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Cholesterol metabolism in mouse models of atherosclerosis and adrenal steroidogenesis

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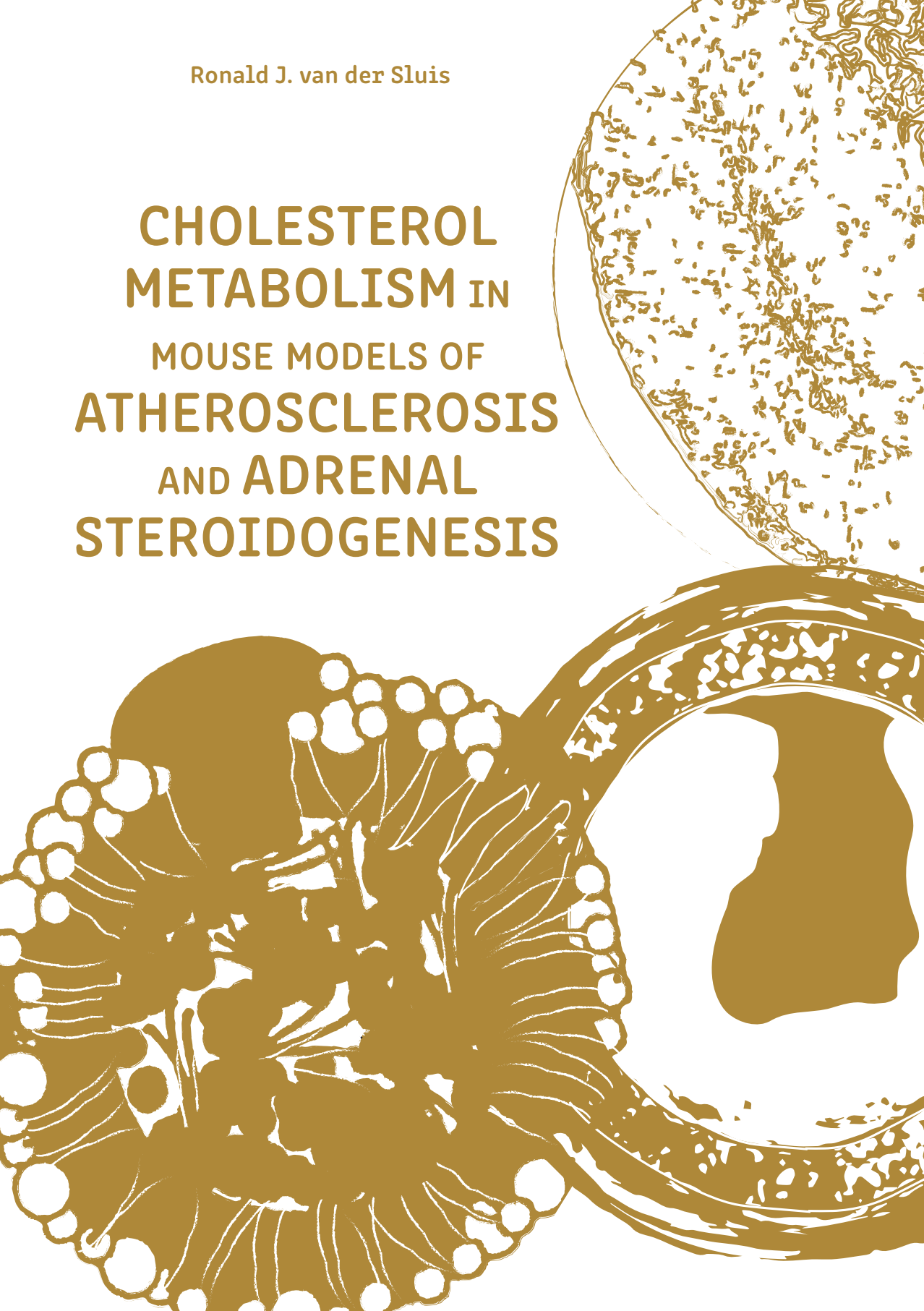
Ronald J. van der Sluis

CHOLESTEROL METABOLISM IN MOUSE MODELS OF ATHEROSCLEROSIS AND ADRENAL STEROIDOGENESIS



Ronald J. van der Sluis

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CHOLESTEROL METABOLISM IN MOUSE MODELS OF ATHEROSCLEROSIS AND ADRENAL STEROIDOGENESIS

Proefschrift

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de graad van Doctor aan de Universiteit Leiden,
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in 1979

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Voor Mariëlle, Eveline & Thomas

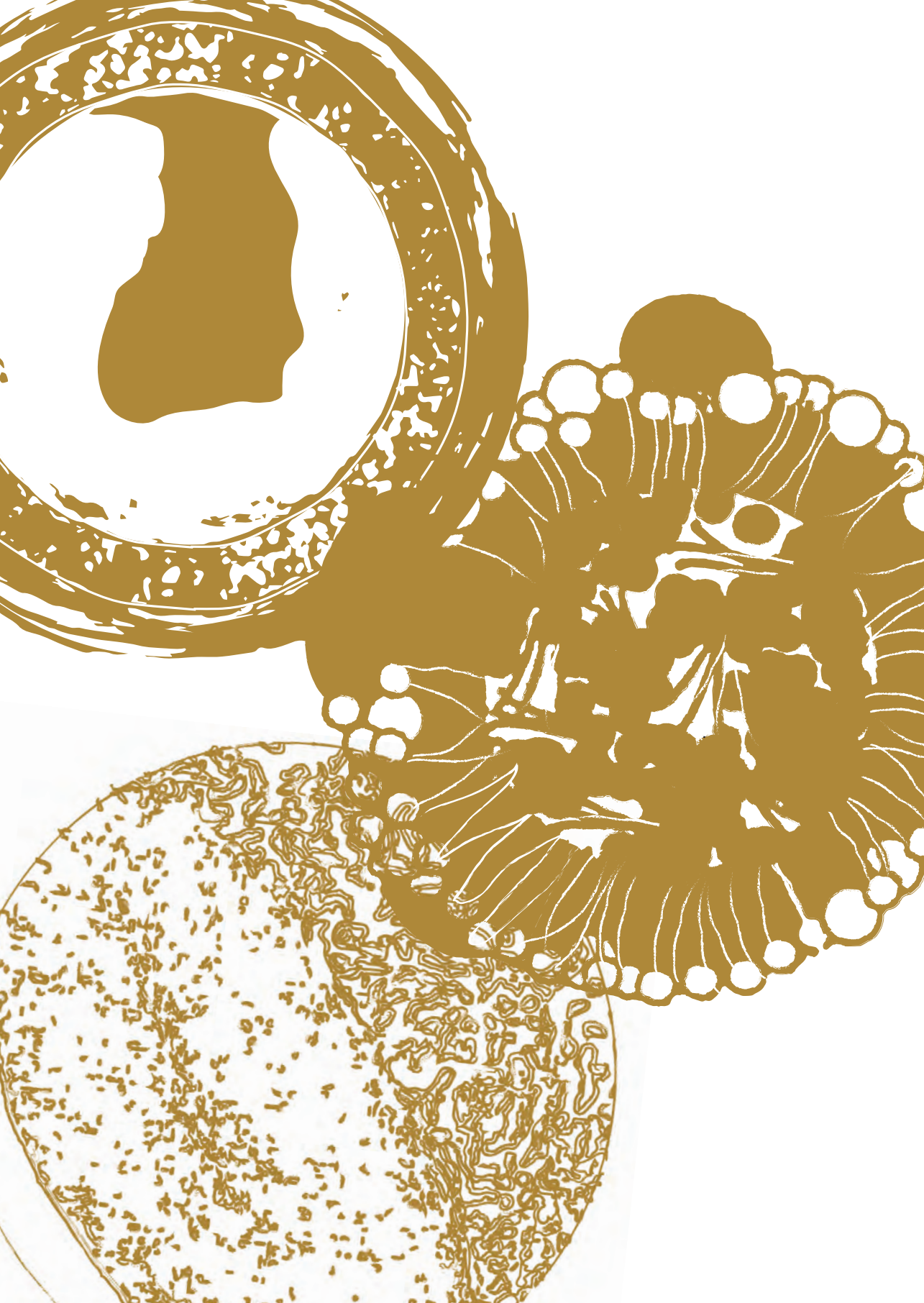

TABLE OF CONTENTS

Chapter 1	General introduction and thesis outline	**9**
Chapter 2	Disruption of PLTP-mediated HDL maturation reduces SR-BI deficiency-driven atherosclerosis susceptibility despite unexpected metabolic complications. *Accepted as: Arterioscler Thromb Vasc Biol.* 40(3):611- 623 (2020)	**39**
Chapter 3	HDL is essential for atherosclerotic lesion regression in ApoE knockout mice by bone marrow ApoE reconstitution. *Atherosclerosis.* 278:240-249 (2018)	**71**
Chapter 4	VLDL/LDL serves as the primary source of cholesterol in the adrenal glucocorticoid response to food deprivation. *BBA - Molecular and Cell Biology of Lipids* 1865:7 158682 (2020)	**93**
Chapter 5	Elimination of adrenocortical apolipoprotein E production does not impact glucocorticoid output in wild-type mice. *Mol Cell Endocrinol.* 490:21-27 (2019)	**111**
Chapter 6	General discussion and future perspectives	**131**
	Dutch Summary (Nederlandse samenvatting)	**150**
	Curriculum Vitae	**162**
	Scientific publications	**164**
	PhD portfolio	**168**
	Index of abbreviations	**170**

1

GENERAL INTRODUCTION AND THESIS OUTLINE

CHOLESTEROL

Cholesterol, the most prominent sterol in mammals, was originally discovered as a major component of human gallstones by F. Pouletier de la Salle in 1769. In 1815 M.E. Chevreul named the organic molecule "cholesterine" (chole for bile, stereos for solid), which was later adjusted with the chemical suffix of -ol for the alcohol component [1]. Over the past 100 years, cholesterol has been extensively studied and linked to a variety of pathologies and tightly regulated metabolic pathways. The structure of cholesterol consists, in its free form (free cholesterol; FC), of four linked hydrocarbon rings with on one side a hydrocarbon tail, opposing a hydroxyl group [2](**Figure 1A**). The two ends create an amphipathic molecule with a hydrophobic and hydrophilic side. This structure of the cholesterol molecule influences the properties of cellular membranes in animals. The hydrophilic hydroxyl group binds to the phospholipid heads in the cell membrane, turning the hydrophobic hydrocarbon tail towards the core of the membrane bilayer. This improves the membrane fluidity and permeability and allows the cell to change shape [3](**Figure 1C**). An optimal membrane FC/phospholipid ratio is thus essential for membrane rigidity. Any misbalance could influence cellular mobility and eventually induce cell death [4]. Accumulation of membrane-bound FC's are associated with cytotoxic events including intracellular cholesterol crystallization, oxysterol formation [5], and apoptotic signaling pathway activation [6,7]. It is therefore that the majority of cholesterol found in the body exists in its more stable, less cytotoxic, esterified form (cholesteryl esters (CE)). In the plasma compartment, 2/3 of the cholesterol pool exists in the form of CE. Lecithin-cholesterol acyltransferase (LCAT) drives the esterification of FC molecules in plasma, adding a single fatty acid derived from phosphatidylcholine to the hydroxyl group [8](**Figure 1B**). The conversion of FC into CE enables cells to store and/or transport cholesterol, without the risk of FC-induced cytotoxicity. Upon hydrolyzation by cholesteryl ester hydrolase, cholesterol and free fatty acids can be regained for further biosynthesis and metabolism [9].

Besides the eminent role in cell membrane functionality, cholesterol influences a range of pathways i.e. as the precursor for the synthesis of steroid hormones [11] and bile acids [12], and plays a significant role in transmembrane signaling [13] and cellular proliferation [14]. Despite the functional diversity between cholesterol-using pathways, acquisition of cholesterol follows, for most mammalian cells, a comparable pattern. Cholesterol is either synthesized de novo or derived from the blood circulation by cellular uptake.

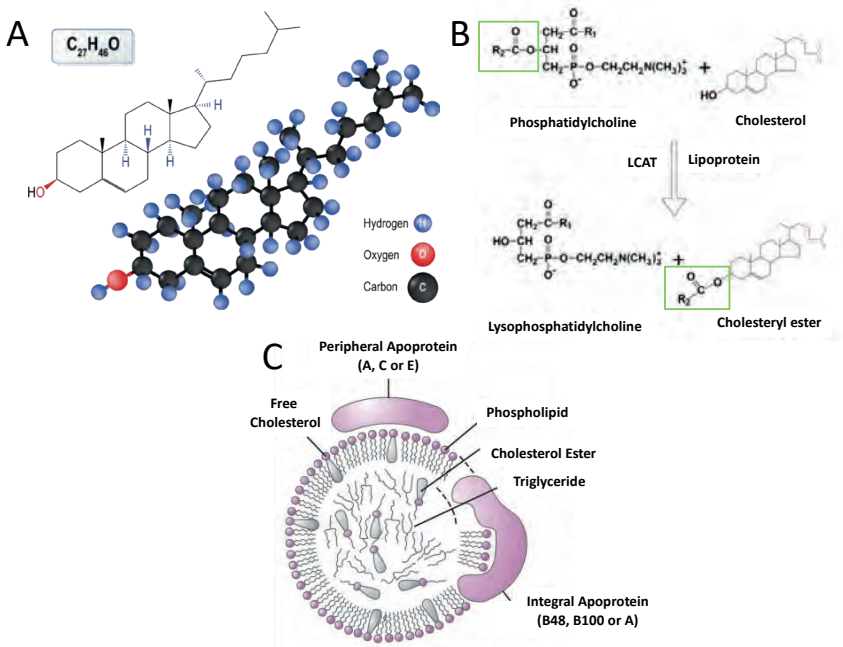

Figure 1: The structural chemical formula and molecular illustration of cholesterol (A). Conversion of free cholesterol (FC) and phosphatidylcholine towards cholesteryl ester (CE) by the enzyme Lecithin-cholesterol-acyltransferase (LCAT) (B). Lipoprotein structure depicting the primary location of FC in the shell of the particle and CE in the core (Adapted from [10] (C)).

LIPID METABOLISM

DE NOVO SYNTHESIS OF CHOLESTEROL

De novo synthesis of cholesterol is mainly found in vertebrates and in lower amounts in plants (not in prokaryotes) [15] and acquired via the mevalonate (MVA) pathway. The MVA pathway is a highly controlled enzymatic process, resulting in the stepwise formation of FC [16]. Furthermore, the MVA pathway is a fundamental metabolic network providing several essential elements for normal cellular metabolism and is executed in the endoplasmic reticulum (ER) and cytoplasm of a cell. Despite the presence of the MVA pathway in almost all animal cells, the amount of de novo synthesized cholesterol differs per species and organ. The total cholesterol pool of mice and man are equal, ~2.150 and ~2.200 mg/kg, respectively. However, the human brain holds approximately 23 % (490 mg/kg) of the total cholesterol pool, whereas a mouse brain comprises only 15 % (330 mg/kg) of the total pool of cholesterol. Interestingly, the brain's cholesterol pool consists mainly of CE's that are derived via novo synthesis and are primarily located in myelin sheaths that insulate axons [17-19].

The hepatic contribution to the cholesterol pool derived from de novo synthesis also varies per species: hepatic cells in mice contribute approximately 40 % to whole body cholesterol synthesis, while human liver cells add approximately 10 % to the total pool [20,21].

The newly formed cellular cholesterol is used as a precursor for the formation of metabolites (bile acids, steroids, water-soluble vitamins) included in cellular membranes, or converted to CE by acyl-Co A acyl transferase (ACAT) and stored in lipid droplets [22]. The CE stored within lipid droplets can be reconverted into FC by hormone-sensitive lipase (HSL)[23] and used as a precursor upon demand or fluxed towards the plasma compartment.

Since appropriate cellular cholesterol levels are critical for normal cell metabolism, the regulation of intracellular cholesterol levels are tightly controlled by feedback mechanisms that operate at both transcriptional as well as post-transcriptional levels [24,25]. Low cellular cholesterol triggers the MVA-pathway to upregulate the rate-limiting enzymes, i.e. 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) [25] and stimulates receptor-mediated exogenous uptake. In contrast, high cellular cholesterol levels activate nuclear hormone receptors that in turn trigger transcription of cholesterol efflux-related genes, i.e. ATP-binding (ABC) transporters, and reduce HMGCR expression [26].

The MVA-pathway is an established target for Statins, an extensively prescribed lipid-lowering drug. Statins inhibit activation of HMGCR, resulting in a reduction of hepatic cholesterol synthesis. Furthermore, statins increase the low-density lipoprotein receptor (LDLr) activity and subsequently reduce plasma cholesterol levels in patients that suffer from hypercholesterolemia.

LIPOPROTEINS AND CHOLESTEROL TRANSPORT

Due to the hydrophobic character of CE and triglycerides (TG), their transport throughout the body is facilitated by lipoproteins. Lipoproteins are spherical macromolecular particles consisting of a hydrophobic core and a hydrophilic shell. The lipoprotein shell contains a monolayer of phospholipids, amphipathic molecules, FC and apolipoproteins, enfolding the hydrophobic core of CE and TG. The major apolipoprotein classes are synthesized by the intestine and liver and located in the membrane of lipoproteins. The amphipathic apolipoproteins serve as enzymatic cofactors and receptor ligands, regulating lipoprotein metabolism [27,28]. The function

and presence of apolipoproteins differ per lipoprotein class. Five lipoprotein classes can be distinguished based on their buoyant density: chylomicrons (CM), very low-density lipoproteins (VLDL), intermediate low-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). The characteristics of the five lipoprotein classes are described in **Table 1** and the pathways for metabolism of the individual lipoprotein classes are depicted in **Figure 2**.

EXOGENOUS CHOLESTEROL METABOLISM

Approximately 70 % of the total cholesterol pool is derived from endogenous production in the liver, while 30 % is of exogenous cholesterol origin and obtained via dietary intake [30]. Exogenous dietary lipid absorption is achieved via several complex functional steps along the whole digestive track. In short, solubilisation of dietary lipids starts in the duodenum and proximal jejunum parts of the intestine where bile acid micelles hydrolyse CE into FC and fatty acids (FA) and triglycerides into monoacylglycerol and FA. Micelles absorb the FC and FA and facilitate transport into the enterocytes of the small intestine, were the dietary FAs are reconverted into triglycerides (TG) by coupling to the monoacylglycerol backbone. Exogenous FC is converted into CE in the ER by ACAT [31]. The majority of the lipids absorbed in the intestine are triglycerides (95 %), but also nearly 50 % of the total dietary cholesterol is absorbed, while the remainder is excreted via the faeces [32-34].

CHYLOMICRONS

Chylomicrons are essential in the transport of exogenous cholesterol from the intestine towards the liver. Within the ER of enterocytes nascent chylomicron particles are formed as a result of lipidation of one apolipoprotein B48 (ApoB48) molecule with CE, TG and phospholipids, alongside apolipoproteins (**Table 1**). Once the chylomicrons enter the blood circulation via the lymphatic system, circulating Apolipoprotein C (ApoC) is acquired. ApoCs in the membrane of chylomicrons serve as a cofactor for lipoprotein lipase (LPL) that is present on the endothelial cells of adipose tissue and skeletal muscle and hydrolyses the TG content to FA for energy storage [35]. Upon TG hydrolysis, superfluous membrane phospholipids are transferred by the phospholipid transfer protein (PLTP) towards HDL. The chylomicrons exchange Apolipoprotein E (ApoE) and ApoCs at the expense of Apolipoprotein A1 (ApoA1) and Apolipoprotein A4 (ApoA4) with HDL, resulting in formation of smaller TG-poor and Apo-enriched remnant particles [36]. Furthermore, chylomicrons can exchange TG for HDL-derived CE via an interaction with the cholesteryl ester transfer protein (CETP), that is present in humans but not in mice [37,38].

Table 1: Plasma lipoprotein characteristics, all lipid values are expressed as a percentage of the total weight; abbreviations: CM: Chylomicrons, VLDL: Very low-density lipoproteins, IDL: Intermediate-density lipoproteins, LDL: Low-density lipoproteins, HDL: High-density lipoproteins, FC: Free cholesterol, CE: cholesteryl ester, TG: Triglyceride, PL: phospholipids, P: Protein, Apo: Apolipoprotein (Adapted from [27,29]).

Lipoprotein class	Size (Å)	Density (g/ml)	Diameter (nm)	FC	CE	TG	PL	P	ApoA	ApoB	ApoC	ApoE
CM	800-5000	<0.95	75-1200	1-3	2-4	80-95	3-9	2	A1,A3,A4	B48	C1-C4	E
VLDL	300-800	0.95-1.006	30-80	4-8	6-22	45-65	10-20	8	A5	B100	C1-C4	E
IDL	250-350	1.006-1.019	25-35	8	42	20-50	15-25	19	-	B100	C1-C4	E
LDL	180-280	1.019-1.063	18-25	6-8	45-50	5-15	20-25	22	-	B100	-	-
HDL	50-120	1.063-1.210	9-12	3-5	5-20	5-10	20-30	40	A1,A2,A4,A5	-	C1-C4	E

PLTP, a plasma glycoprotein and, like CETP, a family member of the lipopolysaccharide (LPS)-binding proteins [39], is involved in the metabolism of both the ApoB-containing lipoproteins chylomicrons/VLDL/LDL and HDL. PLTP deletion results in a marked decrease in plasma levels of ApoB-containing lipoproteins [40] as well as HDL particles [39].

Hepatic clearance of the remaining chylomicron remnants commences with sequestration in the hepatic space of Disse via an ApoE-dependent route. ApoE, associated with chylomicrons, VLDL and its remnants and subclasses of HDL, binds with a high affinity to the LDL receptor (LDLr), LDLr-related protein (LRP) and heparan sulphate proteoglycan (HSPG) thereby facilitating the uptake and clearance of the ApoE-containing lipoproteins. The liver subsequently converts the remnant cholesterol content either into bile acids or re-uses it for VLDL synthesis.

ENDOGENOUS CHOLESTEROL METABOLISM

A third source for cellular cholesterol is derived from the endogenous cholesterol metabolism pathway. Endogenous cholesterol metabolism starts with the synthesis of VLDL within the ER membrane of hepatocytes. Here a single copy of ApoB100 is lipidated with TG and de novo produced or exogenous derived cholesterol [41-43]. The TGs that are packaged into VLDL particles are free fatty acids that are synthesized in the ER. The free fatty acid influx are either derived from the 1) intestine 2) chylomicron remnants or 3) produced by the adipose tissue and taken up from the circulation by hepatic receptors [41-44]. Since hepatic VLDL metabolism is dependent on the availability of TGs, the de novo synthesized ApoB100 molecules undergo degradation when they are not lipidated. The transport of TGs and CEs between membranes is executed by the soluble protein microsomal triglyceride transfer protein (MTTP). MTTP is vital for the assembly of VLDL and chylomicrons and is found in both the liver as well as intestines. Any disruption in the production of MTTP leads to abetalipoproteinemia, characterized by the absence of lipoproteins containing ApoB, low blood TG and cholesterol levels [45].

Once the VLDL particles enter the circulation newly synthesized ApoE and ApoCs are subsequently incorporated into the particle. Animal models revealed that deletion of ApoE or introduction of dysfunctional ApoE induces hypercholesterolemia and subsequently an increased susceptibility for atherosclerotic lesion development [46-48].

Next, circulating VLDL particles interact with LPL at the surface of the endothelial cells to reduce its TG content in a similar manner as with chylomicrons. The remaining VLDL remnants, also known as intermediate-density lipoproteins (IDL), contain less TGs and are either removed from the circulation through hepatic clearance via the LDLr or LRP1 or converted by LPL and hepatic lipase (HL) into low-density lipoproteins (LDL). The LDL particle still contains the ApoB100 molecule but no other apolipoproteins and is subjected to LDLr-mediated internalization and subsequent degradation of the particle [27].

REVERSE CHOLESTEROL TRANSPORT AND HDL

The process of extrahepatic acquisition of cholesterol and subsequent transport towards the liver is called reverse cholesterol transport (RCT). The sole lipoprotein involved in RCT is HDL and its metabolism involves complex interactions of membrane-bound and circulating plasma proteins that can be divided into five major processes (schematically depicted in **Figure 2**) (**1**) Production and secretion of ApoA1 mainly by the liver and intestine [49,50]. (**2**) Via an ABCA1-dependent pathway the ApoA1 particle incorporates cellular phospholipids leading to the formation of lipid-poor discoidal pre-β HDL particles. Consequently, targeted hepatic ApoA1 deficiency in mice results in 83 % lowering of the plasma HDL level [51,52]. (**3**) Once released into the circulation, lipid-poor pre-β HDL particles take up excess amounts of FC from peripheral cells via ABCA1 and to a lesser extend ABCG1-mediated efflux to form FC-enriched discoidal particles. The pivotal role of ABCA1 in the biosynthesis of HDL is demonstrated in ABCA1 deficient (Tangier disease) patients and ABCA1 knockout mice. These patients and mice display an inadequate transport of cholesterol towards the lipoprotein resulting in hypercatabolism of lipid-poor nascent HDL particles and the subsequent development of virtual HDL deficiency [53,54]. (**4**) Esterification of HDL-FC, initiated by LCAT in the plasma, stimulates maturation of the pre-β HDL particles into spherical HDL3 particles [51]. Next, HDL3 particles are converted into larger HDL2 particles via a PLTP-driven acquisition of phospholipids, alongside the attraction of apolipoproteins released during lipolysis of TGs in VLDL and chylomicrons. (**5**) Circulating HDL2 is transported back to the liver where scavenger receptor class B type I (SR-BI) facilitates selective uptake of CE without parallel internalization and degradation of the HDL particle [55]. Based on this important function SR-BI it is considered to be the main lipoprotein receptor for HDL [56,57]. In addition, it facilitates the bidirectional flux of

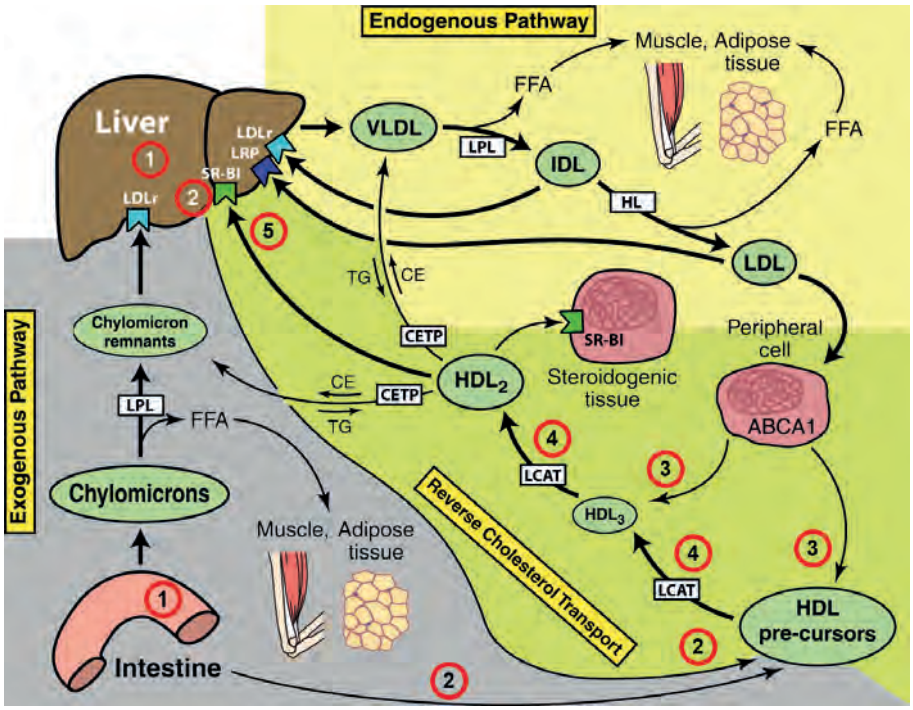

Figure 2: Overview of the basic pathways in plasma lipoprotein metabolism (Adapted from [60]) The numbers 1-5 represent the schematic steps of HDL metabolism during reverse cholesterol transport (see main text for explanation).

FC. In line with its function as main lipoprotein receptor for HDL, in vivo deficiency of SR-BI in mice is associated with impaired serum decay and hepatic uptake of [^{3}HCEt]-HDL, resulting in FC and CE accumulation in HDL particles and the appearance of enlarged HDL particles [58].

In addition to the clearance by SR-BI, HDL-cholesterol (HDL-C) can also be removed from the circulation via alternative routes. Firstly, HDL particles can be enriched with ApoE obtained from extrahepatic tissues or the blood circulation. ApoE on HDL enables removal from the circulation via hepatic LDLr or LRP1-mediated whole particle uptake [59]. A second alternative HDL-cholesterol clearing route is, as previously noted, the transfer of CE from HDL2 towards VLDL and LDL through a CETP-mediated exchange. It should, however, be noted that CETP expression is not present in the majority of rodents (mice and rats).

ADRENAL GLUCOCORTICOID SYNTHESIS

Cholesterol serves as the precursor for the synthesis of steroid hormones, including the in the adrenal cortex produced glucocorticoids (e.g., corticosterone in mice and cortisol in human) and mineralocorticoids (e.g., aldosterone), gonads produced androgens (testosterone), ovarian produced progestin and estrogen (e.g., progesterone and estradiol), and neurosteroids produced in the brain. Besides the common precursor for steroidogenesis, cholesterol, steroid hormones have a common precursor steroid, pregnenolone (**Figure 3B**) [61]. In accordance, high expression of genes linked to cholesterol metabolism are found in the steroid hormone producing organs including the expression of LDLr, SR-BI and ApoE. The adrenal derived glucocorticoids (GC) are synthesized within the zona fasciculata of the adrenal cortex. The action of GCs is transduced upon binding to the GC receptor (GCr) and mineralocorticoid receptor (MCr). Both receptors are members of the nuclear hormone superfamily and structurally and functionally highly related. The GCr is expressed ubiquitously throughout the body and is solely activated by GC. Expression of MCr can be found in specific tissues, particularly in kidney, and is activated with a similar high affinity by both the mineralocorticoid, aldosterone, as well as GC. Albeit the similarities between the two receptors, they differ in biological activity. GCr is linked to immune function and stress response, metabolism and cellular proliferation and differentiation, whereas the role of MCr is more restricted, primarily controlling blood pressure and promoting sodium reabsorption in kidney. Whereas both receptors have different biological activities, both GC and aldosterone bind and activate the MCr. This could theoretically result in an unwanted situation where the MCr is occupated and activated by the more abundantly present GC. To avoid the occupation of the MCr by GCs, tissue-specific expression is found of the GC-inactivating enzyme, 11 beta hydroxysteroid dehydrogenase type 2 (HSD11B2). HSD11B2 converts the GC, cortisol into cortisone (corticosterone to 11-dehydrocorticosterone in rodents), thereby increases the circulating levels and allows aldosterone to activate the MCr. Aldosterone by itself is found in a much lower concentration in the blood and has a very low affinity for the GCr [62, 63]. Once GC bind to GCr or MCr they undergo conformational changes that initiates translocation of the GCr with its chaperone complexes towards the nucleus where it triggers genomic mechanisms on the GC response elements (GREs), as reviewed by Kadmiel et al. [64]. Importantly, an discrepancy in the plasma levels of GC results in pathological disorders known as respectively Addison's disease (relatively low plasma GC levels) or Cushing's syndrome (relatively high plasma GC levels).

A classic role of GCs is the initiation of an anti-inflammatory response upon stress activation. This makes treatment with synthetic as well as natural occurring GCs an interesting therapeutic strategy for a variety of inflammatory conditions. As such, since 1940, GCs have been used to treat symptoms of chronic inflammatory conditions like rheumatoid arthritis, asthma, skin infections, ocular infections, multiple sclerosis and have been given as an immunosuppressant to patients following organ transplantation [65,66].

GC synthesis is regulated via dynamic circadian rhythms and upon stress-induced hypothalamic-pituitary-adrenal (HPA) axis activation [67]. Activation of the HPA axis is followed by the release of pituitary gland-derived adrenocorticotropic hormone (ACTH). ACTH interacts with the melanocortin-2 receptor (MC2R) a family member of the G protein-coupled receptors (GPCR) that is primarily present on the adrenal cortex. Binding of ACTH to the MC2R results via activation of adenylyl cyclase (AC) and instant increase of cytoplasmic adenosine monophosphate (cAMP) [68]. The formation of cAMP activates protein kinase A (PKA) leading to the phosphorylation of hormone-sensitive lipase (HSL) and steroidogenic acute regulatory protein (StAR). The intracellular presence of HSL stimulates the conversion of CEs stored in lipid droplets to FC. The released FC is transport into the mitochondria by StAR, where it is subsequently used as substrate for steroidogenesis. cAMP also stimulates HMGCoA synthesis and increases the mRNA expression of genes involved in the receptor-mediated cholesterol uptake pathways (SR-BI / LDLr), thereby priming the adrenal for an adequate synthesis of GCs [69, 70].

FC is a key component in the synthesis of GCs, therefore multiple sources are available to maintain the subsequent influx that is needed upon HPA axis activation. As schematically depicted in **Figure 3A** cholesterol needed for steroidogenesis in the adrenal could be obtained via **1**) SR-BI derived from HDL **2**) de novo synthesis of cholesterol, and **3**) uptake of cholesterol via the LDLr. However we recently showed that the contribution of the cholesterol taken up via the LDLr to the adrenal lipid pool is marginal [71].

Adrenal steroidogenesis starts within the mitochondria of the zona fasciculata of the adrenal where FC is converted via a stepwise enzyme controlled pathway into GC's (**Figure 3A**). Most enzymes involved in the steroidogenesis pathway are either family members of cytochrome P450 or hydroxysteroid dehydrogenases (HSDs) and function uni-directionally. P450scc, encoded by CYP11A1, initiates the first step

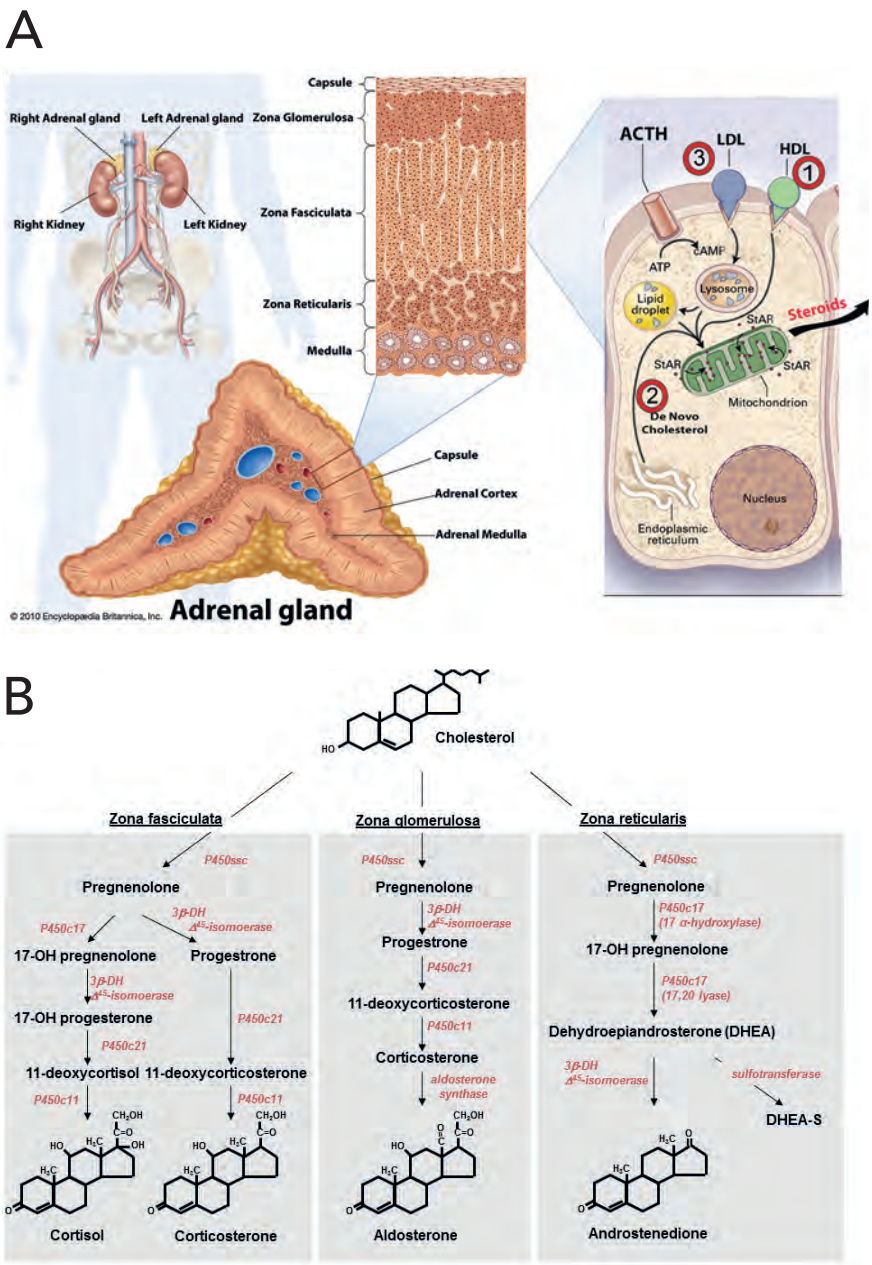

Figure 3: Overview of the pathways involved in adrenal synthesis of steroids, A) Schematic representation of the adrenal and production of the adrenal hormones in the zona fasciculata cells (Reproduced with permission from [78] © Massachusetts Medical Society, adapted and used with permission by the courtesy of Encyclopaedia Britannica, Inc., © 2010 [79]), B) Steroidogenic pathways in the different zones of the human adrenal. Note not all intermediate steroids, pathways and enzymes are shown (Adapted from [80]).

in steroidogenesis, i.e. the conversion of cholesterol into the steroid precursor, pregnenolone. Next, as summarized in **Figure 3A and B**, enzymatic modulation of the intermediate steroids leads to the formation of the cortical hormones: the glucocorticoids cortisol (in humans) and corticosterone (in mice), mineralocorticoid aldosterone as well as androgens (in humans, not in mice) [72]. Limiting factors in the production of GC's are, next to substrate availability, the availability and activity of steroidogenesis-related enzymes [73, 74].

