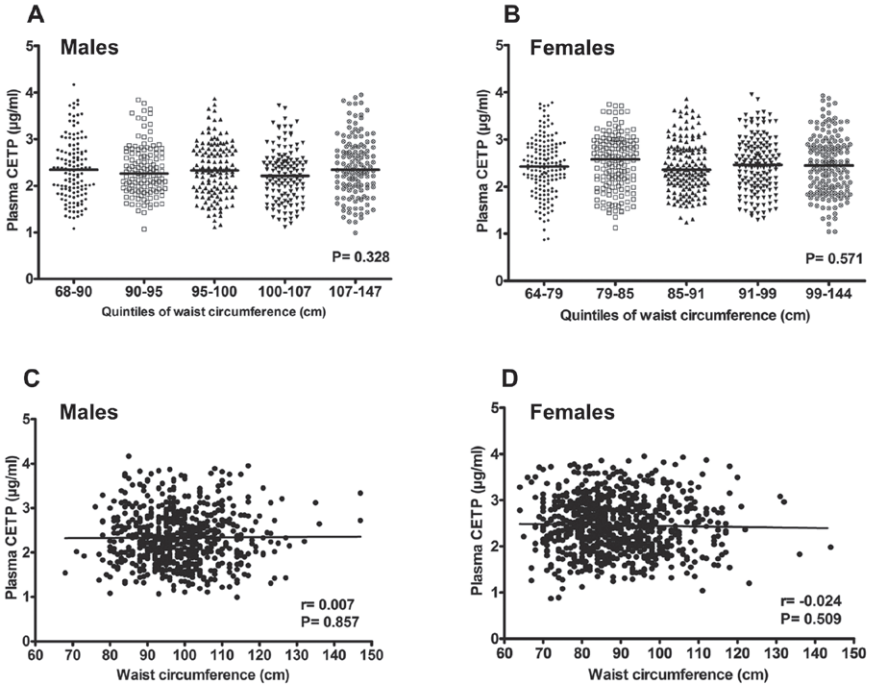



**Figure 1. Waist circumference is not associated with plasma CETP concentration in a general population.** In 1434 subjects that were enrolled in the Rijswijk study, median plasma CETP concentration over quintiles of waist circumference in male (A) and female (B) subjects, associations between plasma CETP concentration and waist circumference in male (C) and female (D) subjects were determined.

## Hepatic CETP expression, specifically in macrophages, determines the plasma CETP concentration and HDL-cholesterol level in humans

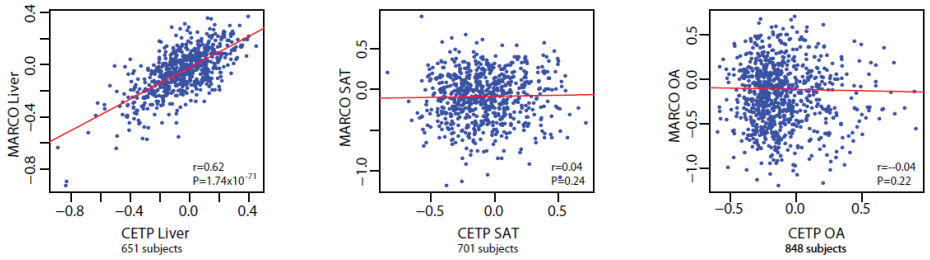
The characteristics of 93 severely obese patients who underwent elective bariatric surgery are shown in Table 1. Biopsies from livers and adipose tissue [i.e. subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT)] were taken during surgery and gene expression profiles were determined by micro-array analyses as described previously.[17] These profiles revealed that the CETP mRNA transcript is much more abundant in liver than in VAT and SAT ($P$=2.22x10$^{-33}$ and $P$=1.9x10$^{-27}$, respectively). Next, the associations between the expression of *CETP* and other genes in VAT, SAT and liver was evaluated. Strikingly, the top genes that correlated highest with hepatic CETP expression are specific macrophage markers including *TIMD4* ($r$=0.631) and *MARCO* ($r$=0.590). In contrast, *CETP* did not correlate with macrophage-specific genes

in adipose tissue. For example, expression of *CETP* and *MARCO* were correlated in liver ($r$=0.590; $P$<0.0001; Fig. 2A) in liver but not in SAT ($r$=-0.092, $P$=0.388; Fig. 2B) or VAT ($r$=0.035, $P$=0.750; Fig. 2C). Using a publicly available considerably larger dataset undergoing bariatric surgery (1008 subjects) [21], we were able to replicate the strong association between *CETP* expression and *MARCO* in liver ($r$=0.624, $P$=1.7391e-71), but not in VAT (e.g. omental adipose tissue, $r$=-0.0423, $P$=0.2183) or SAT ($r$=0.043, $P$=0.2448) (Supplementary Fig. 1). Immunohistochemical stainings revealed that, in the liver, CETP specifically co-localized with $CD68^+$ macrophages (Fig. 2D).

**Table 1 Characteristics of severely obese subjects underwent the bariatric surgery**

| | |
|---|---|
| Age — yr | 44.2±9.7 |
| Male sex — no. (%) | 26 (28.0%) |
| Body mass index† | 46.1±9.5 |
| Glucose- mmol/liter | 46.45±1.98 |
| Cholesterol — mmol /liter | |
| Total | 5.08±1.12 |
| LDL | 3.21±1.02 |
| HDL | 0.98±0.37 |
| Triglycerides — mmol /liter | 2.22±1.98 |
| CETP — µg/ milliliter | 2.70±1.02 |

Importantly, hepatic CETP expression positively correlated with plasma CETP (r=0.519, p<0.0001; Fig 2E), and inversely correlated with plasma HDL-C (r=-0.204, p=0.075; Fig 2H). In contrast, no significant correlation between expression of CETP in VAT and plasma CETP (p=0.140; Fig 2G) or HDL-C (p=0.250; Fig 2J) was noted. Although CETP expression in SAT correlated with plasma CETP (P<0.01; Fig 2F), it did not correlate with plasma HDL-C (p=0.123; Fig 2I). These data suggest that CETP expression in hepatic macrophages determines plasma CETP levels as well as the CETP-induced effects on HDL levels.




**Supplementary Figure 1· Association of expression of CETP and MARCO in liver and adipose tissue**
Scatter plots of the correlation between mRNA level of CETP and MARCO in liver (A), subcutaneous adipose tissue (SAT) (B) and omental adipose (OA) (C) obtained from 1008 subjects undergoing bariatric surgery.[21]

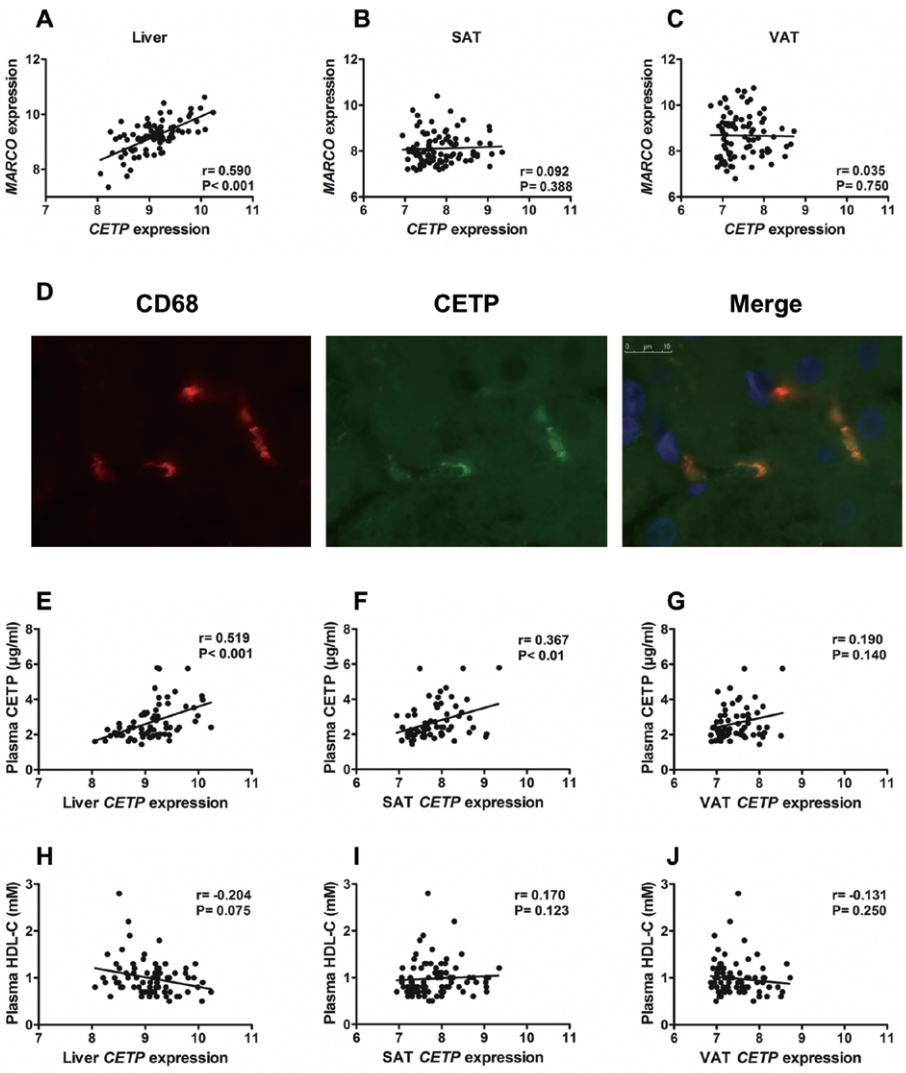



**Figure 2. Hepatic CETP expression in macrophages is associated with plasma CETP concentration and HDL-cholesterol level in humans.** Biopsies from visceral adipose tissues (VAT), subcutaneous adipose tissues (SAT) and livers and were taken during bariatric surgery from 97 severely obese patients, and assayed for genome-wide gene expression profiles. The expression of *CETP* was correlated with *MARCO* in liver (A), but not in SAT (B) and VAT (C). Panel D shows representative pictures of double stainings of CD68 (red) and CETP (green) in a liver section. Hepatic *CETP* expression positively correlated with plasma CETP level (E), and reversely correlated with plasma HDL-cholesterol level (H). The correlation between plasma CETP level and the expression of *CETP* in the SAT (F) and VAT (G), and the correlation between plasma HDL-C level and the expression of *CETP* in the SAT (I) and VAT (J) are shown.

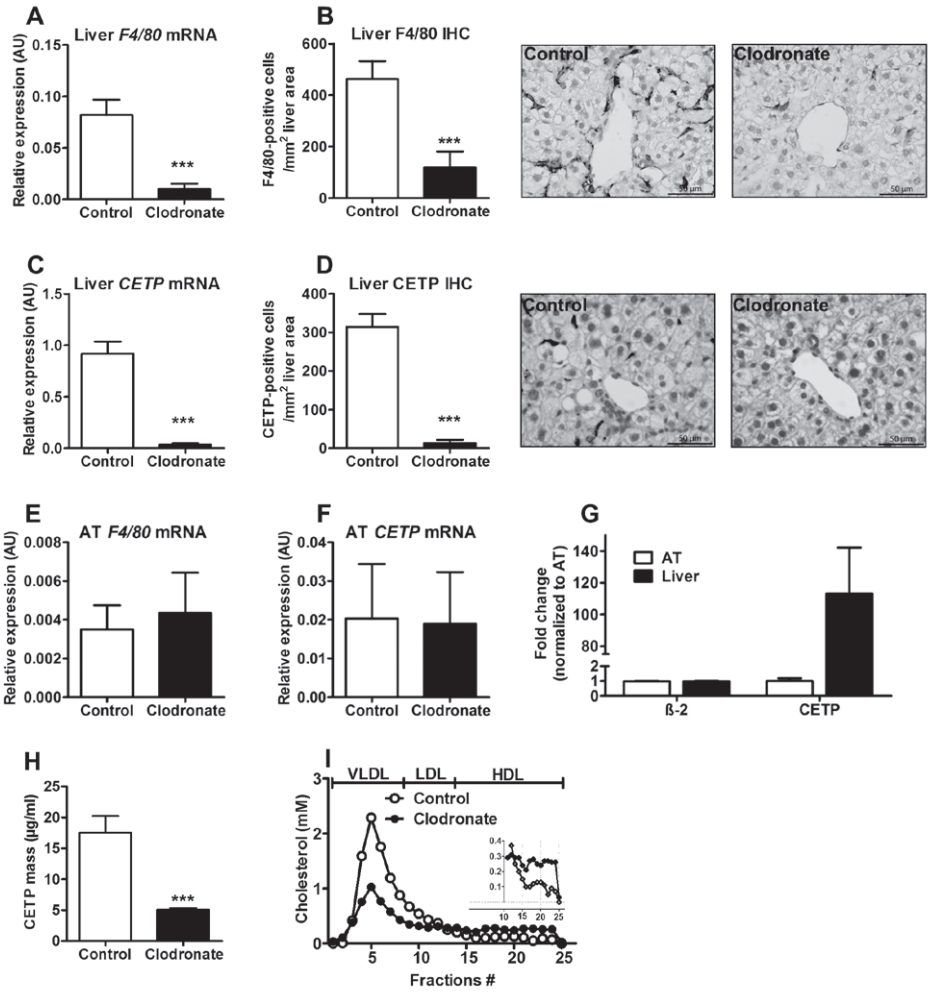



**Figure 3. Elimination of hepatic macrophages abolishes hepatic CETP expression and largely reduces plasma CETP in *E3L.CETP* mice.** *APOE*3-Leiden.CETP* (*E3L.CETP*) mice fed a western-type diet (WTD) were treated with or without liposomal clodronate. Livers were assayed for mRNA expression of *F4/80* (A) and *CETP* (C), as well as for F4/80 (B) and CETP (D)-positive cells. Gonadal adipose tissues were assayed for expression of *F4/80* (E) and *CETP* (F). The expression levels of *ß-2 microglobulin* (*ß-2*) and *CETP* were compared between livers and adipose tissue (G). Plasma was assayed for CETP concentration (H) and the cholesterol distribution over lipoproteins (I).

## Elimination of hepatic macrophages abolishes hepatic CETP expression and largely reduces plasma CETP concentration in *E3L.CETP* mice

To further investigate the contribution of CETP expression in hepatic macrophages to plasma CETP as well as lipoprotein metabolism, *E3L.CETP* mice were injected with

liposomal clodronate, a well-established method to deplete macrophages from liver, but not adipose tissue in rodents [19, 20]. Indeed, as compared with controls, liposomal clodronate markedly reduced hepatic *F4/80* expression (-88%, $P<0.001$; Fig. 3A) and F4/80$^+$ cells (-74%, $P<0.001$; Fig. 3B). Concomitantly, depletion of hepatic macrophages almost completely abolished hepatic *CETP* expression (Fig. 3C, -96%, $P<0.001$) as well as CETP$^+$ macrophages (Fig. 3D, -96%, $P<0.001$). In contrast to the liver, liposomal clodronate did not alter the mRNA expression of *F4/80* (Fig. 3E) or *CETP* (Fig. 3F) in adipose tissue. Strikingly, *CETP* expression in the liver is 113-fold higher than in adipose tissue (Fig. 3G, $P<0.001$), despite a comparable expression of the reference gene *ß-2 microglobulin*. This corroborates the human studies suggesting that the liver, as opposed to adipose tissue, is the main source of CETP. In further support of this notion, elimination of hepatic macrophages largely reduced plasma CETP levels (-71%, $P<0.001$; Fig. 3H), accompanied by a decreased VLDL-C (-49%; Fig. 3I) and increased HDL-C level (+130%; Fig. 3I).



## Lipid-lowering agents reduce plasma CETP concentration by reducing the hepatic macrophage content in *E3L.CETP* mice

Fibrates and niacin, which are used for the treatment of dyslipidemia in humans [22, 23], both decrease plasma CETP and increase HDL-cholesterol [15, 16]. Since our data show that CETP is largely derived from hepatic macrophages, we hypothesized that these lipid-lowering drugs could act by decreasing hepatic macrophages. In line with our previous findings [15, 16], treatment of *E3L.CETP* mice with fenofibrate and niacin for 3 weeks decreased plasma CETP level (-49% and -51%, both $P<0.001$; Fig. 4A) and hepatic expression of *CETP* (-74% and -56%, both $P<0.001$; Fig. 4B). It also lowered plasma TG (-80% and -54%, both $P<0.001$; Fig. 4C) and cholesterol level (-60% and -55%, both $P<0.001$; Fig. 4D), explained by a reduction in VLDL-TG (Fig. 4E) and VLDL-C (Fig. 4F), and an increase in HDL-C (Fig. 4F). Indeed, fenofibrate and niacin decreased hepatic macrophage content reflected by a reduction in hepatic F4/80 expression (-38% and -29%, both $P<0.001$; Fig. 4G), as well as F4/80$^+$ macrophages (-54% and -28%, both $P<0.001$; Fig. 4H and Fig. 4I). Taken together, these data indicate that fenofibrate and niacin reduce the hepatic macrophage content, thereby decreasing hepatic CETP production, resulting in a decreased plasma CETP concentration and increased HDL-cholesterol.

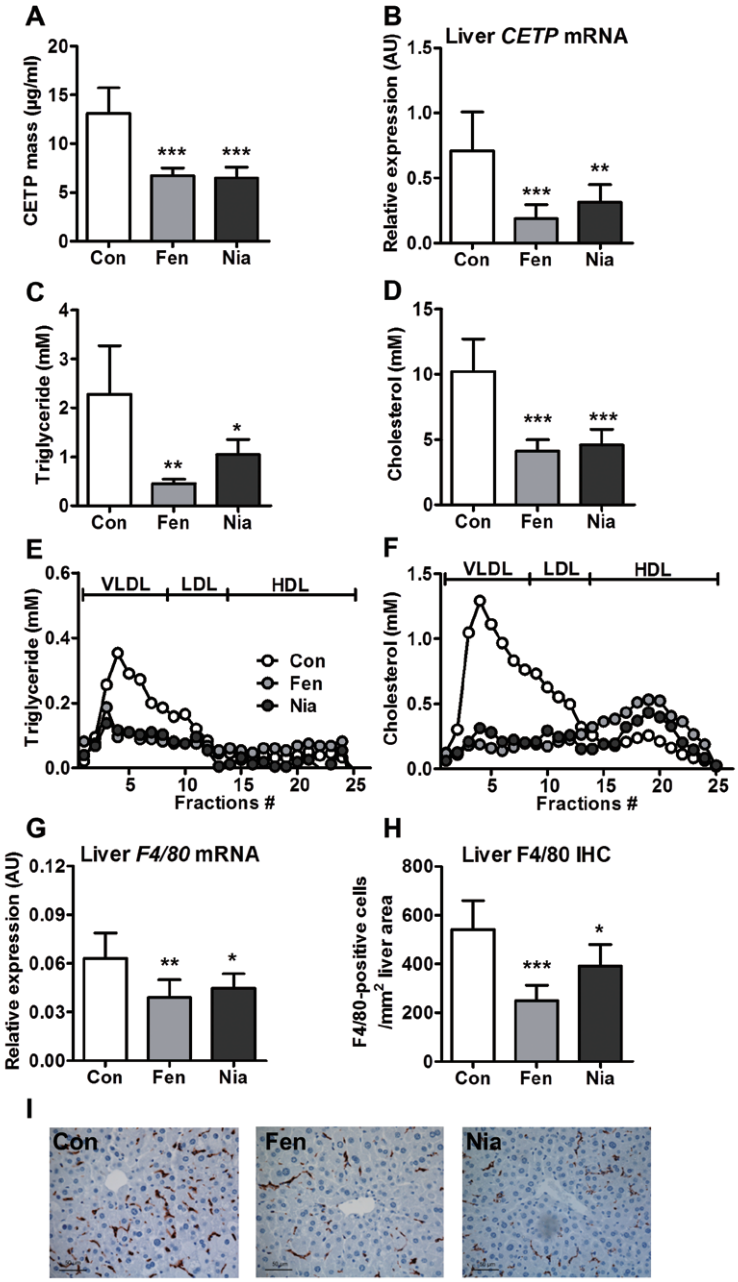



**Figure 4. Lipid-lowering agents reduce plasma CETP concentration by reducing the hepatic macrophage content in *E3L.CETP* mice.** *E3L.CETP* mice fed a WTD were treated without (Con) or with fenofibrate (Fen) or niacin (Nia) for 4 weeks. Plasma was assayed for CETP concentration (A), triglycerides (C) and cholesterol (D) as well as the distribution of triglycerides (E) and cholesterol (F) over lipoproteins. Livers were assayed for expression of *CETP* (B) and *F4/80* (G), and for F4/80-positive cells (H) with representative pictures shown (I).

## DISCUSSION

In this study, we show that liver is the main source of plasma CETP, and that the cells responsible for expression of *CETP* are the hepatic macrophages. Adipose tissue does not appear to contribute significantly to the plasma CETP pool because central adiposity is not associated with the plasma CETP level in the general population.

Previous studies have shown that *CETP* mRNA is abundantly expressed in the liver and adipose tissue in several mammalian species [24]. A small human cohort study also found a correlation between adipose tissue *CETP* expression and plasma CETP concentration [12]. In contrast, our data show much more prominent *CETP* expression in the liver as compared to adipose tissue. In addition, we found no association between waist circumference and plasma CETP level in a large cohort study (n~1,500). Furthermore, by analyzing liver biopsies from obese patients undergoing bariatric surgery, we found that the plasma CETP was strongly correlated with the *CETP* expression in the liver but not in adipose tissue, indicating that liver is the main site of *CETP* expression and is a determinant of the total plasma CETP pool in humans. Some studies have suggested that changes in the degree of adiposity induced by body weight reduction reduced *CETP* expression and improved lipoprotein metabolism, implying that a reduction in adipose tissue reduces plasma CETP [25, 26]. However, in addition to reducing adiposity, body weight reduction significantly attenuates hepatosteatosis [27, 28]. Since we recently demonstrated that a decrease in hepatic lipid content is accompanied by a decrease in plasma CETP level [28], it is thus tempting to speculate that body weight reduction via attenuation of hepatosteatosis reduces the production of CETP by the liver.

Since the liver consists of multiple cell types including hepatocytes, endothelial cells and macrophages, also known as Kupffer cells, we set out to evaluate the cell type responsible for the expression of *CETP*. In the present study, we found that the expression of established macrophage markers strongly associated with the expression of hepatic *CETP*, and CETP was specifically co-localized with the $CD68^+$ macrophages in liver. Mechanistic studies in *E3L.CETP* mice showed that depleting the macrophages from liver by clodronate liposomes virtually abolished hepatic *CETP* expression and largely reduced plasma CETP, fully corroborating our findings in humans that hepatic macrophages, rather than hepatocytes, are the main cellular source of hepatic *CETP* expression and the plasma CETP pool. In contrast to Kupffer cells, we could hardly detect any *CETP* expression in extrahepatic macrophages, including peritoneal macrophages and macrophages in adipose tissue. It has been reported that *CETP* expression is regulated by the activation of liver X receptor α (LXRα) [29], which is highly expressed in multiple organs. Recently, Gautier *et al.* [30] demonstrated that in addition

to an LXRα responsive element in the CETP promotor, the CETP gene contains an ER8 farnesoid X receptor (FXR) response element in the first intron. Therefore, bile acids that are the natural ligand for FXR and are produced by hepatocytes may be essential for maintaining high expression of *CETP* in hepatic versus extrahepatic macrophages. In fact, treatment of *E3L.CETP* mice with the bile acid taurocholic acid greatly increased the hepatic CETP transcript as well as the plasma CETP level [30].

Previously, hepatic expression of *CETP* in mice has been attributed to both macrophages and hepatocytes, based on studies assessing hepatic *CETP* expression 8 weeks after transplantation of bone marrow from wild-type (WT) littermates into human CETP transgenic (Tg) mice and vice versa, suggesting that hepatic macrophages contribute ≈ 50% to the total hepatic *CETP* expression [31]. However, it should be realized that the replacement of liver macrophages after bone marrow transplantation occurs slowly. In the same study, it was found that only 50% of Kupffer cells were replaced by the donor cells 8 weeks after bone marrow transplantation, accompanied by a 50% reduction in plasma CETP as well as a 2-fold lower hepatic *CETP* expression in WT → CETP Tg mice as compared to control transplanted CETP Tg →CETP Tg mice [31]. Interestingly, we found hepatic *CETP* expression decreased by approximately -90% 12 weeks after transplantation when more Kupffer cells were replaced (data not shown), confirming that hepatic macrophages are the predominant source of *CETP* expression.

So far, no plasma biomarkers exist for assessing hepatic macrophage content. Therefore, quantification of the accumulation of hepatic macrophages, for example in studies on non-alcoholic steatohepatitis (NASH), is performed by immunohistochemical analysis of liver biopsies. Our present data, showing that CETP is mainly produced by hepatic macrophages, and that hepatic *CETP* mRNA correlates with plasma CETP, imply that plasma CETP is a plasma biomarker for the hepatic macrophage content. It is therefore attractive to hypothesize that the extent of hepatosteatosis or the extent of inflammation are the main determinants of hepatic macrophage *CETP* expression.

Our findings that *CETP* expression in the hepatic macrophages determines the plasma CETP and affects lipoprotein metabolism provides new avenues for the development of new strategies of CETP inhibition. Although the precise mechanism by which CETP inhibitors (e.g. torcetrapib, dalcetrapib and anacetrapib) decrease CETP activity is not known, they all change the conformation of the plasma CETP protein and induce tight binding of CETP with HDL particles [32]. However, tight binding of CETP with HDL particles by torcetrapib for instance might compromise the function of HDL to generates large CE-rich HDL particles instead of small HDL particles and nascent discoidal HDL [33]. To avoid the potentially adverse effects of the current CETP inhibitors on HDL function, strategies focusing on inhibiting CETP synthesis at its cellular origin may be a promising

alternative. In fact, depletion of hepatic macrophages by liposomal clodronate or decreasing hepatic macrophage content by lipid-lowering drugs including fenofibrate and niacin reduce hepatic expression and plasma level of CETP, consequently generating a less atherogenic lipid phenotype, e.g. decreasing TG and increasing HDL-C.

In conclusion, we demonstrate that hepatic macrophages are the main cellular source of CETP in humans. Therefore, we propose that plasma CETP concentration measurement can be developed as a diagnostic and predictive test for hepatic macrophage content. Moreover, CETP expression in hepatic macrophages determines plasma CETP concentration and affects lipoprotein metabolism. We propose that elimination of hepatic macrophages resulting in decreasing hepatic expression and plasma level of CETP would be a promising strategy for the treatment of dyslipidemia and cardiovascular disease.



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# SUPPLEMENTARY APPENDIX

## Assessment for Rijswijk study

A cross-sectional observational study was performed in a general population in Rijswijk, The Netherlands. All non-diabetic subjects between 40-70 years of age of four general practitioners were identified using computer assisted screening. In addition to known diabetes, persons with known terminal disease, a history of psychiatric disorder or substance abuse were excluded. Screening was carried out in 2079 of the 2942 considered eligible subjects (response rate 70.6%) after written informed consent. During the screening visit, medical history including use of medication was taken, if necessary additional information was retrieved from the medical record. Subsequently, waist circumference was measured in standing position, midway between the lower limit of the rib cage and the iliac crest using a Seca 200 circumference measuring tape (Seca Gmbh, Hamburg, Germany). Venous blood samples were taken after a 12 h overnight fast. Storage of samples for plasma CETP was performed after evaluation of the first 645_subjects. The Rijswijk study was approved by the review board of South West Holland and performed in accordance with the Declaration of Helsinki.

Statistical analysis was performed using SPSS for Windows (version 17.0; SPSS, Chicago, Illinois, USA). Data are expressed as mean ± standard deviation or median (interquartile range). To compare plasma CETP between quintiles of waist circumference a Kruskal-Wallis test for non-parametric continues variables was used.

## Assessment for bariatric surgery study

### Tissue sampling, histology preparation, and mRNA isolation

Tissue sampling and mRNA isolation were performed as described earlier [17]. Wedge biopsies from liver, subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) were taken during bariatric surgery. mRNA was isolated using the Qiagen Lipid Tissue Mini Kit (Qiagen, Hilden, Germany, 74804) and mRNA quality and concentration were assessed with an Agilent Bioanalyzer (Agilent Technologies,Waldbronn, Germany, 5067-1521). mRNA integrity numbers (RIN) of these samples ranged between 4.5 and 9.3 (average 7.5), 5.8 and 8.7 (average 7.5), and 6.2 and 9.4 (average 7.6) for liver, SAT, and VAT respectively.

### mRNA pre-hybridization processing and hybridization

mRNA pre-hybridization processing and hybridization were performed as described earlier [17]. Starting with 200 ng of mRNA, the Ambion Illumina TotalPrep Amplification

Kit was used for anti-sense RNA synthesis, amplification, and purification, according to the manufacturer's protocol (Applied Biosystems/Ambion, Austin, TX, USA). 750 ng of complementary RNA was hybridized to Illumina HumanHT12 BeadChips (Illumina, San Diego, CA, USA) and scanned on the Illumina BeadArray Reader. These micro arrays contain 48813 different probes targeting 37812 different genes; some genes are targeted by more than one probe.

## Data normalization and quality control

Quantile-quantile normalization was applied to all genome-wide data from liver, VAT and SAT using LIMMA package (version 3.4.5) in R (version 2.11.1) (R foundation for statistical computing, Vienna, Austria). Only samples were included that passed quality control filtering, which was based on the median probe intensity, general behavior of known housekeeping genes, and principal component analysis over the samples. Available genome-wide genotype data were used to rule out sample mix-ups [34], and qRT-PCR was performed to estimate the technical quality of the micro array [17]. We obtained reliable RNA measures for 82 liver samples, 90 SAT samples and 84 VAT samples. All expression data has been made freely available by submission to GEO under GSE22070 (SAT data), and GSE22071 (VAT data). Liver expression data will be made available soon.

## Calculations and data visualization

Two probes targeting CETP were present on the micro array. Values of these probes were very strongly correlated (r= 0.82, 0.87, and 0.80 in liver, SAT and VAT respectively). We determined the expression of CETP as the average value of these two probes.

## Plasma CETP concentration, lipid and lipoprotein profiles analysis

Plasma CETP concentration was measured using the DAIICHI CETP ELISA kit according to manufacturer's instructions (Daiichi, Tokyo, Japan).

Plasma cholesterol and triglycerides were assayed using the commercially available enzymatic kits 236691, 11488872 (Roche Molecular Biochemicals, Indianapolis, IN, USA), respectively. The distribution of lipids over plasma lipoproteins was determined using fast protein liquid chromatography (FPLC). Plasma was pooled per group, and 50 µL of each pool was injected onto a Superose 6 PC 3.2/30 column (Äkta System, Amersham Pharmacia Biotech, Piscataway, NJ, USA) and eluted at a constant flow rate of 50 µL/min in PBS, 1 mM EDTA, pH 7.4. Fractions of 50 µL were collected and assayed for cholesterol and triglycerides as described above.

## RNA extraction, reverse transcription and quantitative RT-PCR

Total RNA was extracted from frozen liver and adipose tissue pieces using the Nucleospin RNAII kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. RNA quality was examined by the lab-on-a-chip method using Experion Std Sens analysis kit (Biorad, Hercules, CA) and RNA concentration was determined by Nanodrop technology (Thermo Scientific, Wilmington, USA). Total RNA was reverse-transcribed with iScript cDNA synthesis kit (1708891, Bio-Rad), and the obtained cDNA was purified with Nucleospin Extract II kit (636973, Macherey-Nagel, Bioké). Real-time PCR was performed on a CFX96 machine (Bio-Rad), the reaction mixture consisting of iQ™ SYBR® Green Super mix (Bio-Rad), cDNA, primers (Biolegio, Nijmegen, The Netherlands; see Supplementary Table 2 for primer sequences), and nuclease-free water in a total reaction volume of 10 µL. mRNA values of each gene were normalized to mRNA levels of ß-2 microglobulin and hypoxanthine ribosyltransferase (Hprt). Data were expressed as relative expression using the dCt method (Pfaffl, 2001, Nucleic Acids Res).



**Supplementary Table 2 primer sequences used for RT-qPCR**

| Gene | Forward primer | Reverse Primer |
|---|---|---|
| *ß-2m* | TGACCGGCTTGTATGCTATC | CAGTGTGAGCCAGGATATAG |
| *Cd68* | ATCCCCACCTGTCTCTCTCA | TTGCATTTCCACAGCAGAAG |
| *CETP* | CAGATCAGCCACTTGTCCAT | CAGCTGTGTGTTGATCTGGA |
| *F4/80* | CTTTGGCTATGGGCTTCCAGTC | GCAAGGAGGACAGAGTTTATCGTG |
| *Hprt* | TTGCTCGAGATGTCATGAAGGA | AGCAGGTCAGCAAAGAACTTATAG |

*ß-2m*, ß-2 microglobulin; *Cd68,* cluster of differentiation 68; *CETP*, cholesteryl ester transfer protein; *Hprt*, hypoxanthine ribosyltransferase.

## Immunohistochemistry (IHC)

Paraffin-embedded human liver sections (5 µm) were stained for macrophage marker CD68 (M0814; 1/800, Dako, California, USA) and CETP (ab51771; 1/1000, Abcam, Cambridge, UK).

Paraffin-embedded sections of *E3L.CETP* mouse liver and adipose tissue (5 µm) were stained for macrophage marker F4/80 (MCA497; 1/600, Serotec, Oxford, UK) as described previously [35], and human CETP (ab51771; 1/1000, Abcam, Cambridge, UK).

