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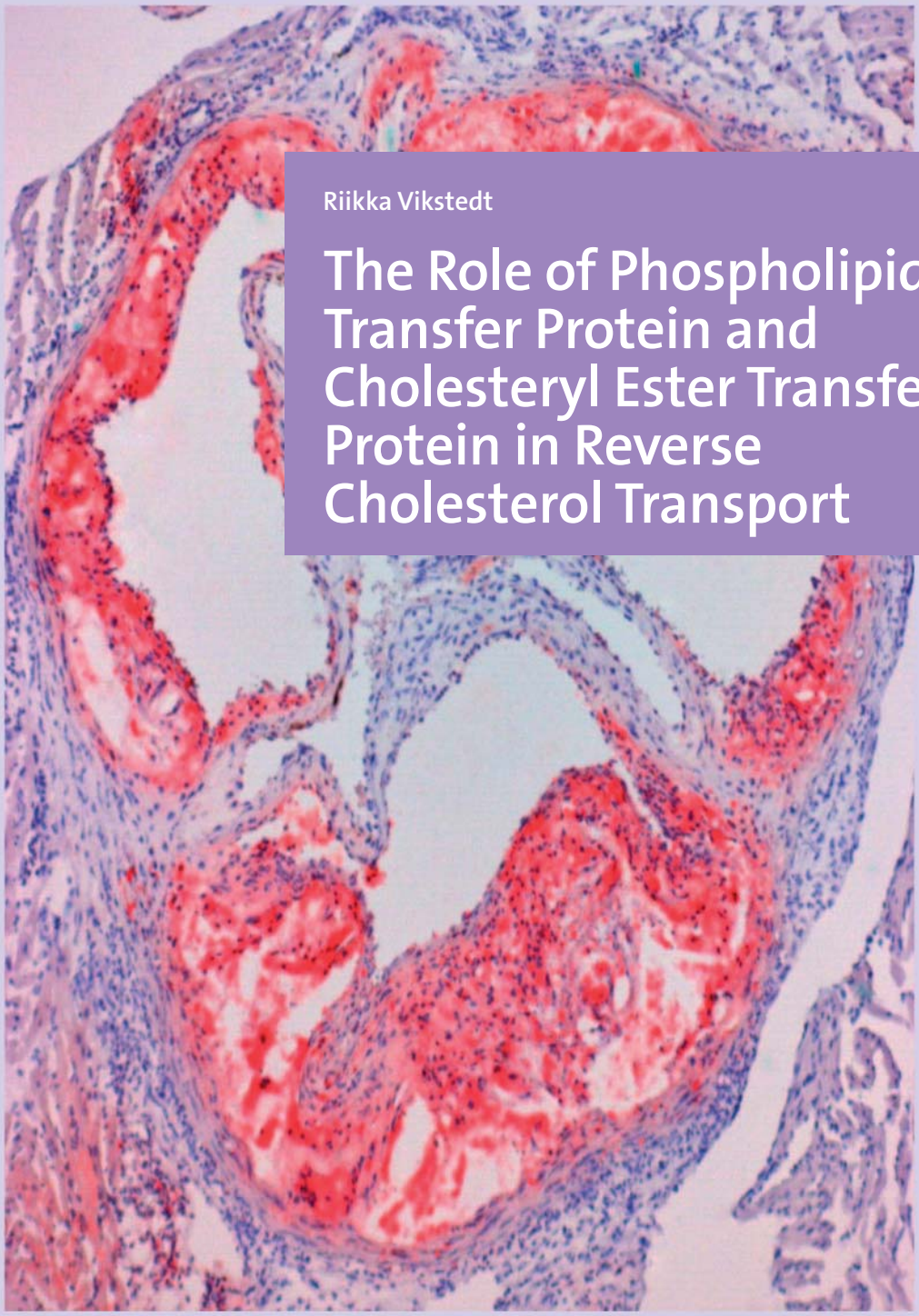

Riikka Vikstedt

The Role of Phospholipid Transfer Protein and Cholesteryl Ester Transfer Protein in Reverse Cholesterol Transport

Riikka Vikstedt

THE ROLE OF PHOSPHOLIPID TRANSFER PROTEIN AND CHOLESTERYL ESTER TRANSFER PROTEIN IN REVERSE CHOLESTEROL TRANSPORT

A C A D E M I C D I S S E R T A T I O N

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ABSTRACT

In atherosclerosis, cholesterol accumulates in the vessel wall, mainly in the form of low-density lipoprotein (LDL). Macrophages of the vessel wall scavenge cholesterol, which leads to formation of lipid-laden foam cells. Several pathways and transporter proteins are involved in the regulation of cholesterol balance in macrophages. High plasma levels of high-density lipoprotein (HDL) protect against atherosclerosis, as HDL particles are able to accept cellular cholesterol and transport it to the liver for excretion in a process called reverse cholesterol transport.