HPA axis activation is a potent and ad hoc system that primes the body within minutes in response to a stressor. However, chronic exposure is harmful resulting in pathophysiological changes including, obesities, depression, and increased susceptibility to atherosclerosis [75, 76]. It is therefore that a properly functioning feedback-mechanism is required in response to the release of GC's. GC's execute a direct inhibitory role on the expression of corticotropin-releasing factor (CRF) in the brain and pituitary gland, thereby suppressing the stimulation of ACTH synthesis and adrenal steroidogenesis [77]

ATHEROSCLEROSIS

An imbalance in the circulating cholesterol levels has been implicated in many diseases, such as cancer [81], diabetes mellitus type 2 (T2DM) [82,83], and Alzheimer's disease (AD) [84,85]. Among the cholesterol-associated diseases, cardiovascular diseases (CVD) are the most frequent cause of death in the Western society [86]. The underlying pathology driving CVD is atherosclerosis, an ongoing process of thickening of the vessel wall leading to deprivation of oxygen and nutrients in distally located tissues. Atherosclerosis is characterized as a chronic inflammatory disease, driven by high cholesterol levels. As depicted in **Figure 4**, development of atherosclerotic lesions starts (**1**) with the infiltration of LDL particles into the vascular wall driven by physical forces (hypertension), chemical insults (hyperglycemia) or genetic alterations [87]. Within the vessel wall, LDL particles become oxidized (**2**) through either non-enzymatic or enzymatic pathways. In addition, it has been proposed that the interaction with endothelial cells, smooth muscle cells or (monocyte-derived) macrophages drives the oxidation of the LDL particle (oxLDL). Notably, the general hypothesis is that oxidation of LDL particles is not possible in the circulation due to strong anti-oxidant defense present in plasma and on the lipoproteins [88].

Modified LDL particles stimulate the expression of adhesion molecules such as P- and E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on the endothelial lining of the arterial wall (**3**) [89,90]. Subsequently, monocytes react to the inflammatory trigger and migrate into the sub-endothelial space via diapedesis (**4**) [91]. Upon entering the sub-endothelial space, monocytes differentiate into macrophages and internalize the modified LDL particles via e.g. scavenger receptor A- or CD36-mediated uptake (**5**) [92,93]. To maintain cellular cholesterol homeostasis and avoid cellular cholesterol-induced toxicity, lipid-laden macrophages actively efflux excess cholesterol via ABCA1, ABCG1 and SR-BI towards HDL particles thereby inducing the RCT process, as previously described [94]. As soon as cholesterol influx exceeds cholesterol efflux, macrophages turn into immobile lipid-laden cells with a "foamy" appearance (foam cells) (**6**). The formation and accumulation of foam cells in the sub-endothelial space are the hallmark for atherosclerotic lesion initiation, called fatty streaks [95]. Lesion progression is a dynamic process characterized by cell proliferation and migration as well as cell death [96]. It is therefore that the lesion content changes during the progression of the disease. Early lesions primarily contain macrophage derived foam cells whereas more advanced lesions are characterized by a variety of foamy cell types e.g. dendritic cells and smooth muscle cell- derived foam cells [97] and the development of a necrotic core. In more advanced lesions, smooth muscle cell (SMC) migration is triggered by the inflammatory response from the media into the intima (**7**). SMCs start proliferating and produce a fibrous cap covering the plaque (**8**) [98, 99]. Over time the plaque advances, narrowing the vessel lumen and hampering the blood flow (**9**) [87]. A common CVD event as a consequence of atherosclerotic lesion development is blockade of the blood flow by a thrombus that is either the result of a ruptured or eroded advanced lesion. The composition between the two advanced lesion phenotypes differs. Ruptured plaques display a thin fibrous cap, more inflammatory cells and contain a lipid core that is exposed to the blood stream upon rupture. Whereas eroded plaques are enclosed with a thick fibrous cap and do not contain a necrotic core [100]. The development of these different types of advanced plaque phenotypes depends on age and sex. Analysis of the carotid artery revealed that women are more prone to develop eroding plaques, whereas men carry more vulnerable rupture-prone lesions [101,102].

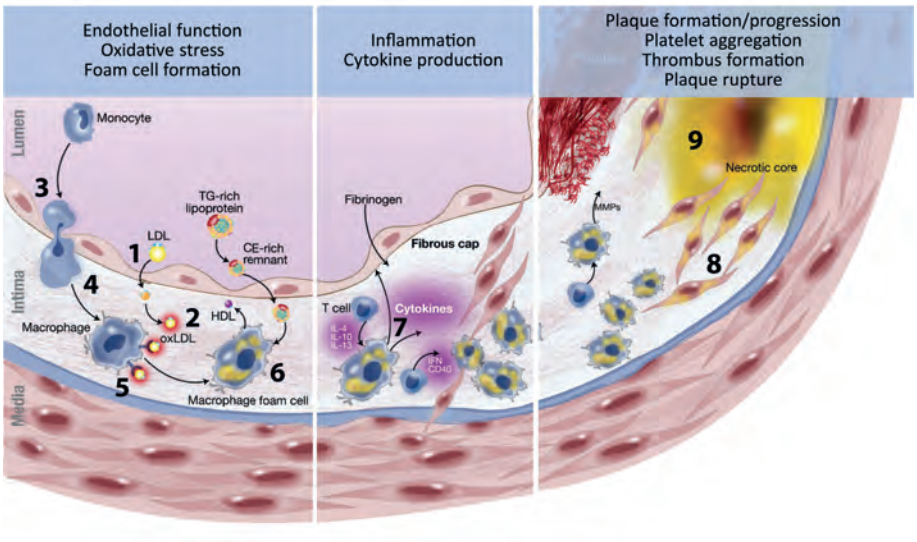

Figure 4: Schematic overview of atherosclerotic lesion development, see text for explanation (Reproduced and adapted with permission from [103], © Massachusetts Medical Society).

Table 2: Differences between human and mouse plasma lipoprotein content.

Parameter	Human	Mouse	
		Wild type	ApoE KO
HDL-C	50 mg/dl	50 mg/dl	48 mg/dl
LDL-C	150 mg/dl	10 mg/dl	100 mg/dl
CETP	Present	Absent	Absent
Atherosclerosis	Susceptible	Resistant	Spontaneous lesions

ATHEROSCLEROSIS MOUSE MODELS

Development of atherosclerosis is under the influence of various environmental as well as genetic modulating factors. Studying atherosclerosis in unregulated human cohorts is therefore difficult. The development of animal models has provided a controlled setting that enables studying the mechanisms and processes driving atherosclerotic lesion development. The most extensively used animal in CVD research is the mouse, chosen for its low maintenance costs, quick reproduction rate, and high availability of models with an atherosclerotic phenotype [104]. However, it should be acknowledged that the lipoprotein metabolism of mice differs from man in crucial aspects, influencing the overall pathogenesis atherosclerosis [105] (**Table 2**).

A major difference in lipid metabolism between mice vs humans is the lipoprotein profile. In wildtype mice the predominant lipoprotein is HDL, whereas humans display an LDL phenotype. A possible reason for this difference is the absence of CETP in mice [106,107]. Importantly, as a result of the major difference in the plasma lipoprotein profile, mice as opposed to humans do not develop atherosclerosis without major interference like genetic modification or atherogenic diet feeding.

APOE KNOCKOUT MICE

One of the most commonly used models for atherosclerosis is the total body ApoE knockout (ApoE KO) mouse, published in 1992 by two separate groups; by N. Maeda [46,47] and J. Breslow [108]. ApoE is synthesized by many tissues and cell types including liver, brain and macrophages [109-111]. The most profound role of the ApoE ligand is facilitating binding of ApoE-containing lipoproteins to the hepatic receptors; LRP1 and LDLr [112]. An additional key role for ApoE is found in the brain and adrenals, where ApoE facilitates intercellular cholesterol transport [12,114]. A targeted mutation of the ApoE gene in mice results in severe hypercholesterolemia, driven by the accumulation of ApoB-containing lipoproteins [46,47]. In line with the notion that relatively high levels of VLDL/LDL-cholesterol are pro-atherogenic, ApoE knockout mice display spontaneous development of atherosclerotic lesions already upon feeding a standard diet low in cholesterol and fat. Early stage lesion formation is measured in young mice around 8 weeks of age, with a strong progressive development of the lesions between 12 and 38 weeks [115]. Feeding ApoE knockout mice a high-fat, high-cholesterol diet further increases plasma cholesterol levels and accelerates lesion development [116,117].

LDLR KNOCKOUT MICE

A second commonly used model for atherosclerotic lesion formation is the LDLr total body knockout mouse. The LDLr is a cell surface receptor expressed primarily on mammalian hepatic cells that binds and internalizes lipoproteins carrying ApoE and/or ApoB100, thereby regulating the plasma cholesterol levels [118-120].

The association between elevated LDL-C level and an increased CVD occurrence is reflected in patients with familial hypercholesterolemia, an autosomal disorder caused by mutations in either the LDLr, ApoB or PCSK9 genes [121]. Mice with a homozygous deficiency of the LDLr gene (LDLr knockout) display a 2 to 3-fold increase in plasma cholesterol levels. Despite the relatively high plasma cholesterol levels, LDLr knockout mice do not spontaneous develop atherosclerotic lesions. However, feeding LDLr knockout mice either a high cholesterol diet (1 % cholesterol 4.4 % fat) or a Western-type diet (0.2 % cholesterol and 21 % fat) increases the plasma cholesterol concentration to a level that does stimulate atherosclerotic lesion development [122].

SR-BI KNOCKOUT MICE

SR-BI is a key player in the metabolism of the anti-atherogenic HDL particles [123]. High expression of SCARB1, the gene encoding for SR-BI, is primarily found in the liver, steroidogenic tissues and endothelial cells [124-126]. Subjects with specific mutations in the gene encoding for SR-BI display elevated levels of HDL [127-132]. To study the role of SR-BI in cholesterol metabolism and in particularly HDL and the RCT pathway in more detail, SR-BI knockout mice were generated [133]. These total body SR-BI knockout mice develop a range of pathologies, including reticulocytosis [134], reduced platelet counts [135,136], increased serum oxidative stress levels [137], as well as a reduced maximal output of adrenal derived glucocorticoids [138]. The most profound phenotype of SR-BI knockout mice is, however, the appearance of cholesterol-enriched large HDL particles. Paradoxically, the increase in HDL-cholesterol levels in SR-BI knockout mice does not confer atheroprotection, but actually results in an increased susceptibility to atherosclerosis development upon Western-type diet feeding (0.25 % cholesterol and 15 % fat) [139]. It is generally assumed that this controversy is due to the loss of the (atheroprotective) functionality of the HDL particles.

The aforementioned strains are either widely used for studying lesion progression or described in this thesis. Yet there are numerous other strains used to study CVD, either spontaneous mutated or genetically created, with or without a dietary trigger and all with their specific characteristics [140-142].

LESION REGRESSION MODELS

The concept that existing atherosclerotic lesions are capable to regress dates back ~60 years [143]. Since then, several rodent and non-rodent models have been developed to prove this concept. Many rodent atherosclerosis regression models are based on the ApoE knockout or LDLr knockout progression models [144]. The general strategy to induction of lesion regression in mouse models with established atherosclerotic lesions is lowering of the proatherogenic ApoB-containing lipoprotein in the circulation. This can be achieved by reintroduction ApoE in ApoE knockout mice via e.g. transplantation with ApoE containing bone marrow from wildtype mice [152] or via transduction with ApoE-encoding viral vectors [150, 151, 153]. A more invasive approach is the aortic arch transplantation model where a lesion-containing aortic arch segment of a hypercholesterolemic ApoE knockout mouse is transplanted into a normolipidemic recipient [154]. Subsequent regression has been achieved with either early and advanced lesions within respectively 3 days or 9 weeks post-transplantation [155-157].
The development of the Reversa mouse by Feig et a.l. (LDLr(-/-) ApoB (100/100) Mttp (flox/flox) Mx1-Cre), resulted in a model where a diet-induced hypercholesterolemia can be reversed by the expression of the MX1-Cre transgene that inactivates the gene encoding for MTTP [158,159]. The associated disruption of hepatic VLDL production resulted in a decrease of the plasma non-HDL fraction, a reduction in the plaque lipid content and egression of CD68-positive macrophages from the plaque.

More recently, a less technically demanding or time-consuming atherosclerosis regression model has been developed using an antisense oligonucleotide (ASO) that is targeted to the LDLr mRNA, inducing hypercholesterolemia and subsequent lesion formation in C57BL/6 wild type mice. By subsequently applying sense oligonucleotides (SO's) targeted to the antisense region of the targeted LDLr mRNA the hypercholesterolemia can be reversed, inducing regression of established atherosclerotic lesions [160].

A second theme in the lesion regression strategy is to increase the efficacy of the anti-atherogenic RCT route via augmenting ApoA1 and subsequently HDL levels. These increased ApoA1 levels significantly reduced the presence of early lesions in ApoE and LDLr knockout mice [145, 146] and decreased foam cell content of existing lesions in LDLr knockout mice [147]. The potential role of HDL in lesion regression was further underlined by the infusion of ApoA1/Milano/PC complex. Increasing the HDL levels in ApoE knockout mice reduced the foam cell content of existing plaques within 48 hours [148,149].

Table 3: A selection of atherosclerotic regression mouse models summarized by their characteristics. ApoE / B / A-I: Apolipoprotein E / B / A-I, ASO: antigen sense oligonucleotides, SO: sense oligonucleotides, FC: free cholesterol, HDL: high-density lipoprotein, LDLr: Low-density lipoprotein receptor, MQ: macrophage, MTTP: Microsomal triglyceride transfer protein, mRNA: messenger ribonucleic acid, RCT: reverse cholesterol transport, TC: total cholesterol, WT: wild type.

Mouse	Model	Progression	Regression method	Lipoprotein profile	Lesion	Reference
ApoE	Reintroduction of ApoE via bone marrow transplantation	Spontaneous or diet induced	Reintroduction of ApoE containing bone marrow	Normalisation of ApoB containing lipoproteins	Lesion size ↓ MQ content ↓ Collagen (advanced) =	152
ApoE	Reintroduction of ApoE via viral vectors	Spontaneous or diet induced	Transduction with ApoE-encoding viral vectors	Normalisation of ApoB containing lipoproteins	Lesion size ↓ MQ content = Collagen ↑	150, 151, 153
ApoE	Reintroduction of ApoE via aortic arch transplantation	Spontaneous or diet induced	Transplantation of an aortic segment containing plaque into a wild type mouse	Lipid levels according to the WT mice	Lesion size ↓ MQ content ↓ Collagen ↓	154-157
LDLr	LDLr(-/-) ApoB (100/100) Mttp (flox/flox) Mx1-Cre) "Riversa mouse"	Diet-induced hypercholesterolemia driven atherosclerosis	Inactivation of MTTP by activating MX1-Cre transgene	Normalisation of ApoB containing lipoproteins after inactivation of MTTP gene	Lesion size ↓ MQ content ↓ Collagen↑	158, 159
C57BL/6 wild type mice	LDLr mRNA targeting oligonucleotide model	Antisense oligonucleotide targeting the LDLr mRNA & diet, induce hypercholesterolemia driving lesion formation	Sense oligonucleotides (SO's) reverse the hypercholesterolemia	ASO's induced increase in ApoB containing lipoproteins, SO's induced reverse in ApoB containing lipoproteins	Lesion size = MQ content ↓ Collagen↑	160
LDLr / ApoE	Adenovirus gene transfer of helper-dependent adenovirus containing human ApoA-I gene targeting the liver	Diet induced	Helper-dependent adenovirus containing human ApoA-I gene induced long-term overexpression of hepatic ApoA-I	ApoB containing lipoproteins remain; efficacy of RCT is increased by increase of HDL	Lesion size ↓ MQ content ↓	145-147
ApoE	ApoA1/Milano/PC	Spontaneous or diet induced	Administration of single high / multiple dosses of recombinant ApoA1/ Milano/PC increase RCT	TC/FC conc. rise; efficacy of RCT is improved by increase of HDL	Lesion size ↓ MQ content ↓	148, 149

THESIS OUTLINE

The first part of this thesis focuses on the role of lipid metabolism, and especially HDL, in the development and regression of atherosclerotic lesions. Low plasma HDL levels are associated with increased risk for CVD events. Furthermore, the anti-atherogenic properties of HDL play a central role in the current hypothesis that HDL is essential for lesion regression. SR-BI is a key player in HDL metabolism, facilitating the bi-directional flux of cholesterol between the particle and (hepatic) tissue. Functional loss of SR-BI in mice results in accumulation of abnormally large HDL-particles and, interestingly, increased susceptibility for atherosclerotic lesion development. The increased susceptibility can theoretically be driven by the functional loss of anti-atherogenic properties of the HDL particle. A key modulator in HDL maturation is the phospholipid transfer protein (PLTP). We hypothesized that the normalization of the particle size could recover HDL's anti-atherogenic function and decrease the susceptibility for lesion development. To validate this hypothesis, we generated SR-BI x PLTP double knockout mice that genetically lack both functional SR-BI and PLTP proteins. In **Chapter 2** the effects of functional ablation of the PLTP gene in SR-BI knockout mice on HDL particle size and functionality and atherosclerotic lesion formation are described.

The standard approach to reduce the risk for a CVD event in patients is statin-induced lowering of specifically the non-HDL fraction. Previous studies in animals and patient cohorts revealed that normalizing hyperlipidemia equals diminishing of the substrate driving lesion progression, resulting in a halted lesion development [144]. The golden bullet in the CVD field, inducing regression of existing lesions, is not yet established in humans. Animal models showed that by extensively lowering lipid levels, regression of early and advanced lesions is possible [143]. We recently introduced a new model for regression of existing lesions by bone marrow-derived reconstruction of ApoE in hyperlipidemic ApoE knockout mice [152]. In **Chapter 3** we monitored over time the changes in lesion size and morphology upon normalizing cholesterol levels in ApoE knockout mice using this technique. In addition, we studied the importance of the presence of HDL during regression of existing lesions in this model.

The second part of the thesis concentrates around the role of ApoE in adrenal glucocorticoid synthesis. Given that (1) high expression levels of ApoE are found within the adrenal gland [161,162] and (2) ApoE can modulate cholesterol metabolism at both the systemic and cellular level [162,163] the hypothesis is that systemic or local ApoE could influence steroidogenesis differently. The current hypothesis is that

CE transported by HDL form the primary substrate for steroidogenesis upon stress activation in mice and that non-HDL fraction is irrelevant. However, previous in vivo studies in mice showed that the different lipoprotein fractions (non-HDL versus HDL) independently affect either the adrenal cholesterol pool or GC output [164,165]. In this context it is of interest that hypercholesterolemic ApoE knockout mice display not only increases in plasma non-HDL levels but also elevated GC levels [108]. In **Chapter 4** we validated the hypothesis that the abundantly present ApoB-containing lipoproteins could act as a source for steroidogenesis in ApoE knockout mice. We therefore drastically lowered the non-HDL levels in ApoE knockout mice via a bone marrow transplantation-mediated reconstruction of ApoE functionality and assessed the outcome on adrenal GC production.

In **Chapter 5** we focus on a possible role for ApoE in cholesterol transport locally within the adrenals. We tested the hypothesis that this local cholesterol transport function can significantly influence the synthesis of GC by the adrenals. Hereto we used our recently established in vivo "whole adrenal transplantation" model to modulate exclusively the adrenal ApoE genotype in C57BL/6 wild-type mice.

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2

DISRUPTION OF PLTP-MEDIATED HDL MATURATION REDUCES SR-BI DEFICIENCY-DRIVEN ATHEROSCLEROSIS SUSCEPTIBILITY DESPITE UNEXPECTED METABOLIC COMPLICATIONS

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ABSTRACT

BACKGROUND AND AIM: Functional loss of scavenger receptor class B, type I (SR-BI) in mice increases atherosclerosis susceptibility and stimulates accumulation of abnormally large HDL particles. A key modulator of HDL enlargement is phospholipid transfer protein (PLTP). In this study the importance of PLTP in SR-BI knockout (sKO) mice and the influence on the atherosclerosis susceptibility was investigated.

METHODS: Mice were fed a Western-type diet for 20 weeks to induce atherosclerosis.

RESULTS: In response to PLTP deficiency, a 1.7-fold ($P<0.001$) and 1.4-fold ($P<0.001$) decrease in serum cholesterol and phospholipid levels was found in sKO mice.
The lipoprotein distribution of cholesterol and phospholipids shifted from HDL to the VLDL/LDL fraction. Moreover, restoration of the HDL particle size was noted in the SR-BI/PLTP double KO (dKO) mice. Additional PLTP deficiency, however, did not normalize the impaired delivery of [^{3}H]cholesteryl esters from HDL to the liver. In line with the observed increase in VLDL levels, dKO mice also displayed a 2.2-fold ($P<0.001$) increase in serum triglycerides, which was accompanied by glucose intolerance and 1.4-fold increased body weight ($P<0.001$). Interestingly, despite the presence of a metabolic syndrome-like phenotype in dKO mice, atherosclerosis susceptibility was significantly lower as compared to sKO mice (dKO 63.0 ± 9.7*10^3 μm^2 vs sKO 117.2 ± 32.4*10^3 μm^2).

CONCLUSIONS: PLTP is essential for the accumulation of the abnormally large HDL particles in SR-BI KO mice. Importantly, deletion of PLTP does reduce the susceptibility to atherosclerosis in SR-BI KO mice despite the increase in levels of pro-atherogenic VLDL particles and the development of a metabolic syndrome-like phenotype.

INTRODUCTION

Low levels of high-density lipoprotein cholesterol (HDL-C) are associated with an increased risk for developing cardiovascular disease (CVD) and its underlying pathology, atherosclerosis [1-6]. Modulation of HDL-C in animal models, however, did not result in clear evidence that ApoA1/HDL raising strategies prevent atherosclerotic lesion development. Increasing the total amount of circulating HDL-C or ApoA1 (the main apolipoprotein on HDL) via, endothelial lipase inhibition [7,8] or by genetic modifications of genes involved in metabolism of HDL (lecithin cholesterol acyl transferase (LCAT)) overexpression [9] and scavenger receptor type B class 1 (SR-BI) deletion [10]) stimulated atherosclerotic lesion formation. In contrast, hepatic overexpression of human ApoA1 in mice and infusing the recombinant ApoA1milano phospholipid complex in cholesterol-fed rabbits did lower the risk for CVD [11-15]. Combined, these data indicate that interference early in the HDL metabolism lowers CVD risk, while increasing the circulating levels of HDL-C by blocking cholesteryl ester (CE) delivery to the liver (SR-BI KO) stimulates atherosclerotic lesion development. Clinical trials in humans using HDL-raising therapies did not give the desired reduction in the amount of cardiovascular events [16], including cholesteryl ester transfer protein (CETP) inhibition in the ILLUMINATE trail (Torcetrapib and Atorvastatin)[17], dal-OUTCOMES trail (Dalcetrapib)[18], AIM-HIGH trial (Niacin)[19] and the HPS2-THRIVE trail (Niacin+Laropiprant)[20]. In the DEFINE trail, the CETP inhibitor Anacetrapib did result in a minor reduction of the coronary lesion burden and a lower incidence of major coronary events [21]. Although Anacetrapib was effective in increasing HDL-cholesterol (+104 %) it cannot be excluded that the risk reduction was the consequence of an 18 % reduction in non-HDL-cholesterol.

The current hypothesis is that HDL's athero-protective properties are dependent on its functionality, not simply the amount of HDL-C in the circulation [21]. Although the knowledge on HDL metabolism and the link to atherosclerosis susceptibility has increased substantially in the past 2 decades, currently there are still large gaps in the general knowledge.

In human and rodent HDL metabolism, cell surface receptor SR-BI plays a predominant role. SR-BI is highly expressed in several cell types and tissues, like liver, macrophages [22] and steroidogenic tissues [23,24]. SR-BI facilitates the selective uptake of HDL-cholesteryl esters (HDL-CE) by hepatocytes [25] and mediates efflux of free cholesterol (FC) from peripheral tissues towards HDL [26] during the reverse cholesterol transport

(RCT) route. Screening of human subjects with elevated HDL-C levels revealed several mutations in the SR-BI gene. Vergeer et al. ([27] 2011) described a family with elevated HDL-C levels due to a missense mutation (P297S) in SR-BI. This mutation was associated with a reduction in cholesterol efflux from macrophages and decreased steroidogenesis. The number of carriers was, however, too low to demonstrate effects on cardiovascular events or carotid intima-media thickness. Various other identified mutations in the human SR-BI gene in subjects with elevated HDL-C levels further underline the role of SR-BI in human HDL metabolism (Zanoni et al. 2016 [28], Chadwick and Sahoo et al. 2012 [29], Brunham et al. 2011 [30], West et al. 2009 [31], Hsu et al. 2003 [32]).

To further understand the role of SR-BI in atherosclerotic lesion development and the RCT route, a total body knockout (KO) mouse was developed by the group of Monty Krieger [33]. They used a neo cassette mutagenesis strategy on the murine SCARB1 gene to generate knockout mice lacking functional SR-BI. SR-BI single knockout (sKO) mice display an impaired HDL-CE delivery to the liver and as a result increased HDL particle size and increased circulating HDL-C levels [33]. In line with these findings, studies using hepatic SR-BI overexpressing mice showed a reduction of HDL-C levels [34].
Despite the substantially increased HDL-C, SR-BI single knockout (sKO) mice are more susceptible to the formation of atherosclerotic lesions, indicating that its HDL is no longer athero-protective and displays most likely an altered functionality [35].

Currently, it is unknown if the increased susceptibility to atherosclerosis of SR-BI sKO mice is the consequence of the impairment of the last step of the RCT pathway and/or of the accumulation of the dysfunctional HDLs with an increased particle size. Therefore in this study, we aimed to block the remodelling of the HDL particles to specifically investigate the effects of the accumulation of the enlarged particles. A key modulator of HDL remodelling is phospholipid transfer protein (PLTP)[36-38]. PLTP belongs to a gene family of lipid transfer/lipopolysaccharide-binding proteins and facilitates the transfer of phospholipids, released during the lipolysis of triglyceride (TG)-rich particles, to pre-β-HDL particles. PLTP thereby enables the conversion of pre-β-HDL particles towards mature HDL particle subtypes [39,40].

We hypothesized that prevention of HDL particle enlargement in SR-BI sKO mice by deletion of PLTP function, will influence atherosclerosis susceptibility. By generation of SR-BI/PLTP double knockout (dKO) mice we show that PLTP is indeed essential for

the generation of the large CE-rich HDL particles in SR-BI sKO mice, but does not completely prevent atherosclerotic lesion development. Moreover, for the first time we provide proof that functional PLTP is essential for the lipolysis of TG-rich lipoproteins and that in absence of PLTP, VLDL triglycerides accumulate in the circulation of SR-BI sKO mice combined with the presence of a metabolic syndrome-like phenotype.

MATERIALS AND METHODS

ANIMALS

Heterozygous female SR-BI single knockout mice (SR-BI sKO, C57Bl/6 background) were kindly provided by M. Krieger. PLTP single KO mice (PLTP sKO, C57Bl/6 background) were kindly provided by M. Jauhiainen and crossbred to generate SR-BI/PLTP double KO (SR-BI/PLTP dKO) mice. Two independent animal experiments were performed: A) the lesion development study (wildtype (WT) n=5, SR-BI single knockout (sKO) n=5, SR-BI/PLTP dKO n=8) and B) the metabolic syndrome-like (MetS) phenotype study (WT n=8, SR-BI sKO n=5, PLTP sKO n=4, SR-BI/PLTP dKO n=7). Mice were given unlimited access to food and water and subjected to a 12 hour light/dark cycle. During the lesion development experiment (study A) mice were maintained on a regular chow diet (CHOW), containing 4.3 % (w/w) fat and no added cholesterol (RM3, Special Diet Services, Witham, UK) until the age of 14 weeks and subsequently challenged for a period of 20 weeks with semi-synthetic Western-type diet (WTD), containing 15 % (w/w) cacao butter, 1 % corn oil, 0.25 % (w/w) cholesterol, 20 % casein, 40.5 % sucrose, 5.95 % cellulose, 20 % corn-starch, 1 % choline chloride, vitamins and minerals (Diet W, Special Diet Services, Witham, UK) to induce atherosclerosis. During the MetS phenotype experiment (study B), all mice were on a regular CHOW diet up to the sacrifice at average 34 weeks of age. Animal experiments were performed at the Gorlaeus Laboratories of the Leiden Academic Centre for Drug Research. All animal work was approved by the Dutch Ethics Committee and regulatory authority at Leiden University and was carried out in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

HISTOLOGICAL ANALYSIS

To induce the development of atherosclerosis in the three valve area of the aortic root, WT, SR-BI sKO and SR-BI/PLTP dKO mice were fed WTD for 20 weeks. After sacrifice, the mice were perfused in situ with phosphate-buffered saline (100 mm Hg)

for 10 minutes via a cannula in the left ventricular apex. The heart plus aortic root were excised and stored in 3.7 % neutral-buffered formalin (Formal-fixx®, Shandon Scientific Ltd., UK) until further analysis. The mean atherosclerotic lesion area of each mouse was quantified from 10 oil red O-stained cryo-sections (10 µm), starting at the appearance of the tricuspid valves up to 300 µm of the ascending aorta using a Leica image analysis system, consisting of a Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging software (Leica Ltd., Cambridge, UK).

After perfusion, a selection of organs (liver, spleen, adrenals, perigonadal white adipose tissue (pWAT), subcutaneous WAT (sWAT), interscapular brown adipose tissue (iBAT) were excised and weighed.

PLASMA LIPID ANALYSES

After a 4 hour fasting-period, approximately 100 µL blood was drawn from each individual mouse by tail bleeding and collected in ethylene diaminetetraacetic acid (EDTA)-coated tubes (Sarstedt, Numbrecht, Germany). Free cholesterol concentration in plasma was determined by an enzymatic colorimetric assay with 0.025 U/mL cholesterol oxidase (Sigma) and 0.065 U/mL peroxidase (Roche Diagnostics, Mannheim, Germany) in reaction buffer (1.0 KPi buffer, pH=7.7 containing 0.01 M phenol, 1 mM 4-amino-antipyrine, 1 % polyoxyethylene-9-laurylether, and 7.5 % methanol). Total cholesterol content was determined after addition of 0.003 U/mL cholesteryl esterase (Seikagaku Corporation, Japan), final absorbance was read at 490 nm. The distribution of cholesterol over the different lipoprotein particles in plasma was determined by fractionation of 30 µL plasma of each mouse using a Superose 6 column (3.2x300 mm, Smart-system, Pharmacia, Uppsala, Sweden). Total cholesterol content of the effluent was determined as above. VLDL-C, LDL-C, and HDL-C in plasma were calculated from the respective fractions (VLDL-C 2-6, LDL-C 7-11, and HDL-C 12-22). Percentual HDL and VLDL lipid composition was calculated after analysis of the free cholesterol, cholesteryl ester, phospholipids, and triglyceride content of pooled FPLC fractions corresponding to the HDL or VLDL peak. Phospholipids (Instruchemie, Delfzijl, The Netherlands) and triglycerides (Roche Diagnostics, Mannheim, Germany) were determined by enzymatic colorimetric assays according to manufacturer's instructions.

BLOOD CELL ANALYSIS

Blood was collected in ethylene diaminetetraacetic acid (EDTA)-coated tubes (Sarstedt, Numbrecht, Germany) by tail bleeding of mice after a 4 hour fasting-period. Whole blood samples (85 µL) were measured from mice on CHOW diet and on WTD,

using an automated veterinary hematology analyzer, Sysmex XT-2000iV (Goffin Meyvis, Etten-Leur, the Netherlands) to investigate blood cell counts.

ANALYSIS OF GENE EXPRESSION BY REAL-TIME QUANTITATIVE PCR

Quantitative gene expression was performed as previously described by Hoekstra et al. [41]. In summary, total RNA was isolated using a standard phenol/chloroform extraction method and reversely transcribed using RevertAid Reverse Transcriptase. Gene expression of analysis was performed by using SYBR-Green technology (Eurogentec). Primers were validated for an equal efficiency. Ratios of the target gene expression were determined by subtracting the Ct of the target gene from the Ct of the housekeeping gene and raised by 2 to the power of this difference. The target gene expression is thus relative to the housekeeping gene expression. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribosomal protein lateral stalk subunit P0 (36b4) were used as the housekeeping genes.

GLUCOSE TOLERANCE TEST

Mice were weighed and fasted 4 hours prior to administration of the glucose bolus of 2 g/kg per orally. Blood glucose was measured in time at 15, 30, 60, 120, 180, and 360 minutes after administration with a Roche accu-check mobile system.

ISOLATION OF PERITONEAL CELLS AND CHOLESTEROL EFFLUX

A cohort of chow-fed 12-week old female C57Bl6 mice were intra-peritoneally injected with 3 % Brewer's thioglycollate medium to induce the infiltration of macrophages into the peritoneal cavity. At 5 days after injection, the peritoneal cavity of the mice was lavaged with 10 mL cold PBS to collect thioglycollate-elicited peritoneal leukocytes [42].

To measure the cholesterol efflux capacity of the cells, thioglycollate-elicited peritoneal macrophages were incubated with 0.5 μCi·mL−1 [^{3}H]-cholesterol in DMEM/0.2 % BSA (fatty acid free) for 24 hours at 37 °C. Cholesterol efflux was studied by incubation of the cells with DMEM/0.2 % BSA or with 2.5 % serum of either WT, SR-BI sKO of SR-BI/PLTP dKO as lipid acceptors. Radioactivity in the medium and the cells was determined by scintillation counting after 24 hours of incubation. Cholesterol efflux was calculated as the amount of radioactivity in the medium compared with the total amount of radioactivity measured in medium plus cells.

MEASUREMENT OF ISOPROSTANES AND CARBONYLS

Serum, hepatic, splenic, and aortic arch isoprostane 8,12-iso-iPF2α-VI levels were measured by gas chromatography-mass spectrometry as described previously [43]. Blood samples were drawn, centrifuged at 7.000 rpm for 10 min, and serum was separated and stored at -80 °C until analysis. Samples were spiked with a known amount of internal standard, extracted and purified by thin-layer chromatography, and analysed by negative ion chemical ionization gas chromatography-mass spectrometry. The aortic arch, liver, and spleen from each individual mouse was obtained, weighed, minced, and homogenized in PBS containing EDTA (2 mM) and butylated hydroxytoluene (2 mM), pH 7.4, and total lipid was extracted using Folch solution (chloroform-methanol, 2:1, v/v). Next, base hydrolysis was performed using 15 % KOH at 45 °C for 1 hour, and the total levels of 8,12-iso-iPF2α-VI were processed before analysis as described above. Total protein carbonyls were determined by using the Zenith test kit according to the manufacturer's instructions (Zenith Technology, Dunedin, New Zealand) [44].

ISOLATION AND [^{3}H]CET-LABELING OF HDL

HDL needed for the [^{3}H]CEt -HDL clearance was isolated from healthy subjects and isolated via differential ultracentrifugation as described by Redgrave, Roberts and West [45]. Hereto the blood was dialyzed against PBS with 1 mM EDTA and the HDL fraction ($1.063<d<1.21$) was labeled with [^{3}H]CEt via exchange from donor particles. Donor particles were formed by sonication of egg yolk phosphatidylcholine supplemented with 50 µCi [^{3}H]CEt. Sonication was carried out with a Soniprep 150 (MSE Scientific instruments UK) for 40 min at 52 °C under a constant stream of argon in a 0.1 M KCl, 10 mM Tris, 1 mM EDTA, 0.025 % NaN_3 buffer (pH 8.0). Donor particles with a density of 1.03 g/ml were isolated by density gradient centrifugation in a Beckman SW 40Ti rotor. HDL was labeled by incubating HDL with donor particles (mass ratio of HDL protein/particle phospholipid 8:1) in the presence of human lipoprotein-deficient serum as the CE transfer protein source (1:1, v/v) for 8 h at 37 °C in a shaking-water bath under argon. Ethylmercurithiosalicylate (thimerosal; 20 mM) was added to stimulate CE transfer and to inhibit phospholipid transfer and lecithin:cholesterol acyltransferase activity. Radiolabeled HDL was then isolated by density gradient ultracentrifugation. The specific activity varied between 5 and 8 dpm/ng protein.

IN VIVO CLEARANCE AND LIVER UPTAKE OF [^{3}H]CET LABELED HDL

To study the clearance of the [^{3}H]CEt labeled HDL, a dose of 215 µg apolipoprotein (± $1.2x10^6$ dpm) of [^{3}H]CEt-HDL (total volume of 200 µL) was injected into the tail vein of WT, SR-BI sKO, and SR-BI/PLTP dKO mice. At 5 min after injection, a blood sample

was drawn to verify the injected dose. At 1, 2, 4, 6, 8 and 24 hours after injection blood samples were drawn to measure serum decay. Lipoprotein distribution of [^{3}H]CEt-HDL was determined at 8 hours after injection by fractionation of 110 µL serum of a pool of three mice using a Superose 6 column (3.2x300 mm, Smart-system, Pharmacia, Uppsala, Sweden). For analysis of liver association, the liver was excised at 24 h after tracer injection and overnight dissolved at 55 °C in Solvable (PerkinElmer), and [^{3}H] activity was quantified. Uptake of [^{3}H]CEt-HDL derived radioactivity by the liver was expressed per gram wet tissue weight and a correction was made for the radioactivity in the blood present in the liver at the time of sampling as determined by injection of screened [^{3}H]BSA (84.7 µL/g wet weight).

PREPARATION OF RADIOLABELED TRIGLYCERIDE-RICH LIPOPROTEINS (TRL)-MIMICKING EMULSION PARTICLES

Radiolabeled TRL-mimicking emulsion particles were prepared from 100 mg of total lipid including triolein (70 mg), egg yolk phosphatidylcholine (22.7 mg), lysophosphatidylcholine (2.3 mg), cholesteryl oleate (CO, 3.0 mg), and cholesterol (2.0 mg), with addition of [^{3}H]triolein (TO) (100 µCi) and [^{14}C]CO (10 µCi). Sonification was performed using a Soniprep 150 (MSE Scientific Instruments, UK) that is equipped with a water bath for temperature (54 °C) maintenance, at 10 µm output [46]. The emulsion was fractionated by consecutive density gradient ultracentrifugation steps in a Beckman SW 40 Ti rotor. After centrifugation for 27 min at 20,000 rpm at 20 °C, an emulsion fraction containing chylomicron-like particles (average size 150 nm) was removed from the top of the tube by aspiration and replaced by NaCl buffer (1.006 g/ml). After a subsequent centrifugation step for 27 min at 40,000 rpm, large VLDL-like particles (average size 80 nm) were obtained in a similar manner. Emulsions were stored at 4 °C under argon and used for in vivo kinetic experiments within 5 days following preparation.