# 7

# ACUTE CENTRAL NEUROPEPTIDE Y ADMINISTRATION INCREASES FOOD INTAKE BUT DOES NOT AFFECT HEPATIC VERY LOW-DENSITY LIPOPROTEIN (VLDL) PRODUCTION IN MICE

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# ABSTRACT

Central neuropeptide Y (NPY) administration stimulates food intake in rodents. In addition, acute modulation of central NPY signaling increases hepatic production of very low-density lipoprotein (VLDL)-triglyceride (TG) in rats. As hypertriglyceridemia is an important risk factor for atherosclerosis, for which well-established mouse models are available, we set out to validate the effect of NPY on hepatic VLDL-TG production in mice, to ultimately investigate whether NPY, by increasing VLDL production, contributes to the development of atherosclerosis. Male C57Bl/6J mice received an intracerebroventricular (i.c.v.) cannula into the lateral ventricle (LV) or third ventricle (3V) of the brain. One week later, after a 4 h fast, the animals received an intravenous (i.v.) injection of Tran$^{35}$S (100 µCi) followed by tyloxapol (500 mg/kg BW), enabling the study of hepatic VLDL-apoB and VLDL-TG production, respectively. Immediately after the i.v. injection of tyloxapol, the animals received either an i.c.v. injection of NPY (0.2 mg/kg bodyweight (BW) in artificial cerebrospinal fluid; aCSF), synthetic $Y_1$ receptor antagonist GR231118 (0.5 mg/kg BW in aCSF) or vehicle (aCSF), or an i.v. injection of $PYY_{3-36}$ (0.5 mg/kg BW in PBS) or vehicle (PBS). Administration of NPY into both the LV and 3V increased food intake within one hour after injection (+164%, $P<0.001$ and +367%, $P<0.001$, respectively). NPY administration neither in the LV nor in the 3V affected hepatic VLDL-TG or VLDL-apoB production. Likewise, antagonizing central NPY signaling by either $PYY_{3-36}$ or GR231118 administration did not affect hepatic VLDL production. In conclusion, in mice, as opposed to rats, acute central administration of NPY increases food intake without affecting hepatic VLDL production. These results are of great significance when extrapolating findings on the central regulation of hepatic VLDL production between species.

# INTRODUCTION

The metabolic syndrome is referred to as a cluster of physiological abnormalities correlated with obesity and type 2 diabetes mellitus [1]. Hallmarked by insulin resistance, hyperglycemia, hypertension, low high-density lipoprotein-cholesterol (HDL-C) and elevated very low-density lipoprotein-triglyceride (VLDL-TG) levels, this cluster of cardiometabolic risk factors is a strong risk factor for type 2 diabetes and cardiovascular disease [1, 2]. Furthermore, due to the strong interlinkage between its individual components, effective treatment of the metabolic syndrome has shown to be extremely challenging [2].

Obesity develops when long-term energy intake exceeds energy expenditure. The brain plays an important role in mediating energy intake, with the hypothalamus being its key regulator [3, 4]. Two major neuronal populations within the hypothalamic arcuate nucleus (ARC) exert opposing effects on energy intake. Proopio-melanocortin (POMC) neurons are activated upon food intake to exert anorectic effects by inhibiting food intake and promoting a negative energy balance. In contrast, when energy levels are low, neuropeptide Y (NPY)/Agouti-related peptide (AgRP) neurons are activated to stimulate food intake and promoting a positive energy balance [5-7].



The 36-amino acid peptides NPY, peptide YY (PYY) and pancreatic polypeptide, collectively called the NPY family of peptides, affect food intake by interacting with G-protein-coupled Y receptors [8, 9]. NPY is widely expressed in both the brain and the peripheral nervous system. Within the brain, NPY is highly expressed in the hypothalamus, especially in the ARC [8]. NPY-neurons co-expressing AgRP are only found in this hypothalamic nucleus, as AgRP is uniquely expressed in the ARC [10]. NPY/AgRP neurons can be activated by a diversity of signals, such as leptin and insulin [11]. Upon activation, NPY stimulates its Y receptors to activate circuits that increase food intake and fat storage [5]. Concomitantly, by antagonizing the melanocortin 3 and 4 (MC3/4) receptors in the paraventricular nucleus (PVN), AgRP prevents the catabolic drive initiated by the melanocortin system [5]. In this fashion, NPY/AgRP neurons exert a so-called double-anabolic drive.

In addition to modulation of food intake, NPY may also be involved in the regulation of lipid metabolism. A recent study in rats showed that acute modulation of central NPY signaling, either by NPY or by an Y5 receptor agonist, increased hepatic VLDL-TG production. Accordingly, central administration of a Y1 receptor antagonist decreased hepatic VLDL-TG production [12]. In mice, central NPY administration prevented the peripheral insulin-induced inhibition of glucose production by the liver, and reversed the insulin-induced inhibition of hepatic VLDL-TG production

under hyperinsulinemic conditions [13]. Hypertriglyceridemia, associated with increased hepatic VLDL-TG production and/or decreased VLDL-TG clearance, is an important risk factor for cardiovascular diseases such as arterial atherosclerosis (for review [14]). Since atherosclerosis is generally studied in hyperlipidemic mice rather than in rats, we set out to validate the effect of NPY on hepatic VLDL-TG production in mice, with the ultimate goal to investigate whether NPY, by increasing VLDL-TG production, contributes to the development of atherosclerosis.

## MATERIALS AND METHODS

### Animals



For all experiments, 15 weeks old male C57Bl/6J mice were used, housed in a temperature and humidity-controlled environment with free access to food and water. Experiments were performed after 4 h of fasting at 12:00 pm with food withdrawn at 8:00 am, unless indicated otherwise. Food intake and body weight were measured weekly during experiments. All animal experiments were approved by the Animal Ethics Committee of the Leiden University Medical Center, Leiden, The Netherlands.

### Intracerebroventricular surgery

For i.c.v. cannula implantation, mice were anaesthetized with 0.5 mg/kg BW Medetomidine (Pfizer, Capelle a/d IJssel, The Netherlands), 5 mg/kg BW Midazolam (Roche, Mijdrecht, The Netherlands) and 0.05 mg/kg BW Fentanyl (Janssen-Cilag, Tilburg, The Netherlands) and placed in a stereotactic device (TSE systems, Homburg, Germany). A 25-gauge guide cannula was implanted into the left lateral ventricle using the following coordinates from Bregma: 1.0 mm lateral, 0.46 mm posterior and 2.2 mm ventral. For third ventricle cannulations the following coordinates from Bregma were used: 0.0 mm lateral, 1.3 mm posterior and 5.7 mm ventral. The guide cannula was secured to the skull surface with dental cement (GC Europe N.V., Leuven, Belgium) and the anesthesia was antagonized using 2.5 mg/kg BW Antipamezol (Pfizer, Capelle a/d IJssel, The Netherlands), 0.5 mg/kg BW Flumazenil (Roche, Mijdrecht, The Netherlands) and 1.2 mg/kg BW Naloxon (Orpha, Purkersdorf, Austria). Animals were single housed after the surgery.

### Food intake measurement

After a recovery period of at least 1 week, the mice received a pre-weighed amount of food after which basal food intake was measured for two hours, starting from

09:00 a.m. One day later, mice received an i.c.v. injection of NPY (0.2 mg/kg in 1 µL of artificial cerebrospinal fluid, aCSF) under light isoflurane anesthesia (1.5% in air). Food was weighed before and one and two hours after waking up from the anesthesia to determine NPY-induced food intake.

## Hepatic VLDL-TG and VLDL-apoB production

In experiments performed under complete anesthesia, 4 h fasted mice were anesthetized with 6.25 mg/kg Acepromazine (Alfasan, Woerden, The Netherlands), 6.25 mg/kg Midazolam (Roche, Mijdrecht, The Netherlands), and 0.31 mg/kg Fentanyl (Janssen-Cilag, Tilburg, The Netherlands). In other experiments, mice were awake throughout the whole experiment, except for the lateral ventricle (LV) or third ventricle (3V) injections, which were performed under light isoflurane sedation (1.5% in air).



A basal blood sample was taken from the tail tip in a chilled capillary, and mice received an intravenous injection of 100 µl PBS containing 100 µCi Tran$^{35}$S label (MP Biomedicals, Eindhoven, the Netherlands) via the tail vein, resulting in incorporation of $^{35}$S into newly produced VLDL-apolipoprotein B. After 30 min, the animals received an intravenous injection of tyloxapol (500 mg/kg body weight; Triton WR-1339, Sigma), as a 10% (w/w) solution in sterile saline, to prevent systemic lipolysis of newly secreted hepatic VLDL-TG [15].

Immediately after the tyloxapol injection, mice received an injection of either NPY (0.2 mg/kg BW, Bachem, St. Helens, UK in 1 µL aCSF) or vehicle (aCSF, 1 µL) into the lateral ventricle (LV) or third ventricle (3V). In the dose-finding study, mice received an LV injection of NPY (0.0002, 0.002, 0.02, 0.2 or 2.0 mg/kg BW in 1 µL aCSF) or vehicle. In the antagonist study, mice received either an LV injection of Y1 antagonist GR231118 (0.5 mg/kg in 1 µL aCSF) or vehicle (aCSF, 1 µL) or an i.v. injection of $PYY_{3-36}$ (0.5 mg/kg in 100 µL PBS) or vehicle (PBS, 100 µL).

Blood samples were taken from the tail tip into chilled capillaries at the indicated time points up to 90 min after tyloxapol injection. The tubes were kept on ice after which they were centrifuged at 4°C. Plasma TG concentration was determined using a commercially available kit according to the instructions of the manufacturer (no. 11488872, Roche Molecular Biochemicals, Indianapolis, IN) At 120 min, the animals were sacrificed and blood was collected by orbital puncture for isolation of VLDL by density gradient ultracentrifugation [16]. $^{35}$S-activity was measured in the VLDL fraction and VLDL-apoB production rate was calculated as $dpm.h^{-1}$ [17].

### Verification of cannula position

After termination of mice, brains were taken out and fixed by submerging in 4% paraformaldehyde for 48 hours (Sigma-Aldrich, Zwijndrecht, the Netherlands) followed by 30% sucrose (Sigma-Aldrich, Zwijndrecht, the Netherlands) in PBS for at least 24 hours, until the brain has sank to the bottom of the container. Cannula position was verified in 30 µm thick brain cryosections mounted on microscopic slides. The sections were fixated and defatted in CARNOY solution (100% ethanol, chloroform and acetic acid in a 6:3:1 ratio), hydrated by descending ethanol concentrations (100-96-70%) in MilliQ (MQ) water, and a Nissl staining was performed using cresyl violet (Sigma-Aldrich, Zwijndrecht, the Netherlands): 0.9 g cresyl violet, 300 mL MQ, 2.25 mL 10% acetic acid, pH 4.5. The sections were then dehydrated in ascending ethanol concentrations (70-96-100-100%) followed by 2 times isopropanol and 2 times Histo-Clear (National diagnostics, Atlanta, USA). Cover slips were mounted using xylene, and the cannula position was verified by locating the end of the cannula track observed in the tissue.



### Statistical analysis

Differences between two groups were determined with Mann-Whitney non-parametric tests for two independent samples. Differences between multiple groups were determined with the Kruskal-Wallis non-parametric test for *k* independent samples. When significant differences were found, the Dunn's Multiple Comparisons test was used as a follow-up test to determine differences between two independent groups. A *P*-value of less than 0.05 was considered statistically significant. Data are presented as means ± SD.

## RESULTS

### Lateral ventricle NPY administration stimulates food intake in mice

To verify that central administration of NPY stimulates food intake, both basal and NPY-induced food intake were assessed during two hours, starting at 09:00 a.m. with all mice serving as their own control. Administration of NPY (0.2 mg/kg BW) in the left lateral ventricle (LV) increased food intake during the first hour after injection by +164% (0.34±0.19 vs 0.90±0.40 g, $P<0.001$, Fig. 1). Food intake during the second hour after injection was similar to the basal food intake in this specific time frame (0.40±0.17 vs 0.49±0.20 g, *n.s.*, Fig. 1).

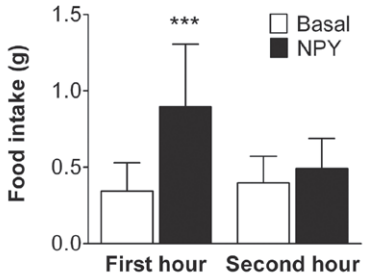



**Figure 1. NPY administration into the lateral ventricle acutely increases food intake.** NPY (0.2 mg/kg) was administered in the left lateral ventricle under light isoflurane anaesthesia, and food intake was measured for two hours, starting at 09:00 a.m. All animals served as their own controls (basal food intake). Values are means ± SD (n = 9), ***$P$<0.001 compared to basal.

## Lateral ventricle NPY administration does not affect hepatic VLDL production

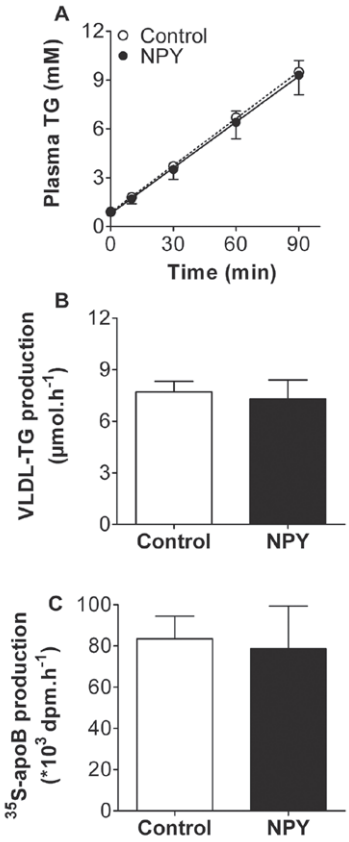


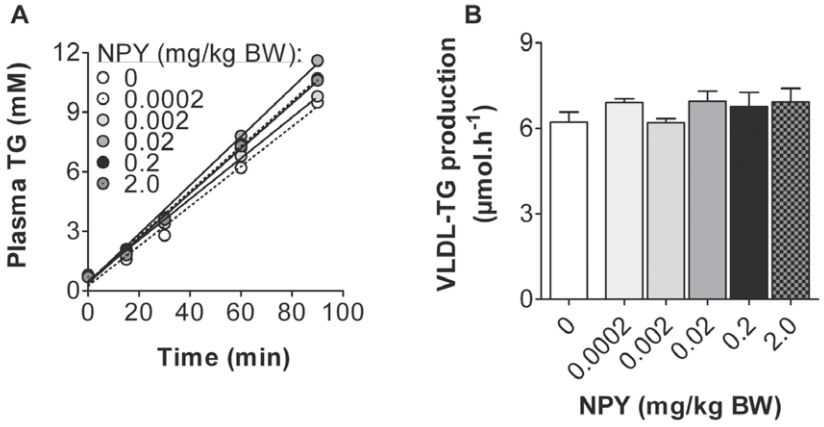

**Figure 2. NPY administration into the lateral ventricle does not affect hepatic VLDL production in anesthetized mice.** After a 4 hour fast, mice were fully anesthetized and hepatic VLDL production was assessed. Mice received an i.v. injection of Tran$^{35}$S label (t=-30 min), followed by an injection of tyloxapol (t=0 min), directly followed by an LV injection of NPY (0.2 mg/kg BW) or artificial cerebrospinal fluid (control). Plasma triglyceride (TG) levels were determined at indicated time points (A). VLDL-TG production rate was calculated from the slopes of the individual TG-time graphs (B). At t=120 min, mice were exsanguinated and VLDL fractions were isolated from serum by ultracentrifugation. $^{35}$S-apoB production was determined by scintillation counting of the isolated VLDL fraction (C). Values are means ± SD (n = 8-10).

Next, we assessed the effects of a single injection of NPY (0.2 mg/kg BW) into the left lateral ventricle on VLDL production in 4 h-fasted anaesthetized mice. Acute central administration of NPY did not affect VLDL-TG production rate in mice (7.7±0.6 vs 7.3±1.1 µmol/h, *n.s.*, Fig. 2A, B). Accordingly, hepatic VLDL-$^{35}$S-apoB production was

also unchanged upon NPY administration (84±11 vs 79±21 x$10^3$ dpm/h, *n.s.*, Fig. 2C). Thus, although this dose of NPY increased food intake, it did not affect hepatic VLDL production.

Subsequently, we performed a dose-finding study to assess whether either higher or lower dosages of NPY (0.0002, 0.002, 0.02, 0.2 or 2.0 mg/kg BW) were capable of increasing hepatic VLDL-TG production. Again, we did not observe any difference between the VLDL-TG production rate in controls (6.2±0.5 µmol/h) and that in mice treated with NPY (6.9±0.1, 6.2±0.1, 6.9±0.3, 6.8±0.5 or 6.9±0.5 µmol/h at 0.0002, 0.002, 0.02, 0.2 or 2.0 mg /kg BW, respectively, *n.s.*, Fig. S1). Since the use of anesthetics theoretically could interfere with the modulation of central NPY signaling, we repeated the experiment in conscious mice. However, NPY (0.2 mg/kg BW) did not increase hepatic VLDL-TG or VLDL-apoB production in conscious mice (data not shown).






**Supplemental Figure S1. Higher nor lower dosages of NPY administered in the lateral ventricle affect hepatic VLDL production in anesthetized mice.** After a 4 hour fast, mice were fully anesthetized and hepatic VLDL production was assessed using the tyloxapol method. Mice received an i.v. injection of Tran$^{35}$S label,(t=-30 min), followed by an injection of tyloxapol (t=0 min), directly followed by an LV injection of NPY (0.0002, 0.002, 0.02, 0.2 or 2.0 mg/kg BW) or artificial cerebrospinal fluid (control; 0 mg/kg). Plasma triglycerides were determined at indicated time points (A). VLDL-TG production was calculated from the slopes of the individual TG-time graphs (B). Values are means ± SD (n =2-5).

## Antagonizing central NPY signaling does not affect hepatic VLDL production

Since other modulators of NPY signaling have previously been shown to acutely interfere with VLDL-TG production in rats [12], we next assessed the effects of $PYY_{3-36}$ and of GR231118, a synthetic $Y_1$ receptor antagonist, on hepatic VLDL-TG and VLDL-apoB production. Central administration of GR231118 did not affect the hepatic production

of VLDL-TG (8.6±1.8 vs 8.7±1.4 µmol/h, *n.s.*, Fig. 3A, B) or VLDL-apoB (55±11 vs 59±9 x$10^3$ dpm/h, *n.s.*, Fig. 3C). In line with this finding, intravenous administration of $PYY_{3-36}$, the endogenous antagonist of NPY, was also ineffective in lowering the hepatic production of VLDL-TG (8.5±0.9 vs 7.5±0.9 µmol/h, *n.s.*, Fig. 3D, E) and VLDL-apoB (73±18 vs 75± 13 x$10^3$ dpm/h, *n.s.*, Fig. 3F).

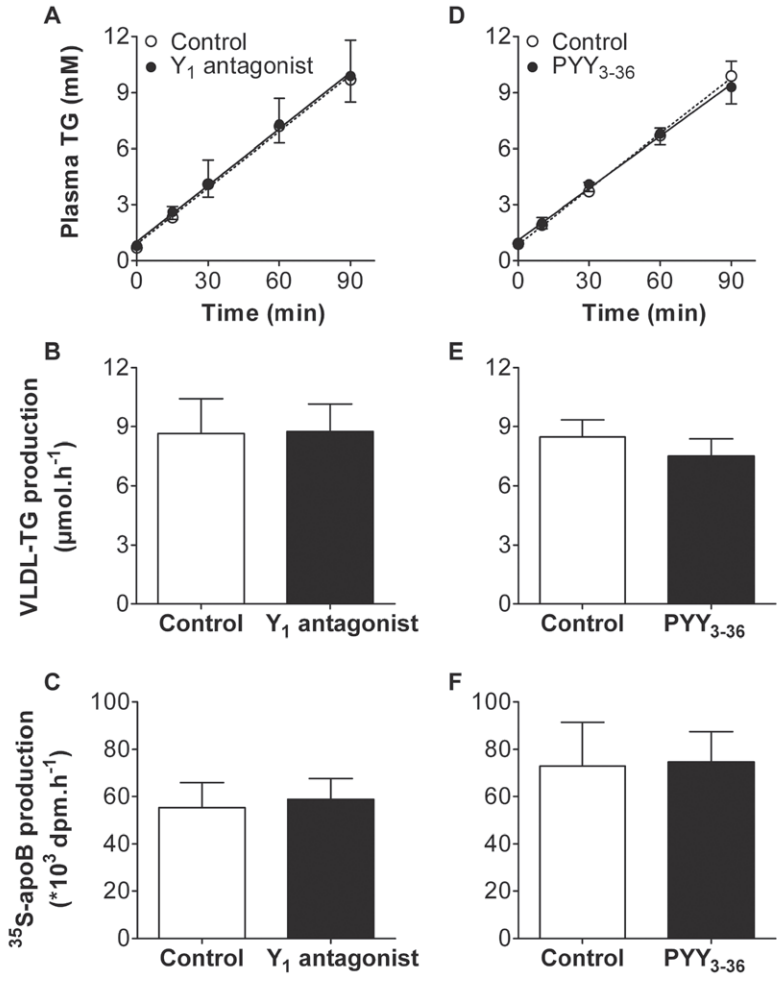



**Figure 3. Lateral ventricle nor peripheral administration of NPY antagonists affects hepatic VLDL production in anesthetized mice.** After a 4 hour fast, mice were fully anesthetized and hepatic VLDL production was assessed. Mice received an i.v. injection of Tran$^{35}$S label (t=-30 min), followed by an injection of tyloxapol (t=0 min), directly followed by an LV injection of GR231118 (0.5 mg/kg BW) or artificial cerebrospinal fluid (control; A-C), or by an i.v. injection of $PYY_{3-36}$ (0.5 mg/kg BW) or PBS (control; D-F). Plasma triglyceride (TG) levels were determined at indicated time points (A+D). VLDL-TG production rate was calculated from the slopes of the individual TG-time graphs (B+E). At t=120 min, mice were exsanguinated and VLDL fractions were isolated from serum by ultracentrifugation. $^{35}$S-apoB production was determined by scintillation counting of the isolated VLDL fraction (C+F). Values are means ± SD (n = 7-11).

## Third ventricle NPY administration stimulates food intake in mice

In contrast to the LV, the third ventricle (3V) is located at the base of the hypothalamus, the brain area that mediates NPY-induced feeding. To exclude that the absence of effect of modulation of central NPY signaling was due to LV versus 3V injection, we next performed 3V cannulations in mice. We first assessed the effects of 3V NPY (0.2 mg/kg BW) on food intake. NPY significantly increased food intake not only during the first hour after injection by +367% (0.21±0.08 vs 0.98±0.44 g, $p<0.001$, Fig. 4), as observed with LV injection of NPY (Fig. 1), but also during the second hour after injection by +105% (0.22±0.11 vs 0.45±0.19, $p<0.05$, Fig. 4), suggesting that 3V NPY administration is more effective than LV NPY administration. However, the effect of NPY is both acute and transient irrespective of the specific location of i.c.v. injection.



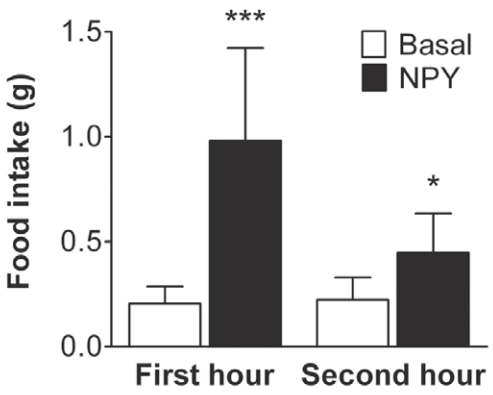



**Figure 4. NPY administration into the third ventricle acutely increases food intake.** NPY (0.2 mg/kg) was administered in the third ventricle under light isoflurane anaesthesia, and food intake was measured for two hours, starting at 09:00 a.m. All animals served as their own controls (basal food intake). Values are means ± SD (n = 11), *p<0.05, ***p<0.001 compared to basal.

## Third ventricle NPY administration does not affect hepatic VLDL-TG production

Albeit that 3V injection of NPY increased food intake to a greater extent than LV injection, administration of NPY (0.2 mg/kg BW) in the 3V was still unable to increase hepatic VLDL production in conscious mice, as both the hepatic production rate of VLDL-TG (6.5±0.6 vs 6.0±0.9 µmol/h, *n.s.*, Fig. 5A, B) and VLDL-apoB (22±3 vs 22±2 x$10^3$ dpm/h, *n.s.*, Fig. 5C) were unchanged. Collectively, these data thus show that acute modulation of central NPY signaling does not affect hepatic VLDL production in mice.

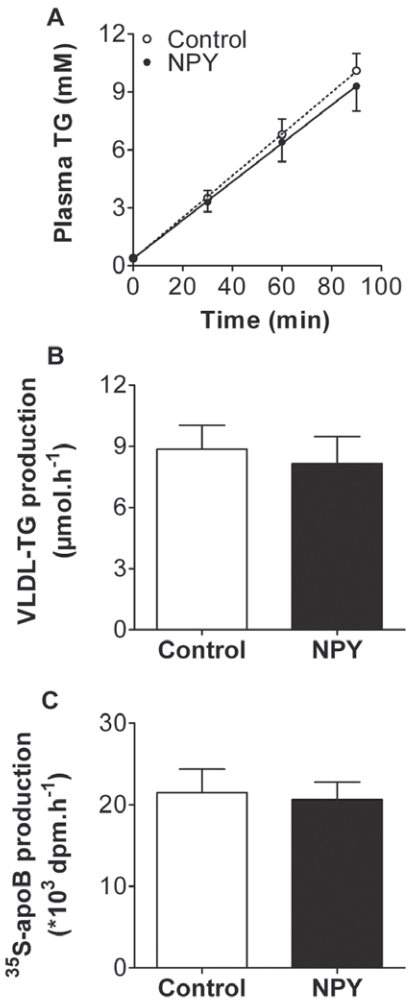



**Figure 5. NPY administration into the third ventricle does not affect hepatic VLDL production in awake mice.** Hepatic VLDL production was assessed after a 4h-fast. Mice received an i.v. injection of Tran$^{35}$S label (t=-30 min), followed by an injection of tyloxapol (t=0 min), directly followed by a 3V injection of NPY (0.2 mg/kg BW) or artificial cerebrospinal fluid (control). Plasma triglyceride (TG) levels were determined at indicated time points (A). VLDL-TG production rate was calculated from the slopes of the individual TG-time graphs (B). At t=120 min, mice were exsanguinated and VLDL fractions were isolated from serum by ultracentrifugation. $^{35}$S-apoB production was determined by scintillation counting of the isolated VLDL fraction (C). Values are means ± SD (n = 9-12).



## DISCUSSION

Since modulation of central NPY signaling acutely increases VLDL-TG production in rats, we initially set out to investigate the acute effects of central NPY administration on VLDL-TG production in mice, ultimately aimed at investigating the contribution of central NPY administration, by modulating VLDL production, to the development of atherosclerosis. We confirmed that central administration of NPY acutely increases food intake in mice, similarly as in rats. In contrast to the effects in rats, central administration of a wide dose range of NPY was unable to increase VLDL-TG production in mice. Moreover, inhibition of NPY signaling by $PYY_{3-36}$ or Y1 receptor antagonism was ineffective. In contrast to

rats, in mice acute modulation of NPY signaling thus stimulates food intake but without affecting hepatic VLDL-TG production.

NPY is a well-known stimulant of food intake in both rats [18] and mice [19] and this feeding response is mediated via the hypothalamic NPY system (for review [20]). The present study confirms this effect of NPY on food intake in mice, as administration of NPY in both the LV and 3V markedly increased food intake (Fig. 1 and 4, respectively). This effect was most pronounced in the first hour after injection, which is in line with previous observations [21]. 3V injection was somewhat more effective than LV injection, which might be explained by a higher hypothalamic NPY concentration possibly reached by 3V NPY injection. Collectively, these data indicate that NPY acutely increases food intake irrespectively of the rodent species.



Interestingly, neither LV nor 3V administration of NPY affected hepatic VLDL production in mice (Fig. 2 and 5, respectively). Furthermore, inhibition of central NPY signaling by $PYY_{3-36}$ or the Y1 antagonist GR231118 also failed to affect VLDL production by the liver (Fig. 3). In contrast, in rats, central NPY administration was reported to acutely stimulate hepatic VLDL-TG production [12]. Bruinstroop et al [22] recently confirmed that central NPY administration acutely increases VLDL-TG production in rats. In addition, they demonstrated that the regulation of hepatic lipid production by the central NPY system in rats is guided via the sympathetic nervous system, as selective sympathetic denervation of the liver abolished the effect of central NPY administration [22].

We questioned whether differences in the experimental design between our VLDL production studies with those reported in rats [12] could have accounted for different outcomes. In mice, VLDL production experiments are commonly performed under anesthesia, whereas the studies by Stafford *et al.* [12] and Bruinstroop *et al.* [22] were performed in conscious rats. In theory, anesthesia could interfere with the effects of central NPY administration. For example, the μ-opioid receptor agonist fentanyl acts by inhibiting the release of multiple neurotransmitters, including the chief inhibitory transmitter gamma-aminobutyric acid (GABA) [23]. A subpopulation of NPY neurons in the ARC co-produces GABA [24]. Furthermore, NPY can act in concert with GABA to augment food intake mediated by the PVN [25]. Hence, using an inhibitor of GABA release might interfere with the effects of the centrally administered NPY. However, in the current study we show that central NPY administration also failed to increase VLDL production by the liver in conscious mice (Fig. 5). Importantly, the VLDL-TG production rates were comparable in both anesthetized and conscious mice, indicating that anesthesia did not affect baseline hepatic VLDL-TG production. Hence, the divergent regulation of hepatic VLDL production and food intake by NPY in mice cannot be explained by the use of anesthesia.

A second difference in experimental design between the rat studies and our initial setup, was the site of i.c.v. administration of NPY. Initially, we cannulated the LV in mice for obvious practical reasons, whereas Stafford *et al.* [12] and Bruinstroop *et al.* [22] cannulated the 3V which is more easily accessible in rats. As the third ventricle is located at the base of the hypothalamus, one could speculate that this difference in injection site might interfere with the results obtained. However, whereas 3V NPY administration induces a potent and longer-lasting effect on food intake (Fig. 4) as compared to LV administration, it still did not affect hepatic VLDL-TG nor VLDL-apoB production in our hands (Fig. 5).

Interestingly, our group previously reported that LV administration of NPY was able to reverse the inhibition of hepatic VLDL-TG production in hyperinsulinemic euglycemic clamp conditions in mice [13]. This led us to conclude that insulin suppresses hepatic VLDL production at least in part by inhibiting central NPY signaling. Together with the present data, this suggests that in mice, NPY has no direct effect on hepatic VLDL production, whereas it is a downstream mediator in the suppression of hepatic lipid production by insulin.



In our study, as in previous studies [18, 19], the effects of NPY on food intake were measured in a satiated state. In contrast, hepatic VLDL production was assessed after a period of fasting, both in our study and in the previous rat studies [12, 22]. Fasting induces hypothalamic NPY mRNA expression [26]. Consequently, food intake and hepatic VLDL production were assessed during different states of endogenous NPY production, possibly leading to a different degree of sensitivity for exogenous NPY. However, the dose-finding study assessing the effects of both lower and higher dosages of NPY did not reveal any dose affecting hepatic VLDL production. Moreover, antagonizing central NPY signaling by $PYY_{3-36}$ or an Y1 antagonist also did not affect VLDL production. Collectively, these data further support the notion that in mice, acute modulation of the central NPY system affects food intake but not hepatic VLDL production.

In addition to food intake, NPY also regulates hepatic glucose production in a similar fashion in mice and rats [13, 27]. Hence, it is tempting to speculate why NPY exerts different effects in rats versus mice on hepatic VLDL production specifically. Based on the reports of Stafford *et al.* [12] and Bruinstroop *et al.* [22], rats display lower basal hepatic VLDL-TG production rates when compared to those currently reported in mice. Whereas in control rats, plasma TG levels increased by ~2 mM [12] and ~3.5 mM [22] within one hour after tyloxapol injection, we observed that in control mice plasma TG levels are increased by ~6 mM within the same period of time. This suggests that hepatic VLDL metabolism in itsel is differentially regulated in rats versus mic.

However, the apparent species difference concerning the regulation of hepatic

VLDL-TG production by NPY might also be caused by a difference in the expression of its receptor. In mammals, NPY is one of the most abundant peptides found and its receptors are widely expressed in both the central nervous system and peripheral tissues [28, 29]. Central expression of Y1-Y5 receptors is similar in rats and mice [28]. Interestingly, in addition to the Y1-Y5 receptors, mice also express the Y6 receptor. This receptor, which is a functional receptor in mice and is expressed in various brain sites including the hypothalamus [30, 31], is not expressed in rats [32]. Even though a role for the Y6 receptor in appetite regulation has been doubted [30], the exact function of the Y6 receptor remains elusive. If activation of this receptor by NPY would exert an opposing effect specifically on hepatic VLDL production, this might explain our negative findings in mice. Obviously, further investigation is needed to confirm this hypothesis. Therefore, the Y6 receptor might be an interesting target for future research investigating the role of the central NPY system in the regulation of hepatic VLDL production in mice.



Genetic association studies in humans have reported conflicting results on the role of NPY in serum TG metabolism. A polymorphism in the untranslated region between the Y1 and Y5 receptor genes was associated with lower serum TG levels in obese subjects [33]. In addition, the Leu7Pro polymorphism in the signal peptide part of the NPY gene has been linked with higher serum TG levels in preschool-aged boys [34]. However, this polymorphism was not associated with serum TG levels in female coronary heart disease patients [35]. Furthermore, studies on a variation in the 5'-flanking region of the Y2 receptor gene [36] and on the NPY signal peptide polymorphism T1128C [37] both report no association with serum TG levels. Collectively, these data emphasize the need of further research into the role of NPY in the regulation of peripheral TG metabolism. However, in light of the apparent species difference at least with respect to VLDL-TG production suggested from our study, caution should be taken when suggesting a common mechanism in humans based on findings resulting from animal studies.

In conclusion, acute central administration of NPY increases food intake without affecting hepatic VLDL production in mice, whereas NPY increases both food intake and VLDL production in rats. This apparent species difference in the effects of NPY, specifically on hepatic VLDL-TG production, is of great significance for future animal studies on the central regulation of hepatic VLDL production and underscores a general concern in animal research in view of extrapolating findings from specific animal studies to explain observations done in humans.

