Novel modulators of lipoprotein metabolism : implications for steatohepatitis and atherosclerosis

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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.

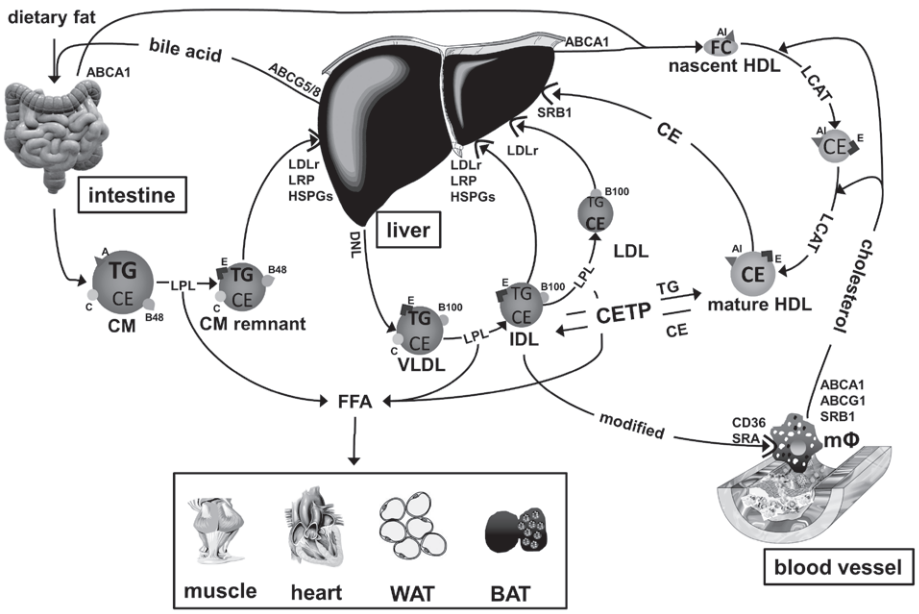

Figure 1. Schematic overview of lipoprotein metabolism. See text for explanation.

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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