IN VIVO CLEARANCE AND ORGAN UPTAKE OF RADIOLABELED TRL-MIMICKING EMULSION PARTICLES

To study the in vivo clearance of radio-labeled TRL-mimicking emulsion particles, mice were fasted for 4 hours and injected intravenously with 200 µl of emulsion particles with an average size of 80 nm (0.2 mg triglyceride (TG) per mouse). Blood samples were taken from the tail vein at 2, 5, 10, and 15 min after injection to determine the plasma decay of either [^{3}H]TO and [^{14}C]CO [47]. After taking the last blood sample, mice were euthanized by cervical dislocation and perfused with ice-cold PBS containing via the left ventricle of the heart to remove blood and non-internalized TRL-mimicking

particles from the organs. Subsequently, the liver, heart, spleen, hind limb muscle, gonadal WAT, subcutaneous WAT, interscapular BAT, adrenals and thymus were collected. Organs were dissolved overnight at 55 °C in Solvable (PerkinElmer), and [^{3}H] and [^{14}C] activity were quantified. Uptake of [^{3}H]TO- and [^{14}C]CO-derived radioactivity by the organs was expressed per gram wet tissue weight.

STATISTICAL ANALYSES

Statistical analyses were performed utilising the unpaired Student's t-test and one-way analysis of variance (ANOVA) after confirmation of Gaussian distribution using the test of Kolmogorov and Smirnov (Instat GraphPad software, San Diego, USA). A Grubbs outlier test was performed via Graphpad Quickcalcs online software. A Bonferroni post comparison test was executed after the ANOVA. The probability level for statistical significance (P) was set at 0.05 and the results are expressed as an average of standard error of the mean (SEM).

RESULTS

HDL particle size reduction and increased plasma triglycerides in CHOW-fed SR-BI/PLTP dKO mice.

In accordance with previously described studies [48,49] CHOW-fed SR-BI sKO mice displayed increased serum total cholesterol levels (TC 2.5-fold; P<0.005 vs WT), free cholesterol (FC 5.4-fold, P<0.005 vs WT), phospholipids (PL 1.3-fold; P<0.001 vs WT), triglycerides (TG 1.3-fold; P<0.05 vs WT) and free cholesterol/cholesteryl ester ratio (FC/CE ratio 4.5-fold; P<0.001 vs WT) (**Table 1**). Furthermore, lipoprotein fractionation (by FPLC) confirmed an increased HDL-C amount (area under the curve (AUC) SR-BI sKO 138 ± 4.2 vs WT 50 ± 1.7; P<0.001) alongside a left-shifted HDL peak, indicative for enlarged HDL particles (**Figure 1A CHOW**). These specific SR-BI sKO phenotypes were partly normalized in the SR-BI/PLTP dKO group. Compared to SR-BI sKO mice, serum TC and FC levels were significantly lower in the SR-BI/PLTP dKO group (TC 0.7-fold; P<0.01; FC 0.7-fold; P<0.01 vs SR-BI sKO). However, the FC/CE ratio in the SR-BI/PLTP dKO mice was not reduced (FC/CE ratio 4.0-fold vs WT; P<0.001). Circulating TG (TG 0.8-fold; P<0.05 vs SR-BI sKO) as well as the serum phospholipids (PL 0.75-fold; P<0.005 vs SR-BI sKO) were also decreased in the SR-BI/PLTP dKO mice versus the SR-BI sKO mice (**Table 1**). Furthermore, FPLC analysis showed a lower HDL-C peak with a right shift towards smaller sized particles and a decreased AUC in SR-BI/PLTP dKO

mice as compared to SR-BI sKO animals (AUC SR-BI/PLTP dKO 69 ± 2.2 vs 138 ± 4.2 SR-BI sKO; P<0.0001) (**Figure 1A CHOW**).

In mice, PLTP regulates the transfer of phospholipids needed for HDL particle maturation during the lipolysis of triglycerides in ApoB containing lipoproteins, like VLDL [50]. In line with this, a concomitant 3.5-fold increase in the AUC of the non-HDL fraction was observed in the SR-BI/PLTP dKO mice (AUC: SR-BI/PLTP dKO 19.0 ± 1.6; SR-BI sKO 5.5 ± 0.6; P<0.05) (data not shown).

HDL particles play a distinct role in the reverse cholesterol transport route from non-hepatic peripheral tissues towards the liver. The cholesterol efflux capacity of macrophages on the one hand and hepatic cholesterol uptake capacity on the other hand are important determinants of the effectivity of the reverse cholesterol transport route. Normalization of the cholesterol levels and reduction of the HDL particle size upon deletion of PLTP in SR-BI sKO mice did affect the capacity of serum to stimulate efflux of cholesterol from lipid-loaded wildtype macrophages in vitro (**Figure 2A**). In line with previous data [51], plasma from SR-BI sKO showed a decreased efflux capacity versus plasma from WT mice. Cholesterol efflux towards SR-BI/PLTP dKO plasma was improved, although not fully restored to WT level (WT 42.5 %, SR-BI sKO 30.3 %, SR-BI/PLTP dKO 35.9 %, all data sets are pooled plasma) (**Figure 2A**).

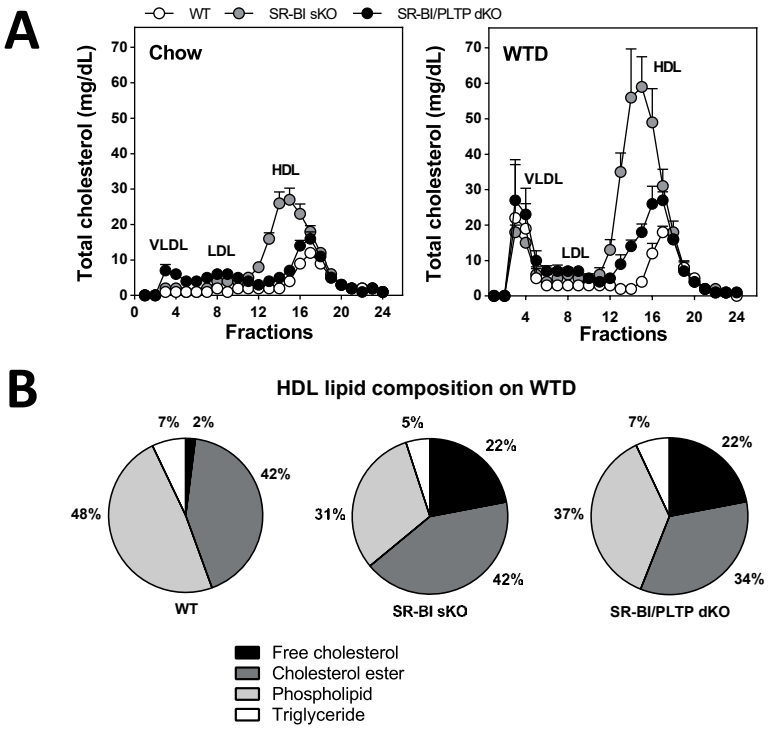

Figure 1: Plasma cholesterol lipid distribution of wild type (open circle), SR-BI sKO (grey circle),SR-BI/PLTP dKO (black circle) on either CHOW (WT n=4, sKO n=5, dKO n=8) or 20 weeks on Western type diet (WTD; amount of animals in all groups was 4), data represents mean+SEM (A). HDL composition (fractions 12-22) upon WTD challenge in % (B).

In vivo, labeled [^{3}H]-cholesteryl ether ([^{3}H]CEt) HDL injections showed that the disturbed serum clearance and hepatic uptake as a result of hepatic SR-BI deficiency was not restored by deletion of PLTP (% injected dose (ID) [^{3}H]CEt in the circulation at 24 hours after injection: SR-BI sKO 19.1 ± 1 %; SR-BI/PLTP dKO 17.4 ± 2 %; WT 3.6 ± 2 %; SR-BI sKO and SR-BI/PLTP dKO P<0.005 vs WT) (**Figure 2B**) (Hepatic uptake as % ID [^{3}H]CEt; SR-BI sKO 9 ± 0.4 %; SR-BI/PLTP dKO 9 ± 1 % vs WT 46 ± 2 %; P<0.0001) (**Figure 2C**).

In conclusion, under CHOW-fed conditions, deletion of PLTP in SR-BI KO mice limits the supply of phospholipids for the formation of the enlarged HDL particles caused by the absence of SR-BI on the liver. Moreover, HDL-C normalization in SR-BI KO mice by deletion of PLTP resulted in a slight improvement of plasma efflux capacity in vitro but did not restore the [^{3}H]CEt hepatic uptake in vivo.

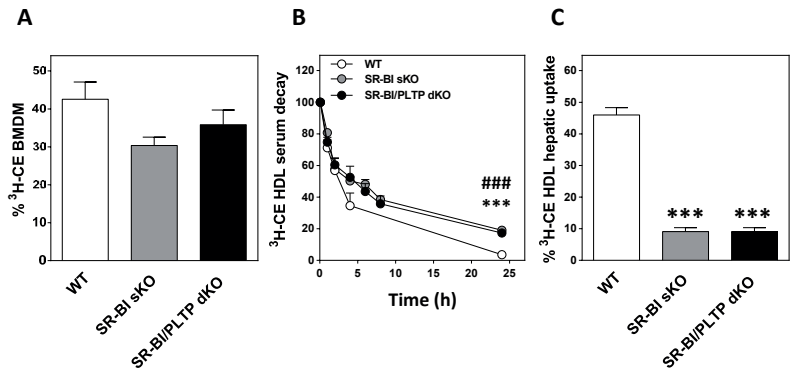

Figure 2: In vitro efflux from cholesterol loaded C57Bl6 bone marrow derived cells (BMDM) towards serum of either WT, SR-BI sKO or SR-BI/PLTP dKO mice on CHOW diet (A). Serum decay of [^{3}H]CE labeled HDL post IV injection of wild type (WT; open circles, n=3), SR-BI sKO (SR-BI sKO; gray circles, n=3) and SR-BI/PLTP dKO (SR-BI/PLTP dKO; black circles, n=3) data represents mean+SEM. Significant difference at the 24 hr time point; ***P<0.005 vs wild type; ###P<0.005 vs SR-BI sKO (B). Liver uptake of [3H]CE labeled HDL, data represents mean+SEM (WT n=5, SR-BI sKO n=5, SR-BI/PLTP dKO n=8, ***P<0.005 SR-BI vs WT, ***P<0.005 SR-BI vs WT) (C). Data represents mean+SEM.

HDL particle size reduction in SR-BI sKO mice did not recover SR-BI sKO blood phenotype on WTD.

To induce atherosclerotic lesion formation, mice were subsequently challenged with a Western-type diet (WTD) for a period of 20 weeks. Over time the serum TC levels increased in the SR-BI sKO group by 2.4-fold (P<0.001) (**Table 1 WTD**) and WTD feeding aggravated the SR-BI sKO HDL-C phenotype (AUC SR-BI sKO 269.5 ± 14.6; WT 71.0 ± 3.2 P<0.001) (**Figure 1 WTD**). The additional PLTP deletion resulted in significantly lower TC (1.7-fold; P<0.005), FC (1.7-fold; P<0.005), and PL (1.4-fold; P<0.005) levels versus SR-BI sKO WTD-fed mice (**Table 1 WTD**). Notably, deletion of PLTP also did not normalize the augmented plasma FC/CE ratio during the WTD challenge (4.0-fold; P<0.001 versus WT). On WTD, however, the clear reduction in HDL-C particle size and amount remained evident (AUC SR-BI/PLTP dKO 126.0 ± 5.0 vs SR-BI sKO 269 ± 14.6; P<0.001). The HDL particle lipid composition analysis showed an 11-fold increase in FC content in HDL of SR-BI/PLTP dKO mice versus WT mice, a phenotype similar to SR-BI sKO animals (**Figure 1B**).

Meurs et al. previously showed that the augmented FC levels in SR-BI sKO mice result in FC-enriched erythrocytes that have an augmented turnover and consequently

leading to an increased spleen size and higher reticulocyte counts in the sKO animals [49]. Another SR-BI sKO-specific blood phenotype are decreased platelet counts due to the increased FC content [52]. Here we confirmed these SR-BI sKO phenotypes, including higher reticulocyte count (SR-BI sKO 21.39 ± 2.4 $*10^{12}$/L vs 3.68 ± 1.0 $*10^{12}$/L in WT; P<0.0001), an enlarged spleen size (SR-BI sKO 301 ± 81 mg vs WT 185 ± 34 mg; P<0.05), and reduced platelet counts (SR-BI sKO 358.3 ± 30 $*10^{9}$/L vs WT 551.8 ± 83 $*10^{9}$/L; P<0.05). These phenotypes were not improved upon additional deletion of PLTP (reticulocyte count SR-BI/PLTP dKO 18.0 ± 1.0 $*10^{12}$/L; spleen SR-BI/PLTP dKO 255 ± 19 mg; platelet count SR-BI/PLTP dKO 317.9 ± 26 $*10^{9}$/L) (**Figure 3A, B, C**). Strikingly, upon WTD challenge, deletion of PLTP in the SR-BI sKO mice resulted in an increased WBC count, driven by a 1.4-fold increase in lymphocytes compared to SR-BI sKO (SR-BI/PLTP dKO 12.3 ± 1.1 $*10^{9}$/L vs SR-BI sKO 8.8 ± 0.9 $*10^{9}$/L and WT 6.7 ± 0.7 $*10^{9}$/L; P<0.01). Besides the increased lymphocyte count, deletion of PLTP in SR-BI KO mice resulted in a 2.2-fold increase in neutrophils (SR-BI/PLTP dKO 2.80 ± 0.24 $*10^{9}$/L; SR-BI sKO 1.50 ± 0.21 $*10^{9}$/L; WT 1.31 ± 0.16 $*10^{9}$/L; WT vs SR-BI sKO P=0.05; WT vs SR-BI/PLTP dKO P<0.005; SR-BI sKO vs SR-BI/PLTP dKO P<0.01) (**Figure 3D, E, F**). Combined these effects on blood cell counts suggest an increased inflammatory status in the SR-BI/PLTP dKO mice.

HDL particle size reduction by PLTP deletion in SR-BI KO mice lowers WTD-induced atherosclerosis susceptibility.

Next, the effect on atherosclerotic lesion development was investigated. In line with our previous study [35], WTD feeding for a period of 20 weeks induced the development of atherosclerotic lesions in the aortic root of SR-BI sKO mice, but not in WT controls (SR-BI sKO: 117 ± 32 $*10^{3}$ μm2; WT 1 ± 0.1 $*10^{3}$ μm^2; P<0.01). Normalization of the HDL-C levels, HDL particle size and the partly recovered efflux capacity in the SR-BI/PLTP dKO mice, was associated with 46 % smaller aortic root lesions in SR-BI/PLTP dKO mice versus SR-BI sKO mice (SR-BI/PLTP dKO 63 ± 7 $*10^{3}$ μm^2; t-test P<0.05 vs sKO, ANOVA P=NS) (**Figure 4A, C**), despite the increased inflammatory status. The lesion composition analysis revealed that the decreased lesion size in dKO group was paralleled by a diminished collagen content (SR-BI/PLTP dKO 16 ± 6 $*10^{3}$ μm^2; SR-BI sKO 44 ± 15 $*10^{3}$ μm^2; WT 1 ± 0.4 $*10^{3}$ μm^2, P=0.45 dKO vs sKO, P=0.98 dKO vs WT, P=0.13 WT vs dKO) (**Figure 4B, D**).

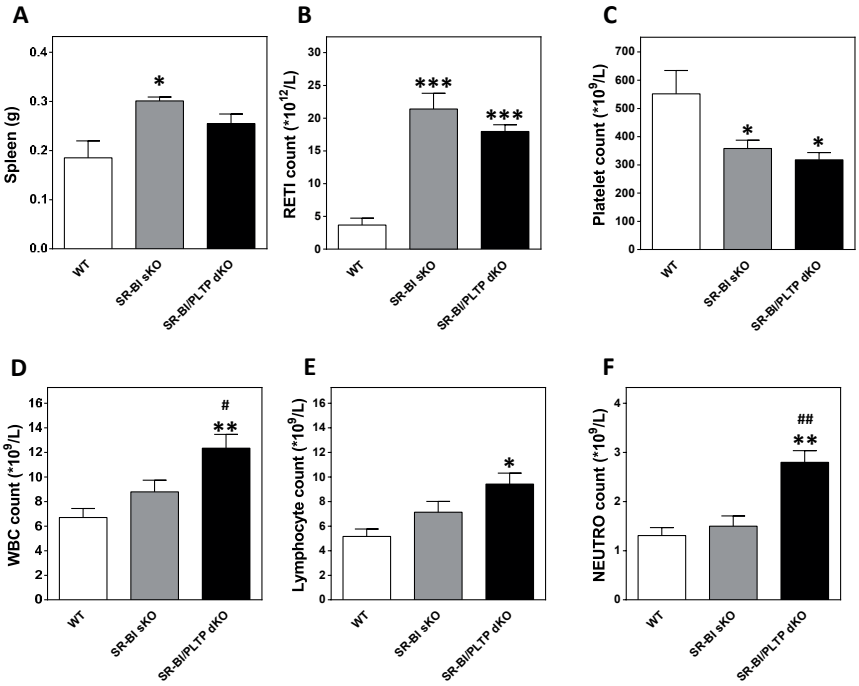

Figure 3: The effect of total body depletion of SR-BI sKO and additional PLTP depletion (SR-BI/PLTP dKO) post overnight fasted conditions on spleen weight (A), reticulocyte counts (RETI) (B),platelet counts (C), white blood cell count (WBC)(D), lymphocyte counts (E), neutrophil counts (NEUTRO)(F). All data represents mean+SEM, *P<0.05, **P<0.01 ***P<0.005 vs Wild type (WT) plasma, #P<0.05, ##P<0.01 vs SR-BI sKO.

In SR-BI sKO mice, the increased susceptibility for atherosclerosis is not only attributed to the impairment of the reverse cholesterol transport process, but also to increased oxidative stress as a result of the dysfunctional anti-oxidant activity of its abnormal HDL particles [53]. To investigate if reduction of HDL-C size by deletion of PLTP in SR-BI KO mice also restored in vivo oxidative stress levels as a measure of increased HDL functionality, we measured F2-isoprostane and carbonyl levels as markers of lipid and protein oxidation, respectively [54]. As previously described [53], SR-BI sKO animals showed increased plasma F2-isoprostanes (SR-BI sKO 415 ± 64 pg/mL compared to 153 ± 7 pg/mL in WT; P<0.01). The additional PLTP deletion in the SR-BI KO mice did not normalize the plasma F2-isoprostane levels (SR-BI/PLTP 338 ± 12 pg/mL, P<0.01 vs WT). Interestingly, local oxidative stress levels in the aortic arterial wall were normalized

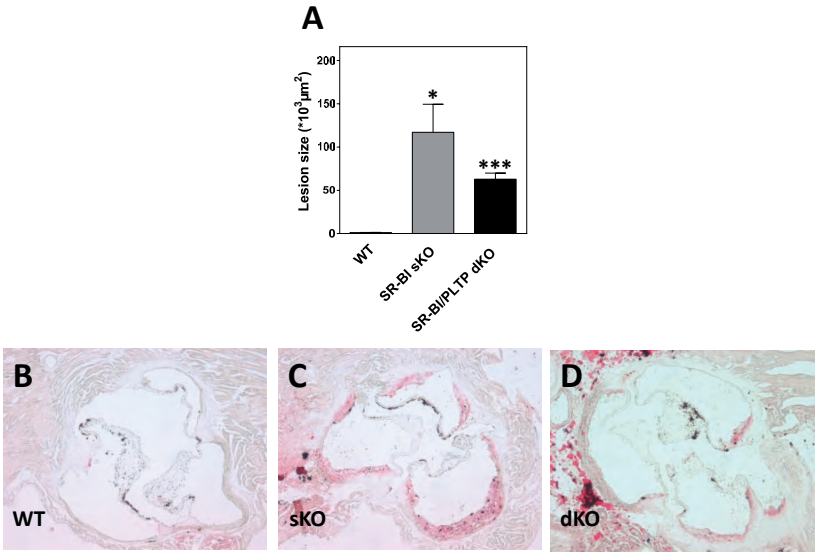

Figure 4: The effect on atherosclerosis lesion development in 20 weeks on Western type diet and overnight fasted conditions (A). Representative ORO positive lesions in the tree valve area per group at a 5x magnification (B). Massons trichrome positive collagen content in the lesion (C). Representative MT positive lesions in the tree valve area per group at a 5x magnification (D). WT (white bar n=5), SR-BI sKO (grey bar n=5) and SR-BI/PLTP dKO (black bar n=8). Statistically significant difference is at *P<0.05 **P<0.01 vs WT; #P<0.05 vs SR-BI sKO).

in the SR-BI/PLTP dKO group. Aorta F2-isoprostane levels were lowered 3.4-fold from 41 ± 3 pg/mg tissue in SR-BI sKO to 12 ± 2 pg/mg tissue (P<0.001) in the SR-BI/PLTP dKO mice as compared to 14 ± 3 pg/mg tissue in WT animals. An identical fold change was seen for carbonyls in aortic tissue, where SR-BI sKO mice had increased carbonyls (SR-BI sKO 0.48 ± 0.08 nmol/mg tissue; P<0.05) as compared to WT (WT 0.22 ± 0.04 nmol/mg tissue). PLTP deficiency normalized the carbonyl level to 0.2 ± 0.04 nmol/mg tissue (P<0.01 compared to SR-BI sKO group) in SR-BI/PLTP dKO mice (**Figure 5A, B, C**).

PLTP deletion in SR-BI sKO mice resulted in a metabolic syndrome-like phenotype driven by a misbalance in TG metabolism.

A prominent finding was that the SR-BI/PLTP dKO mice became severely obese over the period of 20 weeks WTD challenge (**Figure 6B**). At the end of the diet feeding period the body weight (BW) of the SR-BI/PLTP dKO mice as compared to both WT

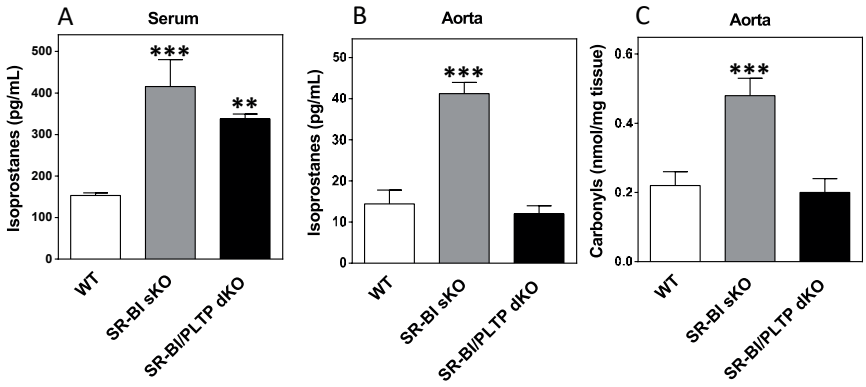

Figure 5: The effect of total body depletion of SR-BI (SR-BI sKO) and additional PLTP depletion (SR-BI/PLTP dKO) on (A) oxidative stress levels measured in 20 weeks on Western type diet female mice in serum and (B&C) aorta tissue. Data represents mean+SEM (WTD n=5, SR-BI sKO n=5 and SR-BI/PLTP dKO n=8). Statistical difference of **P<0.01, ***P<0.01 vs WT.

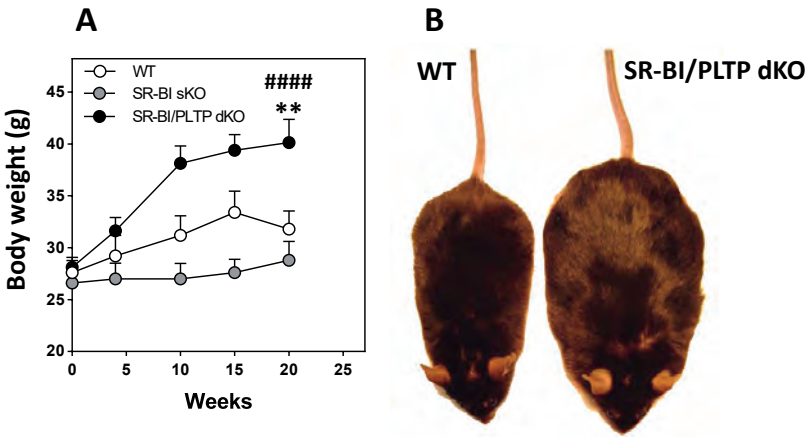

Figure 6: Over time WTD feeding altered the body weight of wild type (WT, open circle, n=5), SR-BI sKO (SR-BI sKO, grey circle, n=5) mice, and SR-BI/PLTP dKO (SR-BI/PLTP dKO, black circle,n=8) mice. At 20 weeks WTD feeding statistically significant difference was **P<0.01 vs WT; #### P<0.001 vs sKO (A). Visual differences between representative SR-BI sKO and SR-BI/PLTP dKO mice at the 20 weeks' time point (B).

and SR-BI sKO mice was 1.2 and 1.4-fold higher (SR-BI/PLTP dKO 40.1 ± 2.3 g; SR-BI sKO 28.8 ± 1.8 g; WT 31.8 ± 1.7 g; P<0.001 for SR-BI/PLTP dKO vs SR-BI sKO and WT). Consistent with Lino et al. ([55] 2015), throughout the experiment the SR-BI sKO mice remained significantly lighter (AUC SR-BI sKO 546.7 ± 10.6; WT 619.3 ± 13.5; P<0.001) (**Figure 6A**).

The rise in BW in the SR-BI/PLTP dKO mice was mainly the result of increased gonadal WAT (gWAT absolute weight; WT 1.30 ± 0.15 g; SR-BI sKO 1.22 ± 0.38 g; SR-BI/PLTP dKO 5.01 ± 0.46 g; P<0.001 vs WT, P<0.001 vs sKO respectively) (**Figure 7A)** and subcutaneous WAT (sWAT absolute weight; WT 0.38 ± 0.09 g; SR-BI sKO 0.33 ± 0.07 g; SR-BI/PLTP dKO 0.70 ± 0.047 g; P<0.01 vs WT, P<0.01 vs sKO) (**Figure 7B**). Well established is the link between obesity and increased plasma TG.

Here we measured a 2.0-fold (P<0.005) increase in circulating TG levels upon WTD feeding in the dKO group compared to the SR-BI sKO mice (**Table 1**). These augmented TG levels in serum of the SR-BI/PLTP dKO mice coincided with an increased AUC in the VLDL fraction (AUC SR-BI sKO 12.5; SR-BI/PLTP dKO 34.0, based on VLDL-C) (**Figure 1A WTD**). Analysis of the lipid content of the VLDL particles showed a dramatic increase in TG content at the expense of CE in VLDL of SR-BI/PLTP dKO mice as compared to VLDL from SR-BI sKO mice (TG 1.5-fold; CE 0.7-fold) (**Figure 7C**).

Table 1: Plasma cholesterol levels of total body depleted SR-BI KO mice with and without additional PLTP function. Data represents mean+SEM; significant difference *P<0.05, ***P<0.005, ****P<0.001 versus WT: #P<0.05, ##P<0.01, ###P<0.005, ####P<0.001 versus SR-BI KO.

	TC (mg/dL)	FC (mg/dL)	TG (mg/dL)	PL (mg/dL)	FC/CE ratio
CHOW					
WT	69±1.3	16±1.6	191±6.0	191.1±6.0	0.18±0.03
SR-BI sKO	175±5.4***	86±2.8***	251±6.1 *	251.4±6.1 ****	0.58±0.02****
SR-BI/PLP dKO	121±3.0***###	56±1.6***##	195±6.4 #	187.7±7.3 ####	0.52±0.03****
WTD					
WT	139±11.3	26±1.4	20.8±3.9	252.1±15,5	0.14±0.01
SR-BI sKO	338±22.4***	173±13.0 ***	48.6±3.3 ###	370.3±12.9 ****	0.62±0.04****
SR-BI/PLP dKO	195±6.8*###	97.1±3.1 ***###	99.7±8.4 ****	267.5±7.7###	0.57±0.03****

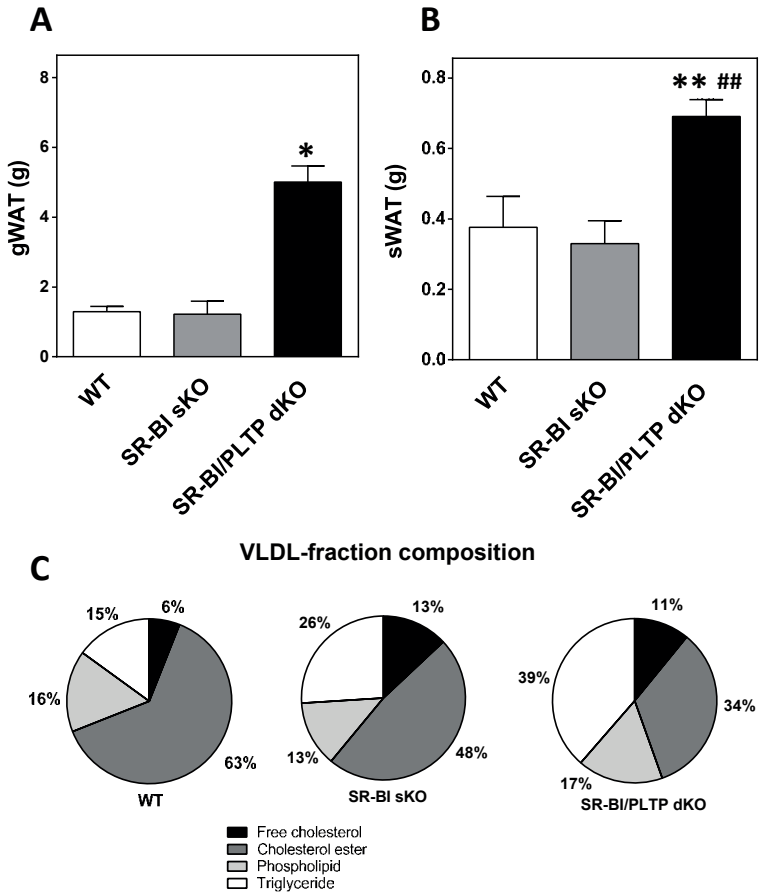

Figure 7: 20 weeks WTD fed SR-BI and PLTP depleted (SR-BI/PLPT dKO) mice displayed increased gonadal white adipose tissue (gWAT)(A), subcutaneous WAT (sWAT)(B). All data represents mean+SEM and statistically significant difference was *P<0.05, **P<0.01 vs WT; ## P<0.01 vs SRBI sKO. Very large density lipoprotein composition (fractions 2-6) upon WTD challenge in % (C).

Despite the elevated TG levels in the circulation and the increased gWAT and sWAT volume, no signs of nonalcoholic fatty liver disease were observed in the SR-BI/PLTP dKO mice. Moreover, the liver weights did not differ between the groups (absolute liver weight; SR-BI/PLTP dKO 1.8 ± 0.18 g, SR-BI sKO 1.4 ± 0.26 g; WT 1.7 ± 0.23 g; P<0.05 SR-BI/PLTP vs SR-BI sKO (**Figure 8A**). In addition, a clear SR-BI sKO hepatic lipid composition phenotype was observed, characterized by decreased levels of TC, FC, CE, PL and TG versus the WT group (**Figure 8B**).

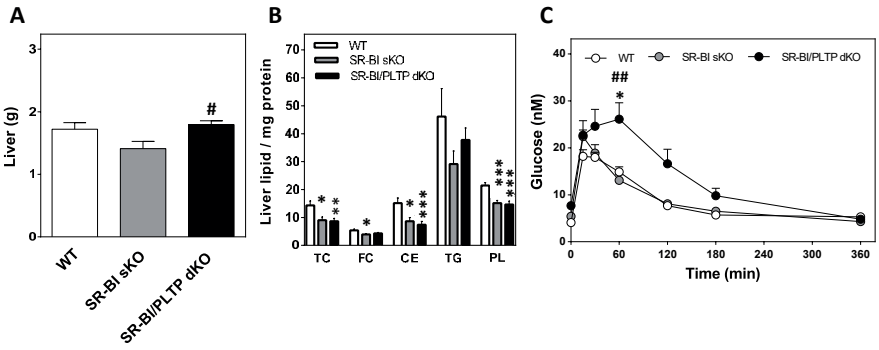

Figure 8: The effect of total body depletion of SR-BI (SR-BI sKO) and additional PLTP depletion (SR-BI/PLTP dKO) after 20 weeks WTD feeding on liver weight (A) liver lipid composition (B). The time course for a glucose tolerance test in response to a glucose bolus (C). Data represents mean+SEM (WTD n=5, SR-BI sKO n=5 and SR-BI/PLTP dKO n=8). Statistical difference of *P<0.05, **P<0.01, ***P<0.005 vs WT, ##P<0.01 versus SR-BI sKO.

Obesity is often associated with an disturbed glucose tolerance and an increased risk for type 2 diabetes mellitus [56]. In a glucose tolerance test, the SR-BI/PLTP dKO mice with an increased WAT volume displayed a worsened glucose handling with a 1.8-fold higher peak concentration at 60 min (t=60min SR-BI/PLTP dKO 26.1 ± 3.5 nM, SR-BI sKO 13.1 ± 1.2 nM, WT 14.9 ± 1.1 nM; SR-BI/PLTP dKO vs WT P<0.05, SR-BI/PLTP dKO vs SR-BI sKO P<0.01). At 360 minutes post glucose bolus, a normalization of blood glucose was recorded in all groups (t=360min SR-BI/PLTP dKO 4.8 ± 0.4 nM; SR-BI sKO 4.3 ± 0.3 nM; WT 5.3 ± 0.2 nM; P=NS) (**Figure 8C**). These data imply that the dKO mice display important signs of MetS [57] after 20 weeks WTD challenge, including elevated plasma TG levels, increased WAT volume and worsened glucose tolerance.

Next, we aimed to understand the role of PLTP in lipolysis and the link with the MetS-like phenotype observed in our SR-BI/PLTP dKO mice. Hereto a dedicated second experiment was performed on regular CHOW diet. In addition to WT, SR-BI sKO, and SR-BI/PLTP dKO mice also PLTP sKO mice were included, as to our knowledge until now no link between single PLTP deficiency and TG levels has been described. Consistent with the first study, TC values were lowered upon deletion of PLTP in SR-BI sKO mice (**Table 1 and 3**). Plasma TC levels of the PLTP sKO mice group were at WT level (**Table 3**). Under these CHOW-fed conditions TG levels (**Table 3**) and body weights (**Table 2**) were not significantly different amongst the different groups. Moreover, an oral glucose tolerance test showed no altered glucose sensitivity amongst the groups (AUC WT 69.5; SR-BI sKO 60.95; PLTP sKO 65.4; SR-BI/PLTP dKO 70.9) (**Figure 9A**).

Table 2: Organ and body weights of total body depleted SR-BI KO mice with and without additional PLTP function. Data represents mean+SEM; significant difference *P<0.05, **P<0.01, ***P<0.005 versus WT: ##P<0.01, ###P<0.005 versus SR-BI KO: $$$P<0.005 versus PLTP KO.

	BW	Liver	Spleen	Thymus	Adrenal	gWAT	iBAT	Heart
CHOW								
WT	25.8±0.88	1.25±0.044	0.10±0.008	0.03±0.005	0.02±0.001	0.82±0.014	0.06±0.004	0.14±0.006
SR-BI sKO	27.8±1.00	1.54±0.029	0.16±0.026*	0.04±0.006	0.02±0.003	0.68±0.092	0.07±0.004	0.17±0.011
PLTP sKO	28.8±0.09	1.35±0.083	0.11±0.013	0.06±0.0002**	0.07±0.001	1.35±0.300	0.06±0.005	0.15±0.015
SR-BI/PLTP dKO	31.0±1.98	1.59±0.085**	0.15.010*	0.05±0.006*	0.02±0.002	1.84±0.278**##	0.10±0.005***###$$$	0.15±0.010

Table 3: Plasma cholesterol levels of CHOW fed total body depleted SR-BI KO, PLTP KO and SR-BI/PLTP dKO mice. Data represents mean+SEM; significant difference *P<0.05, ****P<0.001 versus WT: ##P<0.01, ####P<0.001 versus SR-BI KO: $P<0.05, $$$P<0.005, $$$$P<0.001 versus PLTP KO.

	TC (mg/dL)	FC (mg/dL)	TG (mg/dL)	FC/CE ratio
CHOW				
WT	60.7±2.0	22.9±2.2	105.9±7.7	0.55±0.06
SR-BI sKO	162.9±5.1****	96.7±2.7****	88.5±9.5	1.49±0.03****
PLTP sKO	59.6±6.6####	28.3±2.8####	128.8±14.5	0.91±0.05* ##
SR-BI/PLTP dKO	114.1±7.6**** $$$	63.8±3.9**** #### $$$$	106.7±6.9	1.31±0.11**** $

Thus, the MetS-like phenotype in the SR-BI/PLTP dKO mice or underlying pathologies are not evident while feeding a regular CHOW diet with a low fat content. This allowed us to investigate if disruption of PLTP function in the SR-BI sKO mice was associated with any inherent disturbances in the metabolism of TG-rich lipoproteins independent of secondary effects.

Previous studies by Out et al. [58] showed that SR-BI facilitates the clearance of chylomicron remnants. In line with Out et al. a delayed TG response was observed in the SR-BI sKO after intragastric administration of an olive oil bolus after a 4 hour fasting period (t=3 h plasma ΔTG response; SR-BI sKO 0.29 ± 0.08 μg/μl; WT -0.07 ± 0.05 μg/μl; P<0.05), although the AUC was not affected (AUC SR-BI sKO 3.7 ± 0.2, WT 3.2 ± 0.2; P=NS). Interestingly, the PLTP sKO mice showed an even more dramatically increased plasma TG accumulation versus WT over time after olive oil administration (t=3 h plasma ΔTG response; PLTP sKO 0.45 ± 0.12 μg/μl; P<0.005 vs WT). Deletion of PLTP in the SR-BI sKO mice led to a further aggravation of the phenotype as evidenced by a 12-fold higher accumulation of TG in the circulation at the 3 hour time point (t=3 h plasma ΔTG response; SR-BI/PLTP dKO 0.85 ± 0.16 μg/μl; P<0.001 vs WT, P<0.05 vs PLTP sKO) (**Figure 9B**). In line with the augmented TG buildup over time in the PLTP sKO mice, a 1.4-fold increase was found in the AUC versus WT mice (AUC PLTP sKO 4.5 ± 0.5; P<0.05 vs WT). The SR-BI/PLTP dKO mice showed a further aggravated phenotype with a 1.7-fold increased AUC (AUC PLTP sKO 4.5 ± 0.5; P<0.001 versus WT, P<0.005 vs SR-BI sKO, P<0.05 vs SR-BI sKO) (**Figure 9C**)

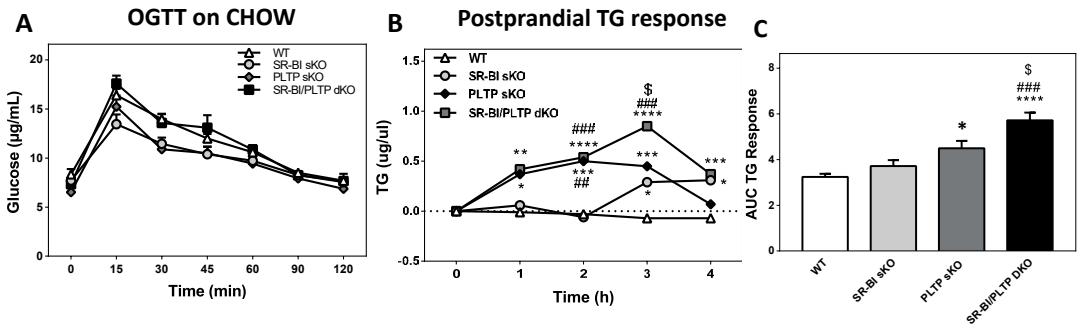

Figure 9: The time course of (A) glucose levels in response to a glucose bolus. TG levels upon a post prandial challenge with olive oil; over time (B), area under the curve (C). Data represents mean+SEM, **P<0.01, ***P<0.005, ****P<0.001 versus WT, #P<0.05, ##P<0.01 versus SR-BI sKO, $P<0.05, $$P<0.01 versus PLTP sKO.