## ACKNOWLEDGEMENTS

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# 8

# GLP-1 RECEPTOR ACTIVATION INHIBITS VLDL PRODUCTION AND REVERSES HEPATIC STEATOSIS BY DECREASING HEPATIC LIPOGENESIS IN HIGH-FAT-FED APOE*3-LEIDEN MICE

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## ABSTRACT

In addition to improve glucose intolerance, recent studies suggest that glucagon-like peptide-1 (GLP-1) receptor agonism also decreases triglyceride (TG) levels. The aim of this study was to evaluate the effect of GLP-1 receptor agonism on very-low-density lipoprotein (VLDL)-TG production and liver TG metabolism. The GLP-1 peptide analogues CNTO3649 and exendin-4 were continuously administered subcutaneously to high fat diet-fed *APOE*3-Leiden transgenic mice*. After 4 weeks, hepatic VLDL production, lipid content, and expression profiles of selected genes involved in lipid metabolism were determined. CNTO3649 and exendin-4 reduced fasting plasma glucose (up to -30% and -28% respectively) and insulin (-43% and -65% respectively). In addition, these agents reduced VLDL-TG production (-36% and -54% respectively) and VLDL-apoB production (-36% and -43% respectively), indicating reduced production of VLDL particles rather than reduced lipidation of apoB. Moreover, they markedly decreased hepatic content of TG (-39% and -55% respectively), cholesterol (-30% and -55% respectively), and phospholipids (-23% and -36% respectively), accompanied by down-regulation of expression of genes involved in hepatic lipogenesis (*Srebp-1c*, *Fasn*, *Dgat1*) and apoB synthesis (*Apob*). In conclusion, GLP-1 receptor agonism reduces VLDL production and hepatic steatosis in addition to an improvement of glycemic control. These data suggest that GLP-receptor agonists could reduce hepatic steatosis and ameliorate dyslipidemia in patients with type 2 diabetes mellitus.

## INTRODUCTION

Type 2 diabetes mellitus (T2DM) has become a major metabolic disorder in both developed and developing countries, with impaired glucose tolerance and insulin resistance as hallmarks [1, 2]. In addition to glucose metabolism, lipid metabolism is disturbed in T2DM patients, reflected by increased plasma levels of low-density lipoprotein, VLDL-TG, and decreased levels of high-density lipoprotein. Moreover, T2DM is strongly associated with fatty liver disease (i.e. hepatic steatosis) [3], for which no effective pharmacotherapeutic options are yet available.

GLP-1 is an incretin hormone produced by intestinal L cells and the brain [4, 5]. GLP-1 is released in response to food intake to stimulate glucose-dependent insulin secretion by the pancreas [4, 6]. Additionally, GLP-1 exerts multiple other effects, including inhibition of food intake [7], slowing gastric emptying [8], and inhibition of glucagon secretion [9]. Thus, GLP-1 was considered as a good target for the treatment of T2DM. However, therapeutic application of GLP-1 is hampered due to its short circulating half-life (<2 minutes), because it is rapidly degraded by dipeptidyl peptidase 4 (DPP-4) that is widely expressed in endothelium and intestinal mucosa [10]. Therefore, pharmaceutical GLP-1 analogues that are resistant to inactivation by DPP-4 have been developed with an improved pharmacokinetic profile related to a longer half-life, of which exenatide (a synthetic version of exendin-4) was approved in 2005 for the treatment of T2DM [11]. We have previously described that CNTO736, a GLP-1 Mimetibody™ receptor agonist that incorporates a GLP-1 peptide analogue genetically fused by a unique linker to a domain that includes the Fc portion of an antibody, has an even longer circulating half-life than exendin-4 and retains the beneficial effects of GLP-1 on glucose metabolism [12]. The long-acting GLP-1 analogue CNTO3649, a more recent version of CNTO736 with two point mutations introduced to improve protein solubility, retains this advantageous pharmacokinetic profile.

In addition to improving glucose metabolism, preliminary studies suggested that GLP-1 receptor agonism decreases plasma TG levels in patients with T2DM [13, 14]. However, the mechanism underlying these beneficial effects on TG metabolism remains unclear. Therefore, the objective of the present study was to evaluate the effects of GLP-1 receptor agonism via CNTO3649 and exendin-4 on VLDL-TG production and liver TG metabolism, and further to explore the underlying mechanisms, in *APOE*3-Leiden* (*E3L*) transgenic mice fed a high fat diet (HFD) [15].

## MATERIALS AND METHODS

### Animals

For all experiments, 8-10 weeks old male *E3L* mice [16] were used, housed in a temperature and humidity-controlled environment with free access to food and water. Experiments were performed after 7 h of fasting at 14:00 pm with food withdrawn at 7:00 am. Body weight was measured weekly during experiments. All animal experiments were performed in accordance with the regulations of Dutch law on animal welfare, and the Institutional Ethics Committee for Animal Procedures from the Leiden University Medical Center, Leiden, The Netherlands, approved the protocol. All surgery was performed under isoflurane anesthesia

### Experiments



Two experiments were conducted, each of which was designed to investigate a specific aspect of the overall hypothesis.

In the first experiment, mice were fed a HFD (44 energy% fat, derived from bovine fat; Hope Farms, Woerden, The Netherlands) for 22 weeks. After 18 weeks of HFD feeding, mice were divided into 5 groups, matched for fasting body weight and plasma glucose levels. An osmotic minipump (model 1004, Alzet DURECT Corp., Cupertino, CA) was implanted subcutaneously in the left back region under light isoflurane anesthesia for the continuous delivery of CNTO3649 (1.0 or 3.0 mg/kg/day, dissolved in PBS), exendin-4 (15 or 50 µg/kg/day, dissolved in PBS) or PBS as a control for up to 4 weeks, while continuously feeding mice the HFD. Additionally, one group of mice received PBS while being fed a chow diet throughout the whole experiment as a control for HFD feeding. After 4 weeks of drug treatment, hepatic VLDL-TG and VLDL-apoB production were determined.

In the second experiment, mice were fed the HFD for 13 weeks. After 9 weeks of HFD feeding, mice were divided into 5 groups, matched for fasting body weight and plasma glucose levels. Osmotic minipumps were implanted subcutaneously for the continuous delivery of CNTO3649 (0.3 or 1.0 mg/kg/day, dissolved in PBS), exendin-4 (15 or 50 µg/kg/day, dissolved in PBS) or PBS as a control for up to 4 weeks, while continuously feeding the mice the HFD. Additionally, one group of mice received PBS while being fed a chow diet as a control for HFD feeding. After 4 weeks of drug treatment, mice were perfused with ice-cold PBS via the heart, and livers were isolated to investigate hepatic lipid content and determine expression of selected genes involved in lipid metabolism. In addition, skeletal muscles from the hind leg were isolated to determine expression of selected genes involved in thermogenesis and fatty acid oxidation.

## Compounds

CNTO3649 (molecular weight = 68,000 g/mol) was constructed by fusing a GLP-1 peptide analogue to a flexible Gly/Ser linker and a fragment of a V region heavy chain (VH) domain linked directly to the $CH_2$ and $CH_3$ domains of an Fc as described previously for CNTO736 [17]. Exendin-4 (molecular weight = 4186.6 g/mol) was purchased from Sigma (St. Louis, MO).

## Plasma glucose and insulin analysis

Blood was collected by tail bleeding into chilled capillary tubes. The tubes were placed on ice and centrifuged, and the obtained plasma was snap-frozen in liquid nitrogen and stored at -20 °C for further measurements. Plasma was assayed for glucose using a commercially available enzymatic kit according to the manufacturer's protocol (Instruchemie, Delfzijl, The Netherlands), and insulin was measured by ELISA (Mercodia AB, Uppsala, Sweden).



## Hepatic VLDL-TG and VLDL-apoB production

Mice were fasted for 7 hours, with food withdrawn at 7.00 am and anesthetized by intraperitoneal injection of 6.25 mg/kg acepromazine (Alfasan, Woerden, The Netherlands), 6.25 mg/kg midazolam (Roche, Mijdrecht, The Netherlands), and 0.3125 mg/kg fentanyl (Janssen-Cilag, Tilburg, The Netherlands). Mice received an intravenous (iv) injection of 100 µl PBS containing 100 µCi Tran$^{35}$S label (MP Biomedicals, Eindhoven, the Netherlands) resulting in incorporation of $^{35}$S into newly produced apoB required for hepatic VLDL production. After 30 min, the animals received an iv injection of tyloxapol (500 mg/kg body weight; Triton WR-1339, Sigma), as a 10% (w/w) solution in sterile saline, to prevent systemic lipolysis of newly secreted hepatic VLDL-TG [18]. Blood samples were drawn before (t=0) and at 15, 30, 60, and 90 min after tyloxapol injection. Plasma was assayed for TG concentration using the commercially available enzymatic kit 11488872 (Roche Molecular Biochemicals, Indianapolis, IN). At 120 min, mice were euthanized, and blood was collected by orbital puncture for isolation of VLDL by density gradient ultracentrifugation [19]. $^{35}$S-apoB was measured in the VLDL fraction and VLDL-apoB production rate was calculated as dpm.h$^{-1}$ [20]. TG and total cholesterol (TC) concentrations in the VLDL fractions were determined using the commercially available enzymatic kits 11488872 and 236691 (Roche) respectively, and phospholipid (PL) concentration was measured using a commercial kit (phospholipids B, Wako Chemicals, Neuss, Germany).

## Hepatic lipid content

Liver lipids were extracted according to a modified protocol from Bligh and Dyer [21]. Briefly, small liver pieces were homogenized in ice-cold methanol. After centrifugation, lipids were extracted by addition of 1800 µl $CH_3OH$: $CHCl_3$ (1:3 v/v) to 45 µl homogenate, followed by vigorous vortexing and phase separation by centrifugation (5 min at 2,000 rpm). The $CHCl_3$ phase was dried and dissolved in 2% Triton X-100. TG, TC, and PL concentrations were measured using commercial kits as described above. Liver lipids were expressed as nmol per mg protein, which was determined using the BCA protein assay kit.

## Hepatic gene expression analysis

Total RNA was extracted from liver pieces using the Nucleospin RNA II kit (Macherey-Nagel, Duren, Germany) or from muscle pieces using the RNeasy Fibrous Tissue Mini kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. RNA quality of each sample was examined by the lab-on-a-chip method using Experion Std Sens analysis kit (Biorad, Hercules, CA) and RNA concentration of each sample was determined by Nanodrop technology (Thermo Scientific, Wilmington, USA). Then, total RNA was reverse-transcribed with iScript cDNA synthesis kit (1708891, Bio-Rad), subsequently, obtained cDNA was purified with Nucleospin Extract II kit (636973, Macherey-Nagel, Bioké). Real-time PCR was performed on a CFX96 machine (Bio-Rad), the reaction mixture consisting of SYBR-Green Sensimix (QT615, GC Biotech), cDNA, primers (Biolegio, Nijmegen, The Netherlands), and nuclease-free water in a total reaction volume of 10 µl. mRNA values of each gene were normalized to mRNA levels of cyclophilin (*Cyclo*) and hypoxanthine ribosyltransferase (*Hprt*). Primer sequences are listed in supplementary table 1.

## Statistical analysis

Differences between groups were determined with the Kruskal-Wallis non-parametric test for *k* independent samples. When significant differences were found, the Mann-Whitney non-parametric test was used as a post-hoc test to determine differences between two independent groups. A *P*-value of less than 0.05 was considered statistically significant. Data are presented as means ± SEM.

**Supplemental Table 1. Primer sequences used for RT-qPCR**

| Gene | Forward primer | Reverse Primer |
|---|---|---|
| Abcg5 | TGTCCTACAGCGTCAGCAACC | GGCCACTCTCGATGTACAAGG |
| Acox1 | TATGGGATCAGCCAGAAAGG | ACAGAGCCAAGGGTCACATC |
| Apob | GCCCATTGTGGACAAGTTGAT C | CCAGGACTTGGAGGTCTTGGA |
| Cpt1 | GAGACTTCCAACGCATGACA | ATGGGTTGGGGTGATGTAGA |
| Cyclo | CAAATGCTGGACCAAACACAA | GCCATCCAGCCATTCAGTCT |
| Dgat1 | TCCGTCCAGGGTGGTAGTG | TGAACAAAGAATCTTGCAGACGA |
| Fasn | TCCTGGGAGGAATGTAAACAGC | CACAAATTCATTCACTGCAGCC |
| Hmgcoar | CCGGCAACAACAAGATCTGTG | ATGTACAGGATGGCGATGCA |
| Hprt | TTGCTCGAGATGTCATGAAGGA | AGCAGGTCAGCAAAGAACTTATAG |
| Mttp | CTCTTGGCAGTGCTTTTTCTCT | GAGCTTGTATAGCCGCTCATT |
| Pgc1α | TGCTAGCGGTTCTCACAGAG | AGTGCTAAGACCGCTGCATT |
| Pgc1β | TTGTAGAGTGCCAGGTGCTG | CCTCCATAGCTCAGGTGGAA |
| Srebp-1c | GGAGCCATGGATTGCACATT | GGCCCGGGAAGTCACTGT |
| Ucp1 | TCAGGATTGGCCTCTACGAC | TGCATTCTGACCTTCACGAC |

*Abcg5*, ATP-binding cassette sub-family G member 5; *Acox1*, acyl-CoA oxidase 1; *Apob*, apolipoprotein B; *Cpt1*, carnitine palmitoyltransferase 1; *Cyclo*, cyclophilin; *Dgat1*, acyl:diacylglycerol transferase 1; *Fasn*, fatty acid synthase; *Hmgcoar*, HMG-CoA reductase; *Hprt*, hypoxanthine ribosyltransferase; *Mttp*, microsomal TG transfer protein; *Pgc1α*, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *Pgc1β*, peroxisome proliferator-activated receptor gamma coactivator 1-beta; *Srebp-1c*, sterol regulatory element binding protein 1c; *Ucp1*, uncoupling protein 1.

# RESULTS

## GLP-1 receptor agonism reduces body weight and fasting plasma glucose and insulin levels in high fat diet-fed *E3L* mice

*E3L* mice were fed a HFD for 18 weeks and thereafter were treated with the GLP-1 receptor agonists CNTO3649 or exendin-4 via subcutaneous osmotic minipumps for 4 weeks while continuing the HFD. Body weight and fasting plasma glucose and insulin levels before and after treatment are shown in Figure 1. Eighteen weeks of HFD feeding increased body weight (+24%, $P<0.05$) (Fig. 1A), tended to increase fasting plasma glucose (+36%, $P=0.055$) (Fig. 1B) and increased fasting insulin levels (13-fold, $P<0.05$) (Fig. 1C) compared to chow diet feeding. CNTO3649 (1.0 and 3.0 mg/kg/day) and the low dose of exendin-4 (15 µg/kg/day) did not affect body weight, whereas the high dose of exendin-4 (50 µg/kg/day) decreased body weight (-16%, $P<0.01$) (Fig. 1A) as compared to HFD control mice. Both doses of CNTO3649 and exendin-4 decreased fasting plasma glucose levels (up to -30%, $P<0.05$ and -28%, $P<0.01$, respectively) compared to HFD control mice (Fig. 1B). Additionally, the high dose of both CNTO3649 and exendin-4 decreased plasma insulin (-43% and -65%, respectively, $P<0.05$ for exendin-4 only) compared to HFD control mice (Fig. 1C). Collectively, these data confirm that GLP-1 receptor agonism by either CNTO3649 or exendin-4 improved glycemic control in the HFD-fed *E3L* mouse model.



## GLP-1 receptor agonism reduces hepatic secretion of VLDL particles without affecting particle composition

To evaluate the effect of GLP-1 receptor agonism on hepatic VLDL production, mice received an intravenous injection of Tran$^{35}$S to label newly formed apoB, and tyloxapol to block LPL-mediated lipolysis of newly synthesized VLDL. HFD feeding increased the hepatic production rate of both VLDL-TG (Fig. 2A-C) and VLDL-apoB (Fig. 2D) compared to chow diet, which is in line with a previous study [22]. Interestingly, the VLDL-TG production rate induced by HFD was reduced by both doses of CNTO3649 (up to -36%, $P<0.01$) (Fig. 2A, C) and exendin-4 (up to -54%, $P<0.001$) (Fig. 2B, C), as determined from the slope of the curve from the individual mice. Likewise, the VLDL-apoB production rate as induced by HFD was decreased by the high dose of both CNTO3649 and exendin-4 (-36% and -43%, $P<0.01$, respectively) (Fig. 2D). HFD feeding increased the TG/apoB ratio within VLDL as compared to chow feeding by +68% ($P<0.001$) (Fig. 2E), indicating that HFD induces the formation of larger lipid-enriched VLDL particles. However, both CNTO3649 and exendin-4 did not affect the VLDL-TG/apoB ratio compared with HFD control group.

Since each VLDL particle contains a single apoB molecule, GLP-1 receptor agonism apparently decreases the production rate of VLDL particles rather than decreasing the lipidation of VLDL particles. Accordingly, CNTO3649 and exendin-4 did not affect the composition of VLDL with respect to TG, TC, PL, and protein content as compared with HFD control group (Fig. 2F).

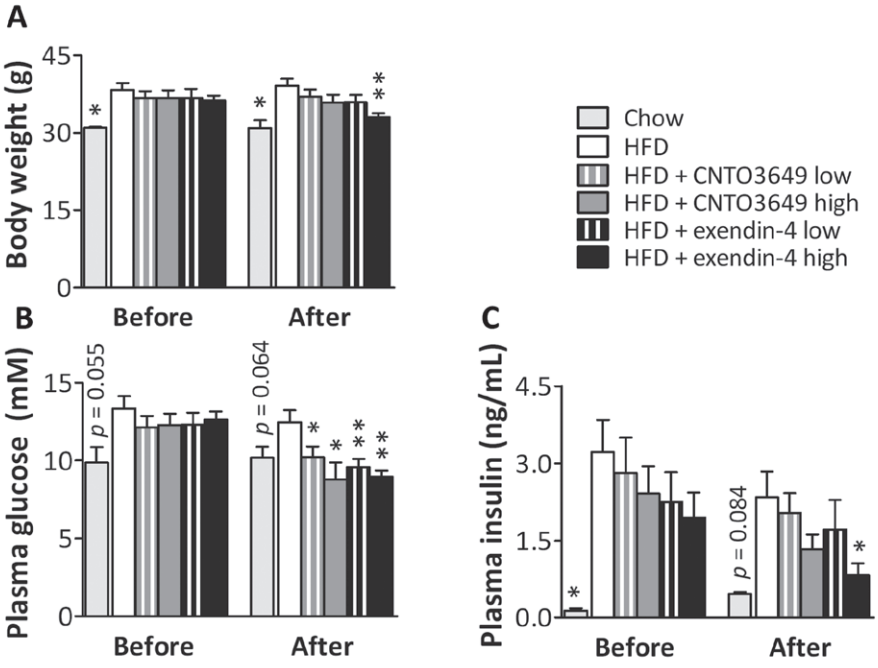



**Figure 1. GLP-1 receptor agonism reduces body weight and fasting plasma glucose and insulin levels.** *APOE*3-Leiden* (*E3L*) mice were fed a high fat diet (HFD) for 22 weeks. The last 4 weeks, mice were treated with either vehicle (HFD control), CNTO3649 (1.0 or 3.0 mg/kg/day) or exendin-4 (15 or 50 µg/kg/day). As a control for HFD feeding, an additional group of mice was included fed a chow diet that received vehicle (chow control). Blood was collected by tail bleeding after 7 h of fasting. Just before drug treatment (week 18) and after treatment (week 22), body weight (A), plasma glucose (B) and plasma insulin (C) levels were determined. Values are means ± SEM for at least 6 mice per group. **P*<0.05, ***P*<0.01 compared to HFD controls.

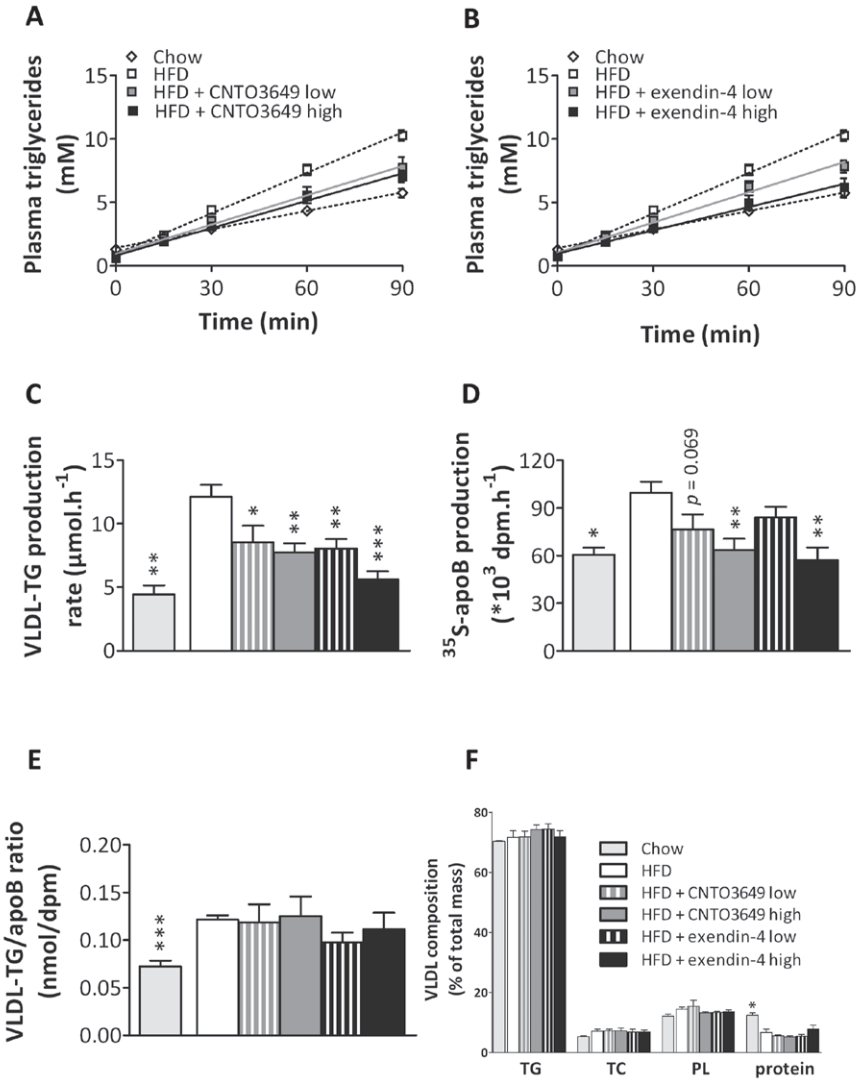



**Figure 2. GLP-1 receptor agonism reduces hepatic VLDL-TG and VLDL-apoB production without affecting VLDL particle composition.** *E3L* mice were fed a HFD for 22 weeks. The last 4 weeks, mice were treated with either vehicle (HFD control), CNTO3649 (1.0 or 3.0 mg/kg/day) or exendin-4 (15 or 50 µg/kg/day). As a control for HFD feeding, an additional group of mice fed a chow diet was included that received vehicle (chow control). After 7 h fasting, mice were injected with Tran$^{35}$S label (t=-30 min) and Triton WR-1339 (t=0 min). Blood was drawn at the indicated time points and plasma TG concentrations were determined (A, B). VLDL-TG production rate was calculated as µmol/h from the slopes of the TG-time curves of the individual mice (C). At t=120 min, mice were exsanguinated, and VLDL was isolated by density gradient ultracentrifugation. $^{35}$S-activity was determined, and VLDL-apoB production rate was calculated as dpm.h$^{-1}$ (D). The VLDL-TG production rate to VLDL-apoB production rate ratio was calculated as nmol/dpm (E). The content of triglycerides, cholesterol, phospholipids and protein in VLDL was determined and calculated as % of total mass (F). Values are means ± SEM for at least 6 mice per group. *$P$<0.05, **$P$<0.01, ***$P$<0.001 compared to HFD controls. TG: triglycerides; TC: total cholesterol; PL: phospholipids; Pro: protein.

## GLP-1 receptor agonism reverses high fat diet-induced hepatic steatosis

To obtain insight into the mechanism underlying the reduction in hepatic VLDL production induced by GLP-1 agonism, we next determined the effect of the GLP-1 receptor agonists on hepatic lipid content in a second set of mice. Consistent with the first experiment, CNTO3649 and exendin-4 decreased body weight and fasting plasma glucose and insulin levels compared to the HFD control group (Supplemental Fig. 1) to a similar extent as in the first study. HFD feeding induced a marked increase in hepatic TG, TC, and PL content compared to chow diet (Fig. 3), indicating that HFD leads to hepatic steatosis. The high dose of both CNTO3649 and exendin-4 largely reduced hepatic TG (-39%, $P<0.05$ and -55%, $P<0.01$, respectively). Hepatic TC was reduced by both doses of CNTO3649 and exendin-4, (up to -32%, $P<0.05$ and -55%, $P<0.01$, respectively). Also, both doses of CNTO3649 and exendin-4 reduced hepatic PL (up to -23%, P<0.01 and -36%, $P<0.01$, respectively). Importantly, hepatic lipid content observed after treatment with the high dosages of CNTO3649 and exendin-4 group did not differ from that of the chow control group ($P>0.05$), suggesting that GLP-1 receptor agonism completely reversed HFD-induced hepatic steatosis.



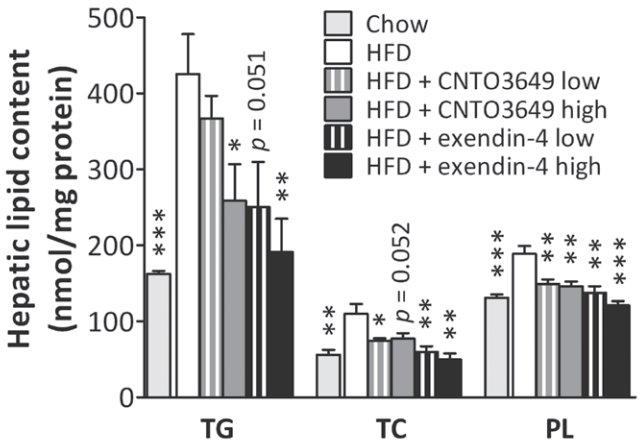
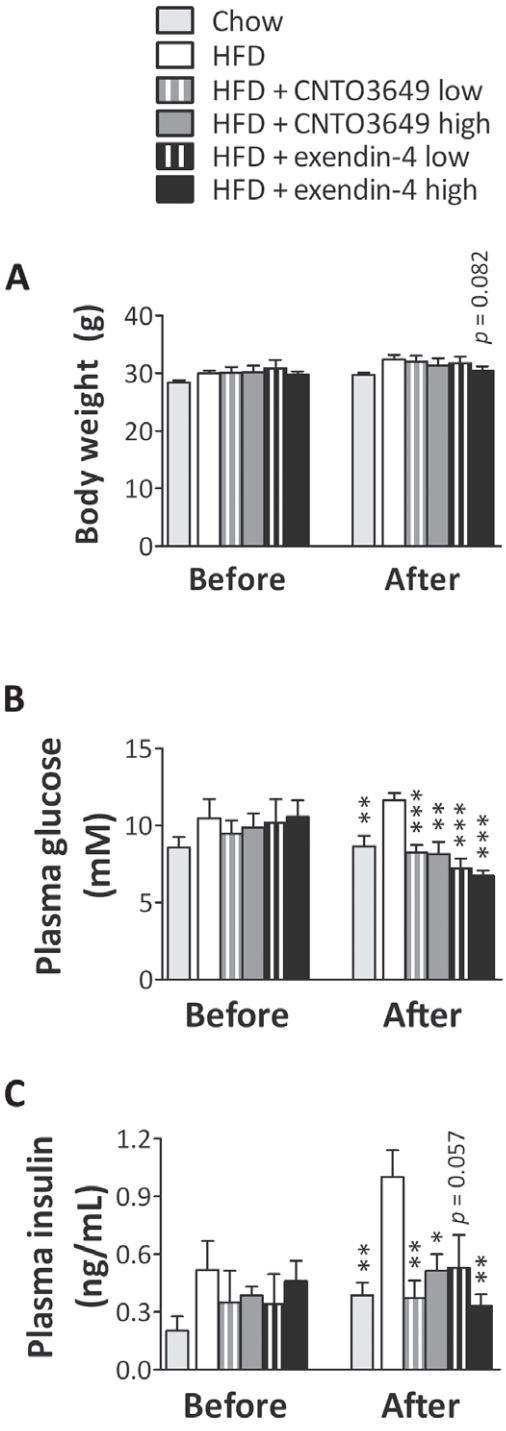



**Figure 3. GLP-1 receptor agonism reverses high fat diet-induced hepatic steatosis.** *E3L* mice were fed HFD for 13 weeks. The last 4 weeks, mice were treated with either vehicle (HFD control), CNTO3649 (0.3 or 1.0 mg/kg/day) or exendin-4 (15 or 50 µg/kg/day). As a control for HFD feeding, an additional group of mice was included fed a chow diet that received vehicle (chow control). Livers were isolated from 7 h fasted mice, liver pieces were homogenized, and triglycerides, cholesterol and phospholipids were determined as nmol per mg protein. Values are means ± SEM for at least 6 mice per group. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to HFD controls.




**Supplemental Figure S1. GLP-1 receptor agonism reduces fasting glucose and insulin levels.** *E3L* mice were fed a high fat diet (HFD) for 13 weeks. The last 4 weeks, mice were treated with either vehicle (HFD control), CNTO3649 (0.3 or 1.0 mg/kg/day) or exendin-4 (15 or 50 µg/kg/day). As a control for HFD feeding, an additional group of mice fed a chow diet was included that received vehicle (chow control). Just before drug treatment (week 13) and after treatment (week 17), body weight (A), plasma glucose (B) and plasma insulin (C) levels were determined. Values are means ± SEM for at least 6 mice. **P*<0.05, ***P*<0.01, ****P*<0.001 compared to HFD controls.

## GLP-1 receptor agonism affects hepatic expression of genes involved in VLDL production, lipogenesis and lipid homeostasis

To elucidate the mechanism how GLP-1 receptor agonism reduces liver lipids and VLDL secretion, we investigated the hepatic expression of genes involved in lipid metabolism (i.e. lipogenesis, VLDL secretion, cholesterol metabolism, and FA oxidation) (Fig. 4). HFD feeding strongly tended to increase the expression of peroxisome proliferator-activated receptor gamma coactivator 1-beta (*Pgc1β*), whereas the high dose of CNTO3649 and both doses of exendin-4 tended to reduce this HFD-induced increase in *Pgc1β*. Next, HFD feeding increased the expression of the lipogenic transcription factor sterol regulatory element binding protein 1c (*Srebp-1c*) (3.7-fold, $P<0.05$) (Fig. 4A) and its target gene FA synthase (*Fasn*) (6.7-fold, $P<0.01$) (Fig. 4B), which plays a role in *de novo* lipogenesis, contributing to HFD-induced hepatic steatosis. The high dose of CNTO3649 and both doses of exendin-4 decreased the expression of *Srebp-1c* (-53%, $P<0.05$, and up to -75%, $P<0.05$, respectively) (Fig. 4A) and the low dose of CNTO3649 and the high dose of exendin-4 decreased *Fasn* (-40%, $P<0.05$ and -53%, $P<0.01$, respectively) (Fig. 4B) compared with the HFD group. Acyl:diacylglycerol transferase 1 (*Dgat1*), which catalyzes the final step in hepatic TG synthesis, was significantly decreased by exendin-4 only (up to -71%, $P<0.05$) (Fig. 4C). In addition, the high dose of CNTO3649 and both doses of exendin-4 suppressed expression of apoB (*Apob*) (-62%, $P<0.05$ and up to -72%, $P<0.01$, respectively) (Fig. 4D), without affecting expression of microsomal TG transfer protein (*Mttp*) (Fig. 4E) that mediates apoB lipidation. In addition, the high dose of CNTO3649 and both doses of exendin-4 suppressed the expression of the FA oxidation gene acyl-CoA oxidase 1 (*Acox1*) (-56% and up to -75%, $P<0.01$, respectively) (Fig. 4F). Moreover, both doses of CNTO3649 and exendin-4 increased the expression of HMG-CoA reductase (*Hmgcoar*) (up to 2.1-fold and 3.4-fold, $P<0.05$, respectively) involved in *de novo* cholesterol synthesis (Fig. 4G). Finally, the expression of ATP-binding cassette sub-family G member 5 (*Abcg5*), involved in bile acid secretion, was significantly decreased for both doses of exendin-4 only (up -58%, $P<0.01$) (Fig. 4H).

Collectively, these data indicate that GLP-1 receptor agonism decreases lipogenesis and apoB synthesis, consequently resulting in suppression of VLDL particle production and a compensatory decrease in hepatic FA oxidation. In addition, the reduction in hepatic cholesterol content results in compensatory mechanisms to increase hepatic cholesterol synthesis and decrease secretion of hepatic cholesterol as bile acids.