Phospholipid transfer protein (PLTP) remodels HDL particles in the circulation, generating preβ-HDL and large fused HDL particles. In addition, PLTP maintains plasma HDL levels by facilitating the transfer of post-lipolytic surface remnants of triglyceride-rich lipoproteins to HDL. Reportedly, PLTP has both antiatherogenic and proatherogenic properties. Most of the cholesteryl ester transfer protein (CETP) in plasma is bound to HDL particles and CETP is also involved in the remodeling of HDL particles. CETP enhances the heteroexchange of cholesteryl esters in HDL particles for triglycerides in LDL and very low-density lipoprotein (VLDL). The role of CETP in the development of atherosclerosis is controversial.

The aim of this thesis project was to study the importance of endogenous PLTP in the removal of cholesterol from macrophage foam cells by using macrophages derived from PLTP-deficient mice, determine the effect of macrophage-derived PLTP on the development of atherosclerosis by using bone marrow transplantation, and clarify the role of the two forms of PLTP, active and inactive, in the removal of cholesterol from foam cells. In addition, the ability of CETP to protect HDL against the action of chymase in CETP-HDL complexes was studied. Furthermore, cholesterol efflux from macrophages derived from low- and high-HDL subjects was studied. Finally, cholesterol efflux potential of sera obtained from the study subjects was compared.

In this thesis project it was demonstrated that the absence of PLTP in macrophages derived from PLTP-deficient mice decreased cholesterol efflux mediated by ATP-binding cassette transporter A1 (ABCA1). The bone marrow transplantation studies demonstrated that selective deficiency of PLTP in macrophages decreased the size of atherosclerotic lesions and caused major changes in serum lipoprotein levels and PLTP activity. It was further demonstrated that the active form of PLTP can enhance cholesterol efflux from macrophage foam cells. Both preβ-HDL and large fused HDL particles enriched with apoE and phospholipids were effective in cholesterol efflux. Besides PLTP, also CETP may enhance the reverse cholesterol transport process, as association of CETP with reconstituted HDL (rHDL) particles prevented chymase-dependent proteolysis of discoidal rHDL and preserved their cholesterol efflux potential. Finally, monocyte macrophages isolated from either low- or high-HDL subjects displayed similar cholesterol efflux to exogenous acceptors. However, serum from high-HDL subjects promoted more efficient cholesterol efflux than did serum from low-HDL subjects. The observed difference was most probably due to differences in the distribution of HDL subpopulations in low-HDL and high-HDL subjects.

The results of this thesis project indicate that both PLTP and CETP can remodel HDL as a cholesterol acceptor, which affects the removal of cholesterol from foam cells present in the vessel wall.

Keywords: atherosclerosis, HDL, reverse cholesterol transport, phospholipid transfer protein, cholesteryl ester transfer protein

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TIIVISTELMÄ

Ateroskleroosissa verisuonten seinämiin kertyy kolesterolia LDL:n (low-density lipoprotein) muodossa. Verisuonten seinämän syöjäsolut eli makrofagit ottavat verenkierrosta kolesterolia sisäänsä ja muuttuvat kolesterolin täyttämiksi vaahtosoluiksi. Verisuonten seinämään kertynyttä kolesterolia voidaan poistaa vaahtosoluista HDL:n (high-density lipoprotein) avulla ja tähän prosessiin osallistuvat erilaiset lipidikuljetusproteiinit. Korkea HDL-pitoisuus verenkierrossa suojaa ateroskleroosilta, sillä HDL-hiukkaset pystyvät vastaanottamaan verisuonten seinämään kertynyttä kolesterolia ja kuljettamaan sitä maksaan elimistöstä poistettavaksi.

Fosfolipidin siirtäjäproteiini (PLTP) muokkaa HDL-hiukkasia verenkierrossa muodostaen pieniä preβ-HDL-hiukkasia ja suuria HDL-hiukkasia. PLTP ylläpitää verenkierron HDL-tasoja kuljettamalla lipolyysissä muodostuneita runsaasti triglyseridejä sisältävien partikkelien fosfolipidijäämiä HDL-hiukkasille. Aikaisemmat tutkimukset ovat osoittaneet, että PLTP:llä on sekä ateroskleroosilta suojaavia että sitä edistäviä ominaisuuksia. Kolesteroliesterin siirtäjäproteiini (CETP) esiintyy verenkierrossa pääasiassa HDL-hiukkasiin liittyneenä ja osallistuu samalla HDL-hiukkasten muokkaukseen. CETP siirtää kolesteroliestereitä HDL-hiukkasilta LDL- ja VLDL (very low-density lipoprotein) -hiukkasille ja triglyseridejä vastakkaiseen suuntaan. CETP:n merkitys ateroskleroosin kehittymisessä on epäselvä.