Accumulation of TG in serum after an intragastric olive oil bolus is the nett effect of 1) absorption of TG in the intestine, 2) lipolysis of the TG-rich chylomicrons once in the circulation, and 3) removal of the particles and released free fatty acids from the circulation by tissues. Next, an in vivo clearance study was performed using triglyceride-rich VLDL-like particles, radiolabeled with glycerol [^{3}H]oleate ([^{3}H]TO) and [^{14}C]cholesteryl oleate ([^{14}C]CO), injected intravenously allowing investigation of the metabolism of these particles and tracing of their core lipids in time. [^{3}H]TO clearance from the plasma compartment was somewhat slower in PLTP KO and DKO mice as compared to both SR-BI KO and WT mice with average plasma half-lives of 5.4 and 4.0 minutes versus 2.5 and 2.3 minutes, respectively (**Figure 10A**). The associated [^{3}H]TO elimination rate constants (K) were 0.30 ± 0.10 for WT mice, 0.28 ± 0.07 for SR-BI KO mice, 0.13 ± 0.04 for PLTP KO mice, and 0.17 ± 0.10 for DKO mice (**Figure 10B**). This observation is in line with our previous findings that the plasma lipoprotein lipase (LPL) and hepatic lipase (HL) activity is similar in SR-BI KO and wild-type mice [60]. Given the general short plasma half-life of [^{3}H]TO, it is not surprising that upon sacrifice, i.e. at 15 minutes after particle injection, no difference was found amongst the different genotype groups in the uptake of [^{3}H]TO by the primary target organs heart and liver (**Figure 10C**). In accordance with the notion that in WT mice VLDL/chylomicron-remnants are rapidly cleared from the circulation, the plasma half-life of the [^{14}C]CO tracer (2.6 minutes; K: 0.26 ± 0.11) was almost similar to that of the [^{3}H]TO tracer (2.3 minutes: K: 0.30 ± 0.10; **Figures 10A & B**). In contrast, [^{14}C]CO plasma clearance was slower than that of [^{3}H]TO in SR-BI KO mice (average half-life: 3.6 minutes; K: 0.19 ± 0.0.09 for [^{14}C]CO versus 2.5 minutes; K: 0.28 ± 0.07 for [^{3}H]TO;

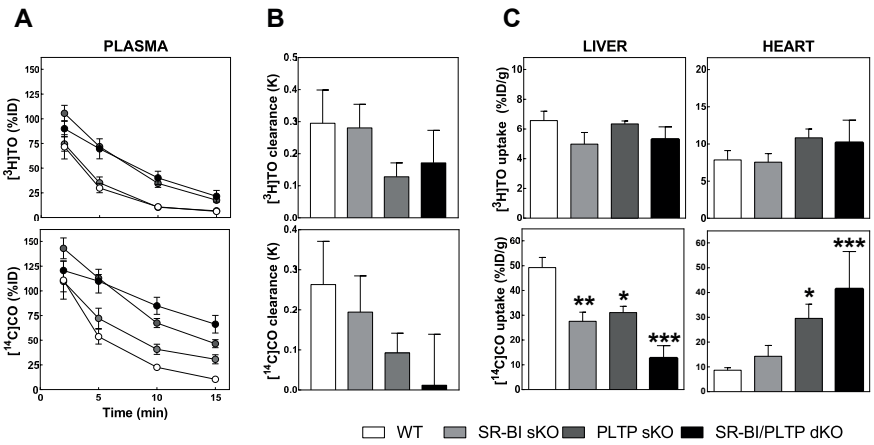

Figure 10: Plasma decay (A) and tissue uptake (C) of radiolabeled triglyceride-rich lipoprotein-associated fatty acids ([^{3}H]TO-derived activity) and cholesteryl esters ([^{14}C]CO) in chow diet–fed male mice. The calculated plasma clearance rates are shown in (B). *P<0.05, **P<0.01, ***P<0.001 vs WT.

Figures 10A & B). In further support of the suggested impact of SR-BI deficiency on the uptake of chylomicron- and VLDL-remnants by hepatocytes [59, 60], a significantly lower amount of [^{14}C]CO was recovered in SR-BI KO livers as compared to WT livers at 15 minutes after triglyceride-rich particle administration (-44 %; P<0.001; **Figure 10C**). The plasma clearance of [^{14}C]CO was also markedly delayed in PLTP KO mice (average plasma half-life: 7.5 minutes; K: 0.09 ± 0.05; **Figures 10A & B**) and this was paralleled by a reduced hepatic remnant particle uptake at 15 minutes after injection (-37 %; P<0.001). Strikingly, remnant particle clearance was even further delayed in DKO mice with an average [^{14}C]CO plasma half-life of 58 minutes (K: 0.01 ± 0.13; **Figures 10A & B**). As a result, livers of DKO mice had taken up only 26 % of the fractional dose of [^{14}C]CO that could be recovered in WT livers at 15 minutes after intravenous particle injection (P<0.001; **Figure 10C**). In contrast, a relatively high level of [^{14}C]CO tracer was detected in hearts of PLTP and DKO mice (**Figure 10C**).

Given that we have previously found that SR-BI deficiency does not alter hepatic expression levels of other receptors involved in lipoprotein remnant uptake, i.e. the LDL receptor and LDL receptor-related protein 1 (LRP1) [60], it can be suggested that in response to the PLTP deficiency a VLDL-remnant particle is generated that is highly dependent on SR-BI for its subsequent clearance by hepatocytes and therefore ultimately accumulates in alternative tissues in a SR-BI deficiency setting.

DISCUSSION

Key findings of this study are A) Deletion of PLTP reduced the SR-BI KO specific enlarged HDL particle size and B) SR-BI/PLTP dKO mice displayed a lower susceptibility to atherosclerosis despite the development of a MetS-like phenotype driven by a defect in TG lipolysis induced by the absence of PLTP. In our unique double knockout mouse model the occurrence of MetS is thus uncoupled from atherosclerosis susceptibility.

Deletion of PLTP in the SR-BI knockout background induced a massive reduction in the amount of large CE-rich HDL particles that accumulate as a result of hepatic SR-BI deficiency in these animals. This clearly indicates that the transfer of phospholipids, released during the lipolysis of TG in TG-rich lipoproteins, to HDL is essential for the enlargement of the HDL particles in SR-BI knockout mice. However, the formation of the HDL particles was not fully ablated. In line with the theory presented by Settasatian [40] we hypothesize that the remaining HDL fraction in SR-BI/PLTP dKO mice is the result of expansion of pre-β HDL particles to their maximum size of available surface PL, followed by fusion into larger CE-enriched particles through electron field differences [61]. This, in combination with the disturbed hepatic CE-uptake due to the SR-BI deficiency, resulted in the remaining levels of HDL in the circulation of SR-BI/PTLP dKO mice [62]. Further maturation of the HDL particles requires the intervention of PLTP and hence is no longer possible [63, 40].

In the general discussion about HDL, the balance tipped towards HDL functionality, not HDL-C quantity, especially for its` anti-atherogenic function [64]. HDL functionality in the reverse cholesterol transport route differs per subclass [65]. Here the in vitro data showed that plasma of the SR-BI/PLTP dKO mice with a smaller HDL particle size and quantity tends to be more effective in inducing macrophage cholesterol efflux over plasma of SR-BI sKO. Moreover, oxidative stress levels locally in the arterial wall were reduced, indicating that both the anti-oxidant and cholesterol efflux functionality of HDL was, at least partly, restored upon deletion of PLTP in the SR-BI KO mice. Atherosclerotic lesion development was however not fully prevented. While smal pre-β HDL particles effectively interact with ATP binding cassette reporter A1 (ABCA1) on macrophages to stimulate cholesterol efflux, ABCG1 and SR-BI facilitate efflux to large mature α-HDL particles [66]. The fact that atherosclerotic lesion development is not completed prevented might be a direct consequence of the absence of macrophage SR-BI. In line our group [67] and others [68,69] previously showed that deletion of SR-BI on bone marrow-derived cells induces atherosclerosis susceptibility of LDL receptor

knockout mice. On the other hand, one can argue that the impaired delivery of HDL's cholesterol cargo to the liver in absence of SR-BI and thus impairment of the last step of the RCT pathway might have been a causative factor in determining atherosclerosis susceptibility in the SR-BI/PLTP dKO mice. However, previous studies in ABCA1/SR-BI knockout mice showed that impairment of macrophage RCT is not sufficient to induce atherosclerotic lesion development [70]. The presence of pro-atherogenic lipoproteins, such as VLDL in the circulation is a prerequisite for atherosclerotic lesion development. Strikingly, SR-BI/PLTP dKO mice showed reduced atherosclerosis as compared to SR-BI sKO mice, despite increased levels of TG-rich VLDL and the development of a MetS-like phenotype (increased WAT, TG and altered fasting glucose levels) [71]. Generally, the presence of MetS is associated with an increased risk for CVD in both humans and mice [72,73]. Our study is one of the first to show an uncoupling between MetS phenotype and atherosclerosis susceptibility.

We hypothesize that the augmented levels of plasma TG triggered the expansion of WAT and subsequently led to the glucose intolerance. Wang et al. indicated an important role for SR-BI in the hepatic clearance of TG enriched ApoB containing lipoproteins (VLDL and chylomicrons)[70,74]. In line, deletion of SR-BI in mice results in elevated levels of these TG-rich lipoproteins [75]. Previous findings of Karavia et al. and our current study show that this is however not associated with weight gain and adiposity of SR-BI sKO mice on WTD [76]. Thus, the additional PLTP deletion in the SR-BI KO background is critical for induction of the MetS phenotype.

High expression levels of PLTP are found in adipose tissue in both men and mice [77,78]. In line, plasma PLTP activity is increased in obese patients [79]. PLTP in adipocytes has been implicated in the promotion of cellular cholesterol efflux [80]. PLTP deletion in adipocytes could thus have led to a disturbed adipose cholesterol balance and subsequently increased body weight and development of a MetS phenotype on WTD. Although, PLTP knockout mice have been extensively studied, until now no effects of PLTP deficiency on body weight gain have been described. It is thus likely that the phenotype is not a direct effect of PLTP deficiency in the adipose tissue, but rather an effect of the massive increase in serum TGs in the SR-BI/PLTP dKOs. Jiang X et al. previously showed that PLTP sKO mice on WTD display no change in TG levels in blood [81]. It is therefore possible that the phenotype becomes only visible on WTD when the clearance of TG-rich lipoproteins and their remnants is impaired, like in the SR-BI KO mice that lack a hepatic recognition site for TG-rich lipoproteins and their remnants [75,82]. Here, we showed that the absence of PLTP did not affect LPL-

mediated triglyceride lipolysis, but rather modified the ability of VLDL/chylomicron remnants to be cleared from the circulation by the liver through receptors other than SR-BI. As a result, livers of DKO mice only cleared 26 % of the fractional dose of [^{14}C]cholesteryl oleate after intravenous triglyceride-rich particle injection. Further mechanistic studies are required to unequivocally establish this two-way dependency of PL transfer and TG lipolysis.

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HDL IS ESSENTIAL FOR ATHEROSCLEROTIC LESION REGRESSION IN APOE KNOCKOUT MICE BY BONE MARROW APOE RECONSTITUTION

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ABSTRACT

BACKGROUND AND AIM: Although studies in mice have suggested that lesion regression is feasible, the underlying mechanisms remain largely unknown. Here we determined the impact of high-density lipoprotein (HDL) on atherosclerosis regression outcome.

METHODS: Atherosclerotic lesion dynamics were studied upon bone marrow transplantation-mediated re-introduction of apolipoprotein E (ApoE) in ApoE knockout mice. Probucol was used to pharmacologically deplete HDL.

RESULTS: Restoration of ApoE function was associated with an initial growth of atherosclerotic lesions and parallel decrease in lesional macrophage foam cell content (47 ± 4 % at 4 weeks versus 72 ± 2 % at baseline: $P<0.001$), despite the fact that cholesterol levels were markedly reduced. Notably, significant lesion regression was detected from 4 weeks onwards, when plasma cholesterol levels had returned to the normolipidemic range. As a result, lesions were 41 % smaller ($P<0.05$) at 8 weeks than at 4 weeks after bone marrow transplantation. Regressed lesions contained an even lower level of macrophage foam cells (33 ± 5 %: $P<0.001$) and were rich in collagen. Probucol co-treatment was associated with a 3.2-fold lower ($P<0.05$) plasma HDL-cholesterol level and a more pro-inflammatory (C-C chemokine receptor type 2: CCR2+) monocyte phenotype. Importantly, probucol-treated mice exhibited atherosclerotic lesions that were larger than those of regular chow diet-fed bone marrow transplanted mice at 8 weeks (186 ± $15*10^3$ μm^2 for probucol-treated versus 120 ± $19*10^3$ μm^2 for controls: $P<0.05$).

CONCLUSIONS: We have shown that probucol-induced HDL deficiency impairs the ability of established lesions to regress in response to reversal of the genetic hypercholesterolemia in ApoE knockout mice. Our studies thus highlight a crucial role for HDL in the process of atherosclerosis regression.

INTRODUCTION

Atherosclerosis is the primary underlying cause of cardiovascular disease morbidity and mortality [1]. Accumulation of cholesterol within macrophages (foam cell formation) as a result of chronic hypercholesterolemia is a hallmark in the development of atherosclerotic lesions. Despite effective lipid-lowering therapies to inhibit the generation of macrophage foam cells and the progression of atherosclerotic lesions, the worldwide incidence of cardiovascular disease remains high. In order to further reduce cardiovascular disease-related mortality and morbidity, inducing regression of established atherosclerotic lesions is considered an important therapeutic challenge. Human trials have shown that regression of existing lesions is possible under intensive lipid lowering conditions. However, the extent of lesion regression achieved with current drug treatments is limited (change in mean percentage atheroma volume: 0.7 to 1.76 %) [2-4]

Despite the fact that only small reductions in lesion size have thus far been observed in the human setting, it has become evident from mouse models that regression of lesions is feasible, i.e. under extreme lipid lowering conditions [5]. Notably, the mechanisms underlying the lesion regression observed in animal models are still subject of debate. Studies by the groups of Ed Fisher and Steve Young have suggested that macrophage emigration from the lesions is the driving force behind lesion regression. More specifically, they showed that acute elimination of hypercholesterolemia through either timed inactivation of the microsomal triglyceride transfer protein gene, i.e. in the so-called Reversa mice, or via transplantation of an atherosclerotic lesion-containing vessel into a normolipidemic wild-type mouse is able to induce a significant decline in the atherosclerotic lesion size [6-8]. In sharp contrast to the macrophage emigration hypothesis, studies in which the hypercholesterolemia in ApoE knockout mice was reversed through adenovirus-mediated reconstruction of ApoE have suggested that impaired recruitment of new monocytes and macrophage apoptosis together actually facilitate the disappearance of macrophages from established lesions [9-12]. Since the mechanism underlying lesion regression could theoretically be dependent on the animal model used, we have recently developed an additional regression mouse model that may aid in uncovering the biological pathways that contribute to lesion regression. Hereto, we used bone marrow transplantation to reintroduce ApoE in ApoE knockout mice and reverse hypercholesterolemia and induce lesion regression [13].

We hypothesize that ApoE-mediated lowering of pro-atherogenic plasma very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL)-cholesterol levels opens up the possibility for macrophage foam cells to unload, i.e. efflux their excess cholesterol to anti-atherogenic high-density lipoprotein (HDL) for subsequent reverse cholesterol transport and initiation of lesion regression [14,15]. Feig et al., already showed with the aorta transplantation model that the presence of HDL-cholesterol is of importance for the emigration of CD68+ macrophages from atherosclerotic lesions [14]. In the current study we aimed to provide additional experimental support for the hypothesis that regression of atherosclerotic lesions requires restoration of macrophage cholesterol homeostasis by reducing the influx of pro-atherogenic lipoproteins in the presence of functional cellular cholesterol efflux pathways. Hereto, we investigated (1) the atherosclerotic lesion dynamics upon bone marrow transplantation-mediated re-introduction of ApoE in ApoE knockout mice and (2) the impact of pharmacological HDL depletion on atherosclerosis regression outcome.

MATERIALS AND METHODS

IN VIVO SETUP

Animal experiments were performed in a temperature and light cycle (12 hour light: 12 hour darkness) controlled room at the Gorlaeus Laboratories of Leiden University, where the Leiden Academic Centre for Drug Research is located. All animal work was approved by the Dutch Ethics Committee and regulatory authority at Leiden University and was carried out in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

The data presented in this manuscript were derived from two independent experiments, one experiment in which we studied the lesion dynamics over time and a second study that involved the effect of probucol-induced HDL lowering on overall lesion regression outcome. In both experiments, 14-week old, in house bred, age-matched homozygous female ApoE knockout mice (C57BL/6 background) were used. At baseline, a group of 12 mice was sacrificed to determine the initial extent of lesion formation. The remaining mice were subjected to bone marrow transplantation with the aim to reverse the hypercholesterolemia. Prior to the bone marrow transplantation, bone marrow recipient mice received a 2 x 4.5 Gy X-ray total-body irradiation (0.19 Gy/min, 200KV, 4 mA) using an Andrex smart 225 Röntgen source (YXLON international).

Bone marrow from the tibias and femurs of in house bred normolipidemic C57BL/6 female donor mice was harvested, filtered to create an unicellular suspension and counted. A total amount of $5*10^6$ bone marrow cells was intravenously injected into the tail vain of the irradiated recipient mice. Throughout the bone marrow transplantation experiments, all mice were housed in individual ventilation cages and received chow diet (RM3 (E) DU: Special Diet Services, Witham, England) with or without probucol supplementation (0.025 % w/w: MP Biomedicals, Santa Ana, USA) and autoclaved water supplemented with antibiotics (83 mg/L Ciprofloxacin, 67 mg/L Polymyxin B sulfate, 6.5 g/L Sucrose) ad libitum. Mice were sacrificed at 2 and 4 weeks post transplantation in the timeline experiment and at 8 weeks post bone marrow transplantation in the probucol experiment. The data from the baseline groups of the two studies were pooled. Data from the experimental group of the probucol study that received a regular chow diet served as the 8 week data point in the timeline analysis.

At the day of sacrifice, mice were anesthetized with a mix of Xylazine (70 mg/kg), Ketamine (350 mg/kg) and Atropine (1.8 mg/kg) prior to in situ perfusion with phosphate buffered saline (pressure 100 mmHg) for 10 min via a cannula in the left ventricular apex. Organs (liver, spleen, adrenals) were harvested and stored for 24 h in 3.7 % formalin (Formal-fixx®, Shandon Scientific Ltd., UK) for tissue sectioning and further analysis.

PLASMA ANALYSES

Throughout both studies, tail vein blood from each individual mouse was obtained and collected in EDTA-coated tubes (Sarstedt, Numbrecht, Germany) for quantification of plasma lipids. Free cholesterol (FC) was determined by using an enzymatic colorimetric assay with 0.048 U/mL cholesterol oxidase (Calbiochem, San Diego, CA, USA) and 0.065 U/mL peroxidase (Sigma-Aldrich, Steinheim, Germany) in reaction buffer (1.0 KPi buffer, pH = 7.7 containing 0.01 M phenol, 1 mM 4-amino-antipyrine, 1 % polyoxyethylene-9-laurylether, and 7.5 % methanol). For the determination of total cholesterol (TC), 0.03 U/mL cholesteryl esterase (Calbiochem, San Diego, CA, USA) was added to the reaction solution. Absorbance was read at 490 nm on a Powerwave 340 from BioTek. Lipoproteins in pooled plasma were separated by means of fast protein liquid chromatography (FPLC: Superose 6 column: 3.2 x 30 mm: Smart-System, Pharmacia, Uppsala, Sweden) and the cholesterol content was analyzed as described above. Fractions 1-13 represented the non-HDL species VLDL and LDL and fractions 13-19 represented HDL. Corticosterone levels were determined in 1:200 diluted EDTA-plasma from orbital vein blood collected at sacrifice using a ^{125}I-corticosterone kit (MP Biomedicals, Santa Ana, USA) following the manufacturer's protocol.

BLOOD CELL ANALYSIS

Routine whole blood cell analysis was performed using the automated Sysmex XT-2000iV Veterinary Haematology analyzer (Sysmex Corporation) on endpoint orbital vein blood, collected in EDTA-coated tubes (Sarstedt, Numbrecht, Germany). To identify specific blood cell subsets, orbital vein blood was subjected to flow cytometric analysis. Hereto, orbital vein blood was isolated and red blood cells were removed using an ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH = 7.3). The leukocyte fractions were stained with the following antibodies: CD4-PerCP (clone RM4-5: BD Biosciences), CD8-APC (clone 53-6.7: eBioscience), Ly6G-FITC (clone 1A8: eBioscience), Ly6C-PE (clone HK1.4: eBioscience). Flow cytometry was performed on a FACS Canto II (Becton Dickinson, Mountain View, CA, US). Data were analyzed using FACS DIVA software (BD Biosciences).

ANALYSIS OF GENE EXPRESSION BY REAL-TIME QUANTITATIVE PCR

Quantitative gene expression analysis was performed as previously described [16]. In brief, total RNA was isolated using a standard phenol/chloroform extraction method and reverse transcribed using RevertAid Reverse Transcriptase. Gene expression analysis was performed using SYBR-Green technology (Eurogentec) on a 7500 Fast Real time PCR system (Applied Biosystems, Foster city, US). Primers were validated for an equal efficiency. Ratios of the target gene expression were determined by subtracting the Ct of the target gene from the average Ct of the housekeeping genes and raised by 2 to the power of this difference. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribosomal protein lateral stalk subunit P0 (36b4) and beta-actin (β-actin) were used as the housekeeping genes. General information of the primers used is summarized in **Table 1**.

Table 1: Primers used for QPCR analysis.

Name	Ref Seq (mRNA)	Primer Forward	Primer Reverse
36B4	NM_007475	5′-CTGAGTACACCTTCCCACTTACTGA-3′	5′-CGACTCTTCCTTTGCTTCAGCTTT-3′
B-ACTIN	NM_007393	5′-AACCGTGAAAAGATGACCCAGAT-3′	5′-CACAGCCTGGATGGCTACGTA-3′
GAPDH	NM_008084	5′-ATCCTGCACCACCAACTGCTTA-3′	5′-CATCACGCCACAGCTTTCCAG-3′
CD68	NM_001291058	5′-TGCCTGACAAGGGACACTTCGGG-3′	5′-GCGGGTGATGCAGAAGGCGATG-3′
ApoE	NM_009696	5′-CCGTGCTGTTGGTCACATTGC-3′	5′-AGCGCAGGTAATCCCAGAAGC-3′

HISTOLOGICAL ANALYSIS

Atherosclerotic lesion quantification was performed in the aortic root. Hereto, 10 µm cryostat sections (Leica CM 350S cryostat) were collected from OCT compound (Tissue-Tek, Sakura Finetek, Alphen a/d Rijn, The Netherlands) embedded hearts starting at the appearance of the aortic valves up to 300 µm into the ascending aorta. Sections were stained for neutral lipids with Oil red O and counterstained with hematoxylin (Sigma-Aldrich, Steinheim, Germany). Collagen was visualized by a Masson's trichrome staining (Sigma-Aldrich, Steinheim, Germany) according to the manufacturer's instructions. Lesional foam cell content was determined based on their foamy appearance in the Masson's Trichrome stained atherosclerotic lesions. Blinded quantification of each staining was performed on 5 sections per mouse using Leica Qwin Imaging software (Leica Ltd., Cambridge, UK). Adrenal morphology and lipid content were examined on 10 µm cryostat sections embedded in OCT compound. A hematoxylin-eosin staining was performed for general morphology and the neutral lipid content was visualized using an Oil red O staining.

IN VITRO MACROPHAGE CHOLESTEROL EFFLUX

To obtain peritoneal macrophages for in vitro cholesterol efflux experiments, C57BL/6 mice were injected intraperitoneally with 1 ml 3 % Brewer's thioglycollate medium. At 5 days after injection, the peritoneal cavity of the mice was lavaged with 10 ml cold PBS to collect thioglycollate-elicited peritoneal macrophages [17]. Thioglycollate-elicited peritoneal macrophages were cultured overnight in DMEM containing 10 % fetal calf serum for optimal adherence. Subsequently, the fetal calf serum was removed and cells were washed with PBS. Next, the cells were exposed to 0.5 µCi [^{3}H]-cholesterol (Perkin Elmer, Waltham, MA, US) in DMEM with 0.2 % fatty acid free BSA which was removed after 24 h. The ability of the macrophages to efflux the incorporated radioactive cholesterol towards plasma lipoproteins, i.e. HDL, was then studied by incubation with 2.5 % (v/v) plasma of ApoE knockout mice transplanted with wild-type bone marrow fed the chow diet with or without probucol supplementation. Cholesterol efflux was measured after 4 h and calculated as the percentage of radioactivity in the medium compared with the total amount of radioactivity in the medium and cells.

STATISTICAL ANALYSES

Statistical analysis was performed using GraphPad Instat Software (San Diego, CA, USA, http://www.graphpad.com). Normality testing was performed using the method of Kolmogorov and Smirnov. Significance was calculated using a two-tailed Student's t-test or two-way ANOVA with Bonferroni's post-test where appropriate. $P<0.05$ was considered significant.

RESULTS

To provide more insight into the dynamics of atherosclerosis regression, we monitored the impact of reversal of the genetic hypercholesterolemia on atherosclerotic lesion size and composition in ApoE knockout mice over time. Hereto, ApoE-containing C57BL/6 wild-type bone marrow was transplanted into lethally irradiated 14-week old ApoE knockout mice with macrophage foam cell-rich early lesions.
ApoE mRNA expression was measured in liver specimens to verify bone marrow transplantation-induced repopulation of hepatic ApoE-expressing macrophages (Kupffer cells) at 8 weeks after transplantation. No functional ApoE mRNA could be detected in the baseline group of mice sacrificed before bone marrow transplantation. However, in line with an effective repopulation of bone marrow-derived cells, a gradual increase in hepatic ApoE expression could be observed over time in mice transplanted with C57BL/6 wild-type bone marrow (**Figure 1A**).

The restored hepatic uptake of VLDL particles upon re-constitution of ApoE production by bone marrow-derived cells in ApoE knockout mice [18] was associated with a time-dependent decline in plasma total cholesterol levels. As can be appreciated from **Figure 1B**, total cholesterol levels were 2-fold lowered ($P<0.001$) at 2 weeks after bone marrow transplantation and reached wild type normolipidemic values of ≤100 mg/dL [19] already at the 4 week time point. Plasma cholesterol levels continued to decrease, translating in a 5.8-fold lowering ($P<0.001$) as compared to baseline levels at 8 weeks after bone marrow transplantation. In contrast, no change in plasma triglycerides (TG) was observed during the course of the study (**Figure 1C**). Lipoprotein distribution analysis validated that the observed decrease in plasma total cholesterol levels could be attributed to a decrease in VLDL/LDL (non-HDL)-cholesterol levels (**Figure 1D**).

Infiltration of monocytes into the arterial wall where they are converted into macrophages and become foam cells is a hallmark of atherosclerosis development. Hence monocyte counts are an important predictor of atherosclerosis susceptibility [20]. Blood specimens collected at each time point were subject to routine hematological analysis to uncover a potential effect of the bone marrow transplantation procedure and the lipid lowering on blood cell composition. In accordance with the notion that proliferating cells are highly susceptible to irradiation-induced death, a clear trend towards a deprivation of blood lymphocytes could be observed at 2 weeks after bone marrow transplantation ($3.54 \pm 0.17*10^9$/L for baseline versus $1.41 \pm 0.19*10^9$/L for T = 2w: $P=0.18$: **Figure 1E**). The initial decrease in blood lymphocyte concentrations

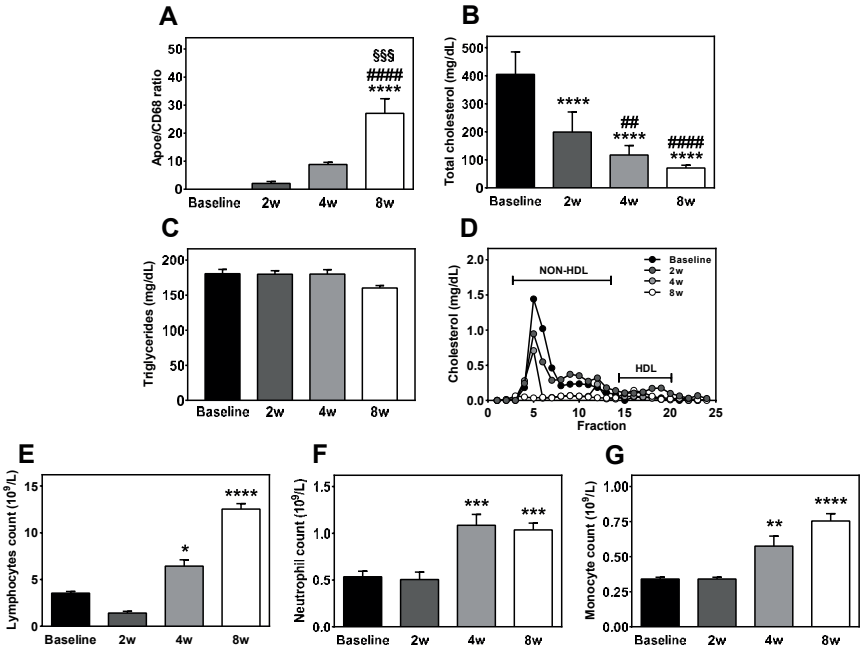

Figure 1: Bone marrow transplantation-induced reconstitution of ApoE in ApoE knockout mice increased hepatic ApoE expression levels over time (A), lowered plasma total cholesterol levels (B), did not change triglyceride levels over time (C), depleted the non-HDL fraction (D) and was associated with a time-dependent effect on blood leukocyte counts (E, F, G). All data represent means+SEM with baseline n=24, T = 2w n=12, T = 4w n=13, T = 8w n=10. *P<0.05, **P<0.01, ***P<0.005, ****P<0.001 vs. baseline: ##P<0.01, ###P<0.005 vs. T = 2w: $$$P<0.005 vs. T = 4w.

was followed by a full restoration at 4 weeks and a marked overshoot at 8 weeks after transplantation (T = 8w: 12.54 ± 0.57*10^9/L: P<0.001 versus baseline). No irradiation-associated decrease in blood neutrophil or monocyte levels was noted in the 2 weeks after the bone marrow transplantation, probably due to the fact that these immune cells are relatively resistant to irradiation damage. Blood counts of these latter two cell types were actually increased from 4 weeks onwards in wild-type bone marrow transplanted ApoE knockout mice as compared to those in ApoE knockout mice at baseline (**Figure 1F and G**).

The extent of atherosclerosis in the different groups of mice was quantified in the aortic root. At baseline, ApoE knockout mice exhibited atherosclerotic lesions of 98 ± 10*10^3 μm^2 that were rich in macrophage foam cells (**Figure 2A and B**). Despite the fact that cholesterol levels were already significantly reduced at 2 weeks after bone

marrow transplantation, significant growth of the atherosclerotic lesions was observed, reaching a size of 202 ± 14*10^3 μm^2 at 4 weeks post bone marrow transplantation ($P<0.005$). Lesion composition analysis indicated that the increase in size was paralleled by a significant decrease in the foam cell core in the 4 weeks after bone marrow ApoE reconstitution (**Figure 2B**). Lesional collagen content was not affected during the lesion progression (**Figure 2C**), but the relative necrotic area was doubled ($P<0.01$: **Figure 2D**). Importantly, as can be appreciated from **Figure 2A**, the initial progression of the lesions up to 4 weeks after ApoE re-introduction was followed by significant lesion regression during the following 4 weeks. As such, the average size of the atherosclerotic lesions at 8 weeks after bone marrow transplantation was 41% smaller ($P<0.05$) than that at 4 weeks post bone marrow transplantation and not significantly different from that at baseline. The relative contribution of macrophage foam cells to total lesion area was even further decreased during the lesion regression phase to 33 ± 5 % at 8 weeks after transplantation (**Figure 2B**). Notably, the collagen content was 1.4-fold higher in regressing lesions (T = 8w) as compared to lesions in the progression phase (T = 4w) (**Figure 2C**). It thus appears that atherosclerosis regression is associated with a switch from a highly cellular (foam cell-rich) lesion towards a more acellular (collagen-rich) lesion phenotype. When taking the blood cholesterol levels and lesion dynamics profile into account, it can be suggested that lesion regression, as defined by a decrease in lesion size, initiates when plasma cholesterol levels are decreased to those found in normolipidemic wild-type mice (≤100 mg/dL).

Apolipoprotein A1 (ApoA1)-containing HDL, through interaction with the membrane proteins ATP-binding cassette transporter A1 (ABCA1) and G1 (ABCG1) and scavenger receptor BI (SR-BI) can acquire excess cholesterol from macrophage foam cells for subsequent transport to and excretion by the liver [21]. The lipid-lowering drug probucol is a potent inhibitor of ABCA1-mediated cholesterol efflux as it interferes with cellular ApoA1 binding [22] and lowers the availability of ABCA1 on the membrane [23]. As such, in vivo probucol treatment is associated with an impairment of ABCA1-mediated lipidation of HDL particles and functional HDL deficiency [24-26]. To show the effect of pharmacological reduction of HDL by probucol treatment on lesion regression, a transplantation of wild-type bone marrow into ApoE knockout mice was combined with administration of probucol (0.25 % w/w) via the chow diet.

As demonstrated in **Figure 3A**, probucol treatment did not change the ability of bone marrow cell-derived ApoE to reverse the genetic hypercholesterolemia. Cholesterol levels decreased rapidly upon transplantation with wild-type bone marrow in both

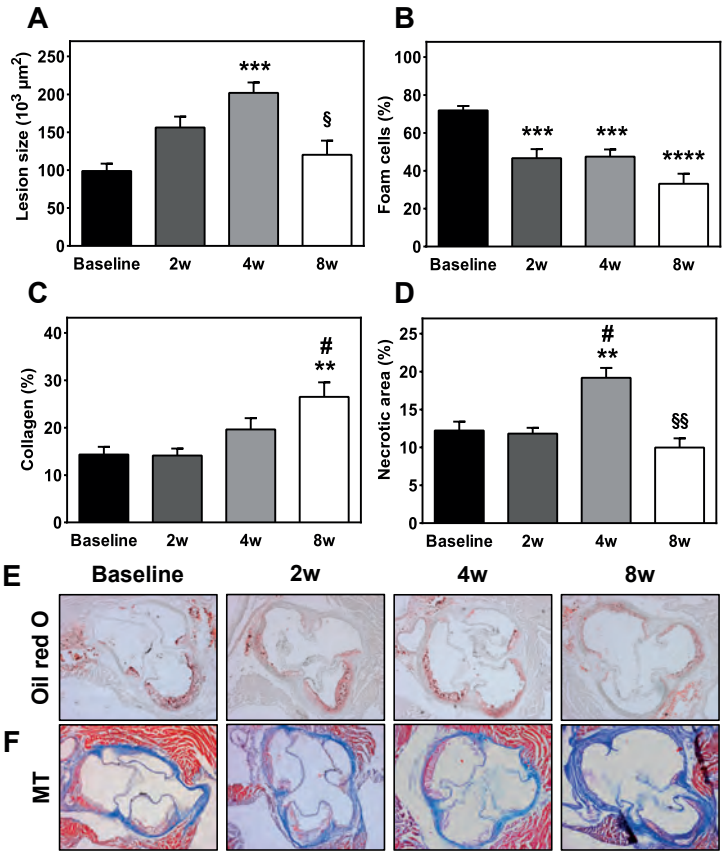

Figure 2: Bone marrow transplantation-induced reconstitution of ApoE in ApoE knockout mice was associated with lesion progression followed by lesion regression (A), a gradual decrease in lesional macrophage foam cell content (B), an increase in lesional collagen content (C), and a time-dependent increase in lesional necrotic core size (D). Representative pictures of Oil red O and Masson's trichrome (MT)-stained aortic root sections used for the quantification of the composition are shown in panels E and F (original magnification 5X). Data represent means + SEM with baseline n=24, T = 2w n=12, T = 4w n=13, T = 8w n=10, **P<0.01, ***P<0.005, ****P<0.001 vs. baseline: #P<0.05 vs. T = 2w: §P<0.05, §§P<0.01 vs. T = 4w.

treatment groups, as expected. However, plasma total cholesterol levels were consistently lower in probucol-treated mice as compared to regular chow diet-fed mice. FPLC-based lipoprotein fractionation performed at sacrifice -8 weeks after bone marrow transplantation - confirmed that the decrease in plasma total cholesterol levels could be attributed to the probucol-induced HDL deficiency (**Figure 3B**). HDL-cholesterol levels were 3.2-fold lower (P<0.001) in probucol-treated mice as compared

to controls. In accordance with the notion that the decrease in HDL levels impairs the ability to induce cholesterol efflux from macrophages, the extent of [^{3}H]-cholesterol efflux from wild-type macrophages to plasma from probucol-treated mice was significantly lower than that to plasma from non-supplemented chow diet-fed mice (10.6 ± 0.8 % for probucol versus 17.2 ± 0.8 % for control: P<0.001: Figure 3C). Our previous studies have suggested that, in normolipidemic C57Bl/6 mice, HDL, via an interaction with SR-BI, delivers the cholesterol substrate for glucocorticoid production to the

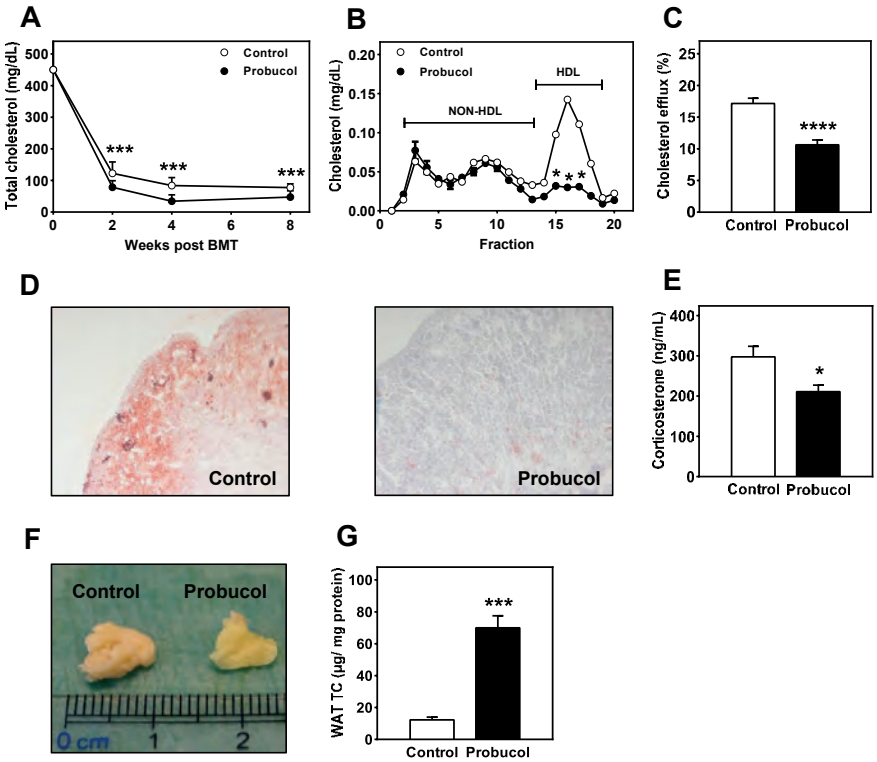

Figure 3: Probucol treatment in ApoE knockout mice transplanted with ApoE-containing bone marrow resulted in a decrease in plasma cholesterol levels (A), which could be attributed to HDL depletion (B: 8 week time point). Plasma from probucol-fed mice as compared to regular chow diet-fed mice exhibits a diminished capacity to induce cholesterol efflux from cultured C57BL/6 wild-type macrophages (C). Probucol supplemented diet feeding is associated with adrenal neutral lipid depletion as compared to control chow diet feeding (D)(original magnification 5X) and a decrease in plasma corticosterone levels (E). Probucol treatment is associated with a yellowish appearance of white adipose tissue (F) and an increase in white adipose tissue total cholesterol levels (G). All data represent means+SEM with control n=10 and probucol n=7. *P<0.05, ***P<0.005, ****P<0.001 vs. control.

adrenals [27]. Histological examination of adrenal sections revealed that control mice displayed neutral lipid positive areas within the adrenal cortex, while this was absent in probucol-fed mice (**Figure 3D**). In addition, levels of the primary glucocorticoid species corticosterone were 29 % lower in sacrifice plasma from probucol-fed mice ($P<0.05$: **Figure 3E**). Probucol treatment thus not only diminished the plasma cholesterol efflux capacity but also effectively impaired HDL's function in the delivery of cholesterol to the adrenals. Interestingly, during sacrifice, it was noted that the white adipose tissue of probucol-treated mice exhibited a yellowish appearance (**Figure 3F**).