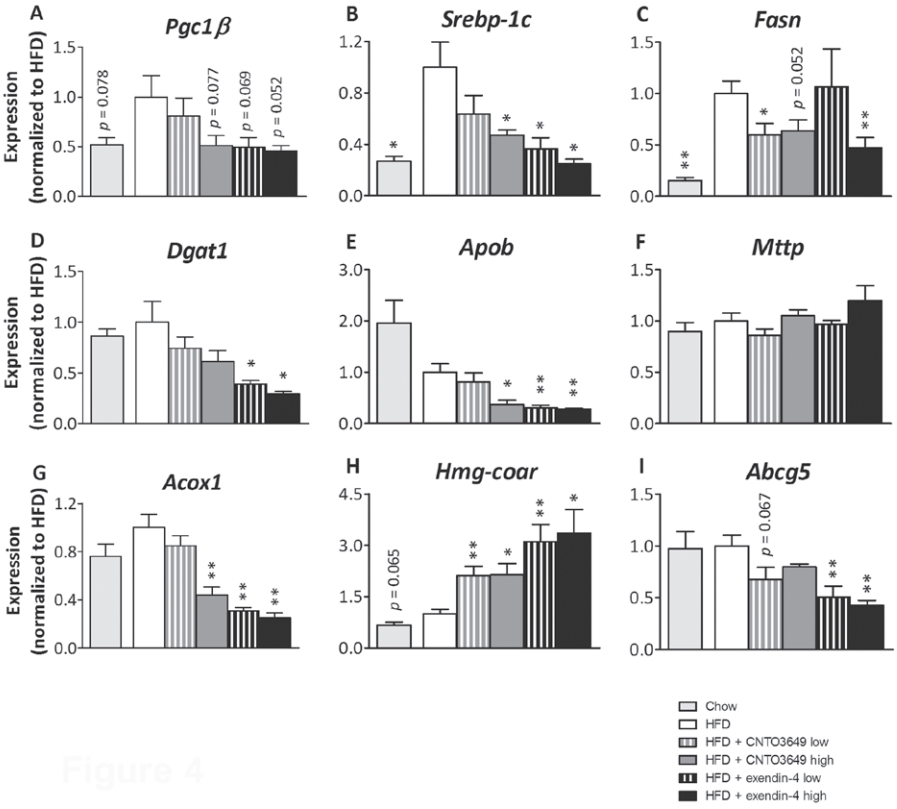
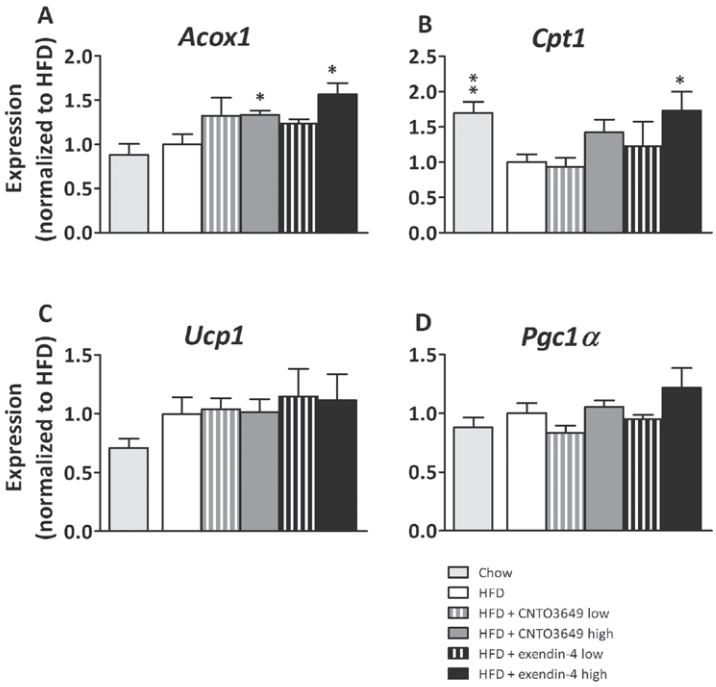



**Figure 4. GLP-1 receptor agonism affects hepatic expression of genes involved in VLDL production, lipogenesis, and lipid homeostasis.** *E3L* mice were fed HFD for 13 weeks. The last 4 weeks, mice were treated with either vehicle (HFD control), CNTO3649 (0.3 or 1.0 mg/kg/day) or exendin-4 (15 or 50 µg/kg/day). As a control for HFD feeding, an additional group of mice was included fed a chow diet that received vehicle (chow control). Livers were isolated from 7 h fasted mice, and mRNA was extracted from liver pieces. mRNA values of indicated genes were normalized to *Cyclo* and *Hprt* mRNA levels. Data were calculated as fold difference as compared with the HFD control group. Values are means ± SEM for at least 6 mice per group. **P*<0.05, ***P*<0.01, ****P*<0.001 compared to HFD controls.

## GLP-1 receptor agonism affects muscle expression of genes involved in fatty acid oxidation

To gain insight into the fate of the FA from the diet we also measured muscle expression of genes involved in thermogenesis and FA oxidation (Supplemental Fig. 2). No differences between groups were found for the thermogenic markers uncoupling protein 1 (*Ucp1*) and peroxisome proliferator-activated receptor gamma coactivator

1-alpha (*Pgc1α*), suggesting that both compounds did not affect energy expenditure by the muscle. However, the expression of *Acox1* was significantly increased for the high dose of CNTO3649 and exendin-4 (+33% and +56%, $P<0.05$, respectively) as compared to HFD controls. Also, the high dose of exendin-4 increased the expression of carnitine palmitoyltransferase 1 (*Cpt1*) (+73%, $P<0.05$). These data indicate an increased FA oxidation in muscle.




**Supplemental Figure S2. GLP-1 receptor agonism affects muscle expression of genes involved in fatty acid oxidation.** *E3L* mice were fed HFD for 13 weeks. The last 4 weeks, mice were treated with either vehicle (HFD control), CNTO3649 (0.3 or 1.0 mg/kg/day) or exendin-4 (15 or 50 µg/kg/day). As a control for HFD feeding, an additional group of mice was included fed a chow diet that received vehicle (chow control). Skeletal muscles were isolated from 7 h fasted mice, and mRNA was extracted from muscle pieces. mRNA values of indicated genes were normalized to *Cyclo* and *Hprt* mRNA levels. Data were calculated as fold difference as compared with the HFD control group. Values are means ± SEM for at least 6 mice per group. *$P<0.05$, **$P<0.01$ compared to HFD controls.

## DISCUSSION

In the present study, we show that GLP-1 receptor agonism both by CNTO3649 and exendin-4 decreases fasting plasma glucose and insulin levels in HFD-fed *E3L* mice. This is in line with earlier reports that GLP-1 and its analogs ameliorate whole-body glucose intolerance in obese animal models [23, 24] and in T2DM patients [25]. More importantly, to our knowledge, this study is the first to demonstrate that CNTO3649 and exendin-4 reduce hepatic VLDL particle production, evidenced by similarly reduced VLDL-TG and VLDL-apoB production rates. In addition, both GLP-1 receptor agonists largely decrease the hepatic lipid content thereby reversing HFD-induced hepatic steatosis.

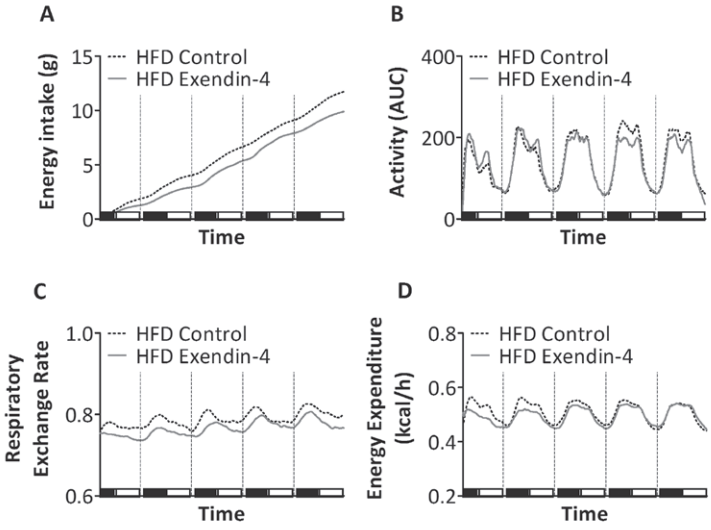
Increased plasma VLDL-TG levels are a central feature of T2DM, and are mainly caused by increased hepatic VLDL-TG and VLDL-apoB production [26]. We observed that HFD feeding increased VLDL-TG and VLDL-apoB production, and in addition increased the VLDL-TG/apoB ratio. As each VLDL particle contains a single molecule of apoB, VLDL-apoB reflects particle number, whereas VLDL-TG reflects the major lipid constituent of the particle. An increased VLDL-apoB production rate with a concomitantly increased VLDL-TG/apoB ratio thus indicates that HFD feeding not only results in overproduction of VLDL particles but also in the formation of larger lipid-enriched VLDL particles. Both CNTO3649 and exendin-4 reduced the VLDL-TG and VLDL-apoB production rates without affecting the VLDL-TG/apoB ratio, suggesting that GLP-1 agonism reduces the VLDL particle production without affecting the lipidation of VLDL-apoB. This was indeed confirmed by VLDL composition analysis. Notably, we have observed the same effects in WT mice treated with exendin-4 (data not shown), ruling out a possible impact of the genetic background of the *E3L* mice on the treatment outcome.

We also observed that both CNTO3649 and exendin-4 completely reversed HFD-induced hepatic steatosis reflected by largely decreased hepatic TG and TC contents to the low levels observed in chow-fed control mice. This corroborates recent studies showing that prolonged infusion of exendin-4 in *ob/ob* mice reduced hepatic TG accumulation [27]. Hepatic gene expression analysis revealed that the GLP-1 receptor agonists decreased the expression of the nuclear transcription factor *Srebp-1c* and its targets *Fasn* and *Dgat1*, which are involved in *de novo* FA and TG synthesis, respectively. At the same time, the GLP-1 receptor agonists decreased *ApoB* expression without affecting the expression of *Mttp*, of which the gene product MTP is involved in the transfer of TG onto apoB. On the other hand, they decreased the expression of *Acox1* and *Cpt1a* (not shown), both of which are involved in FA oxidation.

Collectively, these data strongly suggest that GLP-1 receptor agonism primarily reduces hepatic lipogenesis, thereby causing a reduction in hepatic TG content, with a

compensatory reduction in FA oxidation. Taken together with the concomitantly reduced apoB production, lower hepatic availability of TG results in a reduced production of VLDL particles. It is known that the contribution of *de novo* lipogenesis to total hepatic VLDL secretion strongly increases from 2-5% under healthy conditions up to 25-30% in T2DM patients with hepatic steatosis [28, 29]. Since HFD feeding of *E3L* mice similarly induced hepatic steatosis, the contribution of de novo lipogenesis to the increased VLDL production was likely also augmented by HFD feeding, and reversed by GLP-1 receptor agonism concomitant with attenuating hepatic steatosis. Interestingly, the increased expression of *Acox* and *Cpt1* in the muscle suggests an increase in FA oxidation. In addition, by indirect calorimetry (Supplemental Fig. S3) we observed that exendin-4 treatment reduces the respiratory exchange rate, indicating an increased oxidation of fat. Although this effect was transient, it might have contributed to differences in the observed phenotypes after prolonged treatment. Collectively, these data suggest that GLP-1 receptor agonism not only decreases the production of FA, but also upregulates an oxidative pathway in the muscle to deal with the elevated uptake of FA present in the diet.






**Supplemental Figure S3. Exendin-4 treatment reduces respiratory exchange ratio.** C57Bl/6 mice were fed a HFD for 3 weeks before they were treated with either vehicle (control) or exendin-4 (50 µg/kg/day). Directly after the initiation of the treatment, indirect calorimetry measurements were started. Individual energy intake (A), activity (B), $O_2$ consumption, and $CO_2$ production rates were monitored. Respiratory exchange rate (C) and total energy expenditure (D) were calculated from the $O_2$ consumption and $CO_2$ production rates. Lines represent the mean values of 8 mice treated with vehicle (solid lines) or exendin-4 (dotted lines). Black areas under the x-axis represent the dark (12 hours) and white areas the light periods (12 hours). *$P$<0.05 compared to HFD controls.

The mechanism underlying the reduced hepatic cholesterol content is less clear, although it may be expected from the reduced TG content given the tight relationship between hepatic TG and cholesterol levels. The reduction in hepatic cholesterol content evidently results in a compensatory induction of *Hmgcoar*, involved in *de novo* cholesterol synthesis, and downregulation of *Abcg5*, involved in the elimination of hepatic cholesterol as bile acids into the bile.

It is interesting to speculate on the molecular mechanisms that underlie the reduction in hepatic lipogenesis as induced by GLP-1 receptor agonism. Since *Srebp-1c* plays a crucial role in insulin-mediated *de novo* lipogenesis in the liver [30], it is well possible that the improved HFD-induced glucose intolerance accompanied by reduced insulin levels resulted in downregulation of hepatic *Srebp-1c* expression, thereby attenuating the HFD-induced increase in *de novo* lipogenesis. Beside insulin levels, *Pgc1β* could also be involved as it impacts on *Srebp-1c* expression [31]. Therefore, the observed trend towards a reduction in *Pgc1β* expression might also have contributed to a decreased *Srebp-1c* and consequently a decrease in *de novo* lipogenesis. Interestingly, it has recently been established that human hepatocytes [32, 33] as well as rodent hepatocytes [34] express GLP-1 receptors. In fact, incubation of hepatocytes with GLP-1 and exendin-4 in the absence of insulin directly reduces *Srebp-1c* [34], and reduces hepatocyte steatosis [32]. This indicates that GLP-1 receptor agonism may directly downregulate *Srebp-1c* and lipogenesis through binding of hepatocytic receptors. Third, GLP-1 receptor agonism by exendin-4 or the DPP-4 inhibitor sitagliptin reduces the intestinal production of chylomicron-TG and apoB in hamsters [35], and the DPP-4 inhibitor vildagliptin reduces postprandial chylomicron-TG and apoB in T2DM patients [36]. Indeed, pilot data from our lab confirmed that exendin-4 reduces postprandial TG excursion in mice (unpublished). Since uptake of TG from the diet eventually contributes to VLDL-TG production [37, 38], reduced chylomicron production may contribute to the observed effect of GLP-1 receptor agonism on hepatic VLDL production. Finally, circulating GLP-1 can cross the blood-brain barrier [39] and GLP-1 receptors are abundantly expressed in many brain areas [40]. Several studies have shown that central GLP-1 receptor signaling mediates the effect of GLP-1 on hepatic glucose output [41] and lipid deposition in white adipose tissue [42]. It is therefore reasonable to postulate that the brain-nerve-liver axis might contribute to the beneficial effects of GLP-1 agonism on hepatic lipid metabolism and VLDL secretion.

In our previous study, in which we administered exendin-4 by daily intraperitoneal injections, we were unable to detect any effect of exendin-4 on VLDL production, albeit that exendin-4 did improve glucose tolerance [12]. In that study, daily injections of CNTO736, a previous version of CNTO3649, with a considerably longer half-life than exendin-4, did reduce VLDL production. Since we now demonstrate that continuous

delivery of exendin-4 inhibits VLDL production, it is likely that the ability of GLP-1 agonists to reduce hepatic steatosis and VLDL production is mainly determined by their pharmacokinetic profile. Whereas pulsated exposure of GLP-1 (analogues) is sufficient to improve glucose intolerance, more chronic exposure is required for additional beneficial effects on TG metabolism.

How do the present data obtained in *E3L* mice translate to clinical practice? Recently, it has been reported that treatment of T2DM patients with exenatide on top of pioglitazone resulted in a greater decrease in both hepatic TG and plasma TG levels compared to treatment with pioglitazone only [43]. Based on our present data, it is likely that these observations can be explained by a reduction in *Srebp-1c*-induced lipogenesis, resulting in attenuation of the hepatic TG content, reduction of VLDL-TG production and thus plasma VLDL-TG levels. Therefore, it would be interesting to determine the effect of GLP-1 analogues and DPP-4 inhibitors on VLDL production in future human intervention studies. In addition, we anticipate that the effects of long-circulating GLP-1 receptor agonists such as liraglutide (duration of action ≥ 24 hours) on lipid metabolism will prove to be superior to those of exenatide (duration of action < 24 hours) and in addition will lead to better tolerability due to the necessity of injecting once daily only as compared to twice daily for exenatide [44]. In addition to the beneficial effects on glucose metabolism, VLDL secretion and hepatic lipid content, GLP-1 receptor agonism also reduces blood pressure and the severity of myocardial infarction, while it concomitantly improves left ventricular ejection fraction after infarction [45], enforcing GLP-1 receptor agonism as a valuable therapy to combat T2DM and associated cardiovascular diseases.

In conclusion, our results show that GLP-1 agonism not only decreases bodyweight and improves glycemic control, but also reduces HFD-induced hepatic steatosis, thereby reducing hepatic VLDL biosynthesis and secretion. Therefore, we anticipate that GLP-1 receptor agonism is a valuable strategy to treat T2DM patients, especially those with disturbed lipid metabolism related to hepatic steatosis.



## ACKNOWLEDGEMENTS

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# 9

# EXENDIN-4 DECREASES LIVER INFLAMMATION AND ATHEROSCLEROSIS DEVELOPMENT SIMULTANEOUSLY BY REDUCING MACROPHAGE INFILTRATION

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## ABSTRACT

The etiology of inflammation in the liver and vessel wall, leading to non-alcoholic steatohepatitis (NASH) and atherosclerosis respectively, share common mechanisms including macrophage infiltration. To test both disorders simultaneously, it is highly important to tackle the inflammatory status. Exendin-4, a glucagon-like peptide-1 receptor agonist, reduces hepatic steatosis and has been suggested to reduce atherosclerosis; however its effects on liver inflammation are underexplored. Here, we tested the hypothesis that exendin-4 reduces inflammation in both the liver and vessel wall, and investigated the common underlying mechanism. Female *APOE*3-Leiden.CETP* mice, a model with human-like lipoprotein metabolism that develops human-like atherosclerosis, were fed a cholesterol-containing Western-type diet for 5 weeks and were subsequently treated for 4 weeks with exendin-4 or vehicle. Exendin-4 slightly improved dyslipidemia, but markedly decreased atherosclerotic lesion severity and area (-33%), accompanied with a reduction in monocyte adhesion to the vessel wall (-42%) and macrophage content in the plaque (-44%). Furthermore, exendin-4 reduced hepatic lipid content and inflammation as well as hepatic $CD68^+$ (-18%) and $F4/80^+$ (-25%) macrophage content. This was accompanied by less monocyte recruitment from the circulation as the $Mac\text{-}1^+$ macrophage content was decreased (-36%). Finally, exendin-4 reduced chemokine expression in vivo and suppressed oxidized LDL accumulation in peritoneal macrophages in vitro, dependent on the GLP-1 receptor. In conclusion, exendin-4 reduces inflammation in both the liver and the vessel wall by reducing macrophage recruitment and activation. These data suggest that exendin-4 could be a valuable strategy to treat NASH and arteriosclerosis simultaneously.

# INTRODUCTION

Cardiovascular disease (CVD) due to atherosclerosis is the leading cause of morbidity and mortality in the Western world. There is a strong association between atherosclerosis and non-alcoholic fatty liver disease (NAFLD), which raised the interest of the role of the liver in the development of atherosclerosis. NAFLD embraces a pathological spectrum of liver diseases, from steatosis with virtually no evidence of hepatocellular injury or liver inflammation to non-alcoholic steatohepatitis (NASH) and cirrhosis [1]. NASH is characterized by accumulation of fat in the liver in combination with hepatic inflammation [2]. This inflammatory response was assumed to be the consequence rather than the cause of the disease. However, compelling data point to a central initiating role of monocyte recruitment and macrophage activation in the progression of hepatic inflammation in a similar way as in the development of atherosclerosis as we recently reviewed [3]. The potential of a shared etiology between inflammation in the liver and vessel wall leads to putative intervention therapies to tackle both disorders at the same time by targeting macrophage infiltration. This is particularly interesting, since the current standard therapies to reduce inflammation in NASH have only limited effectiveness. Lifestyle intervention, including exercise, is recommended in patients with NASH. However, it appears to be difficult to achieve improvements in NASH in the long run and pharmacological intervention is ultimately required [4].



Glucagon-like peptide-1 receptor (GLP-1R) agonists, such as exendin-4, are currently being validated for the treatment of type 2 diabetes mellitus and have been shown to increase glucose-dependent insulin secretion, to regulate gastric emptying, and to reduce food intake and body weight [5]. In addition to improving glycemic control, GLP-1R activation improves lipid metabolism. We [6] and others [7-9] have shown that GLP-1R activation reduces hepatic steatosis, increases hepatic lipid oxidation, decreases lipogenesis, and attenuates hepatic VLDL production.Collectively, these data indicate the potential of GLP-1R activation to treat NAFLD, but the impact on liver inflammation and atherosclerosis is still uncertain. Moreover, contrasting results have been reported regarding the effects on atherosclerosis in ApoE$^{-/-}$ mice. While Gaspari *et al.* [10] showed clear inhibiting effects on the progression of atherosclerosis, Panjwahi *et al.* [11] did not observe any effects on atherosclerosis development. It should be noted that these mice do not respond to lipid-lowering therapy and lack apoE, a crucial factor for cholesterol efflux from macrophages. Given this controversy, combined with the reduction in hepatic steatosis, it is of significant importance to further explore the therapeutic potential of GLP-1R activation to impact on inflammation in both the liver and vessel wall.

The aim of the current study was to evaluate the effect of exendin-4 treatment on hepatic inflammation in addition to its effect on the development of atherosclerosis, and to elucidate the underlying mechanisms, in A*POE*3-Leiden.CETP* (*E3L.CETP*) mice fed a Western-type diet. We show that exendin-4 reduces the influx of macrophages into both the liver and vessel wall, and thereby limits the progression of hepatic inflammation and atherosclerosis simultaneously.

## MATERIALS AND METHODS

### Animals

Twelve week old, female *E3L.CETP* transgenic mice expressing human CETP under the control of its natural flanking regions were used [12] and housed under standard conditions in conventional cages with free access to food and water unless indicated otherwise. The animals were fed a semi-synthetic Western-type diet, containing 0.4% (w/w) cholesterol, 1% (w/w) corn oil, and 15% (w/w) cacao butter (Hope Farms, Woerden, The Netherlands) for 5 weeks. After randomization according to body weight, plasma total cholesterol (TC), and triglyceride (TG) levels, an osmotic minipump (model 1004, Alzet DURECT Corp., Cupertino, CA) was implanted subcutaneously in the left back region under light isoflurane anesthesia for the continuous delivery of exendin-4 (50 µg/kg/day, Bachem AG, Bubendorf, Switzerland; dissolved in PBS) or PBS as a control for 4 weeks while the Western-type diet was continued. Experiments were performed after 4 h of fasting at 12:00 pm with food withdrawn at 8:00 am. The Institutional Ethics Committee for Animal Care and Experiments from the Leiden University Medical Center, Leiden, the Netherlands approved all experiments.



### Blood sampling, plasma metabolites, and lipoprotein profiles

Blood was obtained via tail vein bleeding into heparin-coated capillary tubes. The tubes were placed on ice and centrifuged, and the obtained plasma was snap-frozen in liquid nitrogen and stored at -20°C until further measurements. Plasma was assayed for glucose (Instruchemie, Delfzijl, The Netherlands) as well as TC, and TG using the commercially available enzymatic kits 236691, 11488872 (Roche Molecular Biochemicals, Indianapolis, IN, USA), respectively. Plasma insulin was measured by ELISA (Mercodia AB, Uppsala, Sweden). The distribution of lipids over plasma lipoproteins was determined using fast protein liquid chromatography. Plasma was pooled per group, and 50 µL of each pool was injected onto a Superose 6 PC 3.2/30 column (Äkta System, Amersham Pharmacia Biotech, Piscataway, NJ, USA) and eluted at a constant flow rate of 50 µL/min in PBS, 1 mM EDTA, pH 7.4. Fractions of 50 µL were collected and assayed for TC as described above.

## Plasma CETP concentration

Plasma CETP concentration was measured using the DAIICHI CETP ELISA kit according to manufacturer's instructions (Daiichi, Tokyo, Japan).

## Atherosclerosis quantification and monocyte adhesion to the endothelium wall

After 4 weeks of treatment, mice were sacrificed and perfused with ice-cold PBS via the heart. Hearts were isolated and fixed in phosphate-buffered 4% formaldehyde, dehydrated, embedded in paraffin and cross-sectioned (5 µm) through the aortic root area. Cross-sections were stained with hematoxylin-phloxine-saffron to determine lesion area and lesion severity as described before [13, 14]. Briefly, various types of lesions were discerned: no lesions, mild lesions with fatty streak-like lesions containing foam cells, and severe lesions referred to advanced lesions containing foam cells in the media, presence of fibrosis, cholesterol clefts, mineralization and/or necrosis. Additionally, cross-sections were stained with AIA 31420 antiserum (1:3000, Accurate Chemical and Scientific, Westbury, NY, USA) to determine macrophage area and monocyte adhesion to the endothelium wall as described [15]. Lesion area and macrophage area were determined using Leica Qwin-software.



## Hepatic lipid content

After 4 weeks of treatment, mice were sacrificed and perfused with ice-cold PBS via the heart, and livers were isolated. Lipids were extracted according to a modified protocol from Bligh and Dyer [16]. Briefly, small liver pieces were homogenized in ice-cold methanol. After centrifugation, lipids were extracted by addition of 1800 µl $CH_3OH$: $CHCl_3$ (1:3 v/v) to 45 µL homogenate, followed by vigorous vortexing and phase separation by centrifugation (5 min at 2,000 rpm). The $CHCl_3$ phase was dried and dissolved in 2% Triton X-100. TG and TC concentrations were measured as described above. Phospholipids (PL) concentration was measured using a commercial kit (phospholipids B, Wako Chemicals, Neuss, Germany). Liver lipids were expressed as nmol per mg protein, which was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA).

## Hepatic gene expression analysis

Total RNA was extracted from liver pieces using the Nucleospin RNAII kit (Macherey-Nagel, Duren, Germany) according to manufacturer's instructions. RNA quality was examined by the lab-on-a-chip method using Experion Std Sens analysis kit (Biorad, Hercules, CA) and RNA concentration was determined by Nanodrop technology (Thermo

Scientific, Wilmington, USA). Total RNA was reverse-transcribed with iScript cDNA synthesis kit (1708891, Bio-Rad), and the obtained cDNA was purified with Nucleospin Extract II kit (636973, Macherey-Nagel, Bioké). Real-time PCR was performed on a CFX96 machine (Bio-Rad), the reaction mixture consisting of SYBR-Green Sensimix (QT615, GC Biotech), cDNA, primers (Biolegio, Nijmegen, The Netherlands; see Supplemental Table 1 for primer sequences), and nuclease-free water in a total reaction volume of 10 µL. mRNA values of each gene were normalized to mRNA levels of cyclophilin (*Cyclo*) and hypoxanthine ribosyltransferase (*Hprt*). Data were calculated as fold difference as compared with the PBS control group.

**Supplemental Table 1. Primer sequences used for RT-qPCR**

| Gene | Forward primer | Reverse Primer |
|---|---|---|
| *Apoa1* | GGAGCTGCAAGGGAGACTGT | TGCGCAGAGAGTCTACGTGTGT |
| *Cd68* | ATCCCCACCTGTCTCTCTCA | TTGCATTTCCACAGCAGAAG |
| *CETP* | CAGATCAGCCACTTGTCCAT | CAGCTGTGTGTTGATCTGGA |
| *Cyclo* | CAAATGCTGGACCAAACACAA | GCCATCCAGCCATTCAGTCT |
| *F4/80* | CTTTGGCTATGGGCTTCCAGTC | GCAAGGAGGACAGAGTTTATCGTG |
| *Il-1ß* | GCAACTGTTCCTGAACTCAACT | ATCTTTTGGGGTCCGTCAACT |
| *Il-6* | TGTGCAATGGCAATTCTGAT | CTCTGAAGGACTCTGGCTTTG |
| *Hprt* | TTGCTCGAGATGTCATGAAGGA | AGCAGGTCAGCAAAGAACTTATAG |
| *Mcp-1* | AACTGTGTGATTGACAAGCACTTAGAC | TGACAGGATTAATGCAGCAGTGT |
| *Tnfa* | AGCCCACGTCGTAGCAAACCAC | TCGGGGCAGCCTTGTCCCTT |

*Apoa1*, apolipoprotein A1; *Cd68*, cluster of differentiation 68; *CETP*, cholesteryl ester transfer protein; *Cyclo*, cyclophilin; *Il-1ß*, interleukin-1ß; *Il-6*, interleukin-6; *Hprt*, hypoxanthine ribosyltransferase; *Mcp-1*, monocyte chemotactic protein-1; *Tnfa*, tumor necrosis factor α.

## Liver histology

Paraffin-embedded liver sections were stained for F4/80$^+$ macrophages (1/600; Serotec, Oxford, UK) as described [17]. Frozen liver sections (7 µm) were stained for CD68$^+$ resident macrophages (CD68 marker, FA11) and infiltrated macrophages (macrophage marker, Mac-1) as described previously [15].

## LDL isolation, radiolabeling, and oxidation

LDL was isolated from human serum by density-gradient ultracentrifugation as described.[17] LDL was labeled with [$^3$H]cholesteryl oleoyl ether (COEth) by incubation with donor [$^3$H]COEth-containing liposomes in the presence of human lipoprotein-deficient serum. In short, liposomes were created by sonication of 1 mg of egg yolk phosphatidylcholine and 200 µCi of [$^3$H]COEth using a Soniprep 150 (MSE Scientific Instruments, Crawley, UK). Subsequently, LDL was incubated with the liposomes (protein: liposomal phospholipid = 1:8, w/w) for 24 h at 37°C under argon. [$^3$H]COEth-labeled LDL was purified by density gradient ultracentrifugation and dialyzed overnight at 4°C against PBS, pH 7.4. Both LDL and [$^3$H]COEth-LDL were oxidized with 5 µM $CuSO_4$ at 37°C for 20 h. Oxidation was terminated by adding 200 µM EDTA. EDTA and $CuSO_4$ were removed by overnight dialysis at 4°C against PBS, pH 7.4. Proper oxidation of LDL was confirmed by a 2.5-fold increased electrophoretic mobility of oxidized LDL (oxLDL) and [$^3$H]COEth-oxLDL compared to LDL on agarose gel. Protein concentration was determined by the BCA protein assay kit.



## Plasma anti-oxLDL antibodies

An EIA/RIA high binding 96-well Costar plate (Corning Inc., Corning, NY, USA) was coated with oxLDL (7.5 µg/mL) in PBS. IgM, IgG1, and IgG2a antibodies against oxLDL in serum were measured using an ELISA Ig detection kit (Zymed Laboratories, San Francisco, CA, USA) according to the manufacturer's protocol.

## OxLDL uptake by peritoneal macrophages and Oil-red O staining for foam cells

Three days after intraperitoneal injection of 1 mL 4% thioglycollate, peritoneal macrophages from *E3L.CETP* mice were harvested into 10 mL PBS. Subsequently, cells were resuspended in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, 100 units/mL penicillin and 100 mg/mL streptomycin, and incubated at 37°C in a humidified 5% $CO_2$ incubator. Three hours post-plating, cells were washed twice with warm PBS to remove non-adherent cells. Cells were counted and seeded into 24-well plates at a density of 5 x $10^5$ cells per well for three days before the experiment. On the experimental day, peritoneal macrophages were washed three times with PBS and incubated in DMEM supplemented with 1% BSA, 100 units/mL penicillin and 100 mg/mL streptomycin for 1 h, followed by DMEM supplemented with 1% BSA, 100 units/mL penicillin, 100 mg/mL streptomycin), 10 µg/mL [$^3$H]COEth-oxLDL, and exendin-4 (0.05 or 0.5 nM) for 48 h at 37°C in a humidified 5% $CO_2$ incubator. When indicated, exendin-9 (50 nM; Bachem AG, Bubendorf, Switzerland) was added 1 h before addition

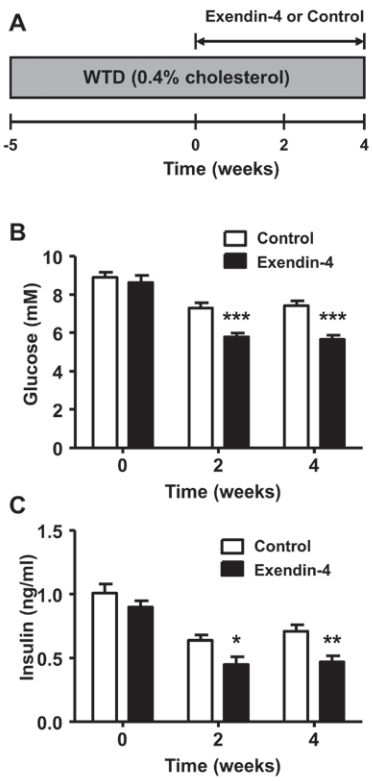
of exendin-4. After incubation, macrophages were washed twice with 500 µL PBS and cell lysates were obtained by adding 500 µL of 0.1 M NaOH. 250 µL of cell lysates was used for quantification of $^{3}$H-radioactivity. Dpm values were normalized to the total amount of protein (mg) present in 250 µL of cell lysates. Protein concentration in cell lysates was quantified with BCA protein assay kit.

For Oil-red O staining, after incubation, cells were washed twice with PBS and fixed for 30 min in 4% formaldehyde in PBS. Cell were incubated in 60% 2-propanol for 2 min, immediately placed in 60% Oil-red O for 30 min, followed by washing with $dH_2O$. Cell nuclei were counterstained with hematoxylin for 1 min.

## Statistical analysis

All data are presented as means ± SEM. Statistical differences between groups were assessed with the Mann-Whitney *U* test for two independent groups. A *P*-value of less than 0.05 was considered statistically significant.






**Supplemental Figure S1. Exendin-4 decreases plasma glucose and insulin levels.** After 5 weeks of feeding a Western-type diet containing 0.4% cholesterol, mice were treated with exendin-4 (50 µg/kg/day) or vehicle (PBS) subcutaneously for 4 weeks. Blood was collected by tail bleeding after 4h of fasting before treatment (T=0) and after 2 (T=2) and 4 (T=4) weeks of treatment (A). Plasma glucose (B) and insulin (C) levels were determined. Values are means ± SEM (n=17 mice per group). **P*<0.05, ***P*<0.01, ****P*<0.001 compared to vehicle.

# RESULTS

## Exendin-4 reduces plasma glucose and insulin levels

*E3L.CETP* mice were fed a Western-type diet containing 0.4% cholesterol for 5 weeks and thereafter were treated with exendin-4 or PBS via subcutaneous osmotic minipumps for 4 weeks while continuing the diet (Supplemental Fig. S1A). After 2 and 4 weeks of treatment, exendin-4 significantly decreased plasma glucose levels (-21%, $P<0.001$ and -24%, $P<0.001$, respectively, Supplemental Fig. S1B) and insulin levels (-30%, $P<0.05$ and -34%, $P<0.01$, respectively, Supplemental Fig. S1C), which is in line with our previous observations in high-fat diet fed *E3L* mice [6].

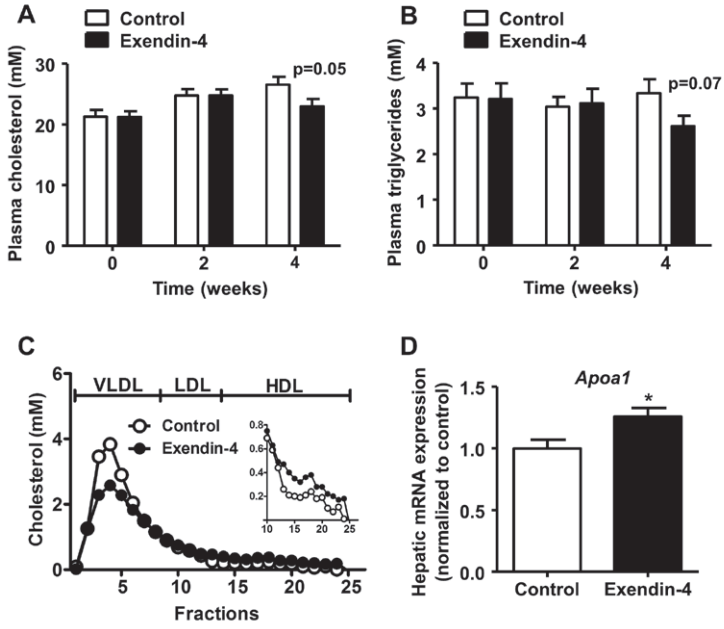



**Figure 1. Exendin-4 decreases (V)LDL and slightly increases HDL.** After 5 weeks of feeding Western-type diet containing 0.4% cholesterol, mice were treated with exendin-4 (50 µg/kg/day) or vehicle (PBS) subcutaneously for 4 weeks. Blood was collected by tail bleeding after 4 h of fasting before treatment (T=0) and after 2 (T=2) and 4 (T=4) weeks of treatment. Plasma cholesterol (A) and triglyceride (B) levels were determined. After 4 weeks of treatment, group-wise pooled plasma was fractionated using FPLC on a Superose 6 column and the individual fractions were assayed for cholesterol (C). Livers were isolated, mRNA was extracted and Apoa1 mRNA was determined as normalized to *Cyclo* and *Hprt* mRNA levels. Data were calculated as fold difference compared to vehicle (D). Values are means ± SEM (n=17 mice per group). **P*<0.05 compared to vehicle.

## Exendin-4 decreases plasma VLDL and slightly increases HDL

Despite clear beneficial effects on glucose metabolism, exendin-4 only tended to decrease plasma cholesterol levels (-14%, *P*=0.05, Fig. 1A) and TG levels (-22%, *P*=0.07, Fig. 1B) after 4 weeks of treatment. Lipoprotein profiling revealed that exendin-4 decreased VLDL-cholesterol and slightly increased HDL-cholesterol levels (Fig. 1C). The latter effect was accompanied by a 26% increased hepatic expression of apolipoprotein A1 (*Apoa1*), encoding the major lipoprotein of HDL (Fig. 1D).

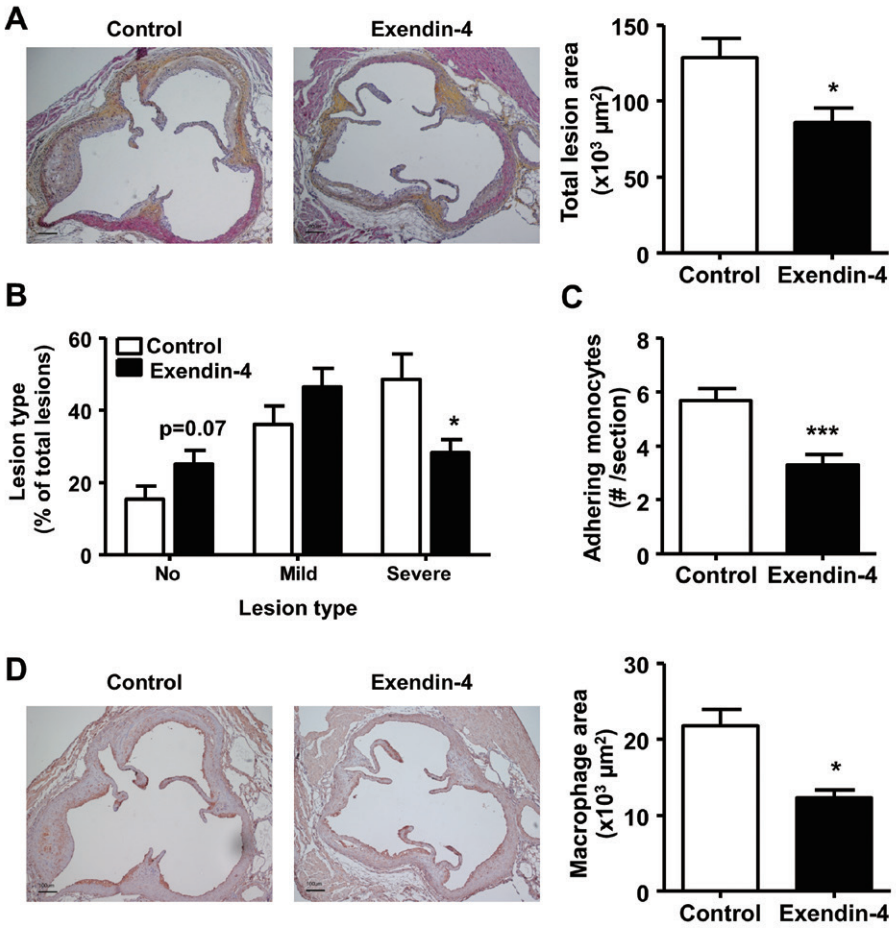



**Figure 2. Exendin-4 reduces aortic atherosclerosis development and monocyte recruitment to the endothelium wall.** After 5 weeks of feeding a Western-type diet containing 0.4% cholesterol, mice were treated with exendin-4 (50 µg/kg/day) or vehicle (PBS) subcutaneously for 4 weeks. Subsequently, hearts were isolated, fixed, dehydrated and embedded in paraffin. Cross-sections of the aortic root were stained with hematoxylin-phoxin-saffron (HPS) (A, B) or anti-AIA serum (C, D). Total lesion area (A), lesion severity (B), the number of adhering monocytes to the endothelium wall (C), and macrophage area (D) were quantified. Values are means ± SEM (n=17 mice per group). **P*<0.05, ****P*<0.001 compared to vehicle.

## Exendin-4 largely suppresses atherosclerosis development and monocyte recruitment in the aortic root

Despite slightly attenuating dyslipidemia, exendin-4 treatment markedly reduced total atherosclerotic lesion area as compared to controls (-33%; $P<0.05$) (Fig. 2A). Additionally, mice treated with exendin-4 showed more lesion-free sections (+63%; $P=0.07$) and less severe lesions (-42%; $P<0.05$) as compared to PBS controls (Fig. 2B). Moreover, exendin-4 significantly reduced the number of monocytes adhering to the endothelium wall in the aortic root (-42%, $P<0.001$, Fig. 2C) as well as the macrophage area in the plaque (-44%, $P<0.05$, Fig. 2D).

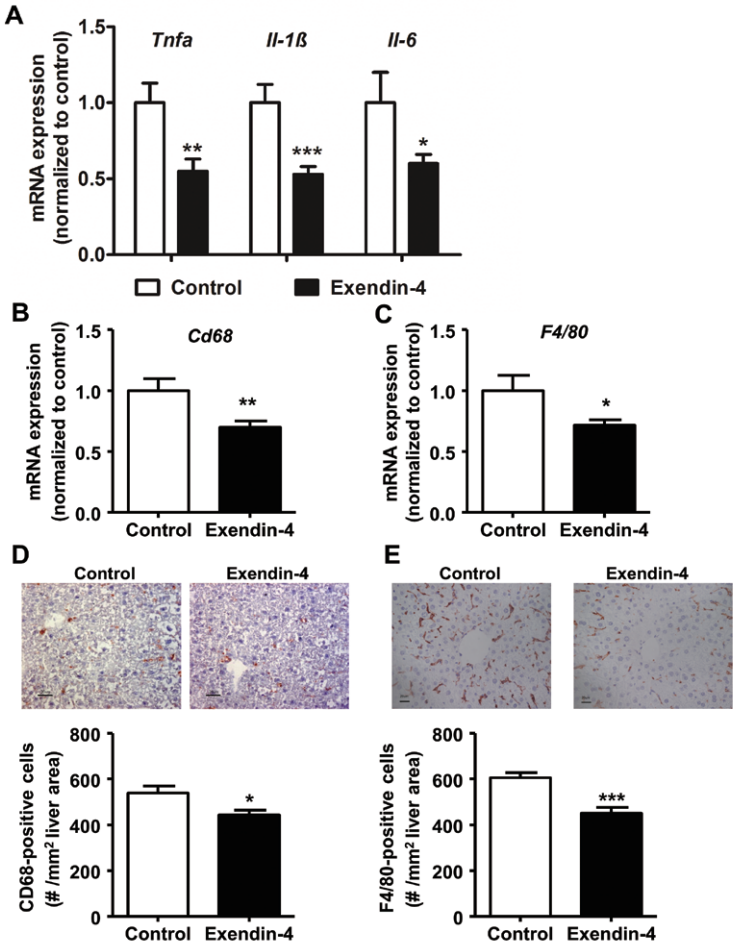



**Figure 3. Exendin-4 reduces liver inflammation and macrophage content.** After 5 weeks of feeding a Western-type diet containing 0.4% cholesterol, mice were treated with exendin-4 (50 µg/kg/day) or vehicle (PBS) subcutaneously for 4 weeks. mRNA was extracted from liver pieces, and mRNA expression of inflammatory markers *Tnfa*, *Il-1β*, and *Il-6* (A), *Cd68* (B) and *F4/80* (C) was determined as normalized to *Cyclo* and *Hprt* mRNA levels. Data were calculated as fold difference as compared to vehicle (A, B, C). Liver sections were immunostained for CD68 and F4/80, and $CD68^+$ (D) $F4/80^+$ (E) macrophages were quantified. Values are means ± SEM (n=17 mice per group). *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to vehicle.

## Exendin-4 reduces hepatic lipids, inflammation and macrophage content

Four weeks of exendin-4 treatment decreased hepatic TG (-11%; *P*=0.057) and TC content (-19%, *P*<0.05), without affecting hepatic phospholipid content. In addition, exendin-4 largely reduced the hepatic expression of the inflammatory markers *Tnfα*, *Il-1β*, and *Il-6* (-45%; *P*<0.01; -47%; *P*<0.001; -40%; *P*<0.05, respectively; Fig. 3A). Moreover, exendin-4 reduced hepatic mRNA expression of the macrophage markers *Cd68* (-30%; *P*<0.01; Fig. 3B) and *F4/80* (-28%; *P*<0.05; Fig. 3C). In line with these data, exendin-4 decreased hepatic CD68$^+$ macrophages (-18%, *P*<0.05; Fig. 3D) and F4/80$^+$ macrophages (-25%, *P*<0.001; Fig. 3E).

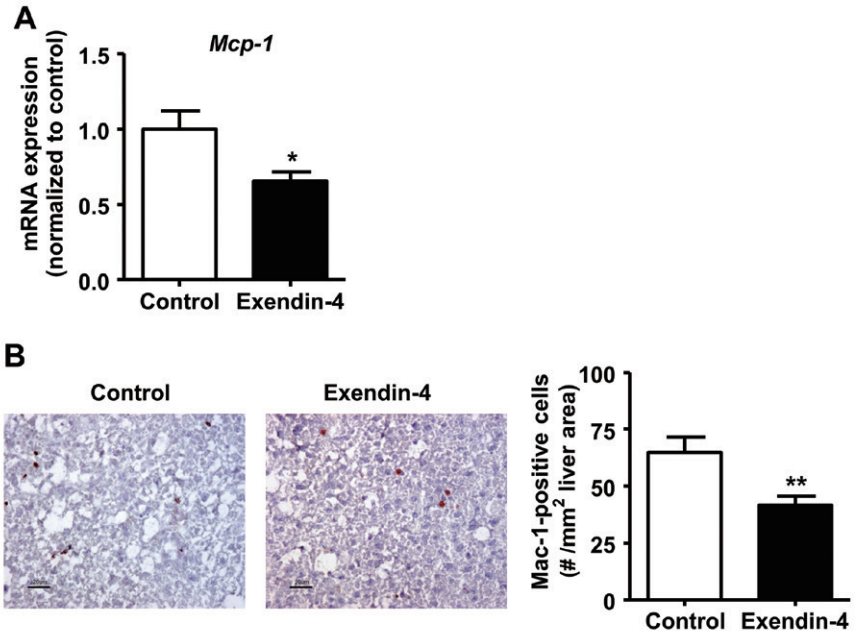



**Figure 4. Exendin-4 reduces macrophage infiltration into the liver.** After 5 weeks of feeding a Western-type diet containing 0.4% cholesterol, mice were treated with exendin-4 (50 µg/kg/day) or vehicle (PBS) subcutaneously for 4 weeks. In the livers, mRNA expression of Mcp-1 (A) were determined as normalized to *Cyclo* and *Hprt* mRNA levels. Data were calculated as fold difference as compared to vehicle (A). Liver sections were immunostained for Mac-1 and Mac-1$^+$ macrophages were quantified (B). Values are means ± SEM (n=17 mice per group). **P*<0.05, ***P*<0.01 compared to vehicle.

## Exendin-4 reduces macrophage infiltration into the liver

To elucidate the mechanism underlying the reduction of liver macrophage content by exendin-4, we first determined the hepatic gene expression of monocyte chemotactic

protein-1 (*Mcp-1*), which mediates monocyte recruitment from the circulation to sites of infection and inflammation. As shown in Fig. 4A, exendin-4 reduced *Mcp-1* expression (-34%; *P*<0.05). This was accompanied by a reduction of macrophages positive for Mac-1, an infiltrating macrophage marker (-36%, *P*<0.01; Fig. 4B).

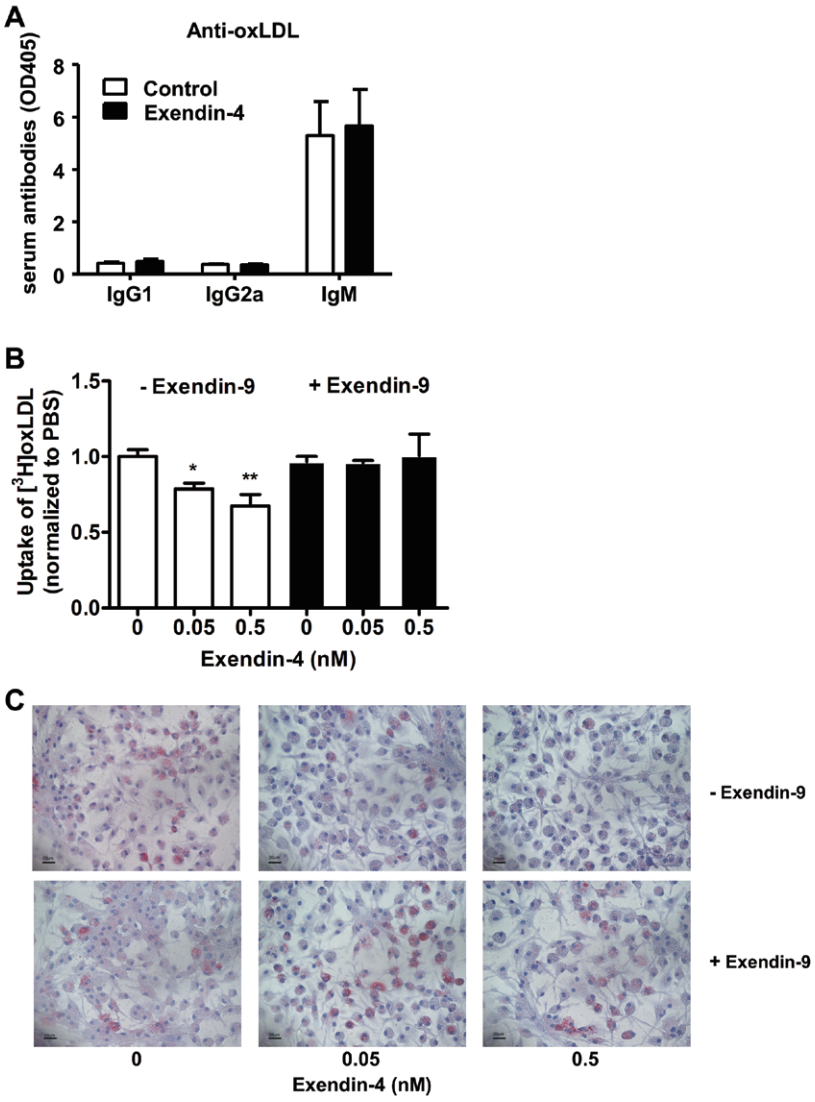



**Figure 5. Exendin-4 has no effect on plasma antibodies against oxLDL, but reduces oxLDL-induced foam cell formation by using peritoneal macrophages.** After 5 weeks of feeding a Western-type diet containing 0.4% cholesterol, mice were treated with exendin-4 (50 µg/kg/day) or vehicle (PBS) subcutaneously for 4 weeks. Plasma anti-oxidized LDL (oxLDL) antibodies were determined (A). Peritoneal macrophages were incubated with exendin-4 and/or exendin-9 for 48 h. The uptake of [$^3$H]COEth-labeled oxLDL was quantified (B). After fixation, lipid accumulation into the cells was visualized by staining with Oil red O (C). Values are means ± SEM (n=17 mice per group). **P*<0.05, ***P*<0.01 compared to vehicle.

### Exendin-4 does not affect circulating antibodies against oxLDL, but reduces oxLDL uptake by macrophages

Since the uptake of oxLDL by macrophages can drive both atherosclerosis and NASH, we first measured specific antibodies against oxLDL in the circulation. Exendin-4 did not affect oxLDL-specific IgG1, IgG2a, and IgM levels in plasma (Fig. 5A). Next, to determine if exendin-4 can impact on oxLDL uptake by macrophages, we incubated peritoneal macrophages with [$^3$H]COEth-oxLDL (10 µg/ml) for 48 h with or without exendin-4 (0.05 and 0.5 nM). Exendin-4 decreased the uptake of [$^3$H]COEth-oxLDL (up to -33%, $P<0.01$) compared to controls (Fig. 5B). This effect was completely abolished by pre-treatment of the cells with exendin-9 (Fig. 5B), which demonstrates that exendin-4 reduces oxLDL uptake by activating the GLP-1R. Oil-red O staining confirmed that exendin-4 reduced foam cell formation, which was blocked by exendin-9 (Fig. 5C).

## DISCUSSION



In this study, we investigated the effect of exendin-4 treatment on liver inflammation in addition to the development of atherosclerosis in *E3L.CETP* mice fed a Western-type diet. Our data show that 4 weeks of exendin-4 infusion markedly decreases total atherosclerotic lesion area, accompanied by a reduction in plaque macrophages. In parallel, exendin-4 caused a marked reduction in hepatic lipids and macrophages as well as hepatic inflammation, hallmarks of NASH.

It is interesting to note that short-term treatment with exendin-4, as compared with vehicle, caused a substantial reduction in atherosclerosis albeit that exendin-4 only slightly affected cholesterol levels and the lipoprotein profile. Therefore, the anti-atherogenic effect of exendin-4 is likely largely independent of modulation of plasma lipid levels. This observation contrasts the effects of classic lipid-lowering compounds including atorvastatin, which reduce atherosclerosis in *E3L.CETP* mice mainly by reducing apoB-containing lipoproteins [14]. We found that exendin-4 inhibited the adherence of monocytes to the vessel wall and decreased the macrophage area of the plaque, suggesting that exendin-4 decreases the recruitment of monocytes into the vessel wall. Our finding add to previous findings demonstrating that 4 weeks of GLP-1 receptor activation decreases atherosclerotic development without reducing plasma lipids in ApoE$^{-/-}$ mice [10, 18, 19]. Although a recent study did not find a reduction in atherosclerosis in ApoE$^{-/-}$ mice [11], this might be related to potential tissue heterogeneity in GLP-1 receptor expression in the ApoE$^{-/-}$ mice as well as variations in the experimental set-up (e.g. animal age, treatment regimen).

Second, we observed that exendin-4 reduced the lipid content of the liver, which

was accompanied by a reduction in inflammatory markers and macrophage content of the liver as judged from hepatic gene and protein expression. It is known that feeding hyperlipidemic mice a diet containing cholesterol increases the hepatic macrophage content already within a few days [20]. Since in our study mice were fed the Western-type diet for 5 weeks before starting treatment, exendin-4 may thus have reduced the hepatic macrophage content either by reducing the infiltration of activated macrophages from the circulation or by inducing the elimination of macrophages from the liver. The current study design does not allow us to discriminate between these possibilities. However, we did observe a decrease in hepatic MCP-1 expression along with a reduction in Mac-1$^{+}$ infiltrating macrophages, suggesting that reduced recruitment of monocytes/macrophages from the circulation to the liver contributes to the reduction in total macrophages. Collectively, we show that exendin-4 treatment affects important features of NASH, including reduction of liver macrophages as well as lipid content.

Historically, NASH was thought to be a causal risk factor for cardiovascular disease (CVD) as patients with NASH have a higher mortality risk than the general population, mainly due to CVD [2]. However, the biological mechanisms linking NASH and accelerated atherosclerosis are still poorly understood. Recently, Bieghs *et al.* put forward the hypothesis that there is a central role for inflammation in the development of NASH and atherosclerosis with common etiologies involving monocyte recruitment and macrophage foam cell formation [3]. From this perspective, we hypothesized that exendin-4 reduces atherosclerosis as well as hepatic inflammation by acting directly on monocyte/macrophage recruitment into both the vessel wall and liver.



So what mechanisms may then be involved? In addition to reducing hepatic MCP-1 as observed in our study, exendin-4 reduces the expression of E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 in the aorta of ApoE$^{-/-}$ mice [18]. These are all processes which likely lead to inhibition of monocyte/macrophage adhesion to the vessel wall and the liver. The importance of blood monocytes in the development of atherosclerosis has been firmly established [21], and accumulating evidence demonstrates the role of hepatic infiltration of blood-borne monocytes in liver inflammation [22, 23]. In fact, the migration of immune cells into the liver appears to be one of the first critical steps in both acute and chronic liver inflammation, mediating innate and adaptive inflammatory responses, which result in ongoing immune cell recruitment, tissue damage, and fibrosis [23].

As a second mechanism explaining the reduction of macrophage recruitment, we showed that exendin-4, via the GLP-1R, reduces the uptake of oxLDL by peritoneal macrophages and, as a consequence, reduced foam cell formation in vitro. These data are in full accordance with previous observations showing that native GLP-1 also

reduces oxLDL uptake by peritoneal macrophages thereby reducing atherosclerosis development from ApoE$^{-/-}$ mice [19]. We did not observe any changes in plasma anti-oxLDL antibodies after exendin-4 treatment, indicating that exendin-4 probably does not affect the oxLDL levels in plasma. Since native GLP-1 decreased the macrophage protein levels of CD36 [19], involved in the uptake of modified LDL, it is likely that in our study exendin-4 exerts its protective effects on macrophage foam cell formation by a similar mechanism. The relevance for oxLDL uptake via scavenger receptors by macrophages in atherosclerosis development has been confirmed by many studies [24]. Recent studies have shown that oxLDL uptake via scavenger receptors is also an important risk factor for the progression to hepatic inflammation [15]. In fact, inhibition of oxLDL uptake and thus foam cell formation by deficiency of the scavenger receptors CD36 and scavenger receptor class A in hematopoietic cells reduces hepatic macrophage infiltration [25], confirming that oxLDL uptake by macrophages not only plays a vital role in atherosclerosis development but also regulates hepatic inflammation.



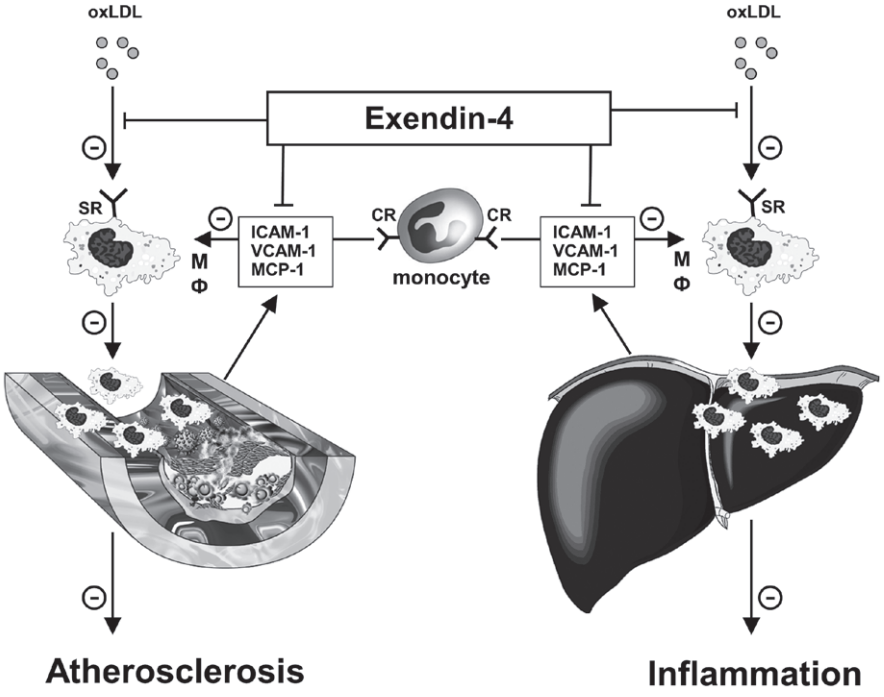



**Figure 6. Proposed mechanism underlying the beneficial effects of exendin-4 on atherosclerosis development and NASH.** Exendin-4 attenuates the development of atherosclerosis and NASH by 1) reducing the expression of chemokines and adhesion molecules, which leads to less recruitment of circulating monocytes/macrophages, and 2) inhibiting the uptake of oxLDL by macrophages and the subsequent formation of foam cells in the vessel wall and liver, respectively. For further explanation see text. CR, chemokine receptor(s); ICAM, intercellular adhesion molecule-1; mΦ, macrophage; MCP-1, monocyte chemotactic protein-1; oxLDL, oxidized LDL; SR, scavenger receptor; VCAM, vascular cell adhesion molecule-1.

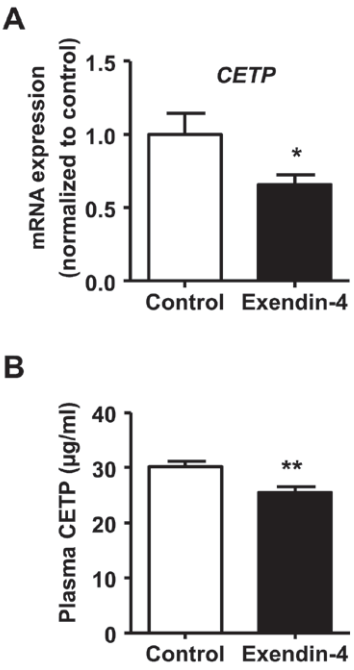
Collectively, we hypothesize that the protective effect of exendin-4 against inflammation in both the vessel wall and liver can be explained by (1) reducing monocyte/macrophage recruitment from the circulation and by (2) inhibiting the uptake of oxLDL by macrophages and the subsequent activation and formation of foam cells at these locations (see Fig. 6).

Interestingly, we also observed that exendin-4 decreased hepatic CETP gene expression (-34%, $P<0.05$) and plasma CETP concentration (-15%, $P<0.01$) (Supplemental Fig. S2). Recently, we demonstrated similar effects for niacin, which were explained by a reduction in macrophages that largely contribute to hepatic CETP expression [26]. In our present study, hepatic CETP expression positively correlated with the hepatic expression of the macrophage markers CD68 ($R^2=0.332$; $P<0.001$) and F4/80 ($R^2=0.607$; $P<0.001$) (Supplemental Fig. S3), indicating that exendin-4 also reduces CETP expression by reducing the hepatic macrophage content. Since CETP is involved in the transfer of cholesteryl esters from HDL to (V)LDL, the reduction in CETP may have contributed to the slight decrease in (V)LDL/HDL ratio, and thereby to reduced atherosclerosis development. Taken together, these data suggest that reducing NASH in general, by reducing the macrophage content, may reduce CETP expression, thereby improving the lipoprotein profile and therefore decrease the risk of developing atherosclerosis.



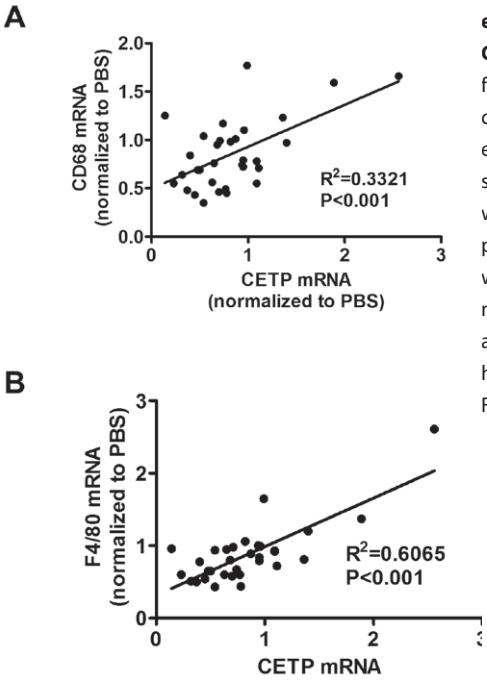
Thus far, there is no established pharmacological compound to treat NASH. Lifestyle modifications, such as weight loss, exercise, and restriction of nutrition intake are still the mainstays for the treatment of NASH [27]. Although lipid-lowering agents (e.g. statins and fibrates) and anti-oxidants (e.g. vitamins C, E) have beneficial effects on atherosclerosis development, none of them have shown adequate and convincing benefits in the treatment of NASH [28, 29]. Exendin-4 has been approved for the treatment of T2DM [5] and has also been shown to possess cardioprotective actions that include, in addition to reducing atherogenesis, suppression of arrhythmias, heart failure, myocardial infarction, and death [30]. Based on our collective findings that exendin-4 reduces high fat diet-induced hepatic steatosis by decreasing lipogenesis [6] and suppresses macrophage content in both vessel wall and liver (in the current study), we propose that exendin-4 is a suitable candidate to concomitantly treat atherosclerosis and NASH.

In conclusion, our findings show that exendin-4 treatment reduces inflammation in both the liver and vessel wall in *E3L.CETP* mice fed a Western-type diet, by reducing monocyte/macrophage recruitment and inhibiting the uptake of oxLDL by macrophages. We anticipate that exendin-4 can be used as a valuable strategy to treat NASH and atherosclerosis in addition to T2DM, especially in patients who display a combination of these diseases.




**Supplemental Figure S2. Exendin-4 decreases hepatic expression and plasma level of CETP.** After 5 weeks of feeding a Western-type diet containing 0.4% cholesterol, mice were treated with exendin-4 (50 µg/kg/day) or vehicle (PBS) subcutaneously for 4 weeks. mRNA was extracted from liver pieces, and mRNA expression of *CETP* (A) was determined as normalized to *Cyclo* and *Hprt* mRNA levels. Data were calculated as fold difference as compared to vehicle. Plasma CETP levels were determined (B). Values are means ± SEM (n=17 mice per group). **P*<0.05, ***P*<0.01 compared to vehicle.






**Supplemental Figure S3. Hepatic CETP expression is positively correlated with hepatic CD68 and F4/80 expression.** After 5 weeks of feeding a Western-type diet containing 0.4% cholesterol, *E3L.CETP* mice were treated with exendin-4 (50 µg/kg/day) or vehicle (Control) subcutaneously for 4 weeks. Subsequently, livers were isolated and mRNA was extracted from liver pieces. mRNA expression of *Cd68*, *F4/80*, and *CETP* was determined as normalized to *Cyclo* and *Hprt* mRNA levels. Data were calculated as fold difference as compared to vehicle and the correlation between hepatic CETP expression and hepatic CD68 (A) or F4/80 (B) expression was linearly plotted.

## ACKNOWLEDGMENTS

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# 10

# BOTH TRANSIENT AND CONTINUOUS CORTICOSTERONE EXCESS INHIBIT ATHEROSCLEROTIC PLAQUE FORMATION IN APOE*3-LEIDEN.CETP MICE

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*Both authors contributed equally

*PLOS ONE 2013; 8: e63882*

## ABSTRACT

The role of glucocorticoids in atherosclerosis development is not clearly established. Human studies show a clear association between glucocorticoid excess and cardiovascular disease, whereas most animal models indicate an inhibitory effect of glucocorticoids on atherosclerosis development. These animal models, however, neither reflect long-term glucocorticoid overexposure nor display human-like lipoprotein metabolism. To investigate the effects of transient and continuous glucocorticoid excess on atherosclerosis development in a mouse model with human-like lipoprotein metabolism upon feeding a Western-type diet. Pair-housed female *APOE*3-Leiden.CETP* (*E3L.CETP*) mice fed a Western-type containing 0.1% cholesterol for 20 weeks were given corticosterone (50 µg/ml) for either 5 (transient group) or 17 weeks (continuous group), or vehicle (control group) in the drinking water. At the end of the study, atherosclerosis severity, lesion area in the aortic root, the number of monocytes adhering to the endothelial wall and macrophage content of the plaque were measured. Corticosterone treatment increased body weight and food intake for the duration of the treatment and increased gonadal and subcutaneous white adipose tissue weight in transient group by +35% and +31%, and in the continuous group by +140% and 110%. Strikingly, both transient and continuous corticosterone treatment decreased total atherosclerotic lesion area by -39% without lowering plasma cholesterol levels. In addition, there was a decrease of -56% in macrophage content of the plaque with continuous corticosterone treatment, and a similar trend was present with the transient treatment. In conclusion, increased corticosterone exposure in mice with human-like lipoprotein metabolism has beneficial, long-lasting effects on atherosclerosis, but negatively affects body fat distribution by promoting fat accumulation in the long-term. This indicates that the increased atherosclerosis observed in humans in states of glucocorticoid excess may not be related to cortisol *per se*, but might be the result of complex indirect effects of cortisol.

# INTRODUCTION

Atherosclerosis develops as a result of a chronic inflammatory response in an injured vessel wall [1], which is preceded by accumulation of leukocytes and fat deposition, leading to plaque formation [2]. The initial mechanisms in atherogenesis, however, are still incompletely understood.