Krinsky et al. [28] have shown that HDL has the capacity to take up and transport carotenoids, a colored pigment anti-oxidant As such, HDL depletion could induce an accumulation of carotenoids in organs like white adipose tissue. Given that probucol is a highly lipophilic substance, it is anticipated that probucol itself also accumulated in fat tissue. The yellowish appearance can thus also be the result of the accumulation of probucol catabolites that appear green/yellowish [23,29]. In support of probucol storage and the associated inhibition of ABCA1 functionality and cholesterol efflux from adipocytes, we observed that the cholesterol content was markedly higher (6-fold: $P<0.005$) in adipose tissue of probucol-treated mice as compared to that of regular chow diet-fed controls (**Figure 3G**).

In addition to HDL anti-atherogenic role in cholesterol efflux, HDL can also directly impact on the pro-atherogenic immune function of monocytes. More specifically, Han et al. have shown that exposure of HDL to monocytes in vitro lowers the expression of the C-C chemokine receptor type 2 (CCR2), leading to a reduced monocyte migration towards the pro-inflammatory cytokine CCL2/MCP-1 [30]. Monocyte numbers in the blood circulation were not changed by probucol exposure (**Figure 4A**). However, in further support of the induction of functional HDL deficiency upon probucol treatment, the fraction of the circulating monocytes expressing CCR2 was significantly higher in probucol-treated mice (61 ± 2 % for probucol-treated mice versus 47 ± 2 % for control mice: $P<0.005$: **Figure 4B**). As indicated by Swirski et al., the spleen acts as a reservoir for CCR2+ monocytes [31]. In line with the change in the circulating monocyte profile, probucol-treated mice displayed an increased splenic size (data not shown).

Next, the primary readout of our experiment was analyzed, the effects on atherosclerotic lesion regression. At 8 weeks after ApoE re-introduction, atherosclerotic lesions in both groups of mice were low in macrophage foam cell numbers (**Figure 5B**) and contained an equally large necrotic core area (**Figure 5C**). Interestingly, lowering HDL resulted in

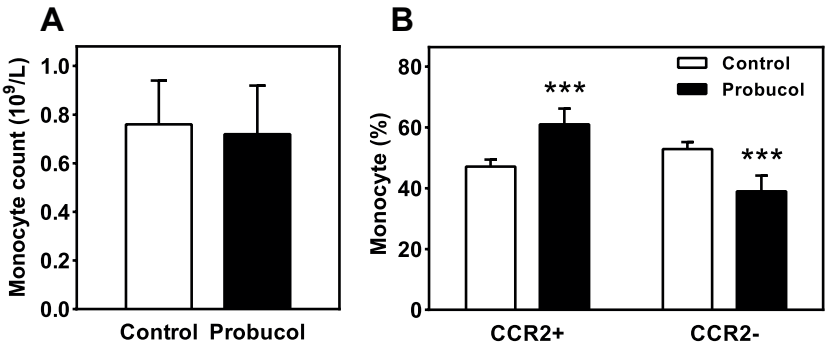

Figure 4: Probucol-induced HDL lowering did not change blood monocyte counts in ApoE knockout mice reconstituted with ApoE-positive bone marrow (A), but did increase the relative fraction of monocytes carrying CCR2 (B). Data represents means+SEM with control n=10 and probucol n=7 mice. ***P<0.005 vs. control.

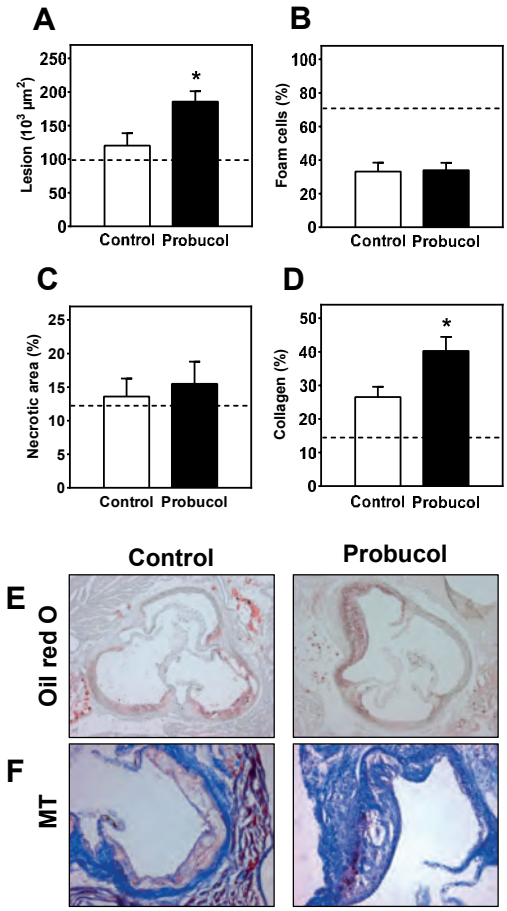

Figure 5: Probucol treatment was associated with larger atherosclerotic lesions versus control at 8 weeks post reconstitution of ApoE-positive bone marrow into ApoE knockout mice (A) an unchanged lesion macrophage foam cell and necrotic area content (B & C) and an enlarged collagen content (D). Data represent means +SEM with control n=10 and probucol n=7. *P<0.05 vs. control. Representative photomicrographs of Oil red O-stained and Masson's Trichrome (MT)-stained aortic root lesions are shown in panels E and F (original magnification Oil Red O: 5X and MT: 10X, respectively).

an increase in the amount of collagen in the lesions (**Figure 5D**). Importantly, as evident from **Figure 5A**, probucol-treated mice exhibited atherosclerotic lesions that were significantly larger than those of regular chow diet-fed bone marrow transplanted mice ($186 \pm 15*10^3 \mu m^2$ for probucol-treated mice versus $120 \pm 19*10^3 \mu m^2$ for control mice: $P<0.05$). The changes in plasma HDL-cholesterol levels, cholesterol efflux capacity, glucocorticoid output and inflammation status in response to probucol treatment were thus apparently accompanied by a marked impairment in the ability of aortic root atherosclerotic lesions to regress.

DISCUSSION

Here we aimed to provide proof for the hypothesis that HDL is essential for the regression of established atherosclerotic lesions observed in response to re-introduction of ApoE in ApoE knockout mice through bone marrow transplantation. The key findings of the current study are that, in our experimental mouse model, regression of existing lesions is initiated when plasma cholesterol levels are lowered to those within the normolipidemic range (<100 ng/mL) and that probucol-induced depletion of HDL impairs the ability of established lesions to regress.

We validated our previous findings that bone marrow transplantation-induced reversal of hypercholesterolemia in ApoE knockout mice is associated with a decrease in lesional macrophage foam cell content [13]. Our lesion dynamics analysis indicated that the initial decrease in macrophage foam cell content became evident as early as 2 weeks after bone marrow transplantation. In line, Feig et al. showed, using the Reversa mice, a gradual decrease in plaque macrophage content up till 4 weeks after the induction of lesion regression [8]. Studies using adenovirus-mediated replenishment of ApoE in ApoE knockout mice have suggested that the decrease in plaque macrophage content in response to lipid lowering can be attributed to a decrease in monocyte infiltration into the lesions in the context of an unchanged macrophage apoptosis rate [9]. Similarly to our findings, Potteaux et al. did not detect a decrease in absolute blood monocyte numbers upon ApoE re-introduction [9]. The decrease in monocyte infiltration observed in the study of Potteaux et al. appeared to be driven by a change in monocyte activation status. Using flow cytometry we could verify the observation of Potteaux et al. that lipid lowering does not change the ratio between $LyC6^{high}$ and $LyC6^{low}$ monocytes (data not shown). Unfortunately, we did not include in our flow cytometry panel the monocyte activation marker CD62L that was changed in the Potteaux study. As such, we cannot not exclude that the monocyte

activation status was changed after bone marrow transplantation-mediated ApoE re-introduction. However, given that (1) cholesterol lowering preceded the inhibition of monocyte recruitment into plaques in the ApoE adenovirus studies by Potteaux et al. and (2) our ApoE knockout mice still displayed significant hypercholesterolemia at 2 weeks after bone marrow transplantation and actually showed lesion progression, we anticipate that the initial decrease in plaque cellularity is not driven by a change in the monocyte infiltration rate. In our experimental setup we subjected the mice to total body irradiation to be able to efficiently transplant ApoE-containing wild-type bone marrow and thereby lower plasma cholesterol levels. Studies by Lorimore et al. have indicated that macrophages become activated and display secondary necrosis as a bystander effect in whole body irradiation-induced tissue damage in vivo [32]. It is therefore suggested that the decrease in lesional cellular content / macrophages in the initial (lesion progression) stage is rather due to irradiation-related necrosis and not primarily the result of a change in the lipid environment.

Given the relatively long time between the irradiation and the start of atherosclerosis regression (>4 weeks), other mechanisms should underlie the continuing decrease in macrophage disappearance from the lesions at the later time points, i.e. in the atherosclerosis regression phase. In this context, it is interesting to note that ApoE can facilitate anti-atherogenic cholesterol efflux [33,34]. It is therefore possible that ApoE secreted by newly infiltrated macrophages within the lesion might locally promote cholesterol efflux via ABCA1 (without intermediary involvement of HDL). However, it should be noted that the in vivo studies by Van Eck et al. have indicated that the change in serum cholesterol levels is the dominant factor in predicting the effect of ApoE on lesion development [34]. To explain the mechanism that underlies regression, it is important to take our findings regarding the effect of probucol treatment on atherosclerotic lesion regression into account. Probucol treatment on top of ApoE re-introduction was associated with functional HDL deficiency, a more pro-inflammatory / infiltrating (CCR2+) blood monocyte phenotype, decreased steroidogenesis and an apparent resistance to atherosclerosis regression. The observed shift in monocyte phenotype towards a more infiltrating subtype may also have contributed to the impairment of the regressive capacity of the lesions in probucol-treated mice. In support, Rahman et al. showed that progressive lesions have the blood signature of a pro-inflammatory monocyte phenotype, while the conversion towards anti-inflammatory monocyte is required to induce regression of lesions [35]. However, previous studies by the group of Ed Fisher have suggested that plasma HDL deficiency alone is already sufficient to disrupt the lesion regression capacity. They showed that transplantation

of an atherosclerotic lesion-rich aorta from hypercholesterolemic ApoE knockout mice into wild-type mice resulted in a rapid disappearance of lesional macrophages [36], an effect that was largely diminished when aortas were transplanted into HDL deficient ApoA1 knockout mice [14].

Further studies by Feig et al. have suggested that HDL, through lowering the cellular lipid content, increases the expression of the chemokine receptor CCR7 that facilitates macrophage emigration [14]. Given that blockade of CCR7 function significantly impairs the ability of macrophages to migrate out of the lesion, limiting the lesion regression potential in the aorta transplantation model [7], we highly likely assume that the functional HDL deficiency is the primary underlying cause of the detrimental effect of probucol treatment on lesion regression. Although we cannot exclude that besides the HDL lowering effect of probucol, additional probucol associated anti-oxidative mechanisms affect the lesion development. Additional studies will be needed to provide proof for the involvement of CCR7 in the effect of HDL on atherosclerosis regression in our current experimental regression mouse model.

Our recently established bone marrow transplantation-based atherosclerosis regression mouse model supplements the aforementioned aorta transplantation-based, Reversa, and ApoE adenovirus-based regression models first generated by respectively the groups of Ed Fisher [38, 36] Steve Young [37] and Dan Rader [10]. The current study highlights that (1) the absence of HDL in the plasma compartment limits the atherosclerosis regression potential in at least two out of 4 of the experimental models and (2) that this effect is not dependent on the exact cause of the HDL deficiency, i.e. genetic ablation of apolipoprotein A1 function versus pharmacological disruption of HDL lipidation through probucol treatment. Intriguingly, probucol treatment has been shown to increase the rate of reverse cholesterol transport [38], which should theoretically enhance the regression capacity. However, the effect of probucol on reverse cholesterol transport described by Yamamoto et al. [38] appears to be mainly driven by effects locally within the liver and not at the level of the macrophages. As such, we anticipate that increasing HDL-mediated cholesterol efflux through pharmacologically stimulating specifically the expression of the cellular cholesterol transport proteins interacting with HDL, i.e. ABCA1, ABCG1 and SR-BI, in macrophages is of clear therapeutic interest in the context of atherosclerosis regression. In further support of this notion, we have previously already shown that liver X receptor-mediated induction of ABCA1 and ABCG1 expression increases the ability of macrophages to disappear from established atherosclerotic lesions in our regression mouse model

[13]. Although we assume that ABCA1 should be considered the primary therapeutic target given its crucial role in the protection against macrophage foam cell formation and atherogenesis [39], additional mechanistic studies in the three regression mouse models are warranted to show the exact impact of the individual gene products involved in macrophage cholesterol efflux on atherosclerosis regression.

In conclusion, we have shown that probucol-induced HDL deficiency impairs the ability of established lesions to regress in response to reversal of the genetic hypercholesterolemia in ApoE knockout mice. Our studies thus highlight a crucial role for HDL in the process of atherosclerosis regression.

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3

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4

VLDL/LDL SERVES AS THE PRIMARY SOURCE OF CHOLESTEROL IN THE ADRENAL GLUCOCORTICOID RESPONSE TO FOOD DEPRIVATION

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Menno Hoekstra

ABSTRACT

The contribution of individual lipoprotein species to the generation of the adrenal cholesterol pool used for the synthesis of anti-inflammatory glucocorticoid species remains unknown. Here we examined the impact of specific lowering of very low-density lipoprotein (VLDL) and low-density (LDL) levels on adrenal cholesterol and glucocorticoid homeostasis. Hereto, lethally-irradiated hypercholesterolemic apolipoprotein E (ApoE) knockout mice received ApoE -containing bone marrow from wild-type mice (n=6) or ApoE knockout control bone marrow (n=10) and were subsequently fed a regular chow diet. Transplantation with wild-type bone marrow was associated with a 10-fold decrease in VLDL/LDL-cholesterol levels. No changes were observed in adrenal weights, adrenal cholesterol content, or basal plasma corticosterone levels. However, food deprivation-induced corticosterone secretion was 64 % lower ($P<0.05$) in wild-type bone marrow recipients as compared to ApoE knockout bone marrow recipients, in the context of similar plasma adrenocorticotropic hormone (ACTH) levels. A parallel 19-29 % decrease in adrenal relative mRNA expression levels of ACTH-responsive genes SR-BI ($P<0.01$), StAR ($P<0.05$), and CYP11A1 ($P<0.05$) was detected. In support of relative glucocorticoid insufficiency, blood lymphocyte and eosinophil concentrations were respectively 2.4-fold ($P<0.01$) and 8-fold ($P<0.001$) higher in wild-type bone marrow recipients under fasting stress conditions.

In conclusion, we have shown that a selective lowering of VLDL/LDL levels in hyperlipidemic ApoE knockout mice through a transplantation with ApoE-containing wild-type bone marrow is associated with a decreased maximal adrenal glucocorticoid output. Our studies provide experimental support for the hypothesis that, in vivo, VLDL/LDL serves as the primary source of cholesterol used for glucocorticoid synthesis under high stress conditions.

INTRODUCTION

Steroid producing cells such as adrenocortical cells have a dual need for cholesterol. Besides their use of cholesterol in general cellular processes like the maintenance of membrane functionality [1,2], adrenocortical cells need to acquire sufficient levels of cholesterol to serve as precursor for the synthesis of steroid hormones, i.e. glucocorticoids [3-6].

Although it is well established that cholesterol is the sole precursor for the synthesis of glucocorticoids such as cortisol in humans and corticosterone in rodents, the exact contribution of different sources to the pool of cholesterol used for adrenal steroidogenesis remains unknown to date. Statin treatment does not impact on cortisol levels in humans [7,8], which argues against an essential role for de novo cholesterol synthesis in the generation of the cholesterol substrate. In contrast, low-density lipoprotein (LDL) deficient human subjects that carry functional mutations in the apolipoprotein B100 (ApoB100) gene display a reduced capacity of the adrenals to produce cortisol [9]. Moreover, subjects carrying a deleterious mutation in the high-density lipoprotein (HDL) receptor scavenger receptor class B type I (SR-BI) gene also display signs of glucocorticoid insufficiency [10]. It thus appears that, in humans, both LDL and HDL supply the adrenals with a significant amount of the cholesterol substrate that is needed for steroidogenesis. In accordance with the assumption that lipoproteins also act as essential cholesterol source in rodents, an estradiol treatment-induced reduction in plasma cholesterol levels is associated with a diminished adrenal glucocorticoid response to an adrenocorticotropic hormone (ACTH) challenge [3]. A specific disruption of HDL-mediated adrenal cholesterol acquisition generally replicates the lipoprotein deficiency-associated reduction in adrenal cholesteryl ester stores and maximal glucocorticoid output in normolipidemic mice [5,6,11] Notably, studies in cultured adrenocortical cells and gene targeted mice by Kraemer et al. and Li et al. have indicated that hormone-sensitive lipase (HSL)-mediated breakdown of lipoprotein-derived cholesteryl esters is a perquisite to effectively generate the steroidogenic cholesterol pool used under high steroidogenic pressure conditions [12,13]. In agreement with the notion that HDL is an effective cholesterol donor in mice carrying a human-like lipoprotein profile, we have recently observed an anticipated depletion of the adrenal cholesteryl ester stores in hypercholesterolemic LDL receptor knockout mice that genetically lack HDL [14]. Interestingly, these HDL deficient ApoA1 x LDL receptor double knockout mice did – however – not display the expected glucocorticoid insufficiency phenotype. More specifically, they exhibited

a normal plasma corticosterone response to both overnight fasting and the induction of endotoxemia [14]. From these latter observations, it can be hypothesized (1) that the adrenal cholesteryl ester pools are not the primary determinant for the overall steroidogenesis rate in mice or (2) that mice can compensate the loss of HDL-derived cholesterol pool by using the non-HDL lipoprotein fractions as source of the steroidogenic cholesterol substrate. Apolipoprotein E (ApoE) knockout mice lacking a functional ApoE gene exhibit relatively high cholesterol levels associated with non-HDL species, i.e. very low-density lipoprotein (VLDL)- and LDL particles, in the context of normal plasma HDL-cholesterol levels [15], which is paralleled by hypercorticosteronemia [16]. To uncover the contribution of VLDL/LDL to the adrenal glucocorticoid output, in the current study we investigated the effect of a specific lowering of VLDL/LDL-cholesterol levels on adrenal cholesterol and glucocorticoid homeostasis in ApoE knockout mice.

MATERIALS AND METHODS

IN VIVO SETUP

Animal experiments were performed at the Leiden Academic Centre for Drug Research within the Gorlaeus Laboratories of Leiden University. All animal work was approved by the Dutch Ethics Committee and regulatory authority at Leiden University and was carried out in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

All experimental mice were held in a 12 hour light /12 hour dark light-cycle and temperature controlled room. From one week prior until two weeks after the bone marrow transplantation, male ApoE knockout recipient mice were provided with autoclaved drinking water supplemented with antibiotics (83 mg/L ciprofloxacin, 67 mg/L polymyxin B sulfate, 6.5 g/L sucrose). Bone marrow aplasia was induced in recipient mice by exposing them to 2x 4.5 Gy total body X-ray irradiation (0.19 Gy/min, 200 kV, 4 mA) using an Andrex Smart 225 Röntgen source (YXLON International, Copenhagen, Denmark) with a 6-mm aluminium filter. The next day, bone marrow from the tibias and femurs of C57BL/6 wild-type and ApoE knockout donor mice was harvested, filtered to create a unicellular suspension and counted. A total amount of $5 * 10^6$ bone marrow cells were subsequently intravenously injected into the tail vain of the irradiated recipient mice. Mice that had received the bone marrow were

individually housed in sterilized filter top cages and fed sterilized chow diet (RM3 (E) DU; Special Diet Services, Witham, England) ad libitum. At 9 weeks after bone marrow transplantation, all mice were bled via a tail cut at 09:00 in the "non-stressed" ad libitum fed state for plasma corticosterone analysis. Food deprivation overnight, i.e. during the regular active eating phase, is considered a stressful event for mice and consistently associated with a rise in adrenal glucocorticoid output and blood glucocorticoids levels (unpublished data and [6,11]). At ten weeks after the bone marrow transplantation, mice were therefore subjected to overnight food deprivation from 05:00 PM and tail bleeding at 09:00 for plasma "fasting stress" corticosterone analysis. Subsequently, mice were injected with a mix of xylazine (70 mg/kg), ketamine (350 mg/kg) and atropine (1.8 mg/kg), bled via the eye for biochemical plasma analysis and haematological analysis, and sacrificed. Organs were perfused in situ with PBS (pressure 100 mmHg) for 10 min via a cannula in the left ventricular apex. Perfused organs were fixated for about 24 hours in 3.7 % neutral-buffered formalin solution (Formal-fixx®, Shandon Scientific Ltd., UK) and subsequently preserved in 0.1 % sodiumazide in PBS solution or snap-frozen and stored at -20 °C for further analysis.

PLASMA MEASUREMENTS

Plasma corticosterone levels were determined in tail blood using a ^{125}I-corticosterone kit (MP Biomedicals, Santa Anna, USA) following the manufacturer's protocol. Plasma adrenocorticotropic hormone (ACTH) levels were measured in tail blood using a ^{125}I-radiolabeled kit following the manufacturer's protocol (Phoenix pharmaceuticals, Burlingame, CA, USA). Plasma free cholesterol and cholesteryl ester levels were determined using colorimetric assays. The cholesterol distribution over the different lipoproteins was determined in pooled plasma via fast protein liquid chromatography-based fractionation (FPLC; Superose 6 column; 3.2 - 30 mm; Smart-System, Pharmacia, Uppsala, Sweden). FPLC fractions 1-13 represent the non-HDL species (VLDL and LDL), while fractions 13-19 represent HDL.

ADRENAL LIPID ANALYSIS

One adrenal per mouse was used for tissue lipid quantification using the extraction method developed by Bligh and Dyer [17]. Free cholesterol and cholesteryl ester levels in the lipid extract were quantified using colorimetric assays. Tissue lipid levels were corrected for the protein input.

ANALYSIS OF GENE EXPRESSION BY REAL-TIME QUANTITATIVE PCR

Total RNA was isolated for quantitative gene expression analysis as previously described [18]. In short, total RNA was isolated using a standard phenol/chloroform extraction method and reverse transcribed using RevertAid Reverse Transcriptase. Gene expression analysis was performed by using SYBR-Green technology (Eurogentec) on a 7500 Fast Real time PCR system (Applied Biosystems, Foster city, US). Primer sequences are available upon request. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribosomal protein lateral stalk subunit P0 (36B4) and beta-actin (β-actin) were used as the housekeeping genes.

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Instat Software (San Diego, CA, USA, http://www.graphpad.com). Normality testing was performed using the method of Kolmogorov and Smirnov. Significance was calculated using a two-tailed Student's t-test or two-way ANOVA with Bonferroni's post-test where appropriate. P values <0.05 were considered significant.

RESULTS

Reintroduction of ApoE through transplantation of wild-type bone marrow is an effective means to induce a specific, long-term, reduction in plasma VLDL/LDL-cholesterol levels and, as a result, lower the associated atherosclerosis susceptibility in ApoE knockout mice [19-21]. To determine the relative impact of VLDL/LDL on glucocorticoid output in vivo, we therefore evaluated adrenal cholesterol and glucocorticoid homeostasis in male ApoE knockout mice transplanted with either bone marrow from ApoE containing wild-type donor mice or with control bone marrow from age- and sex-matched ApoE knockout donors.

As evident from **Figure 1A**, successful recovery of ApoE, i.e. in hepatic macrophages (Kupffer cells), could be verified on the gene expression level in livers of mice that were transplanted with wild-type bone marrow. No functional ApoE transcript was detected in livers of ApoE knockout bone marrow recipients, as expected. The effective restoration of ApoE functionality was associated with a 5- to 7-fold decrease ($P<0.001$) in plasma free cholesterol and cholesteryl ester levels (**Table 1**).

FPLC-based lipoprotein fractionation indicated that the decrease in cholesterol levels was due to a 10-fold decrease in cholesterol associated with VLDL/LDL particles (**Figure 1B**). Importantly, no relevant change in plasma HDL-cholesterol levels was observed in response to ApoE reintroduction (**Figure 1B**).

Adrenals were isolated from the two types of bone marrow recipient mice for morphological and biochemical analysis. No significant difference in absolute adrenal weights was observed (**Figure 2A**). Notably, lipid extraction and subsequent quantification revealed that tissue levels of free cholesterol (**Figure 2B**) and cholesteryl esters (**Figure 2C**) were also not different between the two groups of adrenals.

Table 1: Plasma lipid levels in chow diet-fed lethally irradiated ApoE knockout mice transplanted with wild-type (BM+) or ApoE knockout (BM-) bone marrow.

	Ad libitum fed			Overnight fasted		
	BM-	BM+	P value	BM-	BM+	P value
Free cholesterol (mg/dL)	152±8	27±1	< 0.001	147±9	27±3	< 0.001
Cholesteryl esters (mg/dL)	746±58	130±9	< 0.001	717±51	90±6	< 0.001

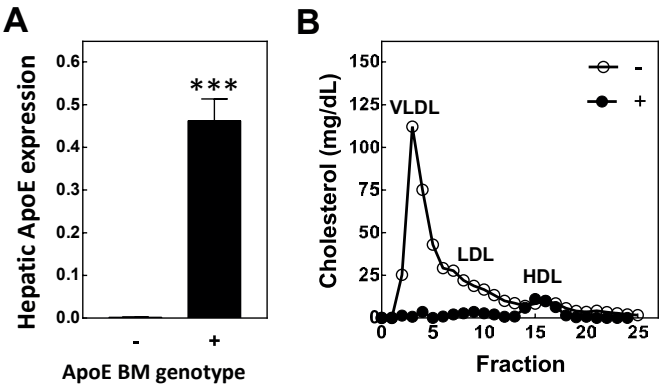

Figure 1: Transplantation of wild-type bone marrow (ApoE BM+) into lethally irradiated ApoE knockout mice restores ApoE mRNA expression levels within the liver (A) and lowers plasma very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) as compared to ApoE knockout bone marrow (ApoE BM-) recipients (B). Data in panel A represent means+SEM of 10 (ApoE BM-; white bars / symbols) and 6 (ApoE BM+; black bars / symbols) mice per group. *** P<0.001. HDL, high-density lipoprotein.

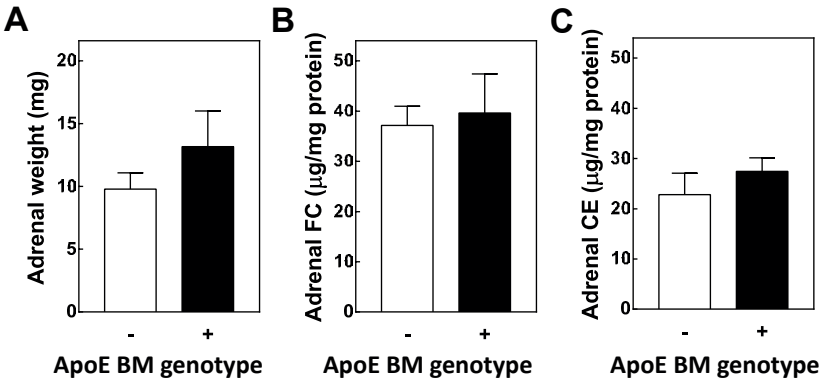

Figure 2: Reintroduction of ApoE into ApoE knockout mice via bone marrow transplantation did not affect the adrenal weight (A) or the adrenal free cholesterol (B) and cholesteryl ester (C) content. Data represent means+SEM of 10 (ApoE BM-; white bars) and 6 (ApoE BM+; black bars) mice per group.

The bone marrow ApoE-induced reduction in plasma VLDL/LDL did not significantly impact on baseline plasma corticosterone levels (**Figure 3A**). The bone marrow genotype did, however, affect the plasma corticosterone response to food deprivation-associated stress (**Figure 3A**). Corticosterone levels increased 8-fold in ApoE knockout bone marrow recipient controls after overnight food deprivation (260 ± 54 ng/ml in the fasted state versus 32 ± 5 ng/ml in ad libitum fed state, respectively; $P<0.001$). In contrast, food deprivation was only associated with a 2-fold rise ($P>0.05$) in plasma corticosterone levels in wild-type bone marrow recipients. Plasma corticosterone levels were 64 % lower (92 ± 11 ng/ml; $P<0.05$) in wild-type bone marrow recipients as compared to ApoE knockout bone marrow recipients in the fasting state (**Figure 3A**). Adrenal transplantation studies have suggested that, in mice, a minimal plasma level of ~100 ng/ml is required to achieve the anti-inflammatory / immunosuppressive glucocorticoid action, while a plasma level of >200 ng/ml corticosterone is required to activate the glucocorticoid receptor-mediated (hepatic) metabolic responses [22]. Food deprived wild-type bone marrow recipients exhibited a relatively high inflammatory status as measured by routine hematological analysis, which suggests that these mice also exhibit a reduced glucocorticoid function as compared to ApoE knockout bone marrow recipient mice. The significant increase in total blood leukocyte concentrations (5.8 ± 1.4 x 10E9/L versus 2.5 ± 0.1 x 10E9/L; $P<0.01$) was primarily driven by a 2.4-fold increase ($P<0.01$) in the blood lymphocyte count (**Figure 3B**). The lymphocytosis was paralleled by a striking 8-fold rise ($P<0.001$) in the number of eosinophils as well as a 3.3-fold increase ($P<0.01$) in blood neutrophil concentrations, while no significant

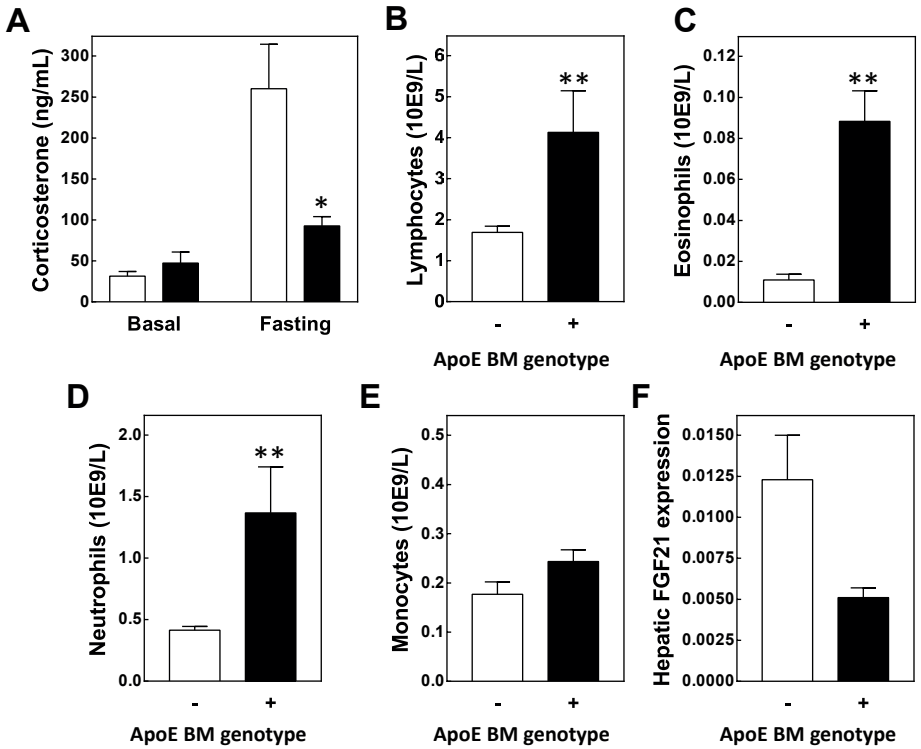

Figure 3: Reintroduction of ApoE into ApoE knockout mice via bone marrow transplantation reduced the fasting-induced plasma corticosterone response while it did not impact the basal adrenal corticosterone output (A). Restoration of ApoE functionality in ApoE knockout mice is associated with increased blood lymphocyte (B), eosinophil (C) and neutrophil (D) concentrations, without a change in blood monocyte counts (E), and a trend towards lower hepatic FGF21 relative expression levels under fasting stress conditions. Data represent means+SEM of 10 (ApoE BM-; white bars) and 6 (ApoE BM+; black bars) mice per group. * $P<0.05$, ** $P<0.01$.

change in blood monocyte counts was observed (Figure 3C, D, E). Importantly, the significant changes in blood leukocyte numbers also coincided with a marked 59 % decrease in the hepatic mRNA expression levels of the glucocorticoid receptor target gene fibroblast growth factor 21 (FGF21) [23-25]. Although the bone marrow genotype-associated difference in hepatic FGF21 expression level failed to reach significance due to the large intra-group variations (Figure 3F), it appears that the decrease in VLDL/LDL-cholesterol levels in wild-type bone marrow recipients is associated with a physiologically relevant decrease in the adrenal glucocorticoid function.

The extent of adrenal glucocorticoid output not only depends on the adrenocortical cell cholesterol availability, but also on the level of activation of intracellular signaling pathways that stimulate the transcription of genes involved in the intracellular mobilization of cholesterol and the conversion cholesterol to corticosterone. The steroidogenic trigger was equally high in the two experimental groups as judged from the observation that ApoE -mediated reversal of the hypercholesterolemia was not associated with a change in the fasting plasma levels of the primary steroidogenic activator ACTH (**Figure 4A**). Strikingly, as can be appreciated from **Figure 4B**, we did – however – measure a significant decrease in the adrenal relative mRNA expression levels of the established ACTH-responsive genes SR-BI [26,27], steroidogenic acute regulatory protein (StAR) [28], and cholesterol side-chain cleavage enzyme (CYP11A1) [29]. Gene expression levels of SR-BI, StAR, and CYP11A1 were respectively 29 % ($P<0.01$), 19 % ($P<0.05$), and 27 % ($P<0.05$) lower in wild-type bone marrow recipients as compared to ApoE knockout bone marrow controls (**Figure 4B**). In accordance with the findings of Ho et al. that, in rats, the 11-beta-hydroxylase (CYP11B1) transcription rate is increased by ACTH treatment [30], CYP11B1 mRNA expression levels also tended to be lower in adrenals of wild-type bone marrow recipients (-32 %; $P>0.05$; **Figure 4B**). No parallel change was observed in the mRNA expression levels of HMG-CoA reductase (HMGCR), the LDL receptor (LDLR), acetyl-Coenzyme A acetyltransferase 1 (ACAT-1), hormone-sensitive lipase (HSL), hydroxy-delta-5-steroid dehydrogenase,

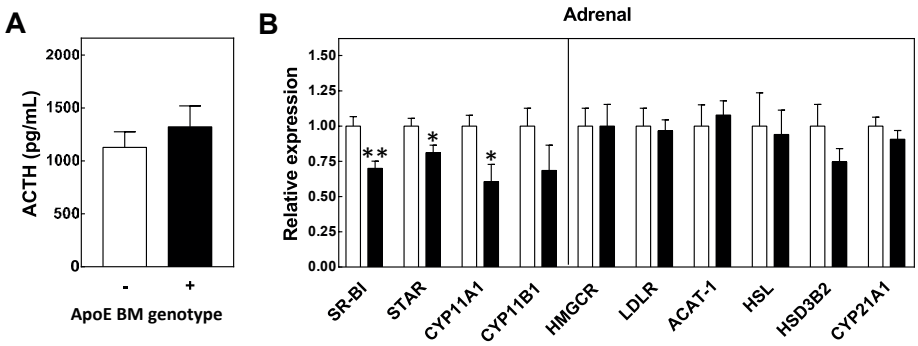

Figure 4: Reintroduction of ApoE into ApoE knockout mice via bone marrow transplantation did not impact fasting plasma ACTH levels (A), but lowered adrenal relative mRNA expression levels of the ACTH-responsive steroidogenic genes SR-BI, StAR and CYP11A1 without altering transcript levels of other key proteins involved in adrenal cholesterol handling and steroidogenesis (B). Data represent means+SEM of 10 (ApoE BM-; white bars) and 6 (ApoE BM+; black bars) mice per group. * $P<0.05$, ** $P<0.01$.