The role of glucocorticoids (GC) in the development of atherosclerosis is not yet clearly established in humans or in animals and is, at least, dependent on individual's [3, 4] or animal's [5] exposure to appropriate levels of adrenal steroids. Human data show an association between increased GC secretion and cardiovascular disease even after long-term successful correction of GC excess [6], whereas previous studies in animals, e.g. rabbits [7-11] and dogs [12] using either natural or synthetic GC, suggest an atheroprotective role of GC. On the other hand, 11β-dehydrogenase type 2 (11βHSD2) deficient mice, in which the activation of the mineralocorticoid receptor (MR) by GCs cannot be prevented, have an increased atherosclerotic plaque development [13], suggesting that increased activation of the MR promotes atherosclerotic plaque formation. However, a recent study demonstrated that adrenalectomy, which removes endogenous GC, stimulated the formation of initial atherosclerotic lesions in low-density-lipoprotein receptor knockout mice [5].



Only a limited number of studies have evaluated the effect of endogenous GC excess on atherosclerosis development in mice. These studies, that used chronic stress to increase endogenous GC, reported either an increase [14, 15] or no effect [16] on atherosclerosis development in ApoE-deficient mice. However, chronic stress, in addition to increasing GC, induces other complex endocrine and metabolic changes, for instance increased sympathetic outflow [17] that may affect atherosclerosis development. These mouse models therefore do not reflect long-term endogenous GC overexposure, like in Cushing's syndrome (CS) in humans. Furthermore, ApoE-deficient mice do not reflect human-like lipoprotein metabolism and have a deviant immune status compared to wild-type mice [18].

In the present study, our aim was to investigate the effects of GC excess on atherosclerosis development in the *APOE*3-Leiden.CETP* (*E3L.CETP*) mouse, a well-established model for human-like lipoprotein metabolism that is prone to develop atherosclerosis upon feeding a cholesterol-containing Western-type diet [19] and is responsive to the hypolipidemic drugs used in the clinic similar to humans [20-23]. The latter is in sheer contrast to other mouse models for hyperlipidemia and atherosclerosis including apoE-knockout and LDL receptor-knockout mice. We administered corticosterone (CORT) non-invasively via the drinking water, and based on the clinical

observation in CS patients, we investigated both transient and chronic effects of CORT on atherosclerosis development.

## MATERIALS AND METHODS

### Ethics statement

This study was carried out in strict accordance with the regulations of Dutch law on animal welfare, and the institutional ethics committee for animal procedures of Leiden University. The protocol was approved by the institutional ethics committee for animal procedures of Leiden University (Permit Number: 10132 and for the pilot experiment: 08221) and all efforts were made to minimize suffering.

### Mice, housing, corticosterone supplementation, and diets

Human CETP expressing transgenic mice, which express CETP under control of its natural flanking regions, were crossbred in our own animal facility with *E3L* mice to obtain the heterozygous *E3L.CETP* mice on a C57Bl/6 background [24]. Female mice (10-16 weeks of age) were pair housed and maintained on a 12 h:12 h light-dark cycle (lights on 7 a.m.) in a climate controlled environment, with ad libitum access to food and drinking water. Mice were fed a Western-type diet containing 0.1% cholesterol (Diet T + 0.1% cholesterol, Arie Blok Diervoeding, Woerden, the Netherlands) for a period of three weeks after which they were matched for age, plasma cholesterol, triglycerides, phospholipids, age and bodyweight and then randomized to receive CORT (Sigma-Aldrich, Manchester, UK) at a concentration of 50 µg/ml in the drinking water with 0.25% ethanol as vehicle for five weeks (transient group, n=17), continuously for the entire duration of the experiment (seventeen weeks) (continuous group, n=21), or vehicle (control group, n=19). Food and bodyweight were recorded weekly. At the end of the experiment mice were decapitated within 90 seconds from disturbing the cage and trunk blood was collected. Gonadal and subcutaneous fat pads were removed from each mouse by the same person to reduce inter-observer variation. The same area of subcutaneous fat was removed from each mouse excluding the inguinal lymph node. Fat pads and adrenals were weighed using a micro scale, and frozen in liquid nitrogen.

### Pilot experiment for the determination of optimal CORT dose

Prior to experiment, we performed a dose finding study in male C57Bl/6J mice fed HFD with 12.5 µg/ml (n=2), 25 µg/ml (n=4) and 50 µg/ml (n=2) of CORT, and control receiving 0.25% ethanol (n=2) as vehicle for four weeks to determine the optimal CORT dose.

These dosages were chosen based upon a previous study [25] that documented profound metabolic effects with CORT 100 µg/ml and less pronounced effects with 25 µg/ml. Based upon our dose finding study, we chose 50 µg/ml CORT in the drinking water for our subsequent experiments as this dose led to the largest increases in food intake and body weight as well as in plasma cholesterol. Because male mice do not readily develop atherosclerosis and the known models are in majority female models, we used female mice for the purpose to study the atherosclerosis development. In males and females, this CORT dose was sufficient to increase food intake and to maintain a higher bodyweight throughout the experiment. In addition, circulating circadian CORT levels at week 5 were 5-8 fold increased in the morning and 3-4 fold in the evening (data not shown).

## Sampling of circadian corticosterone, hormone, and lipid measurements

Plasma CORT was sampled before CORT administration (baseline), and after CORT administration at week 5 during the first light hour at 07.00 h, at 12.00 h, during the last light hour at 18.00 h, and three hours after the onset of the dark phase at 22.00 h. During the dark phase samples were collected in red light conditions. All CORT samples were obtained within 90 seconds from disturbing the cage, via tail incision, allowing the mouse to move freely on top of the home cage [26]. Trunk blood was used to determine plasma CORT at the end of the experiment after CORT administration at week 17 at 09.00 h and 18.00 h. Total plasma cholesterol, triglycerides, phospholipids, insulin and glucose were sampled after 4 hour-fast at baseline, week 5, 8 and 17 of the intervention. Body weight and food intake were measured weekly.

Plasma CORT levels were determined by radioimmunoassay (MP Biomedicals LCC, Orangeburg, NY). Plasma levels of total cholesterol, triglycerides and non-esterified free fatty acids were measured with enzymatic colorimetric reaction (Roche diagnostics GmbH, Mannheim; and Wako Pure Chemical Industries, respectively), plasma insulin was measured with an ELISA (Crystal Chem Inc., Downers Grove, IL, USA) and plasma glucose with a hexokinase method (Instruchemie, Delfzijl, The Netherlands). Homeostasis model index of insulin (HOMA-IR) was calculated by multiplying fasting insulin concentration (µU/ml) with fasting glucose (mmol/l), and dividing with 22.5 [27].

## Lipoprotein profiling

Distribution of cholesterol over plasma lipoproteins was determined using fast protein liquid chromatography. Pooled plasma from each group were used and 50 µl of each pool was injected onto a Superpose 6 PC 3.2/30 column (Äkta System, Amersham Pharmacia Biotech, Piscataway, NJ) and eluted at a constant rate of 50 µl/min in PBS,

1 mM EDTA, pH 7.4. Fraction were collected and assayed for cholesterol as described above.

## Gene expression analysis in adipose tissue

Total RNA was extracted from gonadal fat pads using the Nucleospin RNA II kit (Macherey-Nagel, Duren, Germany) according to manufacturer's instructions. RNA quality of each sample was examined by the lab-on-a-chip method using Experion Std Sens analysis kit (Biorad, Hercules, CA) and RNA concentration of each sample was determined by Nanodrop technology (Thermo Scientific, Wilmington, USA). Then, total RNA was reverse-transcribed with iScript cDNA synthesis kit (1708891, Bio-Rad), and obtained cDNA was purified with Nucleospin Extract II kit (636973, Macherey-Nagel, Bioké). Real-time qPCR was performed on a CFX96 machine (Bio-Rad), the reaction mixture consisting of SYBR-Green Sensimix (QT615, GC Biotech), cDNA, primers (Biolegio, Nijmegen, The Netherlands), and nuclease-free water in a total reaction volume of 10 µl. mRNA values of each gene were normalized to mRNA levels of ß2-microglobulin (*ß2m*) and hypoxanthine ribosyltransferase (*Hprt*). Primer sequences are listed in supplementary table 1.



**Supplemental Table 1. Primer sequences used for RT-qPCR**

| Gene | **Forward primer** | **Reverse Primer** |
|---|---|---|
| *β2m* | TGACCGGCTTGTATGCTATC | CAGTGTGAGCCAGGATATAG |
| *Cd68* | ATCCCCACCTGTCTCTCTCA | TTGCATTTCCACAGCAGAAG |
| *F4/80* | CTTTGGCTATGGGCTTCCAGTC | GCAAGGAGGACAGAGTTTATCGTG |
| *Hprt* | TTGCTCGAGATGTCATGAAGGA | AGCAGGTCAGCAAAGAACTTATAG |
| *Il-6* | TGTGCAATGGCAATTCTGAT | CTCTGAAGGACTCTGGCTTTG |
| *Tnfa* | AGCCCACGTCGTAGCAAACCAC | TCGGGGCAGCCTTGTCCCTT |

*β2m*, β2-microglobulin; *Hprt*, hypoxanthine ribosyltransferase; *IL-6*, Interleukin-6; *Tnfa*, Tumor necrosis factor α.

## Quantification of atherosclerosis

After 17 weeks of intervention, mice were killed by decapitation and the hearts were isolated. Hearts were fixed in phosphate-buffered 4% formaldehyde, dehydrated and embedded in paraffin. Cross-sections (5 µm) throughout the aortic root area were cut. 12 sections per mouse, stained with hematoxylin-phloxin-saffron for histological analysis, with 50 µm-intervals were used for atherosclerosis measurements. Lesions were categorized for severity according to the guidelines of the American Heart Association, adapted for mice [28, 29], as follows: type 0 (no lesions), types 1 through 3 (early fatty streak-like lesions containing foam cells), and type 4 to 5 (advanced lesions containing foam cells in the media, presence of fibrosis, cholesterol clefts, mineralization, and/or necrosis). AIA 31240 antiserum (1:3000, Accurate Chemical and Scientific, Westbury, NY) was used to quantify macrophage content of the plaque as well as monocytes adhering to the endothelium. Lesion area, the macrophage area and the number of monocytes adhering to the endothelium were quantified using Image J software (National Institutes of Health).

## Serum macrophage colony-stimulating factor (M-CSF) and anti-oxidized low-density lipoprotein (ox-LDL) antibodies measurements



Macrophage colony-stimulating factor (M-CSF) was measured using a mouse M-CSF Quantikine ELISA kit according to the manufacturer's instructions (MMC00, R&D Systems Inc, Germany). An EIA/RIA high binding 96-well Costar plate (Corning Inc., Corning, NY, USA) was coated with ox-LDL (7.5 µg/mL) in PBS. IgM and IgG2a antibodies against oxLDL in serum were measured using an ELISA Ig detection kit (Zymed Laboratories, San Francisco, CA, USA) according to the manufacturer's protocol.

## Statistical analysis

Data are presented as means ± SEM. Statistical differences were calculated using Anova with Tukey's post-hoc test, for multiple comparisons, except for plasma CORT measurements transient and continuous groups were compared with the control group individually per time point using an unpaired two tailed *t*-test, with GraphPad Prism, version 5.01. $P<0.05$ was considered as statistically significant.

# RESULTS

## CORT treatment increases plasma CORT concentrations and affects circadian rhythm

Chronic administration of high doses of CORT in the drinking water (50 µg/ml) resulted in significant increases in plasma CORT levels at week 5 (Fig. 1B) in both groups, compared to controls (transient group: 07.00 h 8-fold, 12.00 h 3-fold, 18.00 h 1-fold and 22.00 h 4-fold; continuous group: 07.00 h 5-fold, 12.00 h 4-fold, 18.00 h 1-fold and 22.00 h 3-fold). At week 17 (Fig. 1C) there were no differences between groups at 09.00 h and 18.00 h. At the end of the experiment, thymus weight (Fig. 1D) was not different between the three groups but adrenal weight (Fig. 1E) was significantly reduced in the continuously exposed group by -50%, in agreement with adrenal atrophy secondary to long-term exogenous GC exposure.



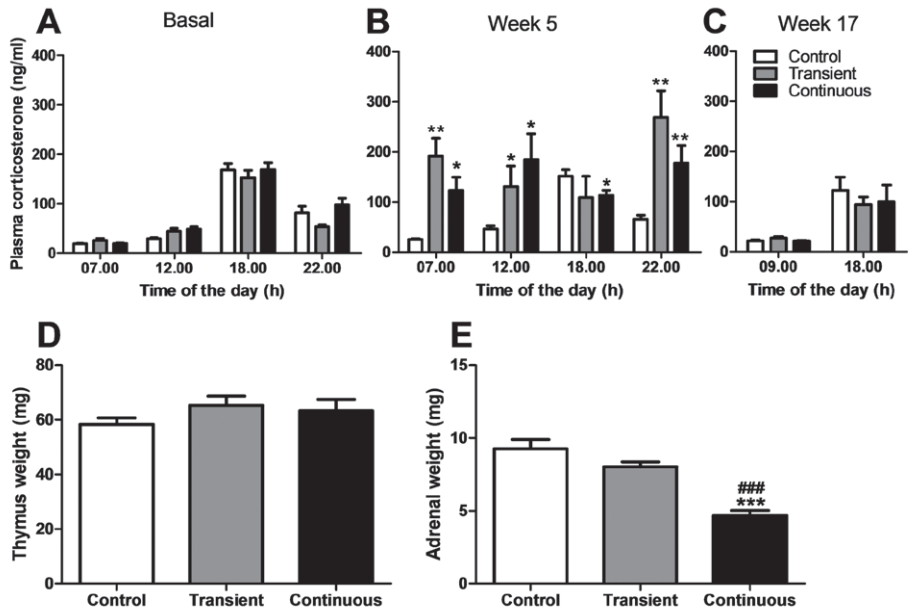


**Figure 1. Effect of transient and continuous CORT treatment on circadian plasma CORT levels in female *E3L.CETP* mice at baseline (A), week 5 (B) and week 17 (C), as well as on thymus weight (D) and adrenal weight (E) at week 17** (Control group: white bars, transient group: grey bars and continuous group: black bars), Data are means ± SEM (n=17-21), $^{*,\#}P<0.05$, $^{**,\#\#}P<0.01$, $^{\#\#\#}P<0.0001$, *versus control group and $^{\#}$transient group.

## CORT treatment affects food intake and bodyweight and induces long-lasting changes in body composition and inflammation in adipose tissue

As expected, CORT treatment increased food intake of the transient and the continuous group during the first three weeks of the experiment after which the transient group returned to the level of the controls and food intake with continuous treatment remained elevated (Fig. 2A). This increase was accompanied by an increase in body weight (Fig. 2B) in both groups. After the discontinuation of CORT treatment, body weight decreased to the level of the controls. The continuously exposed group showed a continuous increase in body weight and maintained a higher body weight to the end of the experiment (Fig. 2B) compared to the other two groups. After 17 weeks, gonadal and subcutaneous fat pad weights (Fig. 2C and D), when compared to controls, were significantly increased by +35% and +31%, respectively, in the transient group, and by +140% and +110% in the continuous group. To evaluate whether the increased fat mass resulted in changes of inflammation in the fat pad, the mRNA expression of markers of the macrophage content (i.e. *F4/80* and *Cd68*) and proinflammatory cytokines [Tumor necrosis factor α (*Tnfa*) and Interleukin-6 (*Il-6*)] in the gonadal fat pad were determined. As compared to control group, transient administration of CORT did not affect the expression of *F4/80* (Fig. 2E) and *CD68* (Fig. 2F), but decreased the expression levels of *Tnfa* (Fig. 2G) and *Il-6* (Fig. 2H) in the long-term by -32% and -47%, respectively; while continuous administration of CORT increased the expression of *F4/80* (Fig. 2E) and *CD68* (Fig. 2F) by +58% and +70%, respectively, decreased the expression of *Tnfa* (Fig. 2G) by -26% and did not affect the expression of *Il-6* (Fig. 2H). These data indicate although excess GC exposure increased fat mass which was accompanied with an increase in macrophage content, expression of proinflammatory cytokines in the adipose tissue was generally reduced.



## CORT treatment does not affect plasma lipids, cholesterol lipoprotein profile, but increases plasma insulin and HOMA-IR

Although both transient and continuous administration of CORT increased food intake to a certain extent, CORT treatment did not increase plasma levels of total cholesterol, triglycerides or phospholipids during the experimental period of 17 weeks (Table 1). Moreover, there were no differences between groups in the distribution of cholesterol over lipoproteins (Fig. 3A-C). Plasma levels of insulin and HOMA-IR, but not plasma glucose levels, were increased (Fig. 3 D-F) in the continuous group at week 17, reflecting GC-induced insulin resistance.

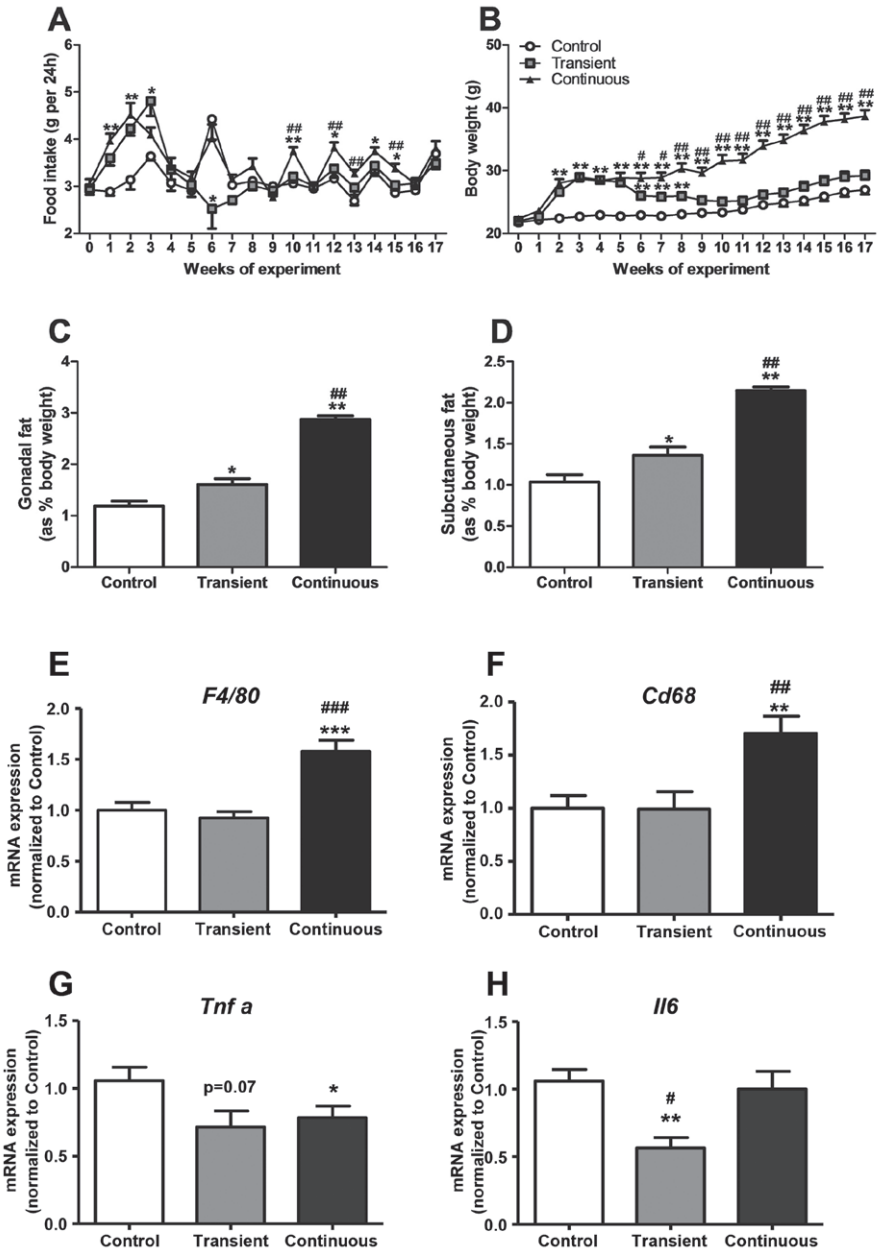


**Figure 2. Effect of transient and continuous CORT treatment on food intake (A), body weight (B)** (Control group: white circles, transient group: grey squares and continuous group: black triangles)**, gonadal fat (C) and subcutaneous fat (D) as % of the body weight, mRNA expression of *F4/80* (E), *CD68* (F), *Tnfα* (G) and *Il-6* (H) in the gonadal fat** (Control group: white bars, transient group: grey bars and continuous group: black bars). Data are means ±SEM (n=17-21), Anova with Tukey's post-hoc test, $^{*,\#}P<0.05$, $^{**,\#\#}P<0.01$, $^{***,\#\#\#}P<0.001$, *versus control group and #versus transient group.

**Table 1. Effect of CORT treatment on fasting plasma lipids.**

| | Total cholesterol (mmol/l) | | | Triglycerides (mmol/l) | | | Phospholipids (mmol/l) | | |
|---|---|---|---|---|---|---|---|---|---|
| week | Control | Transient | Continuous | Control | Transient | Continuous | Control | Transient | Continuous |
| 0 | 10.0±1.5 | 10.2±2.3 | 10.2±2.1 | 3.9±1.1 | 4.3±1.1 | 4.1±1.4 | 3.6±0.4 | 3.7±0.6 | 3.5±0.6 |
| 5 | 13.2±1.8 | 14.0±5.2 | 14.8±4.2 | 4.6±1.6 | 4.8±2.3 | 4.5±1.6 | 4.5±0.6 | 4.5±1.1 | 4.6±1.0 |
| 8 | 13.4±2.0 | 13.7±3.9 | 12.4±4.3 | 3.8±1.5 | 4.0±1.7 | 4.2±0.9 | 4.5±0.5 | 4.6±0.8 | 4.2±0.9 |
| 17 | 12.4±2.8 | 10.2±3.2 | 10.0±3.0 | 3.2±1.4 | 3.3±1.0 | 2.9±0.5 | 3.8±0.9 | 3.4±0.9 | 3.4±0.7 |

Data are means ± SEM (n=17-21), Anova with Tukey's post-hoc test.

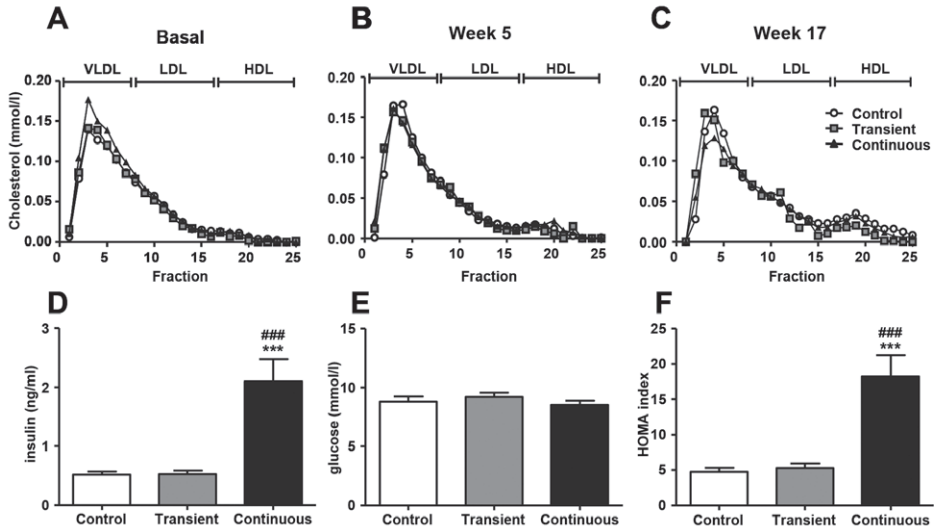


**Figure 3. Effect of transient and continuous CORT treatment on cholesterol distribution over lipoproteins fractioned by FPLC at baseline (A), week 5 (B) and 17 (C)** (Control group: white circles, transient group: grey squares and continuous group: black triangles) **and on plasma insulin (D), plasma glucose (E), HOMA-IR (F) on week 17** (Control group: white bars, transient group: grey bars and continuous group: black bars). Data are means ± SEM (n=17-21), Anova with Tukey's post-hoc test, $^{**,\#\#\#}P<0.001$, *versus control group and #versus transient group.

## Transient and continuous CORT treatment decrease atherosclerosis lesion area to a similar extent

Remarkably, CORT treatment decreased total atherosclerotic lesion area equivalently in both transiently (-39%) and continuously (-39%) treated groups (Fig. 4A and B). Moreover, both transient and continuous groups showed similar trends towards a less severe lesion phenotype as compared to the control group (Fig. 4C), suggesting that CORT treatment reduces atherosclerosis development in a long-lasting manner. CORT treatment, neither transiently nor continuously, affected the number of monocytes adhering to endothelium wall (Fig. 4D and E), yet continuous administration of CORT did reduce the macrophage content (-56%) of the plaque (Fig. 4D and F) as well as the macrophage content as percentage of the total plaque area (-52%) (Fig. 4G).

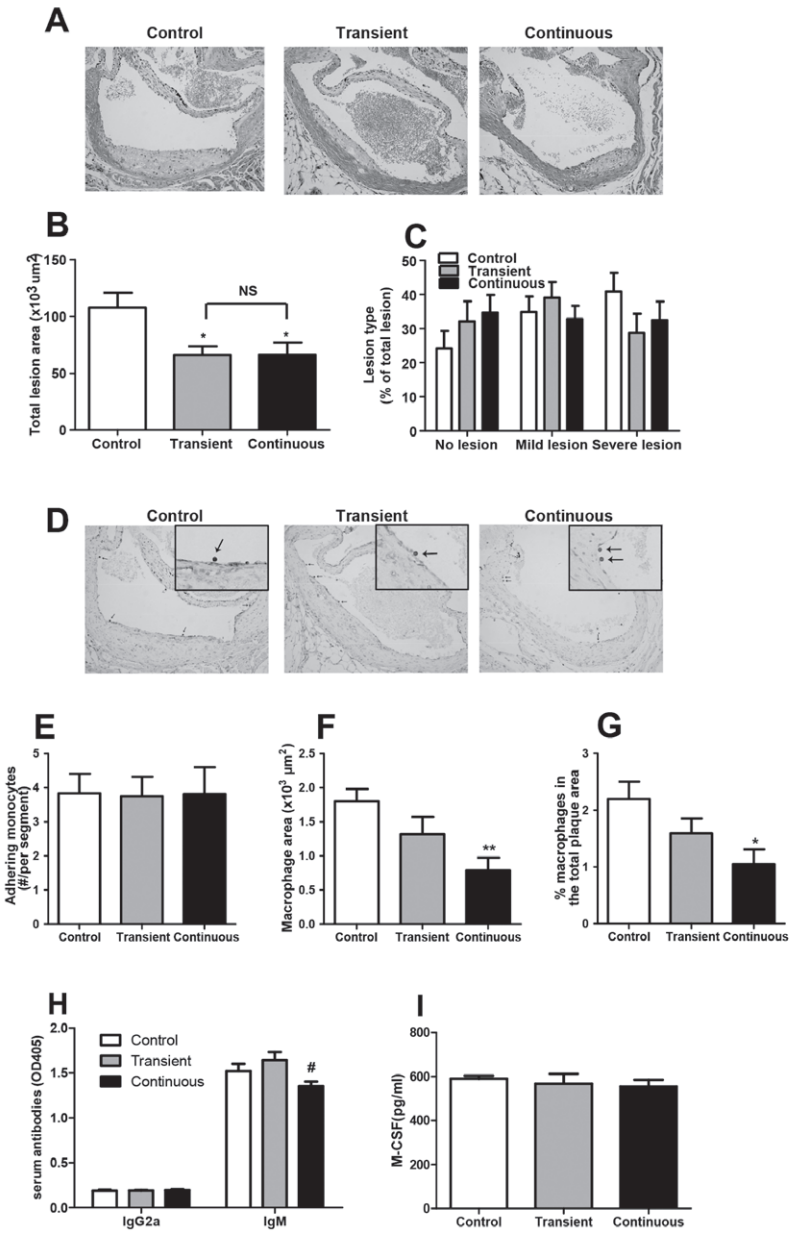


**Figure 4. Effect of transient and continuous CORT treatment on atherosclerosis development: representative HPS-stained pictures of lesions (A), lesion area (B), lesion type as % of the total lesion (C), representative pictures of monocyte/macrophage staining (adhering monocytes shown by arrows) (D), adhering monocytes per segment (E), macrophage content of the plaque (F) and macrophages as % of the lesion area (G).** Serum anti-ox-LDL specific antibodies (H) and serum M-CSF (I) were measured on week 17. (Control group: white bars, transient group: grey bars and continuous group: black bars). Data are means ± SEM (n=17-21), Anova with Tukey's post-hoc test, $^{*\#}P<0.05$, $^{**}P<0.01$, *versus control group and $^{\#}$versus transient group.

A reduction of the macrophage content of the plaque (-27%, *P*=0.125) and percentage macrophages in total plaque area (-27%, *P*=0.145) was observed in the transient group and but these reductions failed to reach statistical significance (Fig. 4F and G). Since uptake of oxidized LDL (ox-LDL) by macrophages to become foam cell plays an important role in the development and progression of atherosclerosis, we measured specific antibodies against ox-LDL in the circulation. As compared to control group, CORT treatment, neither transiently nor continuously, influenced the ox-LDL specific IgG2a and IgM level, indicating that CORT probably does not affect the ox-LDL level in circulation (Fig. 4H). Because macrophage proliferation and differentiation was shown to be linked to the atherosclerotic process [1], an essential factor regulating macrophage growth, macrophage colony stimulation factor (M-CSF) [30] was measured at the end of this experiment. However, no differences in the serum levels of M-CSF were detected among groups (Fig. 4I)

## DISCUSSION

This study demonstrates for the first time that CORT treatment resulting in increased plasma CORT concentrations decreases atherosclerotic plaque formation in mice with human-like lipoprotein metabolism, without affecting either plasma lipid levels or lipoprotein profiles. Interestingly, inhibition of atherogenesis was found both in transiently and continuously exposed animals, whereas continuous treatment with CORT also decreased macrophage content of the plaque despite the normalization of the body weight. In addition, CORT treatment resulted in long-lasting changes in body fat content, which were still present even 12 weeks after abrogation of CORT. This indicates that increased CORT exposure *per se* has beneficial, long-lasting effects on atherosclerosis, but negatively affects body fat distribution and insulin sensitivity, by promoting fat accumulation in the long-term.

The concentration of glucocorticoids (GCs) attained in our experiments is higher than under stress state but well comparable to patients with severe Cushing's syndrome, where cortisol secretion was 7 times higher that in healthy controls and circulation cortisol concentrations was 3-5 times higher [31].

Increased plasma CORT levels were easily induced non-invasively using CORT in the drinking water. CORT affected circadian GC rhythm by reducing the degree of variation in plasma concentrations. The human clinical equivalent of chronic hypercortisolemia, Cushing's syndrome (CS), is characterized by a blunted or even complete loss of diurnal rhythm [3]. In our model we re-capitulate some of these key temporal aspects of CS: although a chronic high circulating cortisol levels is a key aspect of CS, the most reliable

measure for diagnosis is very high late night (i.e. 22:00 –24:00 h) plasma cortisol [3]. Our high- CORT animals parallel these aspects of the syndrome, with both high baseline levels of CORT (200 ng/ml), as well as a peak in CORT at the end of the night (rather than at the beginning). Moreover, the continuously exposed group displayed reduced adrenal sizes, in agreement with adrenal atrophy, secondary to the long-term exogenous CORT administration despite normal plasma CORT levels found at the end of the experiment. This might be explained by increased metabolism of CORT as well as by other pharmacokinetic adaptations like increased clearance and increased distribution volume as a result of increased adipose tissue.

Plasma lipid levels were not affected by transient nor continuous CORT treatment. This is interesting particularly in the case of plasma values of total cholesterol, VLDL-cholesterol and VLDL-triglycerides since these are well known risk factors for the development of atherosclerosis. In fact, cholesterol exposure generally is a good predictor of atherosclerosis development in *E3L.CETP* mice (Princen and Rensen, unpublished observations). Moreover, CORT exposure did not affect lipoprotein profiles. In previous studies, mice were subjected to chronic stress, which causes a complex of endocrine changes, including high CORT levels. These studies have reported inconsistent effects on plasma cholesterol levels, which were increased [15], decreased [16], or not reported [14]. These studies used ApoE-deficient mice in which the ApoE- deficiency causes severe accumulation of cholesterol in macrophages resulting in a high pro-inflammatory state, which could explain the differences observed in atherosclerosis development between our low grade inflammation model of *E3L.CETP* mice fed a low amount of dietary cholesterol (twice human daily intake) [32, 33]. In addition, the use of chronic stress to increase endogenous GCs in previous models will also induce other complex endocrine and metabolic changes [17] that may also affect atherosclerosis development. Humans with high GCs also have inconsistent cholesterol levels. In the literature, the prevalence of hyperlipidemia in patients with CS varies from 38% to 71% [34]. A study by *Mancini et al.* (2004) has shown that hyperlipidemia occurs less frequently than the other metabolic complications of CS and that it was not correlated to the degree of hypercortisolism or duration of the disease [35]. However, the causative role of cortisol excess for hyperlipidemia has not been extensively described in the literature and the findings are, like the animal data, controversial. In some study populations, the prevalence of hypertriglyceridemia was even lower than in BMI-matched controls [36].

Intriguingly, CORT treatment reduced atherosclerotic lesion area, and tended to decrease lesion severity, to a similar extent in transiently vs continuously exposed mice. This suggests that GCs are able to induce long-term effects in the preliminary processes of atherosclerotic plaque formation. It is tempting to speculate on possible underlying

mechanistic explanations for this phenomenon. One possibility is the induction by increased GC exposure of epigenetic mechanisms, like chromatin remodeling and histone modifications [37]. These alterations have been demonstrated in the context of chronic CORT [38], and may lead to long lasting suppression of or changes in macrophage function. Extensive documentation supports a crucial role for macrophages in the initiation of atherosclerosis by entering the vessel wall, taking up oxidized LDL (ox-LDL) [1, 2] and transforming into foam cells that produce a variety of cytokines further driving the process of the plaque formation, as well as macrophage proliferation and differentiation [39, 40]. In the present study, transient and continuous CORT treatment did not affect the plasma ox-LDL level, albeit that continuous CORT treatment significantly reduced the macrophage content of the plaque, and a similar trend was also observed in the transiently exposed group. GCs were shown to decrease the development of atherosclerosis by reducing the monocyte recruitment [41-43], macrophage foam cell formation [44], macrophage growth, as well as macrophage inflammatory action to produce pro-inflammatory cytokines [45]. In this study, no differences were observed in the number of monocytes adhering to endothelium between the groups. It is plausible that GCs excess inhibits macrophage growth instead of monocyte recruitment as GCs inhibit macrophage colony stimulating factor (M-CSF)-induced macrophage differentiation in vitro [45]. Although, we did not detect any differences in serum M-CSF level between groups, it should be realized that M-CSF is a general marker for macrophage growth, and the systemic concentration in plasma may not reflect the local macrophage growth in the vessel. Additionally, GCs can also inhibit macrophage growth by suppressing the granulocyte/macrophage colony-stimulating factor (GM-CSF) production in isolated macrophages [46], confirming that GCs might target the macrophage, the major cellular CORT target, thereby attenuating macrophage proliferation and differentiation, and thus inhibiting atherosclerotic plaque formation.

In humans, increased GC exposure, like in patients with CS, is associated with the metabolic syndrome and cardiovascular disease, even after long-term successful correction of GC excess [6, 47, 48]. Carotid intima media thickness (IMT) is increased and vessel wall plaques are more common in patients with CS [36, 49, 50]. Indeed, CS patients have abnormal fat distribution and suffer from disturbed coagulation and osteoporosis. Although, it is documented that bone mineral density fully recovers after normalization of cortisol levels, other features, like the adverse metabolic profile and the hypercoagulable state, do not completely resolve [51, 52]. The causal relation, however, between the episode of cortisol overexposure and long-term changes in the development of cardiovascular diseases is not established and is difficult to assess in humans because of the rarity and heterogeneity of CS. In agreement, in our mouse model CORT also stimulated

the development of other components of the metabolic syndrome. We observed that CORT increased food intake, body weight, insulin concentrations, and altered body composition. High CORT levels are known to stimulate voluntary food intake dose-dependently [53-55]. GCs are also known to induce insulin resistance [48]. Moreover, insulin and CORT synergistically promote redistribution of energy storage in favor of increased fat tissue [56]. The changes in body weight were accompanied by a significant increase in both gonadal and subcutaneous fat mass in continuous group and, remarkably after 12 weeks of wash out, in transiently exposed group as well. Chronically administered GC facilitates an increase of fat mass in mice [25, 57]. In humans, increased exposure to cortisol (being either endogenous CS or exogenous corticosteroids) induces increased total body fat [58], and is specifically characterized by a redistribution of adipose tissue from peripheral to central sites of the body, mainly in the truncal region and visceral depots [59]. This is accompanied by a greater than two-fold increased risk in insulin resistance/diabetes, hypertension, and hyperlipidemia [39]. In the human equivalent of severe chronic stress, CS, the prevalence of the metabolic syndrome is increased [47, 48], and intriguingly, after remission, these patients still have increased waist circumference [36, 60] and higher visceral fat mass without an effect on the body mass index [61], and their cardiovascular risk remains increased.



Adipose tissue macrophages are the primary source of the proinflammatory cytokines, and the macrophage content of adipose tissue has been shown to correlate positively with adiposity [62]. In the present study, although excess GC exposure increased fat mass and induced obesity which was accompanied with an increase in macrophage content, inflammation in the adipose tissue was not elevated. Moreover, after abrogation of CORT treatment, the macrophage content in the adipose tissue was normalized and the reduction of inflammation was persisted in the long-term despite of the presence of a persistent increase in fat mass. CORT treatment induces prolonged, complex changes in the adipose tissue that reflect both the adverse metabolic effects of glucocorticoids by increasing the adiposity, and the anti-inflammatory capacity by reducing the expression of the proinflammatory molecules in the macrophages. Therefore, we cannot exclude that the protective effects of CORT treatment on atherosclerosis development can, at least partly, be explained by decreased release of cytokines from adipose tissue. It is well possible that at high dose, the anti-inflammatory effects of GCs attenuate potentially adverse metabolic influences. In clinical practice, this means that GC schemes as used for anti-inflammatory indications might benefit from adjustments towards a higher dose for a shorter period of time (or even as a few 'high dose 'pulses' as is used in clinical practice with methyprednisolone instead of lower dosages for a prolonged period of time.

In conclusion, increased CORT exposure in mice with a human like lipoprotein metabolism has beneficial, long-lasting effects on atherosclerosis, despite negatively affecting body fat distribution and insulin sensitivity by promoting fat accumulation in the long term. This indicates that the increased atherosclerosis observed in the human in states of GC excess may not be related to cortisol *per se*, but may be the result of complex effects of cortisol on the endothelium and/or coagulation. The effects of GC excess, therefore, are multiple, and dependent on many factors, but above all, may irreversibly affect many pathophysiological processes, thereby influencing long-term cardiovascular risk.

## ACKNOWLEDGEMENTS

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# 11

# GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Cardiovascular disease (CVD), which is mainly due to atherosclerosis, is currently the leading cause of death in the Western world [1]. One of the most important risk factors for atherosclerosis is dyslipidemia, hallmarked by increased plasma levels of (V)LDL-cholesterol (C) and triglycerides (TG), and decreased plasma levels of HDL-C. Lipid-lowering agents (especially statins) improve dyslipidemia and have proven to reduce major cardiovascular events [2, 3]. However, since these drugs only partially improve the mortality and morbidity due to CVD, other drug targets to combat CVD are being explored. Since HDL-C is inversely correlated with cardiovascular risk [4], novel strategies to raise HDL-C levels are currently under development, aiming at further reduction of atherosclerosis. Because the cholesteryl ester transfer protein (CETP) plays a pivotal role in HDL-C metabolism [5], CETP has become one of the most important targets for development of HDL-raising strategies.

Another risk factor for CVD is non-alcoholic steatohepatitis (NASH), characterized by accumulation of triglycerides and immune cells including macrophages in the liver. The prevalence of NASH is increasing steadily [6]. In contrast to atherosclerosis, no established pharmacological agents have been identified thus far to treat NASH. Lifestyle modifications, such as weight loss, exercise, and restriction of nutrient intake, are still the mainstays for the treatment of NASH [7]. Additionally, the non-invasive diagnosis for NASH is cumbersome in routine clinical practice, and no easily accessible biomarker with sufficient sensitivity and specificity is available to detect increased hepatic macrophage content, a hallmark of NASH.



The studies described in this thesis 1) demonstrated the cellular origin of CETP expression and its implications for NASH and CVD, 2) elucidated the effects of pharmacological and dietary lipid-lowering interventions on plasma CETP levels, 3) investigated a novel target for treatment of atherosclerosis and NASH, and 4) evaluated the role of the brain in peripheral TG metabolism.

## The cellular origin of CETP, and its implications for NASH and CVD

Previous studies have demonstrated that CETP mRNA is abundantly expressed in adipose tissue and liver of several mammalian species, including human, monkey, rabbit, pig and hamster [8]. In addition, CETP is expressed to a lower extent in the spleen, heart, small intestine, adrenal gland, and skeletal muscle [9-12]. A small human cohort study suggested that CETP expression in adipose tissue correlates with plasma CETP concentration [13]. In **Chapter 6**, our data showed much more prominent *CETP* expression in the liver as compared to adipose tissue. In addition, we observed no association between central obesity measured as waist circumference and plasma CETP level in a large cohort of the general population (n~1,500), implying that central adipose tissue does not correlate

with plasma CETP concentration. Rather, by analyzing liver biopsies from obese patients undergoing bariatric surgery, we found that plasma CETP concentration was strongly correlated with CETP expression in the liver but not with that in the adipose tissue, indicating that liver is the main site of *CETP* expression and is a determinant of the total plasma CETP pool in humans. Few studies suggested that changes in the degree of adiposity induced by body weight reduction reduced CETP expression and improved lipoprotein metabolism [14, 15]. However, we (**Chapter 4**) and others [16] showed that body weight reduction, in addition to reducing adiposity, significantly reduced hepatic lipid content and attenuated hepatosteatosis. Moreover, we also showed in this thesis that a decrease in hepatic lipid content was accompanied by a decrease in plasma CETP level (**Chapter 3** and **4**). It is thus tempting to speculate that bodyweight reduction via attenuation of hepatosteatosis reduces the production of CETP by the liver, and via this mechanism reduces plasma CETP concentration.

The liver consists of multiple cell types including hepatocytes and non-parenchymal cells such as endothelial cells and macrophages (i.e. Kupffer cells). Therefore, we set out to evaluate the cell type responsible for the hepatic expression of CETP. In this thesis, we demonstrated that the expression of established macrophage markers (e.g. *Cd68*, *Abcg1* and *Marco*) in the liver strongly correlated with the hepatic expression of CETP both in human CETP transgenic (Tg) mice (**Chapter 5**) and in humans (**Chapter 6**). Moreover, CETP appeared to be specifically co-localized with $F480^+$ macrophages in the mouse liver and with $CD68^+$ macrophages in the human liver (**Chapter 6**). Mechanistic studies in *APOE*3-Leiden.CETP (E3L.CETP)* mice showed that depletion of macrophages from liver following administration of clodronate liposomes virtually abolished hepatic CETP expression and largely reduced plasma CETP level, fully corroborating our findings in humans that hepatic macrophages, rather than hepatocytes, are the main cellular origin of hepatic CETP expression and the plasma CETP pool.



Previously, hepatic expression of CETP has been attributed to both macrophages and hepatocytes. This dogma was mainly derived from studies assessing hepatic CETP expression 8 weeks after transplantation of bone marrow from wild-type (WT) littermates into human CETP Tg mice and vice versa, suggesting that hepatic macrophages contribute ~50% to total hepatic CETP expression [17]. However, it should be realized that the turnover of liver macrophages after bone marrow transplantation occurs slowly. In the same study, it was found that only 50% of Kupffer cells were replaced by the donor cells 8 weeks after bone marrow transplantation, accompanied by 50% reduction in plasma CETP as well as 2-fold lower hepatic *CETP* expression in WT → CETP Tg mice as compared to control transplanted CETP Tg → CETP Tg mice [17]. Interestingly, we found hepatic CETP expression to be decreased by approximately

-90% at 12 weeks after transplantation when more Kupffer cells had been replaced (*Berbée et al.* unpublished), again confirming that hepatic macrophages are the main predominant source of CETP expression.

In contrast to the liver (i.e. Kupffer cells), we could hardly detect CETP expression in extrahepatic macrophage-rich organs, including adipose tissue and spleen, or in isolated peritoneal macrophages. It has been reported that that CETP gene promoter contains a liver X receptor α (LXRα) responsive element [10], and CETP expression is regulated by the activation of LXRα [18], which is highly expressed in multiple organs. Recently, *Gautier et al.* [19] reported that hepatic CETP expression is also upregulated by activation of the farnesoid X receptor (FXR), for which bile acids are the natural ligands. In addition to an LXRα responsive element in the CETP gene promoter, the CETP gene was shown to contain an ER8 FXR response element in its first intron. Since FXR is highly expressed in the liver and its natural ligand bile acids are produced by hepatocytes, the specific liver environment may be essential for maintaining high expression of CETP in hepatic versus extrahepatic macrophages. Although treatment of CETP Tg mice with both LXR agonists [18] and FXR agonists [19] induces CETP expression in the liver, the leading regulator for CETP expression in hepatic macrophages *in vivo* is still unknown yet, which should be investigated by future studies.

Our findings that whole body CETP expression is predominantly derived from hepatic macrophages reveal that plasma CETP may be a biomarker for hepatic macrophage content, a hallmark of NASH. NASH is characterized by accumulation of fat (steatosis) in combination with inflammation (e.g. infiltrated macrophages) in the liver [20]. Although several imaging modalities have been advocated as non-invasive diagnostic method for liver steatosis, they are insufficient to distinguish NASH from simple non-inflammatory fatty liver disease (NAFLD). Liver biopsies are currently the golden standard methods for the diagnosis of NASH. However, there are several severe limitations to liver biopsies, such as sampling error, differences in histopathologic interpretation, as well as patient stress and discomfort, risk of bleeding and long hospitalizations. In this thesis, we showed that hepatic macrophages are the main cellular origin of the plasma CETP pool (**Chapter 6**), and that the plasma CETP level significantly correlates with hepatic macrophage content in CETP Tg mice (**Chapter 5**). More importantly, treatment of *E3L.CETP* mice with niacin (**Chapter 5**) and exendin-4 (**Chapter 10**) reduces hepatic macrophage content accompanied with the reduction in plasma CETP level. Taken together, these data suggest that measurement of the plasma CETP concentration can be developed as a diagnostic and predictive test for the hepatic macrophage content in clinical practice, which should be tested in large population cohorts.

In addition, our findings that CETP expression in the hepatic macrophages

determines the plasma CETP level as well as the metabolism of plasma lipoproteins sheds new light on the development of new strategies involving CETP inhibition for the treatment of dyslipidemia and CVD. Although a past CETP inhibitor (i.e. torceptrapib) and current CETP inhibitors (e.g. dalcetrapib, anacetrapib, evacetrapib) convincingly demonstrate HDL-raising effects, they show uncertain results for the treatment of CVD. For example, although torcetrapib [21] and dalcetrapib [22] increase the plasma HDL-C level effectively, both of them failed to reduce CVD outcome in trials. Torcetrapib even caused a marked increase in deaths [21], and dalcetrapib had no beneficial effects on carotid artery wall index, endothelial function or CVD outcomes [23-25]. Although the precise mechanism(s) by which torcetrapib and dalcetrapib inhibit CETP activity is not known, these CETP inhibitors directly act on the plasma CETP protein and induce tight binding of CETP to HDL particles [26]. However, the tight binding of CETP with HDL particles might compromise the function of HDL to generate large CE-rich HDL particles instead of small HDL particles and nascent discoidal HDL, as observed with torcetrapib [27].

The development of CETP inhibitors to raise HDL-C is based on epidemiological studies showing a strong inverse correlation of HDL-C level with CVD risk [4]. The classical "HDL cholesterol hypothesis" predicted that interventions to raise the HDL-C level will result in reduction of CVD risk. However, recent studies showed that HDL functionality perhaps is a more important consideration than the circulating HDL-C level for the treatment of CVD. Indeed, the reverse cholesterol transport capacity of HDL has been shown a much better predictor of CVD than the concentration of HDL-C [28, 29]. Therefore, within the field of HDL-targeting therapeutics a gradual transition takes places from the simple "HDL cholesterol hypothesis" to the "HDL functionality hypothesis" aimed at increasing the HDL particle number and improving HDL functionality for the treatment of CVD [28-30]. Based on this perspective, to avoid potentially adverse effects of the current CETP inhibitors on the function of HDL, strategies focusing on inhibiting CETP synthesis at its cellular origin may be a promising alternative.



## Regulation of plasma CETP by pharmacological and dietary lipid-lowering interventions

Previously, we have shown that several classical lipid-lowering drugs including statins [31], fibrates [32] and niacin [33], increase the plasma level of HDL-C in addition to decreasing the plasma level of (V)LDL-C and TG by reducing hepatic CETP expression and decreasing plasma CETP activity in preclinical studies using *E3L.CETP* transgenic mice. In this thesis, we again demonstrated in **Chapter 6** that fenofibrate and niacin raise the HDL-C level accompanied by reduced hepatic CETP expression and plasma CETP level in *E3L.CETP* mice. In line with those classical lipid lowering drugs, in **Chapter 10** we observed that

exendin-4, a glucagon like peptide-1 (GLP-1) receptor agonist that was approved in 2005 for the treatment of T2DM, decreased plasma VLDL-C and increased HDL-C also accompanied with decreased hepatic *CETP* expression as well as plasma CETP level in *E3L.CETP* mice. Moreover, in **Chapter 3**, we found that although both pioglitazone (PPARγ agonists) and metformin decreased plasma TG and apoB level equivalently, only pioglitazone significantly increased the plasma HDL-C level associated with a reduction in hepatic triglyceride and plasma CETP level in patients with type 2 diabetes mellitus (T2DM). In addition to pharmacological interventions, in **Chapter 4**, prolonged caloric restriction markedly decreased the plasma CETP level and increased the plasma apoAI level in obese patients with T2DM. These collective results from human studies are in full accordance with the findings in *E3L.CETP* mice, suggesting that reduction of (liver-derived) CETP plays an important role in the HDL-raising effects of both pharmacological and dietary lipid-lowering interventions.

Although accumulating evidence indicates that lipid-lowering interventions exert HDL-raising capacity by reducing the plasma CETP level, the mechanisms underlying the CETP-reducing effects of those lipid-lowering interventions has thus far not been delineated. In **Chapter 6**, we demonstrated that hepatic macrophages are the main cellular origin of CETP expression, and that CETP expression in hepatic macrophages determines the plasma CETP level and modulates plasma lipoprotein metabolism. Thus, in **Chapter 5**, we set out to investigate the role of hepatic macrophages in the CETP-lowering effect of niacin. Interestingly, our observations *in vitro* showed that niacin at various concentrations did not reduce CETP expression in cultured macrophages derived from CETP Tg mice, indicating that niacin does not directly regulate the CETP expression *per se* in macrophages. Rather, we observed that niacin reduced the hepatic cholesterol content *in vivo*. More importantly, in line with attenuated liver cholesterol accumulation, we observed that niacin decreased hepatic mRNA expression of macrophage markers (e.g. *Cd68* and *Abcg1*) and the number of F4/80$^{+}$ macrophages, as well as the hepatic expression of CETP. In fact, hepatic macrophage markers showed a high correlation and association with hepatic CETP expression. These data suggest that the reduction of hepatic CETP expression induced by niacin treatment is a direct consequence of reduced macrophage content in the liver. In addition to niacin, we demonstrated in *E3L.CETP* mice that fenofibrate (**Chapter 6**) and exendin-4 (**Chapter 10**) also reduce the hepatic lipid content and decreased the hepatic macrophage content thereby decreasing the hepatic expression and plasma level of CETP.

In humans, we are able to measure hepatic TG content by proton ($^{1}$H) magnetic resonance spectroscopy, although it is currently impossible to assess the hepatic macrophage content noninvasively. We observed that both pioglitazone (**Chapter 3**)

and prolonged caloric restriction (**Chapter 4**) decreased the plasma CETP level accompanied with a decrease in hepatic TG content in patients with T2DM. It has been reported that the reduction of hepatic TG content induced by e.g. weight loss is associated with a decrease in hepatic inflammation [34], and that the histological severity of inflammation is correlated with the number of $CD68^+$ macrophages in patients with NASH [35]. It is thus tempting to speculate that both pioglitazone (**Chapter 3**) and prolonged caloric restriction (**Chapter 4**) decrease not only the hepatic lipid content but also hepatic macrophage content in humans, thereby reducing hepatic expression and plasma level of CETP. This hypothesis needs to be evaluated in future studies, for which the assessment of macrophage number in the liver will ultimately be required.

As summarized in Figure 1, we propose the current mechanism how lipid-lowering interventions reduce hepatic CETP expression and plasma CETP level. Albeit through different actions, they all reduce hepatic lipid content (i.e. TG and cholesterol). This reduction in hepatic lipid content subsequently attenuates hepatic inflammation, which leads to less macrophage infiltration into and/or increased macrophage efflux/emigration out of the liver. Since liver macrophages are the main cellular origin of CETP expression, the decreased number of hepatic macrophages leads to an overall reduction in hepatic CETP expression, and, consequently, the plasma CETP level. Therefore, these lipid-lowering interventions induce a less atherogenic lipid phenotype, e.g. decreasing (V)LDL-C and TG, and increasing HDL-C.



## Novel strategies for treatment of atherosclerosis and NASH

Given the fact that current strategies are insufficient to reduce CVD and no established pharmacological agents have shown adequate and convincing benefits in NASH outcomes, novel strategies for the treatment of those two diseases are eagerly warranted and under development. Emerging evidence indicates that gut hormones regulating energy homeostasis and food intake, could also beneficially affect lipid metabolism, thus have the potential to treat atherosclerosis and fatty liver disease. Glucagon like peptide-1 (GLP-1) is one of those incretin hormones produced by intestinal L-cells and the brain [36, 37], and released in response to food intake to stimulate glucose-dependent insulin production [38]. In addition, GLP-1 exerts multiple other functions, including inhibition of food intake [39], slowing the gastric emptying [40], inhibition of glucagon secretion [41], and improving glucose metabolism [41, 42]. In addition, we (***Chapter 10***) and others [43, 44] observed that GLP-1 receptor agonists decrease plasma TG and VLDL-C level, and increase HDL-C level. Thus, GLP-1 receptor agonism may be a valuable target for both atherosclerosis and NASH.

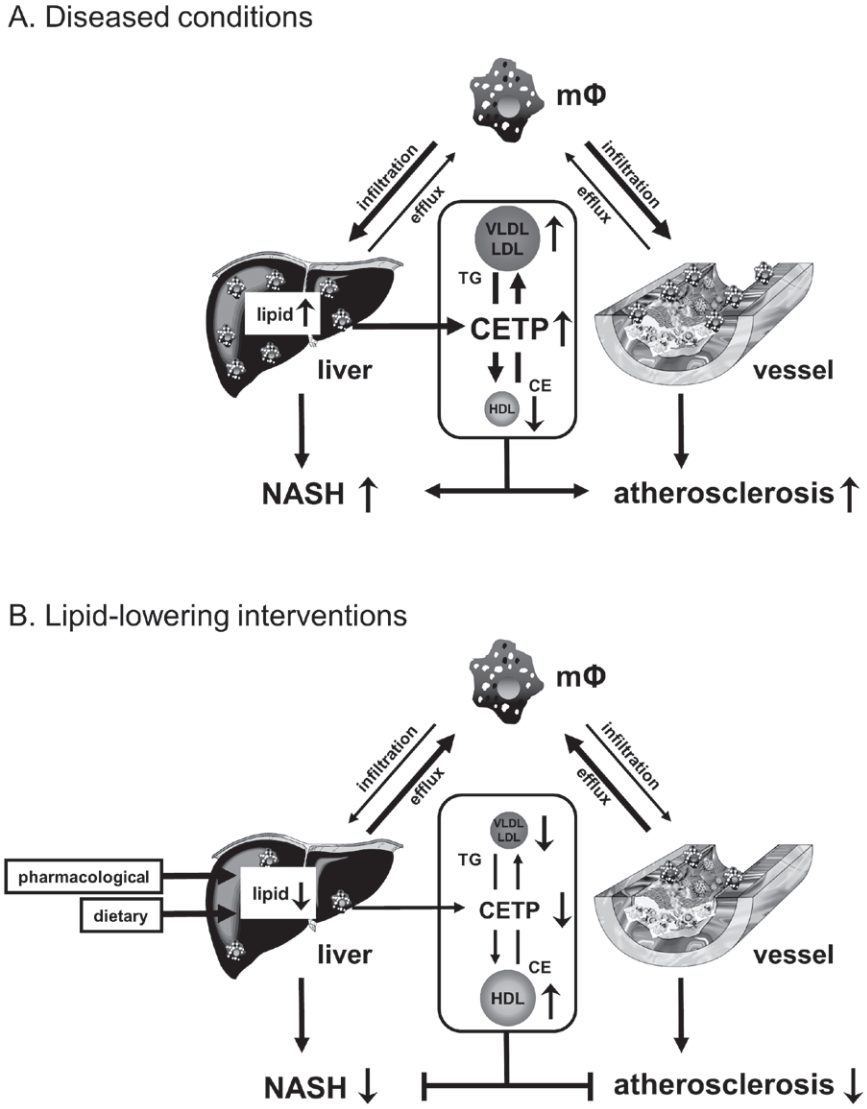


**Figure 1. Proposed mechanism underlying the CETP-lowering effects of lipid-lowering interventions.** (A) Under diseased conditions (e.g. NASH and atherosclerosis), macrophages infiltrate into both the vessel wall and the liver. In the liver, increased hepatic macrophages result in an overproduction of CETP, and consequently to an elevated plasma CETP pool. (B) Lipid-lowering interventions, including both pharmacological (e.g. fenofibrate, niacin, pioglitazone and exendin-4) and dietary (e.g. caloric restriction) interventions, reduce the hepatic lipid content and concomitantly attenuate macrophage infiltration into the liver. The decreased number of hepatic macrophages leads to a reduction in hepatic CETP expression and plasma CETP level, as a result, generating a less atherogenic lipid phenotype. See text for further explanation.

In **Chapter 8**, we first investigated the mechanisms underlying the beneficial effects of GLP-1 receptor agonism on liver TG metabolism in *E3L* mice fed a high fat diet (HFD). We found that GLP-1 receptor agonists decreased hepatic VLDL particle production and completely reversed HFD-induced hepatic steatosis reflected by largely decreasing the hepatic lipid content to the low levels observed in chow-fed control mice. GLP-1 receptor agonists decreased the expression of the nuclear transcription factor *Srebp-1c* and its targets *Fasn* and *Dgat1*, implying that GLP-1 receptor agonism primarily reduces hepatic lipogenesis, thereby causing a reduction in hepatic lipid content. Taken together with the concomitantly reduced *Apob* expression, GLP-1 receptor agonism lowers the hepatic availability of TG, thereby reducing the production of VLDL particles. In addition to decreasing hepatic lipogenesis and increasing fatty acid oxidation, GLP-1 receptor agonists activate AMP-activated protein kinase (AMPK) and SIRT (sirtuin) dependent on GLP-1 receptor expressed in hepatocytes [45], thereby improving hepatic glucose and lipid metabolism and relieving NAFLD. Recently, several clinical trials have confirmed that GLP-1 receptor agonists largely reduce the hepatic TG content in obese patients with T2DM [45, 46], indicating that the GLP-1 receptor is a promising novel target for treatment of NAFLD.

Moreover, in **Chapter 10**, we observe that only 4 weeks of exendin-4 infusion markedly decreases total atherosclerotic lesion area, accompanied by a reduction in plaque macrophage content. Notably, in contrast to classical lipid-lowering compounds that reduce atherosclerosis mainly by improving dyslipidemia, exendin-4 only slightly decreased (V)LDL-C and TG, and increased HDL-C. Furthermore, exendin-4 also decreased hepatic inflammation reflected by reduced expression of inflammatory markers (e.g. TNFα, IL-1β and Il-6), as well as hepatic macrophage content. The GLP-1 receptor is thus not only a promising target for NAFLD, but also NASH and atherosclerosis. It is interesting to note that atherosclerosis and NASH are strongly associated and share common etiologies, involving monocyte recruitment and macrophage foam cell formation [47]. We demonstrated a reduced number of both adhering monocytes to the vessel wall and infiltrated macrophages into the liver after the treatment of exendin-4. In addition, exendin-4, via the GLP-1 receptor, reduced the uptake of oxLDL by macrophages, which may implicate reduced foam cell formation in the vessel wall and in the liver. Taken together, these data corroborate the hypothesis that exendin-4 reduces the development of atherosclerosis and NASH simultaneously by acting directly on monocyte/macrophage recruitment/maturation into both the vessel wall and liver.

So far, exendin-4 has been approved for the treatment of T2DM in the clinical practice. We show that GLP-1 receptor agonism not only suppresses diet-induced NAFLD, but also reduces the development of NASH and atherosclerosis, at least when administered

chronically by using an osmotic minipump. Therefore, we propose that GLP-1 receptor agonism is a novel strategy for treatment of atherosclerosis and NASH in addition to T2DM, in particular in patients who display a combination of these diseases.

## The role of brain in triglyceride metabolism

The brain plays an important role in maintaining energy homeostasis, with the hypothalamus being its key regulator [48]. Two major neuronal populations within the hypothalamic arcuate nucleus are involved in the regulation of energy intake, including pro-opiomelanocortin/cocaine and amphetamine-regulated transcript-expressing neurons and neuropeptide Y (NPY)/agouti-related protein-expressing neurons. Although the role of brain in the regulation of glucose metabolism has been firmly established [49], only a few studies have focused on its function in maintaining TG homeostasis. Recently, accumulating evidence have suggested that various neuronal populations, such as NPY expressing neurons and the melanocortin (MC) expressing neurons, modulate sympathetic outflow from the hypothalamus towards target organs involved in TG metabolism, such as liver, white adipose tissue (WAT) and brown adipose tissue (BAT), and thereby modulate peripheral TG metabolism. For example, a recent study in rats showed that central administration of NPY acutely increases hepatic VLDL-TG production [50]. Also, *Bruinstroop et al.* [51] further confirmed that hypothalamic NPY regulates hepatic VLDL secretion in rats via the sympathetic outflow, as selective sympathetic denervation of the liver abolished the effect of central NPY administration. In contrast to NPY signaling, central administration of melanocortin receptor (MC) receptor agonists decrease hepatic lipogenic gene expression in diabetic mice [52] and decrease hepatic TG content in rats [53].



Hypertriglyceridemia, associated with increased hepatic VLDL-TG production and/or decreased VLDL-TG clearance, is an important risk factor for CVD [54, 55]. Since atherosclerosis is generally studied in hyperlipidemic mice rather than in rats, in **Chapter 7**, we set out to validate the effect of central NPY signaling on hepatic VLDL-TG production in mice, with the ultimate goal to investigate whether NPY, by affecting VLDL-TG synthesis, contributes to the development of atherosclerosis. Although we confirmed that central administration of NPY acutely increases food intake in mice, similarly as in rats [56], surprisingly we were unable to detect any increase in hepatic VLDL-TG production in mice after central NPY infusion. Likewise, antagonizing central NPY signaling by either $PYY_{3-36}$ or Y1 receptor antagonism did not affect VLDL production in mice. Apparently, central NPY signaling exerts different effects on TG metabolism in rats versus mice. One potential explanation is that the hepatic VLDL metabolism in itself is differentially regulated in rats versus mice, as rats display lower basal hepatic VLDL-TG

production rates [50, 51] when compared to that in mice. Secondly, the expression of the several NPY receptors in rats versus mice is different. Both rats and mice express similar Y1-Y5 receptors [57], while only mice express the Y6 receptor [58]. Although the exact function of Y6 receptor in appetite regulation remains elusive, if activation of this receptor by NPY would exert an opposing effect on hepatic VLDL production, this might explain our negative findings in mice. Notably, genetic association studies in humans have reported conflicting results on the association of polymorphisms in the NPY gene with plasma TG levels [59, 60]. Collectively, these data emphasize the requirement of further research, particularly in humans, on the role of hypothalamic NPY in peripheral TG metabolism.

In addition to hypothalamic NPY, gut hormones regulate energy homeostasis via activation of their receptors expressed in the brain. The GLP-1 receptor is abundantly expressed in various tissues [61], not only in the gastrointestinal tract, pancreatic islands, kidneys, and heart, but also in the central nervous system [62]. After showing that GLP-1 receptor agonism decreased hepatic VLDL-TG production and hepatic lipid content in mice (**Chapter 8**), we next investigated whether the effects of GLP-1 receptor agonism on lipid metabolism would be dependent on central GLP-1 receptor signaling. We found that chronic central administration of the GLP-1 receptor antagonist exendin-9 did not counteract the peripherally administered exendin-4-induced decrease in hepatic VLDL-TG production, suggesting that the beneficial effects of exendin-4 on hepatic lipid metabolism is not mediated by the central GLP-1 receptor signaling (unpublished). In contrast to our findings, *Panjwani et al.* [63] recently showed that acute central administration of exendin-4 rapidly decreased hepatic VLDL-TG production, indicating that central GLP-1 signaling might regulate hepatic lipid metabolism. There are several possible explanations for the distinct results obtained from our study and Panjwani's study: (1) the dosage of exendin-9 used in our study is insufficient to block the effects of central GLP-1 receptor activation on hepatic lipid production, and (2) acute activation of the central GLP-1 signaling plays a more important role in hepatic VLDL production than chronic activation. Experiments combining central and/or peripheral GLP-1 administration with hepatic denervations might prove an effective strategy to elucidate the exact role of central GLP-1 signaling in the regulation of hepatic TG metabolism.

The hypothalamus not only regulates, via the modulation of sympathetic outflow, TG metabolism in the liver but also in white adipose tissue (WAT). For example, chronic central NPY infusion promoted lipogenesis in WAT, independent of its effects on food intake [64]. Likewise, inhibition of central MC signaling induced the expression of lipogenic genes in WAT [65]. In contrast, activation of central MC signaling by chronic infusion of an MC3/4 receptor agonist, increased the expression of lipolytic genes in WAT of rats. Additionally, central GLP-1 was implicated to regulate TG metabolism in WAT, as a mouse

study showed that central infusion of GLP-1 decreased TG content in WAT [66].

Unlike WAT, BAT combusts TG in the process of thermogenesis. Recently, evidence has accumulated to suggest that hypothalamic signaling also influence BAT thermogenesis. We and others showed that central infusion of NPY decreased BAT activity and thermogenesis, both in mice (unpublished) and in rats [67]. Also, central infusion of MC4R antagonist resulted in decreased BAT thermogenesis in rats [68]. Since those studies investigating the effect of neuroendocrine factors on BAT thermogenesis have neither focused on lipid metabolism nor performed TG clearance experiments, future studies should therefore emphasize the effect of central signaling on TG clearance by BAT.

Taken together, the brain, in particular the hypothalamus is an important regulator of peripheral TG metabolism. However, the exact role of specific neuroendocrine factors that mediate TG metabolism in liver and BAT needs to be determined by future research.

## Concluding remarks

The current strategies for the treatment of atherosclerosis, i.e. lipid-lowering strategies, are insufficient, and at the start of the studies described in this thesis no pharmacological agents had been identified thus far to treat NASH. Therefore, novel strategies for the treatment of those two diseases were eagerly warranted and currently under investigation. In addition to classical lipid-lowering agents, HDL-raising strategies, e.g. CETP inhibitors, are currently still considered as promising methods to treat dyslipidemia and ultimately CVD. However, current CETP inhibitors may affect the functionality of HDL. Therefore, other ways to reduce CETP levels may be advantageous.