3 beta- and steroid delta-isomerase 2 (HSD3B2), and 21-hydroxylase (CYP21A1) that represent other genes crucially involved in respectively adrenocortical cell cholesterol mobilization and storage and steroidogenesis. It can therefore be anticipated that the observed changes in SR-BI / StAR / CYP11A1 / CYP11B1 gene expression were not resulting from a general change in cellular cholesterol metabolism but rather due to a specific effect on the cellular ACTH response.

DISCUSSION

In the current study we aimed to provide proof for the concept that mice use non-HDL lipoprotein fractions, i.e. VLDL and LDL, as primary source of cholesterol substrate for the production of glucocorticoids. Here we show that selective depletion of VLDL/LDL in hyperlipidemic ApoE knockout mice is associated with a significant decrease in the adrenal expression of genes involved in cholesterol mobilization and steroidogenesis and a reduced maximal adrenal glucocorticoid output under fasting stress conditions.

The marked decrease in the fasting stress-induced adrenal glucocorticoid output upon bone marrow ApoE repopulation in ApoE knockout mice was not paralleled by a change in adrenal cholesterol stores. This finding provides support for the hypothesis, originally derived from our studies in hypercholesterolemic LDL receptor knockout mice [14], that the extent of adrenal cholesteryl ester storage is not causally related to the ability of adrenals to synthesize glucocorticoids. Furthermore, from this finding it can be concluded that in a combined HDL and VLDL/LDL setting, such as in ApoE and LDL receptor knockout mice as well as in humans, VLDL/LDL is not contributing significantly to the generation of the intra-adrenal cholesteryl ester stores. In accordance, our previous studies in HDL deficient ApoA1 x LDL receptor knockout double knockout mice have suggested that rather cholesterol acquired from HDL particles is stored in the adrenocortical cell cholesteryl ester pool [14]. In further support of an important contribution of HDL to adrenal cholesteryl ester accumulation, Plump et al. have shown that a genetic lack of ApoA1 and the associated HDL deficiency is also accompanied by depletion of neutral lipids from adrenals in ApoE knockout mice [31].

Our studies suggest that at least two distinct cholesterol mobilization routes can be discriminated within the adrenals: one in which HDL provides cholesterol to the adrenals for storage and another where VLDL/LDL delivers the cholesterol that is utilized for stress-induced adrenal steroidogenesis (**Figure 5**). Previous studies using

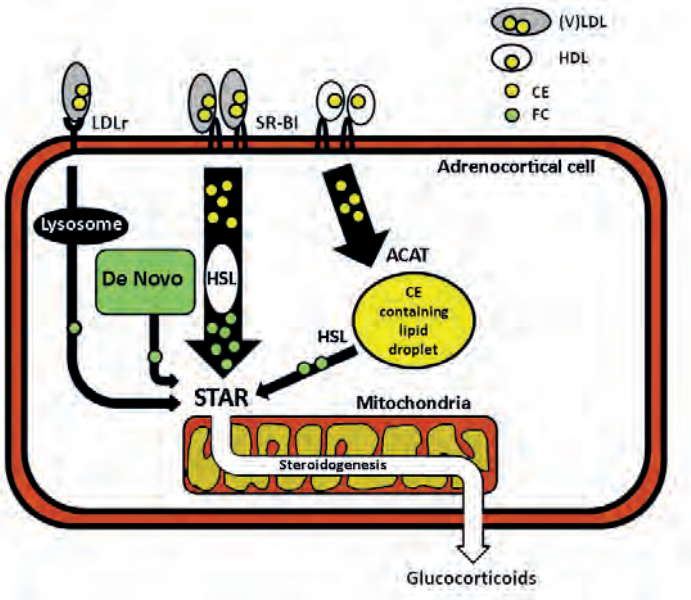

Figure 5: A schematic overview of the suggested contributions of various cholesterol sources to the production of glucocorticoids by adrenocortical cells. (V)LDL-derived cholesterol acts as the major source of cholesterol used for adrenal glucocorticoid synthesis. HDL cholesteryl esters, acquired by SR-BI, are firstly stored in lipid droplets, but can also be regarded an important secondary cholesterol substrate source. Cholesterol acquired through LDL receptor-mediated uptake and de novo synthesis contribute for a very limited extent to steroidogenesis and therefore only become relevant under conditions that the other substrate delivery pathways are defective.

adrenal-specific and total body knockout mice have implied that the cholesterol acquisition from HDL is dependent on selective cholesteryl ester uptake from HDL particles by SR-BI [32]. Given that a genetic lack of the LDL receptor is associated with accumulation of the ApoB100-containing lipoproteins VLDL and LDL in the plasma compartment, it could be assumed that these lipoprotein species deliver the majority of their cholesterol to cells via binding to and subsequent whole particle uptake via the LDL receptor. Lack of the LDL receptor does, however, not impact on the glucocorticoid production rate in adrenocortical cells in vitro [33]. In addition, total body knockout LDL receptor knockout mice display a normal ability of the adrenals to produce glucocorticoids [34,35]. The cholesterol used for steroidogenesis thus seems to be acquired from VLDL/LDL particles by adrenocortical cells in manner that is actually not dependent on the LDL receptor. The LDL receptor-related protein 1 (LRP1) is also considered to be a functional VLDL/LDL receptor, since it is able to bind

and internalize ApoE -containing lipoproteins [36]. LRP1 protein expression has been detected in adrenals and is not different between wild-type and ApoE knockout mice [16]. Unfortunately, total body LRP1 deficiency is embryonically lethal in mice [37]. As a consequence, the relative contribution of LRP1 to the uptake of lipoprotein-associated cholesterol into the adrenals as well the impact of the impact of LRP1 deficiency on adrenal steroidogenesis remains unknown to date. Multiple studies have suggested that SR-BI can mediate selective uptake of cholesteryl esters from VLDL/LDL as well as whole particle clearance [38-41]. Studies in normolipidemic mice have shown that the relative impact of SR-BI deficiency on the adrenal glucocorticoid function (primary glucocorticoid insufficiency) is greater than that observed in response to isolated HDL deficiency (reduced adrenal glucocorticoid function), i.e. in LCAT knockout mice [11] and ApoA1 knockout mice [42]. It is therefore tempting to speculate that SR-BI, next to mediating the adrenal HDL-cholesteryl ester uptake, also controls the flux of the VLDL/LDL-derived cholesterol substrate into the adrenals. Given that mutations in different domains of SR-BI impact differentially on the ability of the protein to respectively bind lipoproteins, mediate the selective uptake of cholesteryl esters, and facilitate cholesterol efflux [43], we consider it of clear interest to determine the effect of different variations in the SR-BI gene on adrenal cholesterol accumulation and steroidogenesis in mice.

An interesting observation of our studies was that bone marrow ApoE-mediated lowering of plasma cholesterol levels did not only reduced the adrenal glucocorticoid output, but also negatively impacted on the mRNA expression levels of SR-BI, StAR and CYP11A1. A decrease in the expression of these three genes, that are essential for adrenal cholesterol mobilization and the subsequent conversion to corticosterone, could theoretically underlie the parallel decrease in glucocorticoid output. However, we consider it likely that the decrease in gene expression is not causally related to the decrease in glucocorticoid output. More specifically, the reduction in the fasting plasma glucocorticoid levels (-64 %) was much greater than the decrease that we observed in the adrenal relative gene expression levels (-19 % to -29 %). Furthermore, plasma corticosterone levels are within the normal range in heterozygous CYP11A1 knockout mice that can only generate 50 % of total amount of CYP11A1 protein present in wild-type controls [44]. Moreover, heterozygous StAR knockout adrenals also do not display an impaired ability to produce corticosterone [45].

ACTH is a potent activator of SR-BI, StAR, CYP11A1 and CYP11B1 transcription in adrenocortical cells in vitro and in vivo [26-29,46]. Given that we did not observe a change in plasma ACTH levels, it can therefore be suggested that restoration of bone marrow ApoE and/or the associated decrease in plasma cholesterol levels impacts directly on adrenal ACTH signaling. Due to a lack of material we unfortunately have not been able to study in depth a possible effect on individual proteins such as the ACTH receptor / melanocortin-2 receptor and cyclic AMP response element-binding protein (CREB) and the second messenger molecule cyclic AMP that together execute the cellular response to ACTH. However, our current in vivo data complement in vitro findings from Reyland et al. which have indicated that ApoE can directly inhibit steroidogenesis through blockade of cyclic AMP-mediated signal transduction in adrenocortical cells [47,48].

CONCLUSION

We have shown that a selective lowering of VLDL/LDL levels in hyperlipidemic ApoE knockout mice through transplantation with ApoE-containing wild-type bone marrow is associated with a decreased adrenal glucocorticoid output. Our studies provide novel experimental support for our working hypothesis that, in vivo, the cholesterol pool used for steroidogenesis under stress conditions is primarily acquired from VLDL/LDL, while HDL-derived cholesterol is mainly used for adrenal storage as cholesteryl esters.

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ELIMINATION OF ADRENOCORTICAL APOLIPOPROTEIN E PRODUCTION DOES NOT IMPACT GLUCOCORTICOID OUTPUT IN WILD-TYPE MICE

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Menno Hoekstra, Miranda van Eck

ABSTRACT

Apolipoprotein E (ApoE) deficient mice exhibit unexplained hypercorticosteronemia. Given that ApoE is also produced locally within the adrenals, we evaluated the effect of adrenal-specific ApoE deficiency on the glucocorticoid function. Hereto, one adrenal containing or lacking ApoE was transplanted into adrenalectomized wild-type mice. Adrenal ApoE deficiency did not impact adrenal total cholesterol levels. Importantly, the ability of the two adrenal types to produce glucocorticoids was also not different as judged from the similar plasma corticosterone levels. Adrenal mRNA expression levels of HMG-CoA reductase and the LDL receptor were decreased by respectively 72 % ($P<0.01$) and 65 % ($P=0.07$), suggesting that cholesterol acquisition pathways were inhibited to possibly compensate the lack of ApoE. In support, a parallel increase in the expression level of the cholesterol accumulation-associated ER stress marker CHOP was detected (+117 %; $P<0.05$).

In conclusion, our studies show that elimination of adrenocortical ApoE production does not impact glucocorticoid output in wild-type mice.

INTRODUCTION

Apolipoprotein E (ApoE) is an important member of the apolipoprotein family of proteins. It provides stability to lipoproteins and contributes to binding of lipoprotein particles by cell surface receptors thereby facilitating their clearance from the circulation [1,2]. ApoE specifically interacts with the low-density lipoprotein receptor (LDLr) and the LDLr-related protein 1 (LRP1) [3,4]. High amounts of ApoE are found on chylomicron- and very-low-density lipoprotein (VLDL) remnant particles [5,6], while some high-density lipoproteins (HDL) species also contain ApoE [7,8]. Total body ApoE deficiency, i.e. in ApoE knockout mice, is therefore associated with an impaired clearance of VLDL/chylomicron remnants from the circulation and marked hypercholesterolemia. Given that hypercholesterolemia is an established risk factor for atherosclerosis, ApoE knockout mice spontaneously develop atherosclerotic lesions already when fed a regular chow diet and represent a commonly used atherosclerosis mouse model [9].

Interestingly, in addition to the observed hypercholesterolemic phenotype, ApoE deficient mice also exhibit an increased plasma level of the primary glucocorticoid species corticosterone [10]. Glucocorticoids are adrenal-derived steroid hormones that play an important role in the physiological response to stress by modulating the expression of genes involved in metabolism and inflammation [11]. Since the cholesterol substrate used for the production of glucocorticoids by the adrenals is primarily derived from lipoproteins, the rise in plasma glucocorticoid levels could theoretically be secondary to the systemic ApoE deficiency-associated hypercholesterolemia. However, it should be noted that in vitro studies have suggested that locally produced ApoE may impact directly on cell cholesterol homeostasis and glucocorticoid output. More specifically, Prack et al. showed that murine Y1 adrenocortical cells expressing human ApoE have an increased cellular cholesterol content and display, in contrast to other ApoE-secreting cell types (i.e. macrophages), a markedly reduced efflux of free cholesterol as compared to control Y1 cells that do not express ApoE [12]. This is paralleled by a significant reduction in the cellular steroid output [13–15]. From these in vitro findings it can be hypothesized that the lack of ApoE within the adrenals is the driving force in generating the hypercorticosteronemia present in total body ApoE knockout mice. In the current study, we validated a potential role of locally produced ApoE on adrenocortical cell cholesterol and steroid metabolism in an in vivo setting using adrenal transplantation.

MATERIALS AND METHODS

IN VIVO SETUP

Female total body ApoE knockout mice (ApoE-/-) on a C57BL/6 background and C57BL/6 mice (ApoE+/+) were bred in house. Throughout the experiment, all mice were group housed in a temperature and light-cycle (12 hour light / dark) controlled room. Bilateral adrenalectomy of the recipient mice and subsequent transplantation of the donor adrenal were carried out as previously described [16]. In brief, adrenal glands of young (10 days postnatal) female donor ApoE -/- (n=9) or C57BL/6 (ApoE+/+; n=10) pups were collected to serve as transplant donors [17]. Bilateral adrenalectomy was performed on 8 week-old female recipient C57BL/6 mice under isoflurane anesthesia through a dorsal midline skin incision and lateral retroperitoneal incisions though the abdominal muscle layer. Subsequently, one donor adrenal was placed per recipient under the renal capsule. The skin wounds were closed using Michel suture clips (size 7.5x1.75 mm, Aesculap, Tuttlingen, Germany). Throughout the experiment, all mice were group-housed and supplied with chow diet (RM3 (E) DU; Special Diet Services, Witham, England), normal water and a 0.9 % NaCl solution ad libitum. For an optimal post-surgery recovery, all cages were supplied with a heating matrass for the duration of one week. Based on our previous studies [16], adrenal transplants were given 8 weeks to become fully mature before subsequent transplant function and morphology analyses were executed.

After the recovery period blood was collected in EDTA coated tubes (Sarstedt, Numbrecht, Germany) at 9:00 AM for basal fed corticosterone measurement. At the end of the study, mice were subjected to overnight food deprivation from 05:00 PM to 09:00 AM to induce physiological stress. Blood was obtained for stressed corticosterone measurement and the mice were subsequently anesthetized with a mixture of Xylazine (70 mg/kg), Ketamine (350 mg/kg) and Atropine (1.8 mg/kg) prior to in situ perfusion with phosphate buffered saline (pressure 100 mmHg) for 10 min via a cannula in the left ventricular apex. Subsequently, organs were collected, weighed, and either snap–frozen (RNA measurements) or stored in 3.7 % formalin (tissue sectioning Formal-fixx®, Shandon Scientific Ltd., UK). Post mortem macroscopic inspection showed no signs of endogenous adrenal regeneration in any of the transplanted mice. All animal experiments were executed at the Gorlaeus Laboratories of Leiden University, where the Leiden Academic Centre for Drug Research is located. All animal work was approved by the Dutch Ethics Committee and regulatory authority at Leiden University and was carried out in compliance with Dutch government guidelines and the Directive

2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

PLASMA LIPID ANALYSIS

At sacrifice orbital whole blood from each individual mouse was collected in EDTA-coated tubes for quantification of plasma lipids. Free cholesterol (FC) was determined by using an enzymatic colorimetric assay with 0.048 U/mL cholesterol oxidase (Calbiochem, San Diego, CA, USA) and 0.065 U/mL peroxidase (Sigma-Aldrich, Steinheim, Germany) in reaction buffer (1.0 KPi buffer, pH=7.7 containing 0.01 M phenol, 1 mM 4-amino-antipyrine, 1 % polyoxyethylene-9-laurylether, and 7.5 % methanol). For the determination of total cholesterol (TC), 0.03 U/mL cholesteryl esterase (Calbiochem, San Diego, CA, USA) was added to the reaction solution. Absorbance was read at 490 nm on a Powerwave 340 from BioTek. Lipoproteins in pooled plasma were separated by means of fast protein liquid chromatography (FPLC; Superose 6 column; 3.2 x 30 mm; Smart-System, Pharmacia, Uppsala, Sweden) and the cholesterol content was analyzed as described above. Fractions 1-13 represented the non-HDL species VLDL and LDL and fractions 13-19 represented HDL.

ANALYSIS OF GENE EXPRESSION BY REAL-TIME QUANTITATIVE PCR

Quantitative expression analysis was performed on equal amounts of RNA as previously described [18]. In brief, total RNA was isolated using a standard phenol/chloroform extraction method and reverse transcribed using RevertAid Reverse Transcriptase. Expression analysis was performed using SYBR-Green technology (Eurogentec) on a 7500 Fast Real time PCR system (Applied Biosystems, Foster city, US). Primers were validated for an equal efficiency. Relative expression values were determined by subtracting the Ct of the target transcript from the average Ct of the housekeeping transcripts and raised by 2 to the power of this difference. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribosomal protein lateral stalk subunit P0 (36B4) and beta-actin (β-actin) were used for normalization. General information of the primers used is summarized in Table 1.

Table 1: Primers used for Real Time Quantitative PCR.

Gene	Ref Seq (mRNA)	Forward sequence	Reverse sequence
36B4	NM_007475	5′-CTGAGTACACCTTCCCACTTACTGA-3′	5′-CGACTCTTCCTTTGCTTCAGCTTT-3′
3βHSD	NM_153193	5′-AGCCTTCCTGTGCCCCTACT-3′	5′-CAGGAGGAAGCTCACAGTTTCC-3′
ABCA1	NM_13454	5′-AGAGCAAAAAGCGACTCCACATAGAA-3′	5′-CGGCCACATCCACAACTGTCT-3′
ACAT1	NM_144784	5′-AGCTGTTTCTCTGGGCCATCCAAT-3′	5′-GAACTCTCCTGGCTTCAGGGCAT-3′
CHOP	NM_007837	5′-CTCTTGACCCTGCGTCCCTAG-3′	5′-TGGGATGTGCGTGTGACCT-3′
CYP11A1	NM_019779	5′-AGAACATCCAGGCCAACATTACCGAG-3′	5′-AGGACTTCAGCCCGCAGCATC-3′
CYP11B1	NM_001033229	5′-TGCCATCCAGGCTAACTCAATGGAACT-3′	5′-AGGGCCTGCTGAACATCTGGGT-3′
CYP21A1	NM_009995	5′-GGGAACTGCCCAGCAAGTT-3′	5′-AGGATGGTGTTCTGGGATTCTTC-3′
GAPDH	NM_008084	5′-ATCCTGCACCACCAACTGCTTA-3′	5′-CATCACGCCACAGCTTTCCAG-3′
HMGCR	NM_008255	5′-CGAGCCACGACCTAATGAAGAATG-3′	5′-TGCATCACTAAGGAACTTTGCACC-3′
HSL	NM_010719	5′-CTGACAATAAAGGACTTGAGCAACTC-3′	5′-AGGCCGCAGAAAAAAGTTGAC-3′
LDLR	NM_010700	5′-TGAGGTTCCTGTCCATCTTCTTCCC-3′	5′-TTGATGTTCTTCAGCCGCCAGTTC-3′
SR-BI	NM_016741	5′-AAACAGGGAAGATCGAGCCAGTAG-3′	5′-CGTAGTGAAGAACCTGGGGCAT-3′
β-actin	NM_007393	5′-AACCGTGAAAAGATGACCCAGAT-3′	5′-CACAGCCTGGATGGCTACGTA-3′

ADRENAL GLUCOCORTICOID FUNCTION TEST

One week prior to the sacrifice, tail blood was collected in EDTA-coated (Sarstedt, Numbrecht, Germany) tubes under low stress conditions to set the basal level. At the sacrifice day, tail blood was collected in EDTA-coated tubes for corticosterone determination under overnight fasting stress conditions. Corticosterone levels were measured in 1:200 diluted EDTA-plasma using a ^{125}I-corticosterone kit (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's protocol.

BLOOD ACTH LEVELS

Plasma adrenocorticotropic hormone (ACTH) levels were determined in tail blood under overnight fasting stress conditions using a ^{125}I-radiolabeled kit following the manufacturer's protocol (Phoenix pharmaceuticals, Burlingame, CA, USA).

HISTOLOGICAL VISUALIZATION

Cellular lipid content was visualized within the transplants. Hereto, 10µm cryostat sections (Leica CM 350S cryostat) embedded in OCT compound (Tissue-Tek, Sakura Finetek, Alphen a/d Rijn, The Netherlands) were collected. Sections were stained for neutral lipids with Oil red O and counterstained with hematoxylin (Sigma-Aldrich, Steinheim, Germany). Free cellular cholesterol was visualized with Filipin III (Cayman chemicals, Ann Arbor, MI, USA) and counterstained with the fluorescent compound Sytox green (Life technologies/Thermo Fisher, Waltham, MA, USA).

STATISTICAL ANALYSES

Statistical analysis was performed using GraphPad Instat Software (San Diego, CA, USA, http://www.graphpad.com). Normality testing was performed using the method of Kolmogorov and Smirnov. Significance was calculated using a two-tailed Student's t-test or two-way ANOVA with Bonferroni's post-test where appropriate. $P<0.05$ was considered significant.

RESULTS

In the current study we evaluated a potential contribution of ApoE produced locally in adrenocortical cells to overall adrenal cholesterol homeostasis and glucocorticoid output. For this purpose we generated mice with a genetic lack of ApoE selectively within the adrenals. Hereto, we transplanted one adrenal from either ApoE knockout (ApoE -/-) mice or C57BL/6 wild-type (ApoE+/+) controls into bilaterally

adrenalectomized C57BL/6 wild-type recipient mice. Our previous studies using SR-BI knockout mice have already proven that this approach represents a valid method to uncover the in vivo role of individual gene products in adrenal cholesterol and steroid metabolism [19].

Adrenal ApoE deficiency does not impact on the plasma lipoprotein profile

Adrenalectomized C57BL/6 mice reconstituted with a wild-type adrenal exhibited plasma free and total cholesterol levels that are in the normal range for (normolipidemic) wild-type mice [20]. Given that the liver is regarded the primary site for the production of ApoE circulating in the plasma compartment, we anticipated that adrenal-specific deletion of ApoE would not impact significantly on overall lipoprotein clearance. In accordance, as evident from **Figure 1A**, plasma free and total cholesterol levels were virtually identical in ApoE knockout adrenal recipient mice. Furthermore, the distribution of cholesterol over the different lipoprotein subclasses was also not different in the two experimental groups. Both types of adrenal recipients carried the majority of their cholesterol in HDL (**Figure 1B**).

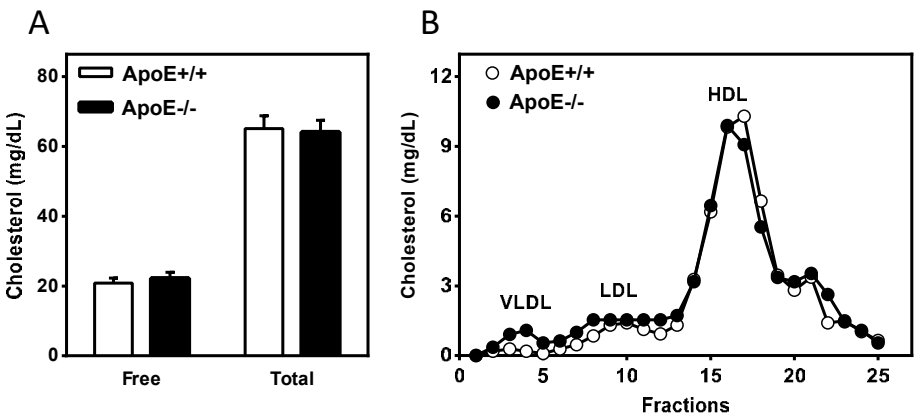

Figure 1: Adrenal-specific ApoE deficiency in C57BL/6 mice did not alter the plasma levels of free or total cholesterol (A). Data represent means+SEM with a group size of ApoE-/- n=9 and ApoE+/+ n=10. The absence of adrenal ApoE also did not change the distribution of cholesterol over the different lipoprotein fractions (B). VLDL, very-low-density lipoprotein; LDL; low-density lipoprotein; HDL, high-density lipoprotein.

Adrenocortical cell-derived ApoE does not modulate cellular cholesterol acquisition

Adrenal transplants were isolated at sacrifice to in detail investigate the effect of adrenocortical cell ApoE deficiency on adrenal cholesterol homeostasis. Our previous studies [19,21,22] have shown that, in mice, the HDL receptor SR-BI is crucial in the generation of the adrenal neutral lipid stores. Adrenal relative mRNA expression levels of SR-BI were not different in the two experimental groups, which suggests that equal amounts of cholesterol esters were acquired from HDL (**Figure 2A**). Gene expression levels of the cholesterol (de-)esterification enzymes ACAT-1 and HSL were also not significantly changed in response to adrenal ApoE deficiency (**Figure 2B & 2C**). This latter finding suggests that the cholesterol acquired from lipoproteins was distributed in

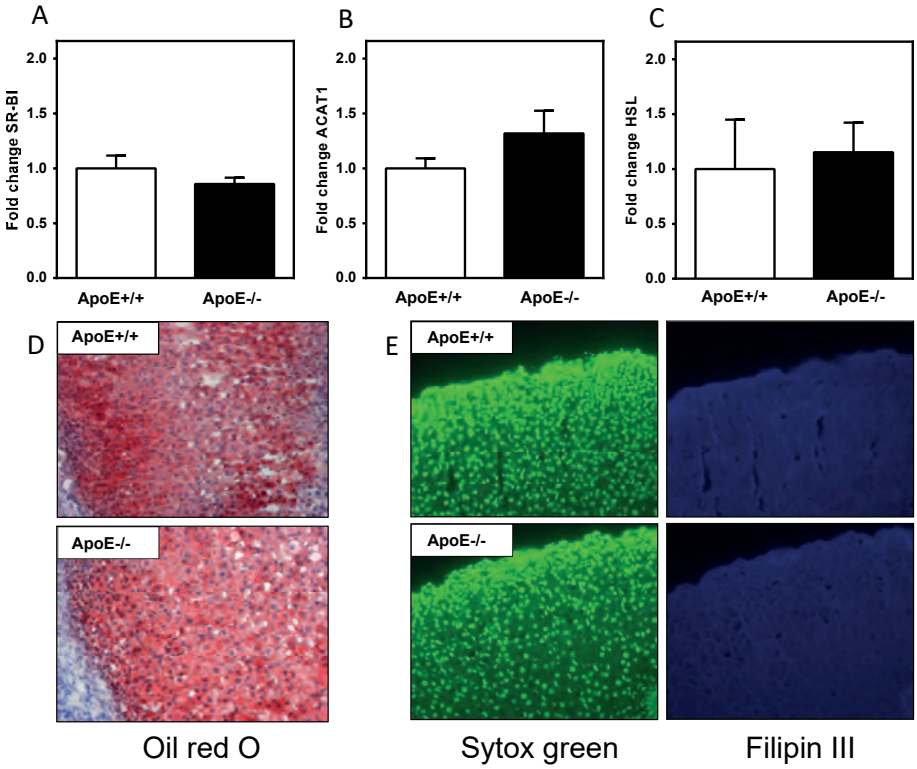

Figure 2: ApoE-/- adrenal transplants showed unaltered mRNA expression levels of SR-BI (A), ACAT1 (B) and HSL (C). Adrenal neutral lipid (Oil red O; D) and free cholesterol (Filipin III; E) content did not differ between ApoE+/+ or ApoE-/- transplant groups (original magnification 400X). All data represent means+SEM with a group size of ApoE-/- n=9 and ApoE+/+ n=10.

a similar manner over the cellular free cholesterol and cholesterol ester pools. Sections of the different adrenal transplants were prepared to visualize the adrenocortical cell cholesterol accumulation. No evident difference in the general morphology of the adrenal transplants could be observed in the two experimental groups. In addition, adrenal ApoE deficiency did not appear to execute a significant effect on the extent of cellular cholesterol ester or free cholesterol accumulation as judged from the stainings depicted in respectively **Figures 2D and 2E**. More specifically, the intensity and cellular distribution of the Oil red O and Filipin III staining was highly similar in the two types of adrenal transplants.

Adrenal-specific ApoE knockout mice exhibit a normal adrenal steroid function

In response to a stress trigger, pituitary-derived adrenocorticotropic hormone (ACTH) stimulates the transcription of a dedicated panel of genes to facilitate an optimal generation of corticosterone from lipoprotein-derived cholesterol by adrenocortical cells [23]. Plasma ACTH levels were similar in the two types of adrenal transplanted mice under stressed conditions (**Figure 3A**). Adrenal-specific ApoE deficiency was also not associated with a change in the mRNA expression levels of the ACTH-responsive genes STAR and steroidogenic enzyme mitochondrial cholesterol side-chain cleavage cytochrome (CYP11A1) that respectively mobilize cholesterol to the steroidogenic machinery within the mitochondria and mediate the conversion of cholesterol into pregnenolone (**Figure 3B**). These combined findings suggest an identical ability of

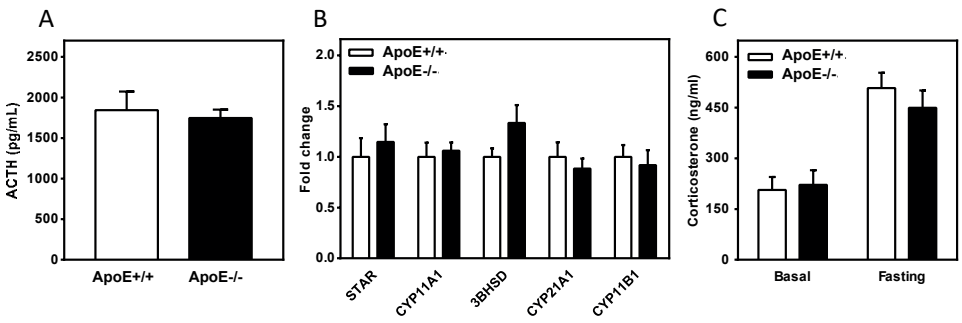

Figure 3: C57BL/6 mice with ApoE-/- and ApoE+/+ adrenal transplants exhibited similar plasma ACTH levels (A) and did not show a change in adrenal mRNA expression levels of the key regulators of steroidogenesis (B), resulting in an identical plasma corticosterone outcome under both basal and fasting stress conditions (C). All data represent means+SEM with a group size of ApoE-/- n=9 and ApoE+/+ n=10.

ACTH to stimulate glucocorticoid production in the two adrenal transplanted groups. In further support, the mRNA expression of the downstream steroidogenic genes 3β-hydroxysteroiddehydrogenase/Δ5-Δ4-isomerase type 2 (HSD3B2), steroid-21-hydroxylase (CYP21A1) and steroid-11β-hydroxylase (CYP11B1) was also unaltered (**Figure 3B**). Interestingly, the null effect of adrenal ApoE deficiency on adrenal steroidogenic gene expression was paralleled by the absence of an effect on plasma glucocorticoid levels. Fasting corticosterone levels were 254 ± 23 ng/ml for wild-type adrenal recipients, while ApoE knockout adrenal transplanted mice exhibited plasma corticosterone values of 224 ± 26 ng/ml (**Figure 3C**). It thus appears that a selective disruption of ApoE only within the adrenals does not recapitulate the stimulating effect on plasma corticosterone levels observed in response to total body ApoE deficiency.

Adrenocortical cell-derived ApoE protects against cholesterol-induced endoplasmic reticulum stress

In vitro overexpression studies have suggested that expression of ApoE in adrenocortical cells executes a negative impact on cellular cholesterol efflux 12,24. In our current in vivo study setup, deletion of ApoE from adrenocortical cells was associated with a 120 % increase (P=0.08; **Figure 4A**) in the expression of ATP-binding cassette transporter A1 (ABCA1) that mediates the efflux of cholesterol from cells to lipid-poor apoA1 to generate nascent HDL particles. Transcription of ABCA1 is generally stimulated by the oxysterol-sensing nuclear receptor liver X receptor (LXR) to overcome the buildup of free cholesterol in cells [25,26]. Notably, the increase in ABC transporter expression coincided with a marked 55 % decrease (P<0.01) in the gene expression of the cholesterol synthesis enzyme HMG-CoA reductase (HMGCR) (**Figure 4B**). Transcription of HMGCR and the LDL receptor is subject to negative feedback in response to cholesterol overload in the endoplasmic reticulum [27, 28]. A clear trend towards a 45 % decrease (P=0.07) was observed in LDL receptor gene expression levels (**Figure 4C**). These combined findings suggest an apparent accumulation of a regulatory free cholesterol pool specifically in the endoplasmic reticulum compartment in response to adrenal ApoE deficiency. Free cholesterol overload has previously been suggested to contribute to endoplasmic reticulum stress [29]. In further support of a potential effect of adrenal-specific ApoE deficiency on specifically ER cholesterol levels, ApoE knockout adrenal transplants exhibited a 117 % increase in the mRNA expression level of the cholesterol accumulation-associated ER stress marker gene CHOP compared to the ApoE producing transplants (P<0.05) (**Figure 4D**).

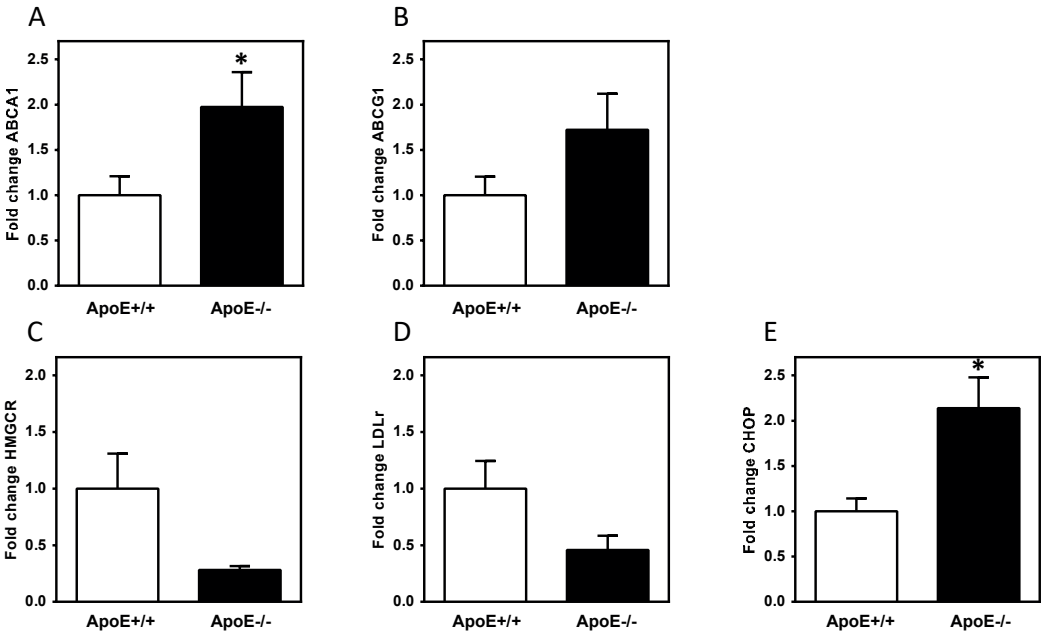

Figure 4: Adrenal-specific ApoE deficiency was associated with a trend towards higher adrenal mRNA expression levels of ABCA1 (A), while respective levels of HMGCR (B) and LDLr (C) were lower or tended to decrease. CHOP mRNA expression levels were significantly increased in ApoE-/- adrenal transplants (D). All data represent means+SEM with a group size of ApoE-/- n=9 and ApoE+/+ n=10. * P<0.05, ** P<0.01.

DISCUSSION

In the current study we determined the effect of adrenal-derived ApoE on cholesterol and steroid metabolism in vivo. Here we show that adrenal-specific ApoE deficiency in C57BL/6 wild-type mice is associated with a downregulation of cholesterol acquisition pathways, whilst the ability of adrenals to produce glucocorticoids is not affected.

This study was based upon the hypothesis, derived from previously published in vitro studies, that ApoE produced within the adrenal executes an inhibitory effect upon the synthesis of glucocorticoids through interfering with the ACTH-induced adrenal cyclic AMP (cAMP) [13, 30–32] response and that total body ApoE knockout mice therefore exhibit higher plasma levels of corticosterone. Our current findings provide clear support for the notion that, in vivo, adrenocortical-cell derived ApoE actually does not impact on the steroidogenic process. An important point to take into account when interpreting our null result on glucocorticoid output is that not only plasma cholesterol levels were similar but that plasma ApoE levels were probably also very similar in both groups of adrenal recipients. In contrast, total body ApoE knockout mice lack adrenal ApoE in the context of a parallel disappearance of ApoE from the plasma compartment when comparing them to wild-type mice. Theoretically a potential effect of the ApoE deficiency within the adrenals on steroid synthesis and output may therefore be nullified for by the fact that our adrenal transplanted mice do have ApoE circulating. However, it should be noted that Thorngate et al. have shown that plasma ACTH levels as well as the adrenal response to an ACTH challenge with respect to tissue cholesterol levels and growth are not different between total body ApoE knockout mice and wild-type controls [33]. In light of our finding that the adrenal expression of ACTH-sensitive genes was not different in mice transplanted with ApoE knockout adrenals as compared to those receiving a wild-type adrenal, we think it is fair to conclude that, as opposed to what was to be expected from the in vitro data, ApoE generally does not impact on the ability of the adrenals to be activated by ACTH in vivo. The fact that the current in vivo findings do not match those previously obtained in vitro provides further support for our working hypothesis - originally derived from studies regarding the role of the LDL receptor [34] - that the relative impact of individual gene products on adrenocortical cell-mediated steroidogenesis depends much on the complexity of the study system.

Given that ApoE apparently does not directly influence the adrenal steroidogenesis rate, it has to be concluded that the hypercorticosteronemia detected in ApoE knockout mice is a secondary effect of the global lack of ApoE. It is easy to speculate that the

ApoE deficiency-associated rise in VLDL/LDL-cholesterol levels generates a larger pool of substrate that can be acquired by the adrenals for use in steroidogenesis. Although we do not regard this option very likely given the already high flux of cholesterol through the VLDL/LDL compartment in wild-type mice, studies in mice that have a normal expression of ApoE in the adrenals but exhibit similar hypercholesterolemia as observed in total body ApoE knockout mice are warranted to exclude that an increased substrate availability is causally related to the overall higher steroid output.

Besides the local effect of ApoE on steroidogenesis, Raber et al. showed that, total body ApoE depletion affects the sensitivity of the negative feedback by glucocorticoids on the hypothalamus – pituitary – adrenal (HPA)-axis within the brain, thereby prolonging the ACTH activation [10]. This misbalance in the HPA-axis feedback mechanism could contribute to the increased glucocorticoids in ApoE knockout mice under basal conditions. In our setting we circumvented this phenotype by the use of C57Bl/6 recipient mice that displayed equal ACTH levels upon stress activation.

Grootendorst et al. have shown that the glucocorticoid phenotype in ApoE knockout mice becomes even more evident upon aging [35]. Pro-inflammatory cytokines such as interleukins 1 and 6 and tumor necrosis factor-alpha are potent activators of the HPA axis and stimulate the secretion of anti-inflammatory glucocorticoids to overcome, for instance, sepsis-related mortality [36-40]. ApoE knockout mice as compared to wild-type mice exhibit a higher basal inflammation status and susceptibility for infections [41], since ApoE stimulates the development of an anti-inflammatory macrophage phenotype [42]. Over time ApoE knockout mice develop atherosclerotic lesions that further stimulate the pro-inflammatory state in these animals, explaining why the hypercorticosteronemia also increases with age in these animals. From these combined findings it can be suggested that the hypercorticosteronemia in total body ApoE knockout mice is secondary to the general higher inflammation status and the fact that the mice develop atherosclerotic lesion that exacerbate the pro-inflammatory state.

An interesting finding of our studies is that adrenal ApoE deficiency is associated with a genetic response that is aimed to reduce cellular cholesterol levels. Based upon this observation it can be suggested that ApoE facilitates the removal of cholesterol from adrenocortical cells. Previous studies have already indicated that ApoE could be an important mediator of cholesterol efflux. Remaley et al. observed that exogenous ApoE can serve as an acceptor for cholesterol transported out of the cells by ABCA1

[43]. Similarly, Huang et al. found that depletion of ApoE from plasma disrupts the efflux of radiolabeled cholesterol from fibroblasts [44]. Our in vitro studies have shown that macrophage ApoE deficiency is associated with a marked reduction in the extent of passive cholesterol efflux to albumin and a mild decrease in the active (ABC transporter-mediated) transport of cholesterol from macrophages towards HDL [45]. In accordance with the notion that ApoE captures endogenous cholesterol for subsequent transport out of the cell, Heeren et al. have shown that cellular recycling of ApoE within cultured hepatocytes is associated with cholesterol efflux [46].

Our data also put forward the suggestion that adrenocortical cell-derived ApoE may be involved in the cellular protection against endoplasmic reticulum stress. The hepatotoxic drug cyclosporine-A is a known inducer of endoplasmic reticulum stress [47–49]. Importantly, Kockx et al. have shown that cyclosporin-A treatment is associated with an accumulation of ApoE specifically in vesicular structures in the endoplasmic reticulum [50]. Niculescu et al. noted that HDL-mediated protection against endoplasmic reticulum stress coincides with a significant increase in cellular ApoE secretion [51]. When taking these latter findings into account, it appears from our findings that ApoE could have a more general role in endoplasmic reticulum physiology as it – by actively mobilizing cholesterol from the endoplasmic reticulum for subsequent transport from the cell – may maintain organelle cholesterol levels within the normal range to overcome the development of ER stress.

CONCLUSIONS

We have shown that elimination of adrenocortical ApoE production does not impact glucocorticoid output in wild-type mice. Furthermore, our studies (1) imply that ApoE probably plays a role in the removal of cholesterol from the adrenals and (2) further highlight the need for proper in vivo testing of the relevance of individual gene products for adrenal steroidogenesis.

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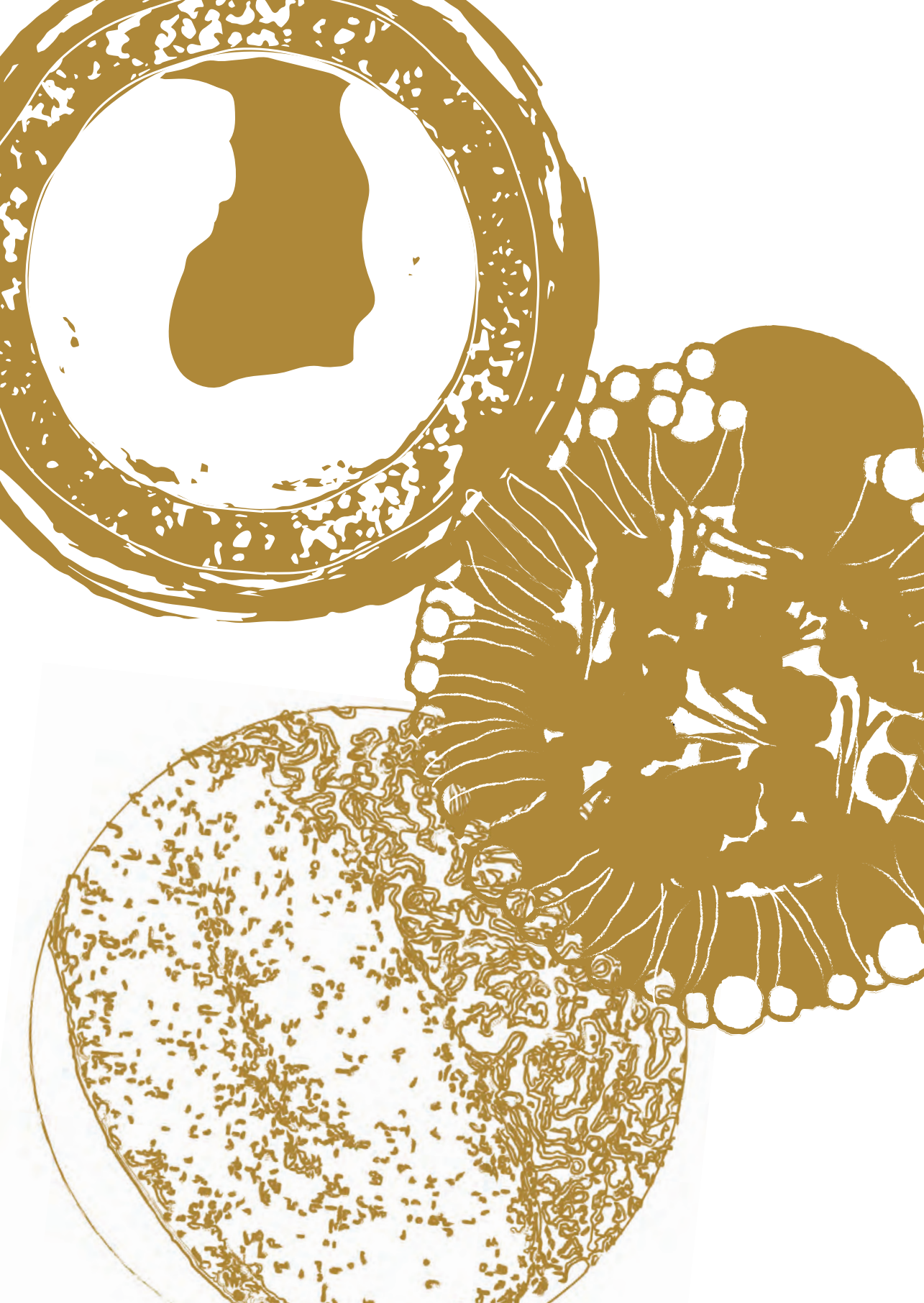

6

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Cholesterol is a vital component in cellular metabolism as well as the building block for steroidogenesis, including the production of adrenal-derived glucocorticoids. Misbalance in the concentration of cholesterol has been linked to a variety of diseases and pathologies, among which are cardiovascular disease (CVD) and metabolic syndrome [1]. The exact interplay between cholesterol levels, glucocorticoids and atherosclerosis is still under debate. In this thesis we discuss the role of cholesterol, specifically high-density lipoprotein cholesterol (HDL-C), during the progression and regression of atherosclerosis. In addition, the effect of intracellular and extracellular cholesterol on adrenal steroidogenesis of glucocorticoids is described.

HDL CHOLESTEROL AND ATHEROSCLEROSIS

CVD events like myocardial infarction, ischemic stroke and co-morbidities including the metabolic syndrome are the leading cause of death in the Western society. The driving pathology behind CVD is atherosclerosis. Atherosclerosis is initiated by a complex interplay between relatively high cholesterol levels and inflammatory components. It is generally accepted that lowering the levels of the low-density lipoprotein cholesterol (LDL-C) fraction reduces the risk for a CVD event. Current interventions strategies achieve this goal mainly via treatment with statins, HMG-CoA reductase inhibitors. However, statin-induced cholesterol reduction lowers the risk of CVD mortality by only approximately 25 % [2], indicating that there is a high need for additional therapies to diminish the CVD burden. Epidemiological studies showed a strong association between low levels of HDL-C and risk for a CVD event. It was therefore hypothesized that increasing HDL might be a valuable strategy to further reduce CVD related mortality and halt progression or even induce regression of atherosclerosis [3-5]. In line, genetically modified animal models revealed that the presence of HDL is of great importance for the initiation of lesion regression [6]. In mice, increased concentrations of HDL, via human ApoA1 overexpression [7,8] or infusion of ApoA1milano, halted progression and initiated atherosclerotic lesion regression. The relationship between HDL and reverse cholesterol transport (RCT) led to the development of HDL increasing drugs and subsequent clinical trials to manipulate lesion development. However, the outcome of these trials was disappointing, showing that increased levels of HDL-C on top of a statin treatment did not stimulate regression of existing lesions [9-11]. The importance of HDL-C quantity was further questioned in humans and mice with a genetically variation in SR-BI, a key receptor in hepatic uptake and metabolism

of HDL cholesteryl esters. SR-BI deficiency results in an augmented level of HDL-C and abnormal enlargement of HDL particles, leading to an increased rather than a decreased susceptibility for atherosclerosis [12]. These observations combined marked a shift in the general discussion around HDL and its anti-atherogenic properties from the importance of HDL-C quantity towards the significance of the functionality of a HDL particle [13,14].

A key modulator of HDL is the phospholipid transfer protein (PLTP) regulating the level, size and composition. PLTP is involved in the transfer of phospholipids from VLDL towards pre-β HDL and α-HDL during lipolysis [15]. Total-body deletion of PLTP in mice led to decreased HDL-C and phospholipid levels and an increase in the non-HDL phospholipid fraction upon a high fat challenge [16].

By additional deletion of PLTP in SR-BI knockout (KO) mice, we provoked normalization of the HDL particle size and reduced HDL-C accumulation (**Chapter 2**). In addition to the lowered amount of cholesterol transported in the HDL fraction, a reduction in atherosclerosis susceptibility was recorded. This finding again stressed that HDL-C quantity is not necessarily predictive for atherosclerotic susceptibility. Under normal conditions, PLTP is also expressed by CD68-positive macrophages in atherosclerotic plaques [19]. Furthermore, in vitro cultured macrophages loaded with acetylated-LDL displayed a paralleled increase in PLTP expression [19]. Our group showed that the absence of macrophage-derived PLTP lowers blood cholesterol levels and decreases the atherosclerotic susceptibility in LDLr KO mice on Western-type diet (WTD) [20]. These data combined suggest that deletion of PLTP locally in macrophages of the atherosclerotic plaque could be of direct influence on macrophage foam cell formation and thus atherogenesis. However, it is most likely that the reduced atherosclerosis susceptibility of PLTP/SR-BI double knockout mice is the result of restored HDL functionality.

The key measure of HDL functionality is defined by its cholesterol efflux capacity from macrophages, which by itself has a strong inverse association with carotid intima thickness independently of HDL-C levels [17,18]. Normalization of HDL-particle size in SR-BI KO mice via the additional deletion of PLTP indeed led to the formation of particles that were more effective in stimulating macrophage cholesterol efflux. In recent years the understanding of HDL metabolism and functionality has improved largely. However, considering that HDL comprises a heterogeneous population of particles with a wide variety of proteins in its shell that influence its function, strategies

aimed at increasing a specific function of the HDL particle without impairing another will remain a challenge. Therefore, it might be more promising to target macrophages in atherosclerotic plaques to stimulate macrophage cholesterol efflux and augment reverse cholesterol transport.

Macrophage cholesterol efflux is mainly established via the ATP-Binding Cassette (ABC) transporter subfamily A1 and G1 (ABCA1 and ABCG1) promoting cholesterol transport towards lipid-poor apolipoprotein A1 (ApoA1) or mature HDL particles, respectively (**Figure 1**)[21-25].

The major regulator of these ABC transporters is the Liver X receptor (LXR). Upregulation of ABCA1 and ABCG1 by LXR agonists stimulates cholesterol efflux from macrophages in vitro. In addition, treatment with LXR agonists results in the disappearance of macrophages from atherosclerotic lesions leading to plaque regression in mice [26-28]. This outcome suggested that LXR could serve as an attractive therapeutic target to reduce CVD incidence. However, LXR's have a more broad role in lipid metabolism. This includes the regulation of hepatic lipogenesis. Administration of synthetic LXR agonists to reduce the CVD incidence in mice increased the levels of plasma triglycerides and very low-density lipoproteins (VLDL) as well as hepatic steatosis in response to LXR agonist treatment [29]. Considering these unwanted systemic effects of LXR agonism, it will be interesting to follow the generation of novel LXR related therapeutic intervention strategies that circumvent the known obstacles and specifically improve macrophage cholesterol efflux capacity, e.g. by nanoparticle-mediated selective delivery of LXR agonist to macrophages [30, 31], to halt the progression and initiate regression of lesions.

Although animal models proved that regression of existing lesions is feasible, the underlying mechanisms remain under debate. Mouse models of lesion regression, either by controlled inactivation of the hyperlipidemia (Reversa mouse) [32] or by transplantation of a lesion containing aortic segment into a normolipidemic mouse (aorta transplantation) [33], suggested that macrophage emigration from the lesion site drives atherosclerosis regression under extreme lipid lowering conditions. Besides lowering cholesterol levels, Rahman et al. showed that for lesion regression a continuous recruitment of Ly6C^{hi} monocytes is required, suggesting a dynamic role for the Ly6C^{hi} monocytes in the lesion [34]. Alternatives to the immigration/emigration hypothesis have been provided by studies executed in ApoE knockout mice with a reversed hypercholesterolemia induced by restoration of ApoE via viral transfection.

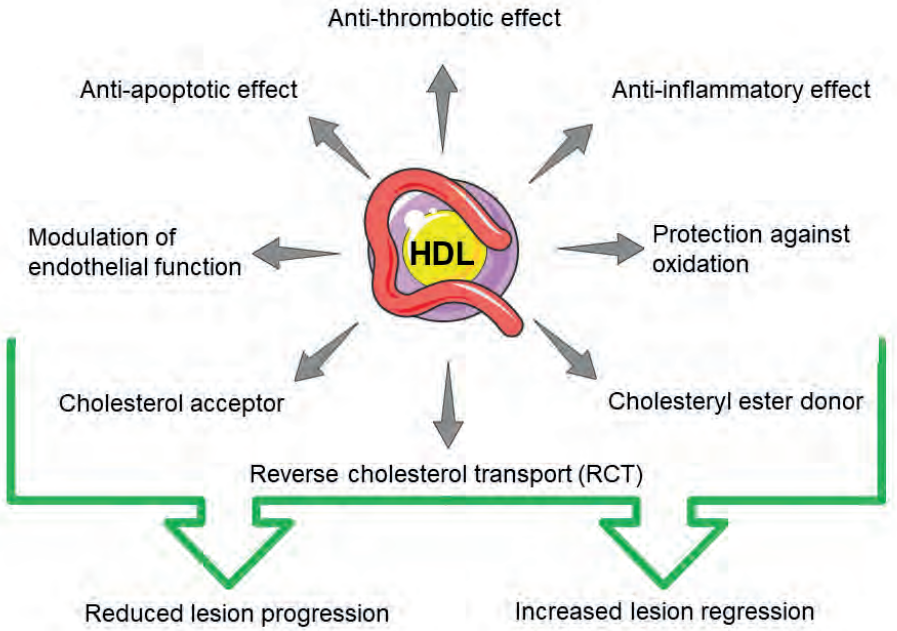

Figure 1: Summary of the anti-atherogenic properties of HDL leading to reduced lesion progression and increased lesion regression.

These studies indicate that regression of existing lesions is facilitated by a combination of impaired recruitment of monocytes and apoptosis of lesional macrophages [35]. The different conclusions of the mechanisms underlying lesion regression could theoretically be dependent on the animal model used. The animal models used are based on lowering the non-HDL fraction while maintaining HDL levels as vehicle for the reuptake of excess cholesterol from the lesions. Our group has recently developed an additional atherosclerosis regression model with the aim to provide more insight in the regression process. More specifically, Van der Stoep et al. showed that reintroduction of bone marrow-derived ApoE in ApoE knockout mice results in a normalization of the hypercholesterolemia and subsequently regression of existing lesions [28]. In **Chapter 3** we tested the hypothesis that the presence of HDL is necessary to initiate lesion regression in this experimental setup. We showed that probucol-induced HDL deficiency impairs the ability of established lesions to regress in response to reversal of the genetic hypercholesterolemia in ApoE knockout mice by reintroduction of ApoE-producing bone marrow-derived cells. Thus independent of the model used, functional HDL is important for stimulating atherosclerosis regression (**Figure 1**).

CHOLESTEROL AND GLUCOCORTICOIDS

In steroidogenesis, cholesterol serves as the sole substrate for the production of steroid hormones including adrenal-derived cortisol in humans and corticosteroids in rodents. However, the contribution of individual lipoprotein species to the generation of the adrenal cholesterol pool remains unknown. In humans, both LDL-C and HDL-C deficiency results in glucocorticoid insufficiency [36,37] suggesting that both LDL and HDL are contributing to the adrenal cholesterol pool. Where the non-HDL fraction is the dominant lipoprotein fraction in humans, the principal lipoprotein fraction in mice is HDL. In **Chapter 4** of this thesis we showed that any alterations to the HDL fraction in murine models results in a reduction of the adrenal cholesteryl ester stores and subsequently leading to a glucocorticoid insufficiency. Also, mice with a human-like lipoprotein profile, transporting the majority of cholesterol in blood by LDL and relatively little by the HDL fraction, display a depletion of the adrenal cholesteryl ester stores [38]. Interestingly, ApoA1 x LDL receptor double knockout mice, lacking the HDL fraction while transporting large amounts of cholesterol in the non-HDL fraction, exhibited a normal plasma corticosterone response despite depletion of the adrenal cholesteryl ester stores [39]. Moreover, in ApoE knockout mice the non-HDL fraction driven hyperlipidemia is paralleled by hypercorticosteronemia and normal cholesteryl ester-stores. These observations let us hypothesize that in mice adrenal cholesteryl ester pools are not the primary source for steroidogenesis and that the loss of HDL can be compensated by the use of cholesterol from non-HDL fractions for steroidogenesis. In **Chapter 4** we examined the impact of the specific lowering of non-HDL levels on adrenal cholesterol and glucocorticoid homeostasis in ApoE knockout mice through transplantation with ApoE-containing wild-type bone marrow. Interestingly, reversal of the hyperlipidemia in ApoE knockout mice was associated with a decreased adrenal glucocorticoid output and accompanied by an unchanged adrenal cholesteryl ester pool. Our studies suggest that in mice at least two cholesterol mobilization routes can be discriminated within the adrenals to maintain a high rate of steroidogenesis (see **Figure 2**): 1) a route in which HDL provides cholesterol to the adrenals for cholesteryl ester storage and that can be subsequently be used for steroidogenesis and cellular metabolism and 2) a route in which cholesterol taken up via the VLDL/LDL (non-HDL fraction) is directly utilized for adrenal steroidogenesis.

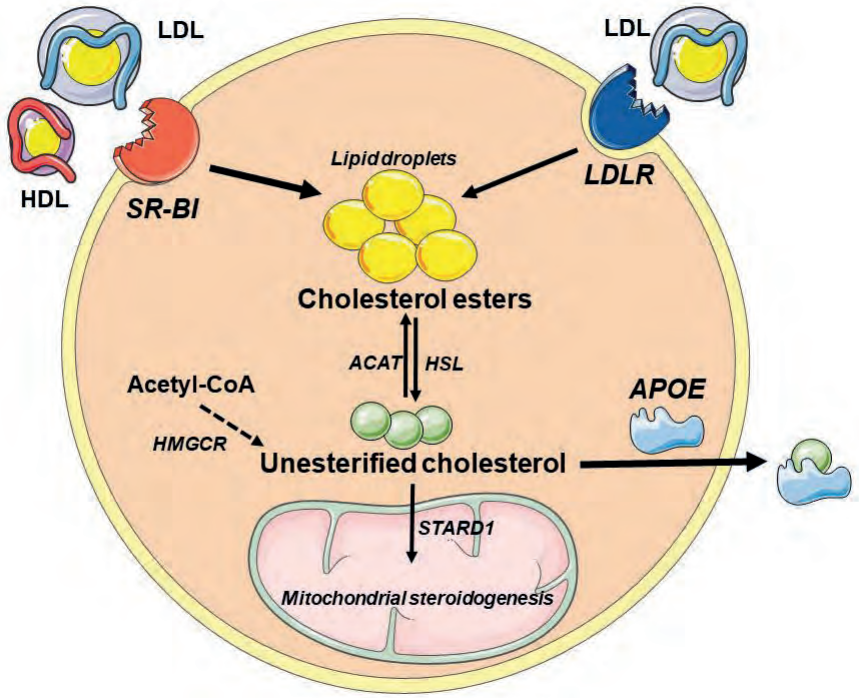

Figure 2: Schematic overview of an adrenal cell with the various routes and sources that provide cholesterol for adrenal steroidogenesis and the suggested role for Apolipoprotein E (ApoE) in the efflux of excess cholesterol from the lipid stores.

The role of the different receptors involved in adrenal cholesterol metabolism is still under debate. The three major lipoprotein receptors available for cholesterol uptake in the adrenal are the LDL receptor, low-density lipoprotein receptor-related protein 1 (LRP1), and SR-BI. We previously demonstrated, via adrenal transplantation, that the LDL receptor is not contributing to the overall adrenal cholesteryl ester pool [40]. Functional deficiency for LRP1 is embryonically lethal and the exact impact of cholesterol uptake via this receptor thus remains elusive. However, in our in vivo study the expression of LRP1 was equal between wild type and ApoE knockout mice (lacking ApoE as important ligand for LRP1-mediated uptake). The third route for adrenal cholesterol uptake is facilitated by SR-BI [41-44]. SR-BI is best known for its affinity for HDL and subsequent selective cholesteryl ester uptake in both the liver and adrenal. Humans with a functional mutation in the SR-BI gene (P297S missense mutation) display increased HDL-C levels and a decreased adrenal steroidogenesis [36]. Yet, the

contribution of SR-BI to cellular cholesterol uptake is not restricted to the HDL fraction but includes selective and whole particle uptake of VLDL/LDL for the acquisition of cholesteryl esters [45-48]. We therefore hypothesize that uptake of VLDL/LDL-derived cholesteryl esters by murine adrenals is also facilitated by SR-BI (**Figure 2 and Chapter 4, Figure 5**). However, further studies need to be executed to elucidate the exact mechanism and its role and relevance for the human situation.

Whereas different circulating lipoprotein fractions affect the steroidogenesis rate, the distribution and presence of intracellular cholesterol could also be of importance. Interestingly, hyperlipidemic ApoE deficient mice exhibit unexplained hypercorticosteronemia. The abundant presence of extracellular substrate for the conversion into steroid hormones could partly explain the hypercorticosteronemia. However, in vitro studies have indicated that locally produced ApoE may also impact directly on adrenal glucocorticoid output. Prack et al. [49] as well as Reyland et al. [50] suggested that intracellular cholesterol transported by ApoE is directly regulating the production of glucocorticoids through interference in the adrenocorticotropic hormone (ACTH)-induced cyclic adenosine monophosphate (cAMP) response [51]. In **Chapter 5** we evaluated the specific effect of adrenal ApoE deficiency on adrenal cholesterol metabolism and steroidogenesis by transplantation of adrenals from ApoE knockout mice into adrenalectomized wild-type animals. The local deletion of adrenal ApoE did not affect glucocorticoid levels in the circulation but did result in the accumulation of cellular cholesterol which was paralleled by endoplasmic reticulum (ER) stress. We therefore hypothesize a novel role for ApoE in the adrenal whereby it actively mobilizes and efflux excess cholesterol to prevent the development of ER stress (**Figure 2**). Yet, further research is required to uncover the exact mechanism that underlies the suggested role of adrenal ApoE.

FUTURE PERSPECTIVES

Development of atherosclerosis and the related cardiovascular events are worldwide the number one cause of death [1]. Scientific milestones like the discovery of the link between cholesterol levels and lesion progression by Nikolay Anitschkow [52] and Russel Ross's establishment of the importance of the inflammatory component in the progression of atherosclerosis [53], largely increased the understanding of the pathobiology underlying the progression of atherosclerotic cardiovascular disease. Targeting a main risk factor, LDL-C, by the introduction of statin-based treatments in

large cohort studies revealed the possibility to reduce overall cardiovascular events and halt further progression of lesion formation. However, actual changes in lesion composition and size are modest with the current interventions [54-56]. Despite the effective statin-induced lowering of LDL-C, in a large group of high-risk patients statin monotherapies inadequately lower serum cholesterol. Furthermore, statin-induced side effects are frequently one of the reasons for discontinuation of the treatment [57]. Therefore, alternative non-statin based therapies have been developed to employ a further reduction in the CVD risk.

The discovery of the enzyme proprotein convertase subtilisin/kerxintype-9 (PCSK9) and the following development of monoclonal IgG2 antibody-based inhibitors of PCSK9 (Alirocumab and Evolocumab) that halt LDL receptor degradation and lower LDL-C [58-60] provided an alternative cholesterol lowering strategy that is effective on top of statin-treatment. The monoclonal antibody approach to silence PCSK9 significantly lowers the LDL-C by ~50 to 60 % and the risk for a cardiovascular event by 9.8 % (Evolocumab) or 15 % (Alirocumab). However, this class of drugs have a downside. High costs and the subcutaneous injections every 2 or 4 weeks made these therapies only available for a selective group of patients. Therefore, alternative therapeutics are under development to inhibit PCSK9 [61], including small interfering RNA (siRNA) [62], vaccine/virus-like particles derived peptides [63-65] and small molecules [66]. These approaches open up a new field to achieve CVD risk reduction via extreme LDL-C lowering therapies and will contribute to the discussion whether a "lower the better" cholesterol strategy will be the future of CVD risk reduction.

Despite the LDL-C lowering associated risk reduction of a CVD event, a residual risk remains. Since lesion progression is an interplay between cholesterol and the inflammatory response of innate and adaptive immune cells, it is hypothesized that it might be beneficial to target the immune response on top op cholesterol lowering. However, anti-inflammatory therapies including corticosteroids and non-steroidogenic anti-inflammatory drugs (NSAID) increase CVD events [67]. The first successful trial targeting the innate immune system, in addition to the conventional therapeutics (LDL-C lowering), was the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) trial by Novartis using an monoclonal antibody-based IL1β inhibitor. This anti-inflammatory treatment dose-dependently lowered IL-6 levels and reduced the risk for primary end points (MI, Stroke, and CVD-related death) while the LDL-C levels remain unchanged [68]. Interestingly, IL1β antibody interventions in ApoE knockout mice with late stage atherosclerosis revealed no effect on lesion size, but

a change in plaque morphology. IL1β antibody-treated mice showed a reduction in smooth muscle cells and collagen and an increased macrophage content [69]. This phenotype in humans could have a Janus faced effect, where in absence of a large collagen-rich cap macrophages can more easily egress from the lesions. Yet, a low collagen content and increases the instability of the lesion leading to more rupture prone plaques. In 2018 Novartis discontinued the development of Canakinumab based on the modest reduction in primary end points and high price per quality-adjusted life-year (QALY) gained ($6.4 million per QALY) [70]. Other alternative trails that test the "inflammatory hypothesis" include the COLCOT trial, using a cheaper and approved anti-inflammatory drug, colchicine. The outcome of this study is positive, with a risk reduction for ischemic cardiovascular related events in patients with a recent myocardial infarction [71]. However, extensive side effects might limit its use in CVD treatment. Overall, anti-inflammatory therapeutics that test the "inflammation hypothesis" could be beneficial in addition to the conventional therapies, however safety and cost effectiveness is a general concern.

Lesion regression of rupture-prone vulnerable / existing lesions includes size reduction (regression) as well as a favorable changes in morphology and composition of the lesions (stabilization). It was a long standing hypothesis that lesion regression of (advanced) plaques was not achievable due to irreversible elements in the composition (necrotic core, fibrosis and calcification). Since the turn of the century, animal models revealed that regression of existing rupture-prone vulnerable lesions was feasible [9,72]. In line, we learned from in vivo models that lesion regression is not simply the reversal of mechanisms that drive progression. It requires distinct processes that initiate the removal of either unwanted cell types or components out of the plaque area, leading to a more favourable composition or shrinkage of the lesion. In addition, non-invasive trials visualizing the lesion progression with IVUS, CIMT ultrasound or angiography showed that upon lipid lowering, lesions in the coronary artery transform into more stable and collagen-enriched lesions [73]. The composition of lesions in the carotid artery could be a predictive biomarker for future events throughout the vascular system [74]. Albeit that stabilization of existing lesions is anticipated to lower the risk for a CVD event, the ideal situation is to deplete all existing lesions from the arterial tree. This is a challenge for future treatments (if feasible at all) and will come with a variety of hurdles to take, including; avoiding atherothrombosis, removal of the collagen layer and the recovery of a healthy endothelial layer.

LABORATORY ANIMAL RESEARCH

For the studies described in this thesis the availability of different knockout mouse models and techniques to modulate gene expression via transplantation techniques have been instrumental. An estimated 14 - 16 million animals, of which 85 - 90 % rodents, are annually used for biomedical research purposes in the U.S. alone [75]. In the Netherlands approximately 530.000 animals, of which 305.000 rodents, were used in 2017 [76]. Despite the elaborate mechanistic lessons learned from the use of in vivo models, the overall problem is that one cannot simply extrapolate in vivo data from animal models towards the human situation. Therefore, the predictive power of animal models for novel drug-based interventions is questioned [77,78]. In the discussion around the predictive power of laboratory animal models, the evolutionary biology and thus genetic (in)equality plays a pinnacle role. Animals that are closely related to humans, the primates, are little used for biomedical research due to ethical objections and high housing costs. The most common animal species used for biomedical research are rodents as they are easy to handle, low in costs and reproduce fast [79]. Although it is clear that understanding a biological mechanism in one species could help to understand the process in a second, the complexity of the biological pathways make that the power of predictions between species for biological function is hard. For CVD, Pasterkamp et al executed a systematic review with the aim to validate atherosclerosis-related genes in ApoE and LDLr knockout mice versus their human orthologues. Interestingly, they found no association with the murine adaptive and innate immunity related genes versus human plaque characteristics, coronary artery disease (CAD) or large artery ischemic stroke (LAS). However, the murine lipid metabolism related genes were significantly associated with human CAD, which is in line with the known role of cholesterol and the risk for CVD. An explanation for the observed discrepancies could be that the reviewed mice models involved primarily total-body knockout mice while mutations for the same genes in humans could have a more mild effect [80]. Nevertheless, it is the complexity of the biological pathways in animals that gives the added value in research fields that cover complex pathologies, like CVD.

Replacement of animals in (drug) research gained interest by the scientific community. During the last decade the use of animals for research is committed to the 3R strategy of Refinement, Replacement, and Reduction. With the rapid progress in the stem cell field it is now possible to skew pluripotent stem cells into almost any cell type. In combination with 3D culturing of different cell types that re-aggregate into organ-like structures, organoids, a potential drug testing and organ replacement platform is created [81]. Furthermore, with the development of the organ-on-a-chip, a 3D

microfluidic based dynamic cell-interaction device [82], several organs and tissue types are mimicked. This platform is designed to function as a 3D in vitro organ model to predict e.g. the pharmacokinetics of drugs. The potential of both platforms could open-up an era where there will be a reduction and replacement of laboratory animals currently used to study complex pathologies. In line, the former Dutch state secretary of economics, Martijn van Dam, set out the ambition in 2016 to make the Netherlands the first animal model free country by 2035 [83]. Recently the U.S. Environmental Protection Agency (EPA) also announced that it will no longer fund studies executed on animals by 2035 [84]. The Dutch ambition was shaped by the public debate around the reduction of laboratory animals and upcoming new techniques. The Dutch committee "Nationaal Comite Advies Dierproevenbeleid (NCAD)" that was assigned to advise the ministry on this topic suggested that a substantial reduction in the use of laboratory animals is an achievable goal [83]. Starting with phasing out the by the government imposed regulatory safety testing and release of biological safety products. However, despite the powerful alternative models described above, the committee agrees that reduction of laboratory animals in pre-clinical and fundamental research cannot be followed at the same pace. Therefore the Dutch scientific community is requested to think along how to achieve this goal in the coming 10 years. It will be interesting to follow the innovations that make this transition possible. Yet, this transition should be accomplished without losing our current expert position in the international research field and coping with the need to study complex biological systems in vivo.

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6

NEDERLANDSE SAMENVATTING

CURRICULUM VITAE

SCIENTIFIC PUBLICATIONS

PHD PORTFOLIO

INDEX OF ABBREVIATIONS

Cholesterol is een essentieel onderdeel voor cellulair metabolisme en een bouwsteen voor de productie van diverse steroïd-hormonen zoals glucocorticoïden. Een verstoorde balans in de concentratie cholesterol in het bloed is gelieerd aan diverse ziektebeelden, waaronder hart- en vaatziekten (HVZ) en het metabool syndroom. In dit proefschrift zijn studies beschreven over de rol van cholesterol, en specifiek de rol van het cholesterol transporterende hoge-dichtheidslipoproteïne (HDL) deeltje, gedurende de progressie en regressie van HVZ. Daarnaast worden de effecten van intra- en extracellulair cholesterol op de productie van steroïd-hormonen afkomstig uit de bijnier uiteengezet.

HDL CHOLESTEROL EN ATHEROSCLEROSE

HVZ zijn de meest voorkomende doodsoorzaak in de Westerse wereld. Hieronder vallen myocard infarcten (MI), kortstondige ischemische aanvallen (TIA), beroertes (cerebrovasculair accidenten; CVA) en andere klinische aandoeningen geïnduceerd door het metabool syndroom. De pathologische aandoening die HVZ drijft is vaatwandverkalking (atherosclerose), plaquevorming in de bloedvatwand. Atherosclerose wordt geïnitieerd door een complex samenspel tussen relatief hoge cholesterolwaarden in het bloed die de vaatwand aantasten en inflammatoire componenten die hierop reageren. Het is algemeen geaccepteerd dat verlaging van de grootste risicofactor, de cholesterol fractie in het lage-dichtheidslipoproteïne (LDL), resulteert in een verminderd risico op een HVZ-gerelateerde aandoening. De huidige interventietherapieën zoals statines realiseren dit voornamelijk door de activiteit van HMG-CoA reductase te remmen. Statine-geïnduceerde cholesterolverlaging leidt tot een 25% vermindering van de kans om te overlijden aan een HVZ-gelieerde doodsoorzaak. Desondanks zijn er nog alternatieve therapieën nodig om het HVZ risico verder te verlagen. Epidemiologische studies laten zien dat er een sterke relatie bestaat tussen een lage concentratie HDL en een verhoogd risico op HVZ. De hypothese was daarom dat het verhogen van HDL-waarden verdere ontwikkeling van atherosclerose zou remmen of zelfs de grootte van bestaande plaques zou kunnen verminderen (atherosclerose regressie). Studies in genetisch gemodificeerde diermodellen hebben laten zien dat de aanwezigheid van HDL essentieel is voor regressie van atherosclerotische plaques. Verhoging van de concentraties van HDL in het bloed, door middel van overexpressie van het humane HDL eiwit Apolipoproteine A1 (ApoA1) of infusie van humaan ApoA1-Milano, resulteert in een afname van

de plaque progressie en leidt zelfs tot een regressie van de plaque in muizen. Daarnaast speelt HDL een belangrijke rol in het transport van cholesterol vanuit de perifere organen naar de lever via een proces genaamd *reverse cholesterol transport* (RCT). Deze bevindingen hebben ertoe geleid dat er verscheidene strategieën zijn ontwikkeld die de concentratie van HDL cholesterol in het bloed van mensen kunnen manipuleren. Helaas waren de uitkomsten van deze therapieën in diverse klinische onderzoeken echter teleurstellend. Er werd aangetoond dat het verhogen van HDL cholesterol, boven op een statinebehandeling, geen vermindering in de plaquegrootte teweeg brengt. Het nut van enkel het verhogen van de HDL cholesterol-concentratie als therapie tegen HVZ werd verder in twijfel gebracht door onderzoeksresultaten uit patiënten en muismodellen met een genetisch defect in de *scavenger receptor B type I* (SR-BI). SR-BI is een belangrijke receptor in het metabolisme van HDL en komt onder andere tot expressie in de lever. Daar faciliteert het de heropname en metabolisme van cholesteryl esters uit HDL. Defecten in het SR-BI-gen resulteren in een verhoging van HDL cholesterol-concentraties in het bloed en een vergroting van de circulerende HDL-deeltjes. Toch leiden deze, in eerste instantie positieve kenmerken, tot een toename in de gevoeligheid voor de ontwikkeling van atherosclerotische plaques. Gecombineerd markeerden de voorgaande observaties een verschuiving in de discussie rond HDL en zijn anti-atherosclerotische eigenschappen, van kwantiteit naar functionaliteit van het HDL-deeltje.
Het eiwit *phospholipid transfer protein* (PLTP) speelt een sleutelrol in het metabolisme van HDL en daarmee de bepaling van de hoeveelheid, grootte en compositie van het HDL-deeltje. PLTP faciliteert de transport van fosfolipiden van het zeer-lage-dichtheidslipoproteïne (VLDL) deeltje naar de beide subgroepen van HDL: pre-β-HDL en α-HDL. Deze functie van PLTP is gebleken uit onderzoek met muizen die gevoed zijn met een vetrijk dieet. Uitschakeling van PLTP in alle weefsels van deze muizen leidde tot een vermindering van de concentratie HDL cholesterol, fosfolipiden en ApoA1 in het bloed en een verhoging van de overige niet-HDL lipoproteïne fracties die fosfolipiden bevatten.