In this thesis, we demonstrated CETP to be involved in the HDL-raising effects of both lipid-lowering agents (i.e. fenofibrate, niacin, pioglitazone and the GLP-1 receptor agonist exendin-4), as well as dietary lipid-lowering interventions (i.e. caloric restriction). In fact, we found that all of these interventions reduced plasma CETP level accompanied by reduction in hepatic lipid content. More mechanistic studies revealed that CETP is predominantly expressed by hepatic macrophages, and that reducing the hepatic macrophage content by lipid-lowering strategies reduces the hepatic CETP expression and plasma CETP level. Therefore, targeting the hepatic macrophage may be a promising alternative for CETP inhibitors to reduce the plasma CETP level and increase the HDL level. In addition, the fact that plasma CETP is mainly derived from hepatic macrophages, a hallmark of NASH, implies that measuring plasma CETP concentration may provide a useful relatively non-invasive biomarker for the hepatic macrophage content in NASH in clinical practice. In addition, we identified, by using *E3L.CETP* mice, the GLP-1 receptor as a novel target for the treatment of atherosclerosis and NASH in addition to T2DM, which need to be confirmed in future human studies.

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# 12

SUMMARY
SAMENVATTING
LIST OF PUBLICATIONS
CURRICULUM VITAE

# SUMMARY

Atherosclerosis and non-alcoholic steatohepatitis (NASH) are the leading causes of cardiovascular disease (CVD) and chronic liver disease, respectively, both of which remain common reasons of morbidity and mortality in the Western world. Atherosclerosis and NASH share the same etiologies, of which disturbed lipid metabolism manifested by dyslipidemia is the most important one, hallmarked by increased plasma levels of (V)LDL-cholesterol (C) and triglycerides (TG), and decreased plasma levels of HDL-C. Pharmacological lipid-lowering agents improving dyslipidemia are effective tools to prevent and treat atherosclerosis. However, since mortality and morbidity due to cardiovascular disease are only partially improved by current lipid-lowering strategies, novel strategies are currently under development aimed at further reduction of atherosclerosis. Since HDL-C levels are inversely associated with cardiovascular risk, one of these strategies is to raise HDL levels. As no pharmacological agents have been identified thus far to treat NASH, and no biomarkers are available to detect NASH, the search for both treatment modalities and biomarkers for NASH is also warranted.

Among HDL-raising strategies, infusion of reconstituted HDL (rHDL) seems to be a promising one for the treatment of CVD, as rHDL has been shown to induce atherosclerosis regression in human studies. Studies in mice indicated that rHDL infusion adversely affects VLDL levels, but this effect is less apparent in humans. This discrepancy may be explained by the fact that humans, in contrast to mice, express CETP. CETP plays a vital role in lipid metabolism by mediating the transfer of TG and CE between (V)LDL and HDL. In **Chapter 2**, we investigated the role of CETP in the effects of rHDL on VLDL metabolism by using *APOE*3-Leiden (E3L)* mice, a well-established model for human-like lipoprotein metabolism, which had been crossbred with mice expressing human CETP under control of its natural flanking regions (*E3L.CETP* mice). At 1 hour after injection, rHDL increased plasma VLDL-C and TG in *E3L* mice, but not in *E3L.CETP* mice. This initial raise in VLDL was caused by competition between rHDL and VLDL for lipoprotein lipase (LPL)-mediated TG hydrolysis, and was thus prevented by the expression of CETP. At 24 hours after injection, rHDL caused a second increase in VLDL-C and TG in *E3L* mice, whereas rHDL even decreased VLDL in *E3L.CETP* mice. This secondary raise in VLDL was due to increased hepatic VLDL-TG production. Collectively, we concluded that CETP protects against the rHDL-induced increase in VLDL, and that treatment of atherogenic dyslipidemia by rHDL should thus not be combined with agents that aggressively reduce CETP activity.

Thiazolidinediones (PPARγ agonists) decrease plasma TG, increase HDL-C and reduce hepatic steatosis. Since previous studies in mice have shown that reduction of hepatic steatosis by lipid-lowering agents is accompanied by reduced hepatic CETP expression and plasma CETP levels, which may explain a secondary increase in HDL-C, we assessed in **Chapter 3** the effects of pioglitazone on plasma CETP levels in patients with type 2 diabetes mellitus (T2DM). Patients with T2DM were randomized to treatment with pioglitazone or metformin and matching placebos, in addition to glimepiride. At baseline and after 24 weeks of treatment, plasma HDL-C and CETP levels were measured, and hepatic TG content was assessed by proton magnetic resonance spectroscopy. Pioglitazone decreased the hepatic TG content, which was indeed associated with decreased plasma CETP levels and increased plasma HDL-C levels, whereas metformin did not significantly change any of these parameters. We concluded that the decrease in hepatic TG content by pioglitazone was accompanied by a decrease in plasma CETP concentration and, therefore, associated with an increase in HDL-C levels.

In contrast to pharmacological interventions, lifestyle interventions such as diet-induced weight loss and exercise are still the mainstays for the treatment of NASH. We recently reported that a 16-week very low calorie diet (VLCD) significantly decreased plasma total cholesterol (TC) and TG levels and markedly reduced the hepatic TG content in obese patients with T2DM and hepatic steatosis, but the potential beneficial effect of a VLCD on plasma CETP and HDL levels had not been studied. In **Chapter 4**, we investigated the effects of VLCD, resulting in a major reduction in hepatic TG content, on plasma CETP and HDL levels in obese patients with T2DM and hepatic steatosis. VLCD markedly decreased plasma CETP concentration and increased plasma apoAI levels, without significantly affecting plasma HDL-C and HDL-phospholipids levels. Although VLCD resulted in HDL that was less lipidated, the functionality of HDL with respect to inducing cholesterol efflux *in vitro* was unchanged. Therefore, we concluded that the marked decrease in hepatic TG content induced by VLCD was accompanied by a decrease in plasma CETP concentration and an increase in apoAI levels, without improving the cholesterol efflux properties of HDL *in vitro*.

Niacin (nicotinic acid) is the most potent HDL-raising drug used in the clinic practice. In addition to raising the level of anti-atherogenic HDL-C, niacin also decreases plasma levels of pro-atherogenic lipoproteins and lipids including VLDL, LDL and TG. Therefore, niacin is regarded as a candidate for the treatment of atherosclerosis. Niacin has recently been shown in *E3L.CETP* mice to decrease the hepatic lipid content, accompanied with a reduction of the hepatic expression and plasma levels of CETP, thereby increasing

HDL-C. In **Chapter 5**, we investigated the mechanisms underlying the CETP-lowering effect of niacin by using human CETP transgenic mice. *In vitro* studies demonstrated that niacin did not directly attenuate CETP expression in macrophages. *In vivo* studies showed that niacin reduced the hepatic cholesterol content and attenuated Western type diet-induced hepatic inflammation. Furthermore, niacin reduced the hepatic gene expression and plasma level of CETP. Concomitantly, niacin decreased the hepatic expression of CD68 and ABCG1, both of which are specific markers for the hepatic macrophage content, as well as the actual hepatic macrophage content. In fact, the hepatic CETP expression was significantly correlated with the hepatic macrophage markers. We concluded that niacin decreases hepatic CETP expression and plasma CETP mass by attenuating liver inflammation and macrophage content in response to its primary lipid-lowering effect, rather than by attenuating the CETP expression level within macrophages.

Since CETP is a current target for treating dyslipidemia, it is crucial to understand the true origin of CETP in humans. Previous studies indicated that adipose tissue and liver are the two major sources of plasma CETP. However, the relative contribution of tissue-specific CETP expression to plasma CETP levels is unknown. Therefore, in **Chapter 6**, we aimed to elucidate the cellular origin of CETP using human cohorts and *E3L.CETP* mice. In a general population study, plasma CETP levels did not correlate with waist circumference, suggesting that central adipose tissue does not contribute to plasma CETP. Microarray analysis of liver and adipose tissue biopsies from bariatric surgery patients showed that CETP expression was highest in the liver, and correlated with inflammatory pathways. Immunohistochemistry revealed that CETP was primarily expressed by hepatic macrophages. CETP expression in liver, but not adipose tissue, positively correlated with plasma CETP levels, and inversely correlated with plasma HDL-C. Selective elimination of macrophages from liver versus adipose tissue in *E3L.CETP* mice virtually abolished hepatic CETP expression, but not adipose tissue CETP expression, accompanied by largely reduced plasma CETP concentration and increased plasma HDL-C. Treatment of *E3L.CETP* mice with lipid-lowering drugs that are known to reduce the plasma CETP concentration and to increase HDL-C in humans, reduced the hepatic macrophage content, thereby reducing plasma CETP and increasing HDL-C. We concluded that plasma CETP is primarily derived from liver macrophages and plasma CETP is a biomarker for the hepatic macrophage content, a hallmark of NASH for which no non-invasive diagnostic tool is currently available.

Accumulating evidence indicates that strategies targeting regulation of energy

homeostasis and food intake also beneficially affect lipid metabolism, and have the potential to treat atherosclerosis and NASH. The brain plays an important role in mediating energy homeostasis, with the hypothalamus being its key regulator. Two major neuronal populations within the hypothalamic arcuate nucleus are involved in the regulation of food intake, including pro-opiomelanocortin/cocaine- and amphetamine-regulated transcript-expressing neurons and neuropeptide Y (NPY)/agouti-related protein-expressing neurons. Previous studies suggested that central NPY administration increases hepatic production of VLDL-TG in rats. In **Chapter 7**, we set out to validate the effects of central NPY on hepatic VLDL production in mice, to ultimately investigate whether NPY, by inducing dyslipidemia, affects the development of atherosclerosis. Administration of NPY into both the lateral and third ventricle of the brain of mice increased food intake within one hour after injection, but had no effects on hepatic VLDL-TG or VLDL-apoB production. Likewise, antagonizing central NPY signaling did not affect hepatic VLDL production. We concluded that in mice, as opposed to rats, acute central administration of NPY increases food intake without affecting hepatic VLDL production. This apparent species difference in the effect of NPY on hepatic VLDL-TG production is of great significance for future animal studies on the central regulation of hepatic VLDL metabolism.

Human studies suggested that glucagon-like peptide-1 (GLP-1) receptor agonism not only modulates energy homeostasis and improves glucose metabolism, but also decreases the plasma TG level. However, the mechanism underlying the reduction in plasma TG remained unclear. In **Chapter 8**, we evaluated the effects of GLP-1 receptor agonism on TG metabolism in high fat diet (HFD)-fed *E3L* mice. Four weeks of treatment with GLP-1 receptor agonists (i.e. CNTO3649 and exendin-4) by using subcutaneous osmotic minipumps improved glycemic control by reducing fasting plasma glucose and insulin levels. In addition, both GLP-1 receptor agonists reduced hepatic VLDL-TG and VLDL-apoB production, indicating reduced production of VLDL particles rather than reduced lipidation of apoB. Moreover, GLP-1 receptor agonism markedly decreased the hepatic content of TG, cholesterol and phospholipids, accompanied by down-regulation of expression of genes involved in hepatic lipogenesis (*Srebp-1c, Fasn, Dgat1*) and apoB synthesis (*Apob*). We concluded that GLP-1 receptor agonism, in addition to improving glycemic control, ameliorates dyslipidemia and reduces hepatic steatosis.

Since our studies showed that exendin-4 improves glycemic control and lipid metabolism, and reverses HFD-induced hepatic steatosis (chapter 8), we anticipated that GLP-1 receptor agonism has the potential to treat atherosclerosis and fatty liver disease.

However, the impact of GLP-1 receptor agonism on NASH, especially with respect to hepatic inflammation, was still uncertain. Since the development of atherosclerosis and NASH share common etiologies, in **Chapter 9** we evaluated the effects of exendin-4 on the development of atherosclerosis and NASH simultaneously by using *E3L.CETP* mice fed a Western-type diet. Although four weeks of treatment with exendin-4 only slightly reduced plasma lipid and lipoprotein levels, it markedly decreased atherosclerotic lesion severity and area, accompanied with a reduction in monocyte adhesion to the vessel wall and macrophage content in the plaque. Furthermore, exendin-4 reduced the hepatic cholesterol content as well as the hepatic $CD68^+$ and $F4/80^+$ macrophage content indicating that exendin-4 attenuated diet-induced NASH. This was accompanied by less monocyte recruitment from the circulation as the hepatic $Mac\text{-}1^+$ macrophage content was decreased. Finally, exendin-4 reduced chemokine expression *in vivo* and suppressed oxLDL accumulation in peritoneal macrophages *in vitro*, dependent on the GLP-1 receptor, suggesting that exendin-4 reduces both atherosclerosis and NASH by reducing macrophage recruitment and activation. We concluded that exendin-4 could be a valuable strategy to treat atherosclerosis and NASH in addition to T2DM, especially in patients who display a combination of these diseases.

In addition to lipid-lowering strategies, anti-inflammatory agents that are aimed at reducing the risk of CVD are now under development. Glucocorticoids are one of the strongest anti-inflammatory drugs widely used as immunosuppressive agents in the clinical practice. However, glucocorticoids excess can also induce adverse metabolic effects in adipose tissue, such as central obesity and insulin resistance, which may attenuate the potentially protective effects of glucocorticoids on CVD. In **Chapter 10,** we investigated the effects of both transient and continuous glucocorticoid treatment on atherosclerosis development by using *E3L.CETP* mice, which display human-like lipoprotein metabolism upon feeding a Western-type diet. Although both 5 weeks (transient) and 17 weeks (continuous) of corticosterone (CORT) treatment increased body weight and food intake for the duration of the treatment, only continuous CORT treatment induced changes in body composition with lower adrenal weight and higher gonadal and subcutaneous fat pad weights at 17 weeks. Moreover, the group that continuously received CORT displayed increased plasma insulin levels and HOMA-IR index, indicating that long-term administration of glucocorticoids induces insulin resistance. Strikingly, both transient and continuous CORT treatment decreased total atherosclerotic lesion area after 17 weeks to a similar extent, without affecting either plasma lipid levels or lipoprotein profiles, accompanied by decreased macrophage content in the atherosclerotic plaque. We concluded that CORT treatment *per se* has

long-lasting beneficial effects on atherosclerosis development. Therefore, in clinical practice, glucocorticoids schemes as used for anti-inflammatory indications might benefit from adjustments towards higher doses for a shorter period of time instead of lower dosages for a prolonged period of time.

Taken together, the studies described in this thesis have contributed to the discovery of CETP as a biomarker for the hepatic macrophage content, a hallmark of NASH for which no non-invasive diagnostic method is currently available, and discovery of novel therapeutic modalities for atherosclerosis and NASH. First of all, we gained more insight into the true cellular origin of CETP (i.e. the liver macrophage), and the mechanisms underlying the CETP-lowering effects of HDL-raising agents (i.e. by reducing the hepatic macrophage content). We extrapolated the association between the reduction of hepatic lipid content and plasma CETP concentration upon lipid-lowering interventions from mice to humans. Furthermore, we demonstrated the role of CETP in discrepant effects of rHDL on VLDL metabolism between mice and humans, and reported a species difference in the central regulation of hepatic VLDL metabolism by NPY between mice and rats, which underscores a general concern in animal research in view of extrapolating findings from specific animal studies to explain observations done in humans. Additionally, we demonstrated that CORT has long-lasting beneficial effects on atherosclerosis development suggesting a possibility for therapeutic application of anti-inflammatory agents in CVD. Finally, we described GLP-1 receptor agonism as a novel strategy to improve lipid metabolism and hepatic inflammation, which may result in novel strategies to treat both atherosclerosis and NASH.

# NEDERLANDSE SAMENVATTING

Atherosclerose en non-alcoholische steatohepatitis (NASH) zijn de voornaamste oorzaken van respectievelijk hart- en vaatziekten (HVZ) en chronische leverziekte. Dit zijn beide belangrijke factoren voor morbiditeit en mortaliteit in de westerse wereld. Atherosclerose en NASH delen een vergelijkbare etiologie, waarbij een verstoord vetmetabolisme de belangrijkste factor is. Dit komt tot uiting door dyslipidemie, gekenmerkt door verhoogde plasmaniveaus van (V)LDL-cholesterol (C) en van triglyceriden (TG), en een verlaagd plasmaniveau van HDL-C. Lipidenverlagende geneesmiddelen die dyslipidemie verbeteren zijn effectieve middelen om atherosclerose te voorkomen en behandelen. Echter, omdat de mortaliteit en morbiditeit geassocieerd met HVZ slechts gedeeltelijk worden verbeterd door de huidige lipidenverlagende strategieën zijn momenteel nieuwe strategieën in ontwikkeling met als doel atherosclerose verder te reduceren. Aangezien het HDL-C niveau omgekeerd gecorreleerd is met het cardiovasculair risico, behoort het verhogen van HDL-C tot deze nieuwe strategieën. Omdat er nog geen farmacologische middelen zijn geïdentificeerd voor het behandelen van NASH en er nog geen biomarkers beschikbaar zijn om NASH te detecteren, is het ook noodzakelijk te zoeken naar behandelingsstrategieën en biomarkers voor NASH.

Omdat gereconstitueerd HDL (rHDL) een regressie van atherosclerose teweeg bracht in humane studies, lijkt infusie van rHDL, als onderdeel van HDL-verhogende strategieën, veelbelovend voor de behandeling van HVZ. Studies in muizen lieten zien dat rHDL het plasmaniveau van VLDL verhoogde, een effect dat minder duidelijk was in mensen. Dit verschil kan mogelijk verklaard worden door het feit dat de mens, in tegenstelling tot de muis, het cholesteryl ester transfer proteïne (CETP) tot expressie brengt. CETP speelt een belangrijke rol in het lipidenmetabolisme door het faciliteren van de overdracht van de neutrale lipiden triglyceriden (TG) en cholesteryl esters (CE) tussen (V)LDL en HDL. In **hoofdstuk 2** hebben we de rol van CETP in de effecten van rHDL op het VLDL metabolisme onderzocht in *APOE*3-Leiden (E3L)* en *APOE*3-Leiden.CETP (E3L.CETP)* transgene muizen die uitstekende modellen vormen voor het lipoproteïnenmetabolisme in de mens. Eén uur na injectie verhoogde rHDL de niveaus van VLDL-C en TG in *E3L* muizen, maar niet in *E3L.CETP* muizen. Deze initiële toename in VLDL werd veroorzaakt door competitie tussen rHDL en VLDL voor de lipoproteïne lipase (LPL)-gemedieerde hydrolyse van TG, en werd dus voorkomen door de expressie van CETP. Vierentwintig uur na injectie van rHDL werd een tweede toename in VLDL-C en TG in *E3L* muizen waargenomen, terwijl het VLDL zelfs was verlaagd in *E3L.CETP* muizen. Deze secundaire stijging in VLDL werd veroorzaakt door een verhoogde VLDL-TG productie. Uit deze studie concludeerden wij

dat CETP beschermt tegen de rHDL-geïnduceerde stijging van VLDL en dat behandeling van atherogene dyslipidemie met rHDL niet gecombineerd zou moeten worden met middelen die CETP activiteit op een agressieve manier verlagen.

Thiazolidinediones (PPARγ agonisten) verlagen het plasma TG niveau, verhogen het plasma HDL-C niveau, en reduceren hepatische steatose. Eerdere studies in muizen hebben aangetoond dat reductie van hepatische steatose door lipidenverlagende middelen samengaat met verlaagde hepatische CETP expressie en een verlaagd plasmaniveau van CETP, wat vervolgens kan leiden tot een verhoging van het HDL-C gehalte. Daarom hebben we in **hoofdstuk 3** het effect van de thiazolidinedione pioglitazon op het CETP niveau in patiënten met type 2 diabetes mellitus (T2DM) onderzocht. Patiënten met T2DM werden gerandomiseerd tot behandeling met pioglitazon of metformine, bovenop behandeling met glimepiride. Aan het begin van de behandeling en na 24 weken behandeling werden de plasmaniveaus van HDL-C en CETP gemeten en werd de hepatische TG inhoud bepaald door middel van proton magnetische resonantie spectroscopie. Pioglitazon verlaagde het hepatische TG gehalte, wat inderdaad geassocieerd was met een verlaagd plasma CETP niveau en een verhoogd plasma HDL-C gehalte, terwijl metformine geen effect had op deze parameters. Wij concludeerden dan ook dat de verlaging van de hepatische TG inhoud door pioglitazon vergezeld ging van een verlaging in plasma CETP concentratie, en daarom geassocieerd was met een verhoging in HDL-C.



Bij gebrek aan farmacologische geneesmiddelen, wordt NASH momenteel nog voornamelijk behandeld door aanpassing van gedrag, d.w.z. afvallen en meer bewegen. Recent toonden wij aan dat een zeer laag calorisch dieet (VLCD) gedurende 16 weken het plasma totaal cholesterol (TC) en TG niveau significant verlaagde, en daarbij het hepatisch TG gehalte verlaagde in obese patiënten met T2DM en hepatische steatose. Een mogelijk gunstig effect van een dergelijk VLCD op plasma CETP en HDL niveaus was echter nog niet onderzocht. In **hoofdstuk 4** onderzochten wij de effecten van een VLCD dat resulteerde in een forse verlaging van het hepatische TG gehalte op het plasma CETP en HDL niveau in obese patiënten met T2DM en hepatische steatose. Het VLCD verlaagde de plasma CETP concentratie en verhoogde het plasma apoAI niveau, zonder een effect te hebben op het plasma HDL-C en HDL-fosfolipidengehalte. Hoewel het VLCD resulteerde in minder gelipideerd HDL was de functionaliteit van het HDL met betrekking tot het induceren van cholesterolefflux vanuit macrofagen *in vitro* onveranderd. Wij concludeerden daarom dat de aanzienlijke verlaging van het hepatische TG gehalte door het VLCD vergezeld ging van een verlaging in plasma CETP

concentratie en een stijging in apoAI niveau, zonder de eigenschappen van het HDL m.b.t. cholesterolefflux te verbeteren.

Niacine (nicotinezuur) is het meest potente HDL-verhogende middel dat in de kliniek wordt toegepast. Naast het verhogen van het HDL-C niveau verlaagt niacine ook het plasmaniveau van proatherogene lipoproteïnen en lipiden waaronder VLDL, LDL en TG. Daarom wordt niacine gezien als een goede kandidaat voor het behandelen van atherosclerose. In *E3L.CETP* muizen verlaagde niacine het hepatische TG gehalte, wat samenging met een verlaging van de hepatische genexpressie en het plasmaniveau van CETP en een verhoging van het HDL-C. In **hoofdstuk 5** hebben we het mechanisme onderzocht waardoor niacine CETP verlaagt door gebruik te maken van transgene muizen die humaan CETP tot expressie brengen. *In vitro* studies toonden aan dat niacine niet direct de CETP expressie in macrofagen verlaagde. *In vivo* studies lieten zien dat niacine in CETP transgene muizen op een westers dieet het hepatische cholesterolgehalte en de ontsteking in de lever verlaagde. Niacine reduceerde ook de hepatische genexpressie van CETP en het plasmaniveau van CETP. Tegelijkertijd verlaagde niacine de hepatische expressie van CD68 en ABCG1 (beide specifieke markers voor de macrofaaginhoud van de lever) als ook het gehalte aan macrofagen in de lever. De hepatische CETP expressie was zelfs significant gecorreleerd met de hepatische macrofaagmarkers. Wij concludeerden dat niacine de hepatische CETP expressie en de plasma CETP concentratie verlaagt door het reduceren van leverontsteking en de macrofaaginhoud van de lever ten gevolge van zijn primaire lipidenverlagende effect, en niet door de expressie van CETP in de macrofaag te verlagen.



Omdat CETP een huidig doel is voor de behandeling van dyslipidemie is het cruciaal om de bron van het CETP in de mens te identificeren. Eerdere studies toonden aan dat CETP vooral gesynthetiseerd wordt in vetweefsel en in de lever. Echter, de relatieve bijdrage van de weefselspecifieke CETP expressie aan het plasma CETP niveau was nog onbekend. Daarom was het doel van **hoofdstuk 6** om de cellulaire bron van CETP op te helderen door gebruik te maken van humane cohorten en *E3L.CETP* muizen. In een algemene populatie, de Rijswijk studie, bleek het plasma CETP niveau niet te correleren met de buikomvang, wat suggereert dat centraal vetweefsel niet bijdraagt aan de plasma CETP pool. Microarray analyse van lever- en vetweefselbiopten van patiënten die bariatrische chirurgie hadden ondergaan, toonden aan dat de CETP expressie in de lever het hoogst was en correleerde met inflammatoire reactiepaden. Via immunohistochemische technieken werd duidelijk dat CETP vooral tot expressie kwam in hepatische macrofagen. De expressie van CETP in de lever, maar niet in

vetweefsel, correleerde positief met het plasma CETP niveau en omgekeerd met het plasma HDL-C niveau. Selectieve eliminatie van macrofagen uit de lever ten opzichte van vetweefsel in *E3L.CETP* muizen deed de CETP expressie nagenoeg teniet in de lever, maar niet in vetweefsel, en leidde tot een sterk verlaagde plasma CETP concentratie en een verhoging van het plasma HDL-C. Behandeling van *E3L.CETP* muizen met lipidenverlagende middelen waarvan bekend is dat zij in de mens de plasma CETP concentratie verlagen en de HDL-C concentratie verhogen, reduceerde de hoeveelheid macrofagen in de lever, verlaagde het plasma CETP niveau en verhoogde het HDL-C. Wij concludeerden dat plasma CETP primair wordt gesynthetiseerd door macrofagen in de lever en dat plasma CETP een biomarker is voor de hoeveelheid macrofagen in de lever, een belangrijke factor van NASH waarvoor nog geen non-invasieve diagnostische middelen voorhanden zijn.

Toenemend bewijs toont aan dat strategieën gericht op het reguleren van de energiehomeostase en voedselopname ook het vetmetabolisme gunstig beïnvloeden en mogelijk zowel atherosclerose als NASH kunnen behandelen. De hersenen spelen een belangrijke rol bij de energiehomeostase, waarbij de belangrijkste rol is weggelegd voor de hypothalamus. Twee belangrijke neuronale populaties in de arcuate nucleus van de hypothalamus zijn betrokken in de regulatie van de voedselinname: de neuronen die het pro-opiomelanocortine/cocaine- and amphetamine-gereguleerde transcript tot expressie brengen, en de neuronen die neuropeptide Y (NPY)/agouti-gerelateerde proteïne tot expressie brengen. Eerdere studies lieten zien dat centrale toediening van NPY de productie van VLDL-TG door de lever verhoogde. In **hoofdstuk 7** wilden we het effect van centraal NPY op de hepatische VLDL-TG productie in muizen valideren om uiteindelijk te onderzoeken of NPY bijdraagt aan de ontwikkeling van atherosclerose door dyslipidemie te induceren. Toediening van NPY in zowel de laterale als derde ventrikel in de hersenen van muizen verhoogde de voedselinname binnen een uur na injectie, maar had geen effect op de hepatische productie van VLDL-TG of VLDL-apoB. Eveneens had het blokkeren van de centrale NPY signalering geen effect op de hepatische VLDL productie. We concludeerden dat acute centrale toediening van NPY in muizen de voedselinname verhoogt net zoals in ratten, maar dat het in tegenstelling tot in ratten de VLDL productie in muizen niet beïnvloedt. Dit diersoortafhankelijke effect met betrekking tot het effect van NPY op de hepatische VLDL-TG productie is van groot belang voor toekomstige studies naar de centrale regulatie van het hepatische VLDL metabolisme.

Studies in mensen suggereerden dat agonisme van de glucagon-like peptide-1 (GLP-

1) receptor niet alleen het energiemetabolisme en het glucosemetabolisme verbetert, maar ook het plasma VLDL niveau verlaagt. Het achterliggende mechanisme van de reductie in het plasma TG was echter nog onbekend. In **hoofdstuk 8** hebben we daarom de effecten onderzocht van GLP-1 receptor agonisme op het TG metabolisme door gebruik te maken van *E3L* muizen die een vetrijk dieet gevoerd werden. Behandeling met de GLP-1 receptor agonisten CNTO3649 en exendin-4 gedurende 4 weken d.m.v. subcutane osmotische minipompjes verbeterde de glucosehuishouding door het plasmaniveau van glucose en insuline in gevaste dieren te verlagen. Daarnaast reduceerden de GLP-1 receptor agonisten ook de hepatische productie van VLDL-TG en VLDL-apoB door een verlaagde productie van VLDL deeltjes en niet door minder lipidatie van apoB. Tegelijkertijd bleek GLP-1 receptor agonisme het hepatische lipidengehalte aanmerkelijk te verlagen. Deze verlaging ging samen met een downregulatie van de expressie van genen die betrokken zijn bij de hepatische lipogenese (*Srebp-1c, Fasn, Dgat1*) en de apoB synthese (*Apob*). We concludeerden dat GLP-1 receptor agonisme, naast het verbeteren van de glucosehuishouding, ook in staat is dyslipidemie en hepatische steatose te verbeteren.

Omdat onze studies aantoonden dat exendin-4 het vetmetabolisme verbetert en vetrijk dieet-geïnduceerde hepatische steatose geheel kan voorkomen (hoofdstuk 8) anticipeerden wij dat GLP-1 receptor agonisme mogelijk ook atherosclerose en ziekte gerelateerd aan een vette lever zou kunnen behandelen. Echter, de impact van GLP-1 receptor agonisme op NASH was nog onduidelijk, zeker met betrekking tot het effect op hepatische ontsteking. Omdat de ontwikkeling van atherosclerose en NASH een gemeenschappelijke etiologie delen hebben we in **hoofdstuk 9** het effect van exendin-4 op de ontwikkeling van atherosclerose en NASH tegelijkertijd onderzocht door wederom gebruik te maken van *E3L.CETP* muizen op een westers dieet. Hoewel vier weken behandeling met exendin-4 slechts een lichte verlaging veroorzaakte van het plasmaniveau van lipiden en lipoproteïnen, bracht het een aanzienlijke verlaging teweeg in de mate en ernst van atherosclerose, vergezeld van een reductie in de adhesie van monocyten aan de vaatwand en het macrofaaggehalte in de atherosclerotische plaques. Exendin-4 bleek ook de hoeveelheid cholesterol en $CD68^+$ en $F4/80^+$ macrofagen in de lever te verlagen, wat aangeeft dat exendin-4 de dieet-geïnduceerde ontwikkeling van NASH remt. Dit ging samen met een verlaagde aantrekking van monocyten uit het bloed aangezien de hoeveelheid $Mac\text{-}1^+$ macrofagen in de lever was verlaagd. Tenslotte reduceerde exendin-4 de expressie van chemokines *in vivo* en onderdrukte het de stapeling van oxLDL in peritoneale macrofagen *in vitro* afhankelijk van de GLP-1 receptor, wat suggereert dat exendin-4 zowel atherosclerose als NASH

vermindert door verlaagde aantrekking/activatie van macrofagen uit het bloed. Wij concludeerden dan ook dat GLP-1 receptor agonisme een waardevolle strategie zou kunnen zijn om naast T2DM ook atherosclerose en NASH te behandelen, met name in patiënten die lijden aan een combinatie van deze ziekten.

Naast lipidenverlagende strategieën zijn ook anti-inflammatoire strategieën in ontwikkeling voor het verlagen van het risico op HVZ. Glucocorticoïden hebben een sterk anti-inflammatoir karakter en worden extensief toegepast in de klinische praktijk als immunosuppressiva. Echter, een overmaat aan glucocorticoïden kan ook negatieve metabole effecten veroorzaken in vetweefsel, zoals centrale obesitas en insulineresistentie, die de mogelijke beschermende effecten van glucocorticoïden op HVZ kunnen tegengaan. In **hoofdstuk 10** onderzochten we de effecten van tijdelijke en continue behandeling met glucocorticoïden op de ontwikkeling van atherosclerose door gebruik te maken van *E3L.CETP* muizen op een westers dieet. Hoewel behandeling met corticosteron (CORT) gedurende 5 weken ('tijdelijk') en 14 weken ('continu') het lichaamsgewicht en de voedselinname deed toenemen gedurende de duur van de interventie, induceerde alleen continue behandeling met CORT een lager bijniergewicht en hoger gewicht van gonadale en subcutane vetkussens na 17 weken. Bovendien nam de insulinespiegel en HOMA-IR index toe in de muizen die continu werden behandeld met CORT, wat erop duidt dat langdurige toediening van glucocorticoïden insulineresistentie induceert. Het was opvallend dat zowel tijdelijke als continue behandeling met CORT na 17 weken atherosclerose in gelijke mate deden afnemen zonder een effect te hebben op het plasmaniveau van lipiden en lipoproteïnen. Deze reductie in plaquegrootte ging vergezeld van een verlaagde hoeveelheid macrofagen in de atherosclerotische plaque. Wij concludeerden dat behandeling met CORT langdurige gunstige effecten heeft op de ontwikkeling van atherosclerose. In de klinische praktijk zouden anti-inflammatoire strategieën met glucocorticoïden dus aangepast kunnen worden van langdurende behandeling met een lage dosis naar kortdurende behandeling met een hoge dosis.



Samenvattend kan gezegd worden dat de studies die beschreven staan in dit proefschrift hebben bijgedragen aan de identificatie van CETP als een biomarker voor de hoeveelheid macrofagen in de lever, een belangrijke factor in NASH waarvoor nog geen non-invasieve diagnostische middelen voorhanden waren, en aan de identificatie van mogelijk nieuwe therapeutische handvatten voor atherosclerose en NASH. Allereerst verkregen we meer inzicht in de cellulaire bron van CETP (de macrofaag in de lever) en het mechanisme waardoor HDL-verhogende geneesmiddelen de plasma CETP concentratie verlagen (door het aantal macrofagen in de lever te verlagen). We waren

in staat de associatie tussen de reductie van de hepatische lipidenconcentratie en de plasma CETP concentratie na lipidenverlagende interventies te vertalen van de muis naar de mens. Ook toonden we de rol aan van CETP in de divergerende effecten van rHDL op het VLDL metabolisme in de muis en in de mens, en onthulden een verschil in de centrale regulatie van de hepatische VLDL productie tussen muizen en ratten. Deze bevinding onderstreept een algemene bezorgdheid over dierproefonderzoek met betrekking tot extrapolatie van bevindingen van specifieke dierstudies om observaties in mensen te verklaren. Eveneens toonden we aan dat CORT langdurige gunstige effecten heeft op de ontwikkeling van atherosclerose, wat een mogelijke toepassing van anti-inflammatoire strategieën in HVZ onderstreept. Tenslotte beschreven we continue GLP-1 receptor agonisme als een nieuwe strategie om zowel het lipidenmetabolisme als leverontsteking positief te beïnvloeden, wat kan resulteren in nieuwe strategieën om zowel atherosclerose als NASH te bestrijden.

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