Door een muismodel te genereren waarin zowel PLTP als de HDL receptor SR-BI waren uitgeschakeld hebben we een normalisatie van de grootte en reductie in de plasmaconcentratie van HDL geforceerd in SR-BI knock-out dieren (**Hoofdstuk 2**). We zagen in dit PLTP x SR-BI dubbel knock-out muismodel naast een vermindering van de hoeveelheid cholesterol die door de HDL-deeltjes getransporteerd werd ook een verminderde gevoeligheid voor het ontwikkelen van atherosclerotische plaques. Deze bevindingen onderstrepen de hypothese dat HDL-kwantiteit niet per

se een voorspellende waarde heeft voor de gevoeligheid voor het ontwikkelen van atherosclerotische plaques. Onder normale condities komt PLTP ook tot expressie in CD68-positieve macrofagen, de immuuncellen die in de vaatwand cholesterol opnemen en afvoeren. Opname van grote hoeveelheden cholesterol door macrofagen verandert ze in schuimcellen, die vervolgens vast komen te zitten in de vaatwand en daar het begin van de atherosclerotische plaque vormen. Macrofagen vertonen onder gekweekte condities een verhoogde expressie van PLTP als ze geladen worden met atherogene LDL deeltjes. Onze groep heeft recent in muizen met een LDL-receptor deficiëntie die een cholesterolrijk "Westers" dieet gevoerd werden aangetoond, dat uitschakeling van de productie van PLTP door macrofagen leidt tot een verlaging van cholesterol concentraties in het bloed en een verminderde gevoeligheid voor atherosclerotische plaquevorming. Deze bevindingen suggereren dat lokaal in de bloedvatwand geproduceerd PLTP van invloed kan zijn op de stapeling van cholesterol in macrofagen en dus op de gevoeligheid voor plaquevorming. In **Hoofdstuk 2** laten we dus zien dat de verminderde gevoeligheid voor plaquevorming in dieren met gecombineerde PLTP- en SR-BI-deficiëntie het resultaat is van de herstelde HDL-functionaliteit.

De functionaliteit van HDL wordt o.a. bepaald door zijn capaciteit om cholesterol op te nemen vanuit macrofagen. De effectiviteit van dit proces hangt sterk af van de dikte van de vaatwand (intima-media-dikte) en is, mede door de heterogeniteit van HDL grootte en proteïne lading, onafhankelijk van de concentratie HDL. Het normaliseren van de grootte van het HDL-deeltje in SR-BI-deficiënte muizen dmv uitschakeling van PLTP, leidde tot de vorming van deeltjes die effectiever cholesterol opnemen vanuit macrofagen. Afgelopen jaren is de kennis rondom het metabolisme en de functionaliteit van het HDL-deeltje exponentieel toegenomen. Hieruit is gebleken dat HDL een heterogene populatie van deeltjes bevat die drager zijn van diverse eiwitten in hun membraan met een directe invloed op de functionaliteit van het deeltje. Dit bemoeilijkt nieuwe strategieën om specifiek de functie van HDL te bevorderen. Het lijkt daarom zinvoller om specifiek de macrofagen in de atherosclerotische plaque te benaderen en zo de macrofaag-cholesterol-efflux te stimuleren.

Macrofaag-specifieke cholesterol efflux wordt voornamelijk bewerkstelligd via de ATP-binding cassette transporters A1 en G1 (ABCA1 en ABCG1). Deze eiwitten faciliteren respectievelijk het cholesteroltransport naar de cholesterol-arme voorlopers van het HDL of reeds volgroeide HDL-deeltjes (**Hoofdstuk 1 Figuur 2**). De voornaamste regulator van de ABC transporterfamilie is de liver X receptor (LXR). LXR-agonist

geïnitieerde op-regulatie van ABCA1 en ABCG1 stimuleert cholesterol efflux vanuit macrofagen in kweek. Daarnaast geeft behandeling met een LXR-agonist als resultaat dat macrofagen uit de atherosclerotische plaque verdwijnen. Dit heeft plaqueregressie tot gevolg. Deze twee bevindingen suggereren dat LXR een potentieel doelwit is om zo het risico op en incidentie van HVZ te verminderen. Helaas heeft LXR een bijzonder brede rol in het lipiden-metabolisme, waaronder de regulatie van lever-specifieke aanmaak van lipiden (lipogenese). Toediening van synthetische LXR-agonisten in muizen leidt daarom tot een vermindering van het risico op HVZ, maar verhoogt daarnaast de triglyceride- en VLDL-concentraties in plasma. Daarnaast ontstaat er als reactie op de LXR-agonist behandeling leververvetting. Nieuwe strategieën gericht op specifieke verbetering van het transport van cholesterol uit macrofagen, bijvoorbeeld door selectieve afgifte van een LXR-agonist aan de macrofagen via nano-deeltjes, moeten de bijwerkingen tegen gaan. Deze macrofaag-specifieke therapieën zouden verdere plaque progressie kunnen stoppen en mogelijk misschien wel regressie van de atherosclerotische plaque kunnen bewerkstelligen.

Menigmaal hebben studies in diermodellen al laten zien dat regressie van de atherosclerotische plaque daadwerkelijk mogelijk is, al zijn de meningen verdeeld over het achterliggende werkingsmechanisme. Muismodellen waar regressie van de plaque is gestimuleerd door gecontroleerde normalisatie van de hyperlipidemie (de Reversa muis) of een transplantatie van een plaque-bevattende aortaboog naar een muis met normale cholesterolwaarden (het aorta transplantatie model), suggereren dat onder extreem cholesterolverlagende condities het verdwijnen van macrofagen de drijvende factor is achter de regressie van de plaque. Daarnaast is aangetoond door Rahman en collega's dat het aanvoeren van nieuwe Ly6C^{hi}-positieve monocyten (de voorlopers van macrofagen) ook essentieel is voor het laten verdwijnen van bestaande atherosclerotische plaques. Dit wekt de suggestie dat er een dynamische rol is weggelegd voor monocyten in de plaque, enerzijds stimuleren ze de ontwikkeling, maar anderzijds zijn ze ook nodig om materiaal in de plaque op te ruimen. Er zijn ook alternatieve theorieën voor de macrofaag-migratie-hypothese. Deze zijn vooral gebaseerd op studies waarbij veelal gebruik is gemaakt van ApoE knock-out muizen waarbij de genetische hypercholesterolemie is genormaliseerd door middel van ApoE herintroductie, bijvoorbeeld door middel van virale transfectie. Deze specifieke studies laten zien dat regressie van bestaande atherosclerotische plaques juist gebaseerd is op een combinatie van een verminderde aantrekking van monocyten en apoptose (het gecontroleerd afsterven) van de macrofagen die in de plaque vastzitten. De verschillende hypotheses en daaruit getrokken conclusies over

het achterliggende mechanisme van regressie zouden theoretisch afhankelijk kunnen zijn van het gebruikte diermodel. Beide hypotheses zijn gebaseerd op diermodellen waarin de non-HDL-fractie is verlaagd en tegelijkertijd de HDL-fractie is behouden om te fungeren als drager voor heropname van cholesterol uit de plaques. Onze groep heeft recent een additioneel diermodel voor plaque regressie ontwikkeld. Van der Stoep et al. hebben laten zien dat door de herintroductie van ApoE-producerende beenmerg-afgeleidde cellen in een ApoE knock-out muis de hypercholesterolemie van deze dieren kan worden genormaliseerd en dat als gevolg daarvan regressie van bestaande plaques kan worden bewerkstelligd. In **Hoofdstuk 3** hebben we in dit model de hypothese getest dat de aanwezigheid van HDL essentieel is om regressie van bestaande plaques te initiëren. We laten zien dat wanneer de hoeveelheid HDL in de circulatie extreem wordt verminderd door toediening van het cholesterol-verlagende medicijn Probucol er geen regressie van bestaande plaques meer optreedt. Uit deze bevinding kan dus geconcludeerd worden dat, onafhankelijk van het gebruikte model, de functionaliteit en aanwezigheid van HDL essentieel is om regressie van bestaande plaques te initiëren in muizen.

CHOLESTEROL EN GLUCOCORTICOÏDEN

Cholesterol is het belangrijkste substraat voor de productie van steroïd-hormonen (steroïdogenese), waaronder het door de bijnier geproduceerde cortisol in mensen en corticosteron in knaagdieren. Helaas is de bijdrage van de verschillende lipoproteïnen-fracties in deze processen nog onduidelijk. In mensen is aangetoond dat een deficiëntie in de aanmaak van zowel LDL als HDL resulteert in een verminderde productie van glucocorticoïden. Dit wekt de suggestie dat zowel LDL-cholesterol als HDL-cholesterol bijdragen aan de cholesterolpool in de bijnier die gebruikt wordt voor steroïdogenese. In mensen is de non-HDL-fractie (LDL en VLDL) de dominante lipoproteïnen-fractie, maar knaagdieren hebben voornamelijk de HDL fractie in hun bloed. In de studies beschreven in **Hoofdstuk 3** hebben we laten zien dat concentratieveranderingen in de HDL-fractie in muizen resulteert in een vermindering van de cholesteryl-ester-pool in de bijnier en daarmee een verminderde productie van corticosteroïden. Het is interessant om te zien dat muizen met een meer humaan lipoproteïnen profiel juist een totale depletie van de bijnier cholesteryl-ester-pool hebben. Dit terwijl ApoA1 x LDL receptor dubbel-deficiëntie muizen (deze dieren missen de HDL fractie en hebben een verhoogde concentratie van de non-HDL fractie) een normale plasma-

corticosteronrespons op stress laten zien, ondanks het missen van een cholesteryl-ester-pool in de bijnier. ApoE-deficiënte dieren tenslotte, met een verhoogde non-HDL fractie, hebben interessant genoeg ook hypercorticosteronemia, terwijl de bijnier cholesteryl-ester-pool van normale grootte is. Vanwege deze observaties hebben wij de hypothese gesteld dat in muizen de bijnier cholesteryl-ester-pool niet de primaire bron van cholesterol is voor de aanmaak van steroïd-hormonen en dat bij het wegvallen van de HDL-fractie de bron voor cholesterol gecompenseerd kan worden door de non-HDL fracties. In **Hoofdstuk 4** zijn wij ingegaan op de impact van non-HDL-specifieke verlaging in het bloed op de cholesteryl-ester-pool in de bijnier en de homeostase van glucocorticoïden. Hiertoe hebben ApoE-deficiënte muizen een beenmergtransplantatie ondergaan met wildtype (ApoE-bevattend) beenmerg. Herintroductie van ApoE in ApoE-deficiënte muizen leidde tot normalisatie van de hypercholesterolemie en een verminderde steroïdeproductie in de bijnier. Dit ging samen met een onveranderde cholesteryl-ester-pool. Deze studies suggereren dat er tenminste twee routes te onderscheiden zijn die de bijnier steroïdogenese van cholesterol voorzien (**Hoofdstuk 6 Figuur 2**): 1) de route waarbij de HDL-fractie de bijnier van cholesterol voorziet voor de cholesteryl-ester-pool die gebruikt kan worden voor steroïdogenese en cellulaire metabolisme doeleinden en 2) de route waarbij cholesterol opgenomen wordt in de bijnier via de VLDL/LDL-fracties. Deze laatste kan direct gebruikt worden voor de aanmaak van steroïd-hormonen.

Naast de rol van de verschillende lipoproteïnen is er ook nog veel onduidelijk over het belang van de verschillende receptoren op de bijniercellen die cholesterol opnemen. De drie voornaamste cholesterol-opname gerelateerde lipoproteïnereceptoren die op de bijnier zitten zijn: 1) de LDL-receptor, 2) LDL-receptor related proteïn 1 (LRP1), en 3) SR-BI. We hebben aangetoond, middels een bijniertransplantatie model, dat de LDL-receptor niet bijdraagt aan de cholesteryl-ester-pool in de bijnier van muizen. Muizen met functionele deficiëntie van LRP1 zijn niet levensvatbaar en dus blijft de exacte impact van cholesterol opname via deze receptor in de bijnier tot nu onduidelijk. De derde route voor cholesterolopname door de bijnier is via SR-BI. SR-BI is niet alleen een receptor met een grote affiniteit voor HDL-deeltjes betrokken bij de selectieve opnamen van cholesteryl-esters door de lever, maar ook in de bijnier. Mensen met een functionele mutatie in het SR-BI-gen (P297S missense mutatie) hebben een verhoogde HDL-concentratie in het bloed en een verlaagde cortisolafgifte. Toch is de contributie van SR-BI in cholesterolopname niet beperkt tot de HDL-fractie, want SR-BI kan de opnamen van hele VLDL/LDL-deeltjes faciliteren. Onze hypothese is dan ook dat in de bijnier van een muis de opname van cholesterol uit VLDL/LDL-deeltjes zou kunnen worden gefaciliteerd door SR-BI (**Hoofdstuk 6 Figuur 2 en Hoofdstuk 4 Figuur 5**). Er is

echter verder onderzoek nodig om het exacte mechanisme, de rol en relevantie in de humane setting te verduidelijken.

Verschillende lipoproteïnenfracties die in het bloed circuleren hebben een direct effect op de cholesterolpool in de bijnier en daardoor op de productie van steroïd hormonen. Daarnaast hebben diverse in vivo studies aangetoond dat de aanwezigheid van cholesterol in de bijnier-cel (intracellulair) van invloed zou kunnen zijn op de aanmaak van glucocorticoïden. Opvallend is dat ApoE-deficiënte muizen naast hypercholesterolemie ook hypercorticosteronemia ontwikkelen. Deels kan dit fenomeen verklaard worden door de overmatige aanwezigheid van het substraat voor steroïdogenese. Echter, in-vitro studies hebben aangetoond dat ApoE geproduceerd in de bijnier zelf ook een direct effect heeft op de steroïd productie. Prack et al. en ook Reyland et al. suggereren dat ApoE een rol speelt bij het intracellulair transport van cholesterol en zodanig ook de productie van steroïd-hormonen kan reguleren. Dit is mede interessant omdat de adrenocorticotropic hormoon (ACTH) gereguleerde respons op cyclic adenosine monophosfaat (cAMP) direct de ApoE expressie kan reguleren. In **Hoofdstuk 5** wordt het effect van een bijnier-specifieke ApoE-depletie besproken en de uitwerking hiervan op het bijnier-cholesterolmetabolisme en de steroïdogenese. In deze studie hebben we een bijnier van een ApoE-deficiënte muis naar een bijnierloos gemaakte wildtype muis getransplanteerd. Deze bijnier-specifieke ApoE-depletie had geen effect op de glucocorticoïdconcentraties in het bloed, maar resulteerde in een accumulatie van cellulair cholesterol tezamen met een verhoogd endoplasmatisch reticulum (ER) stressniveau in de bijnier. Op basis van deze data stellen wij een nieuwe rol voor ApoE voor waarbij het in de bijnier geproduceerde ApoE actief cholesterol mobiliseert en de uitwisseling van overmatige hoeveelheid cholesterol bevordert om zo ER stress in de bijniercellen te voorkomen.

TOEKOMSTPERSPECTIEVEN

Atherosclerose en aan HVZ-gerelateerde ziektebeelden zijn wereldwijd doodsoorzaak nummer één. Wetenschappelijk behaalde mijlpalen, zoals de ontdekking van de link tussen cholesterolniveaus en progressie van de atherosclerotische plaque door Nikolay Anitschkow en het belang van ontstekingscomponenten in de plaque-ontwikkeling door Russel Ross, hebben gezorgd voor een beter begrip van de pathobiologie achter atherosclerose. Cohortstudies naar statine-geïnduceerde LDL-verlaging, hebben aangetoond dat het mogelijk is om het risico op HVZ te verminderen en

verdere progressie van de plaque een halt toe te roepen. Desondanks veranderen de plaquecompositie en -grootte maar nauwelijks met de huidige interventiestrategieën. Alhoewel een groot deel van de patiënten baat heeft bij de verlaging van de LDL-cholesterol concentratie in het bloed middels statines, blijft er een grote groep patiënten die met een monotherapie van statines de cholesterolconcentraties niet of niet afdoende omlaag kan krijgen. Daarnaast zijn de bijwerkingen van statines vaak een reden om de medicatie te stoppen. Mede daarom worden er alternatieve niet-statine-gebaseerde therapieën ontwikkeld die het risico op HVZ verder moeten verminderen. Naar aanleiding van de ontdekking van het enzym proprotein convertase subtilisin/kerxintype-9 (PCSK9) en de daaropvolgende ontwikkeling van monoclonale IgG2-antilichaam met een remmende werking op PCSK9 (alirocumab en evolocumab) is een alternatieve cholesterolverlagende therapie ontwikkeld. Deze monoclonale antilichamen zorgen ervoor dat de LDL-receptor niet afgebroken kan worden en verlagen daarmee de concentratie LDL-cholesterol in het bloed met circa 50 tot 60 procent en verminderen de kans op een HVZ-gerelateerde aandoening met 9.8 % (evolocumab) en 15 % (alirocumab). Toch hebben deze medicijnen een keerzijde. De hoge kosten en subcutane injecties die om de 2 tot 4 weken gegeven moeten worden maken deze therapie alleen geschikt voor een selecte groep patiënten. Alternatieve therapieën die PCSK9 remmen zijn nog in ontwikkeling. Hieronder vallen kleine interfererende RNA moleculen (siRNAs), vaccin/virus-achtige partikels waar peptiden uit kunnen voortkomen en kleine moleculen. Deze technieken openen deuren naar een geheel nieuw tijdperk waarin het risico op HVZ verminderd wordt door extreme verlaging van de LDL-cholesterol-concentratie en dragen bij aan de algehele discussie die gaat over de vraag of in de toekomst een "lager is beter" beleid voor cholesterol concentraties gevolgd zou moeten worden.

Alhoewel het verlagen van de LDL-cholesterol-concentratie een direct verband heeft met het verlagen van het risico op HVZ, blijft er een resterend risico aanwezig. Omdat plaqueprogressie een samenspel is tussen cholesterol en de respons van het aangeboren en verworven deel van het immuunsysteem, is de hypothese dat beïnvloeding van de immuunrespons gunstige effecten kan hebben bovenop strategieën gericht op cholesterolverlaging. Desalniettemin geven therapieën gericht tegen het immuunsysteem, o.a. corticosteroïden en non-steroïdogene anti-inflammatoire drugs (NSAID), juist een verhoging van het risico op HVZ. Een eerste succesvol onderzoek met als doelwit het aangeboren deel van het immuunsysteem was de Canakinumab anti-inflammatory Thrombosis Outcomes Study (CANTOS), uitgevoerd door NOVARTIS. Hierbij werd gebruik gemaakt van een monoclonaal antilichaam dat

de productie van interleukine-1β (IL-1β) remt. Deze ontstekingsremmende therapie verlaagde op een dosis-afhankelijke manier de concentratie interleukine-6 (IL-6) in het bloed en verminderde daarmee de kans op het krijgen van een van de primaire HVZ eindpunten (hartinfarct, beroerte en andere HVZ-gerelateerde doodsoorzaken) terwijl de LDL-cholesterol-concentratie onveranderd bleef. Daarnaast hebben studies in ApoE-deficiënte muizen met verregaande ontwikkeling van atherosclerose laten zien dat een behandeling met een IL-1β-antilichaam leidt tot onveranderde plaquegrootte. Dit terwijl er in de met IL-1β-antilichaam behandelde dieren een vermindering van de gladde spiercellen en collageenvorming en een toename van de hoeveelheid macrofagen gemeten werd. Toch zou dit positieve op het eerste gezicht positieve effect in mensen een tweezijdig effect kunnen geven. Enerzijds omdat het verminderen van de collageenrijke cap over de plaque het vergemakkelijkt om de macrofagen te laten verdwijnen uit de plaque. Anderzijds zorgt collageen juist voor stabiliteit. Dit voorkomt dat er een instabiele plaque ontstaat die eerder kan scheuren. NOVARTIS heeft er uiteindelijk in 2018 voor gekozen om de ontwikkeling van Canakinumab te stoppen. Deze beslissing was gebaseerd op de milde verlaging van de primaire eindpunten en de hoge prijs per quality-adjusted per life-year (QALY) ($6.4 miljoen per QALY). Alternatieve onderzoeken die de ontstekingshypothese testen, zijn onder meer de COLCOT-studie waarin een goedkoper en al goedgekeurd ontstekingsremmende medicijn wordt getest, namelijk Colchicine. De uitkomsten van deze studie lijken positief uit te vallen, met een verlaging van het risico op ischemische HVZ-gerelateerde aandoeningen in patiënten die recent een hartinfarct hebben gekregen. Desalniettemin zijn er ernstige bijwerkingen die de bruikbaarheid van deze ontstekingsremmer in de weg kunnen staan. Samenvattend is het mogelijk dat therapieën die het immuunsysteem targeten een positieve bijdrage kunnen leveren aan het verminderen van het risico op HVZ bovenop conventionele therapieën, mits daarbij de veiligheid en de kosten niet in het geding komen.

Regressie van gevorderde of instabiele kwetsbare plaques kan worden omschreven als a) een reductie van de plaquegrootte (regressie) of b) positieve veranderingen in de samenstelling van de plaque (stabilisatie). In het verleden was de De hypothese dat regressie van (gevorderde) plaques niet mogelijk was door de onomkeerbare elementen in de compositie van de (gevorderde) plaque zoals een necrotische kern, fibrose en calcificaties. Deze zienswijze veranderde rond de eeuwwisseling, toen diermodellen aantoonden dat regressie en stabilisatie van (gevorderde) kwetsbare plaques tot de mogelijkheden behoorde. Daarnaast leerden deze modellen ons dat plaqueregressie niet simpelweg het omgekeerde is van het ontstaan van een plaque. Om regressie

te induceren zijn er specifieke processen nodig die aanzetten tot het verwijderen van ongewilde celtypes of componenten (stabilisatie) uit de plaque om zo een compositie te krijgen die leidt tot het verminderen (regressie) van de plaquegrootte. Non-invasieve onderzoeken die zich richten op het visualiseren van de plaqueprogressie door middel van IVUS, CIMT ultrasound of angiografie hebben laten zien dat door het verlagen van de concentratie cholesterol in het bloed de plaquecompositie in de coronaire vaten collageenrijker werden en dus stabiliseerde. Daarnaast geldt dat het juist de coronaire vaten zijn die als een voorspellende bio-marker aangemerkt kunnen worden voor de staat van het totale vasculaire systeem. Ondanks dat deze plaquestabilisatie goed nieuws is en gelinkt wordt aan een verminderde kans op HVZ, blijft de ideale situatie dat alle plaque verdwijnt uit het vasculaire systeem (regressie). Dit is de uitdaging voor de toekomstige therapieën waartoe een flink aantal horden zullen moeten worden genomen, waaronder het vermijden van atherothrombose, verminderen van de aangroei van collageen lidtekenweefselen het herstel van de beschadigde endotheelcellaag.

PROEFDIEREN IN DE WETENSCHAP

Voor de studies beschreven in dit proefschrift is gebruik gemaakt van diverse muismodellen en technieken (waaronder transplantatietechnieken)om genexpressie te moduleren als instrument om onze hypotheses te testen. Geschat wordt dat jaarlijks alleen al in Amerika 14 tot 16 miljoen dieren, waarvan 85-90 % knaagdieren, gebruikt worden voor biomedisch onderzoek. In Nederland werden in 2017 naar schatting 530.000 dieren, waarvan 305.000 knaagdieren, gebruikt voor dit doel. Ondanks de vele lessen die we als wetenschappers hebben geleerd door het gebruik van proefdieren, blijft het algemene probleem dat de bevindingen uit bijvoorbeeld de muis niet simpelweg geëxtrapoleerd kunnen worden naar de humane situatie. Mede hierdoor wordt de voorspellende waarde van proefdieren voor het testen van nieuwe medicijnen in twijfel getrokken. In de algemene discussie rond het gebruik en de voorspellende waarde van proefdieren speelt de evolutiebiologie en daarmee de genetische (on) gelijkheid tussen verschillende diersoorten een cruciale rol. Dieren die genetisch veel gelijkenissen hebben met mensen, de primaten, worden maar op kleine schaal gebruikt voor biomedisch onderzoek door ethische bezwaren en de dure huisvesting. De meest gebruikte proefdieren (knaagdieren) zijn juist dieren die genetisch verder afstaan van mensen. Desondanks worden ze veel toegepast omdat ze makkelijk te hanteren zijn, tegen een lage kostprijs te huisvesten zijn en zich snel voortplanten. Alhoewel het duidelijk mag zijn dat het begrijpen van een biologisch mechanisme in de ene diersoort helpt om het proces in een andere diersoort te ontrafelen, maken juist de complexiteit

van de biologische mechanismen het lastig om er een voorspellende waarde aan te hangen. Pasterkamp et al. hebben in een systematische review de genen gevalideerd die gelinkt zijn aan atherosclerose in ApoE- en LDL-receptor-deficiënte muizen versus de humane orthologen. Hierbij vonden ze in de plaques van muizen geen associatie in de genen van het verworden en aangeboren immuunsysteem versus de genen gelinkt in de humane plaque compositie van patiënten met coronair vaatlijden of arteriële ischemische beroertes. Desalniettemin kwamen genen die gerelateerd zijn aan het lipiden-metabolisme in muizen significant overeen met hun orthologen in plaques van patiënten. Een verklaring voor deze discrepantie zou kunnen liggen in de gebruikte muismodellen. Voor muizen studies wordt voornamelijk gebruik van totale knock-out modellen terwijl in de humane situatie altijd delen van genen gemuteerd zijn wat dus een milder effect zou kunnen geven. Ondanks deze verschillen verschaffen proefdieren t.o.v. bijvoorbeeld in vitro gekweekte cellijnen juist inzichten in de complexiteit van biologische mechanismen. Hierin ligt ook de kracht voor het ontrafelen van complexe ziektebeelden zoals HVZ.

Het vervangen van proefdieren in de wetenschap heeft de afgelopen periode zijn vlucht genomen binnen de wetenschappelijke gemeenschap. Afgelopen decennium zijn het gebruik van de drie V's, Vervangen Verminderen en Verfijnen, meer ingeburgerd in het ontwerpen van experimenten met dieren. Daarnaast zijn er tegenwoordig betere alternatieven voor het gebruik van proefdieren. Hieronder vallen de ontwikkelingen in het stamcel-veld, waarbij het nu mogelijk is om uit pluripotente stamcellen nagenoeg ieder mogelijk celtype te genereren. Dit, in combinatie met het in 3D kweken van cellen, vormt de basis van de vorming van organoids, mini-organen, waarop medicijnen in kweek getest kunnen worden. Verder is er de ontwikkeling van de organ-on-a-chip modellen, een statische setting waarin verschillende celtypen interactie met elkaar hebben en zo diverse organen kunnen nabootsen. Dit platform is speciaal ontwikkeld als 3D in vitro model voor het farmacokinetisch testen en voorspellen van de effecten van nieuwe medicijnen. De potentie van beide platformen opent een nieuw tijdperk waarin we Vermindering en Vervanging van proefdieren kunnen stimuleren en toch complexe ziektebeelden kunnen blijven bestuderen. Volledig in lijn met deze ontwikkeling ligt het streven van de voormalig staatssecretaris van Economische zaken, Martijn van Dam, die in 2016 de ambitie heeft uitgesproken om Nederland in 2025 als eerste land proefdiervrij te maken. Ook de Amerikaanse Environmental Protection Agency (EPA) heeft verkondigd dat ze per 2035 geen studies meer willen bekostigen die gebruik maken van proefdieren. De Nederlandse ambitie komt voort

uit het publieke debat rondom het verminderen van het gebruik van proefdieren en de opkomende nieuwe technieken die dit zouden kunnen vervangen. De staatssecretaris heeft het Nationale Comité Advies Dierproevenbeleid (NCAD) gevraagd om met aanbevelingen te komen aangaande dit onderwerp. Zij hebben gesteld dat een substantiële vermindering van dierproeven haalbaar is binnen de gestelde termijn. Om deze doelstellingen te halen zou de overheid gefaseerd moeten stoppen met de verplichte toetsing op de biologische veiligheid van producten op proefdieren. Ondanks de ontwikkelde alternatieve platformen is de Commissie er niet van overtuigd dat vermindering van het gebruik van proefdieren in preklinisch en fundamenteel onderzoek op dezelfde voet kan worden gevolgd. Er is daarom aan de Nederlandse wetenschappelijke gemeenschap gevraagd om mee te denken hoe deze doelstelling gehaald zou kunnen worden in de komende 10 jaar. De ontwikkeling van de technieken en voorstellen die nodig zijn om deze transitie te bewerkstelligen zal met grote interesse gevolg worden. De gehele transitie zal namelijk moeten worden afgerond zonder onze huidige expertisepositie binnen de internationale wetenschappelijke gemeenschap te verliezen en moet tegemoetkomen aan de behoefte om complexe biologische systemen te kunnen blijven bestuderen.

CURRICULUM VITAE

Ronald van der Sluis werd geboren op 19 augustus 1979 te Delft. Na het behalen van zijn MAVO diploma in 1996 begon hij aan de Middenkader Laboratorium Opleiding aan het ROC te Leiden met de specialisatie: medisch. Voor deze opleiding heeft Ronald in 2000 stage gelopen bij het Nederlands Herseninstituut te Amsterdam onder begeleiding van dr. Bas Blits en prof. dr. Joost Verhaagen met als onderwerp het laten herstellen en uitgroeien van beschadigde motorneuronen middels AAV-gen transfer van neuro-trofische groeifactoren. Aansluitend heeft hij zijn Bachelor diploma Biologie en Medisch laboratoriumonderzoek met als specialisatie Dierkunde behaald aan de hogeschool van Utrecht. De opleiding werd in 2005 afgerond met een stage aan de onderzoeksorganisatie, Toegepast-Natuurwetenschappelijk Onderzoek (TNO) te Leiden onder begeleiding van dr. Abbey Schepers en prof. dr. Paul Quax. Het onderwerp van deze stage was het opzetten van het vaatwand denudatie model om restenose van gedotterde vaten te bestuderen in muizen. Gedurende de periode 2005 - 2009 was Ronald werkzaam als analist bij TNO Leiden op de afdeling Vascular and Metabolic Diseases onder leiding van prof. dr. Paul Quax waarbij zijn werkzaamheden gefocust waren op het uitvoeren van *in vivo* onderzoek op een contract research basis. In de daarop volgende jaren (2009 - 2013) was hij werkzaam als research analist op de afdeling Biofarmacie van het Leiden Academic Centre for Drug Research (LACDR) te Leiden in de groep van prof. dr. Theo van Berkel en prof. dr. Johan Kuiper. De overkoepelende onderzoek-focus van deze groep was de relatie tussen cholesterol metabolisme en atherosclerose. Samen met dr. Menno Hoekstra heeft hij in deze periode het bijnier-transplantatie model in muizen ontwikkeld waarbij het mogelijk werd om bijnier-specifiek de rol van cholesterol gerelateerde genen in de steroïde productie te bestuderen.

In oktober 2013 kreeg hij de mogelijkheid om een promotieonderzoek, zoals beschreven staat in dit proefschrift, te starten op de afdeling Biofarmacie van het LACDR onder leiding van prof. dr. Miranda van Eck en dr. Menno Hoekstra. Dit onderzoek werd gefinancierd door de Nederlandse Organisatie voor Wetenschappelijk Onderzoek middels een VICI grant toegekend aan prof. dr. Miranda van Eck (VICI 91813603).

Daaropvolgend (2018-2020) was Ronald research analist aan het Leids Universitair Medisch Centrum (LUMC) te Leiden bij de afdeling Nierziekten in de Eilandjes transplantatie groep van prof. dr. Eelco de Koning. Per eind 2020 zal hij werkzaam zijn als stafadviseur binnen de Instantie voor Dierwelzijn van het LUMC.

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PHD PORTFOLIO

COURSES

2016 Writing A Press Release (Leiden University)
On Being A Scientist (Ethics In Science) (Leiden University)
2015 Academic Writing (Leiden University)
Data Management (Leiden University)
2014/7 Introduction To Teaching And Supervision (Leiden University)
2014 Effective Communication (Leiden University)
Time Management (Leiden University)
Dutch Heart Foundation Course: Atherosclerosis & Thrombosis
(Dutch Heart Foundation)
International Atherosclerosis Research School, Hamburg, Germany (iARS)
LACDR PhD Introductory Course (Leiden University)

(POSTER) PRESENTATIONS AT (INTER)NATIONAL MEETINGS

2017 Scandinavian Society for Atherosclerosis Research, Humlebaeck, Denmark (Poster)
European Lipoprotein Club, Tutzing, Germany (Poster)
LACDR Spring Symposium, Leiden, the Netherlands (Poster)
Rembrandt Symposium, Noordwijkerhout, the Netherlands (Poster)
Nederlandse Vereniging voor Endocrinologie, Noordwijk, the Netherlands (Poster pitch)

2016 Lipoprotein Metabolism Gordon Research Conference, Boston, USA (Poster)
LACDR Spring Symposium, Leiden, the Netherlands (Poster)
Rembrandt Symposium, Noordwijkerhout, the Netherlands (Poster)
Cardiovascular Conference/Dutch Atherosclerosis Society, Ede, the Netherlands (Poster)
European Lipoprotein Club, Tutzing, Germany (Poster)

2015 International Society Atherosclerosis, Amsterdam, the Netherlands (Poster)
LACDR Spring Symposium, Leiden, the Netherlands (Poster)
Rembrandt Symposium, Noordwijkerhout, the Netherlands (Poster)
Cardiovascular Conference/Dutch Atherosclerosis Society, Amersfoort, the Netherlands (Poster)

2014 Nederlandse Lipoproteïne Club, Leiden, the Netherlands (Oral)
LACDR Spring Symposium, Leiden, the Netherlands (Poster)
Rembrandt Symposium, Noordwijkerhout, the Netherlands (Poster)
International Atherosclerosis Society HDL, Rome, Italy (Oral)

INDEX OF ABBREVIATIONS

ABCA1/G1	: ATP-Binding cassette transporter A1/G1
ACAT	: Acetyl-coenzyme A acetyltransferase
ACTH	: Adrenocorticotropic hormone
AD	: Alzheimer's disease
Apo	: Apolipoprotein
ApoE/C/A4/B100	: Apolipoprotein E/C/A4/B100
AR	: Adenylyl Cyclase
ASO	: Antisense oligonucleotide
AUC	: Area under the curve
CAD	: Coronary artery disease
cAMP	: Cytoplasmic adenosine monophosphate
CCR2	: C-C chemokine receptor type 2
CD 4/8/68	: Cluster of differentiation 4/8/68
CE	: Cholesteryl ester
CETP	: Cholesteryl ester transfer protein
CHOP	: C/EBP homologous protein
CM	: Chylomicron
CREB	: cAMP-response element binding protein
CRF	: Corticotropin-releasing factor
CVD	: Cardiovascular disease
CYP11A1	: Cytochrome P450 Family 11 Subfamily A Member 1
CYP11B1	: Cytochrome P450 Family 11 Subfamily B Member 1
CYP21A2	: Cytochrome P450 Family 21 Subfamily A Member 2
ER	: Endoplasmic reticulum
FA	: Fatty acid
FC	: Free cholesterol
FGF21	: Fibroblast growth factor 21
GAPDH	: Glyceraldehyde 3-phosphate dehydrogenase
GC	: Glucocorticoid
GCr	: Glucocorticoid receptor
GPCR	: G protein-coupled receptor
gWAT	: Gonadal white adipose tissue
HDL	: High-density lipoprotein
HDL-C	: High-density lipoprotein cholesterol
HL	: Hepatic lipase

HMGCoA	: Hydroxymethylglutaryl coenzym A
HMGCR	: 3-hydroxy-3methylglutaryl coenzyme A reductase
HPA	: Hypothalamic-pituitary-adrenal
HSD11B2	: Hydroxysteroid 11-Beta Dehydrogenase 2
HSD3B2	: Hydroxysteroid 3-Beta Dehydrogenase 2
HSL	: Hormone-sensitive lipase
HSPG	: Heparan sulfate proteoglycan
iBAT	: Interscapular brown adipose tissue,
ICAM-1	: Intercellular adhesion molecule-1
IDL	: Intermediate-density lipoprotein
KO	: Knock out
LCAT	: Lecithin-cholesterol acyltransferase
LDL	: Low-density lipoprotein
LDL-C	: Low-density lipoprotein cholesterol
LDLr	: Low-density lipoprotein receptor
LPL	: Lipoprotein lipase
LPS	: Lipopolysaccharide
LRP1	: LDL receptor related protein 1
LXR	: Liver X receptor
MCP-1	: Monocyte chemoattractant protein 1
MCR	: Mineralocorticoid receptors
MetS	: Metabolic syndrome
mRNA	: Messenger ribonucleic acid
MT	: Massons Trichrome histological staining
MTTP	: Microsomal triglyceride transfer protein
MVA pathway	: Mevalonate pathway
NEUTRO	: Neutrophils
NS	: Non significant
ORO	: Oil red O histological staining
OxLDL	: Oxidized low-density lipoprotein
P	: Protein
PCSK9	: Proprotein convertase subtilisin/kexin type 9
PKA	: Protein kinase A
PL	: Phospholipid
PLTP	: Phospholipid transfer protein
pWAT	: periganodal white adipose tissue
QALY	: Quality-adjusted life-year

RCT	: Reverse cholesterol transport
RNA	: Ribonucleic acid
SEM	: Standard error of the mean
SMC	: Smooth muscle cells
SO	: Sense oligonucleotide
SR-BI	: Scavenger receptor class B type I
StAR	: Steroidogenic acute regulatory protein
sWAT	: subcutaneous white adipose tissue
T2DM	: Type 2 diabetes mellitus
TG	: Triglycerides
VCAM-1	: Vascular cell adhesion molecule-1
VLDL	: Very low-density lipoprotein
VLDL-C	: Very low-density lipoprotein cholesterol
WAT	: White adipose tissue
WBC	: White blood cell count
WTD	: Western-type diet
β-actin	: Beta-actin
[^{14}C]CO	: ^{14}C labeled cholesteryl oleate
[^{3}H]CEt	: ^{3}H labeled cholesteryl ether
[^{3}H]TO	: ^{3}H labeled Tri-oleate
36B4	: Ribosomal protein lateral stalk subunit P0