Tämän väitöskirjatyön tavoitteena oli selvittää makrofagien tuottaman PLTP:n toimintaa kolesterolin poistossa vaahtosoluista PLTP-poistogeenisten hiirten makrofagien avulla, selvittää luuydinsiirtotekniikan avulla makrofagien tuottaman PLTP:n vaikutusta ateroskleroosin kehittymiseen sekä tutkia eri PLTP-muotojen, aktiivisen ja inaktiivisen, osuutta kolesterolin poistossa vaahtosoluista. Lisäksi selvitettiin CETP:n kykyä estää ateroskleroottissa leesioissa esiintyvän proteaasin, kymaasin, aiheuttamaa HDL-hiukkasten pilkkoutumista. Tavoitteena oli myös tutkia, eroaako kolesterolin poistuminen matalan ja korkean HDL-pitoisuuden

omaavien henkilöiden vaahtosoluista ja kuinka heidän seeruminsa toimivat kolesterolin vastaanottajina.

Väitöskirjatyössä havaittiin, että PLTP:n puuttuminen poistogeenisessä hiirimallissa vähensi kolesterolin poistumista vaahtosoluista ABCA1-proteiinin välityksellä. Tutkittaessa luuydinsiirtotekniikan avulla makrofagien tuottaman PLTP:n merkitystä ateroskleroosin kehittymisessä havaittiin, että PLTP:n puuttuminen makrofageista vähensi kolesterolin kertymistä verisuonten seinämään ja ateroskleroottisten leesioiden kokoa sekä aiheutti huomattavia vaikutuksia plasman lipoproteiinien pitoisuuksiin ja PLTP-aktiivisuuteen. Väitöskirjatyössä havaittiin, että vain aktiivinen PLTP-muoto lisää kolesterolin poistumista vaahtosoluista. Aktiivisen PLTP:n muodostamat pienet preβ-HDL-hiukkaset sekä suuret runsaasti apoE:tä ja fosfolipidejä sisältävät HDL-hiukkaset toimivat tehokkaasti kolesterolin poistossa vaahtosoluista. Myös CETP saattaa lisätä kolesterolin takaisinvirtausta, sillä työssä osoitettiin, että CETP muodostaa kompleksin kiekkomaisten HDL-hiukkasten kanssa samalla suojaten näiden HDL-hiukkasten rakennetta ja kykyä poistaa kolesterolia vaahtosoluista. Tutkittaessa kolesterolin poistotehokkuutta matalan ja korkean HDL-pitoisuuden omaavien henkilöiden vaahtosoluista kolesterolin poistotehokkuudessa ei havaittu eroa. Sen sijaan korkean HDL-pitoisuuden omaavien henkilöiden seerumi oli tehokkaampi kolesterolin vastaanottaja kuin matalan HDL-pitoisuuden omaavien henkilöiden seerumi. Ero kolesterolin poistotehokkuudessa johtui todennäköisesti korkean ja matalan HDL-pitoisuuden omaavien henkilöiden seerumien erilaisesta HDL-hiukkasten jakaumasta.

Tämän väitöskirjatyön tulosten perusteella voidaan todeta, että sekä PLTP että CETP muokkaavat kolesterolin vastaanottajahiukkasten rakennetta ja ominaisuuksia, mikä edelleen vaikuttaa kolesterolin poistoon vaahtosoluista verisuonten seinämässä.

Avainsanat: ateroskleroosi, HDL, kolesterolin takaisinkuljetusjärjestelmä, fosfolipidin siirtäjäproteiini, kolesteroliesterin siirtäjäproteiini

CONTENTS

ABBREVIATIONS

ABCA1	adenosine triphosphate-binding cassette transporter A1
ABCG1	adenosine triphosphate-binding cassette transporter G1
ACAT	acyl-CoA:cholesterol acyltransferase
Apo	apolipoprotein
BPI	bactericidal permeability-increasing protein
CAD	coronary artery disease
cAMP	cyclic adenosine monophosphate
CE	cholesteryl ester
C/EBP	CCAAT/enhancer-binding protein
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
CM	chylomicron
CRP	C-reactive protein
CSF	colony-stimulating factor
CVD	cardiovascular disease
EL	endothelial lipase
ELISA	enzyme-linked immunosorbent assay
FC	free cholesterol
FFA	free fatty acid
FXR	farnesoid X-activated receptor
GGE	gradient gel electrophoresis
HA-PLTP	high-activity form of phospholipid transfer protein
HDL	high-density lipoprotein
HL	hepatic lipase
ICAM-1	intercellular adhesion molecule 1
IDL	intermediate-density lipoprotein
IL	interleukin

IMT	intima-media thickness
INF-γ	interferon-γ
KO	knockout
LA-PLTP	low-activity form of phospholipid transfer protein
LBP	lipopolysaccharide-binding protein
LCAT	lecithin:cholesterol acyltransferase
LDL	low-density lipoprotein
LDLr	low-density lipoprotein receptor
LPL	lipoprotein lipase
LPS	lipopolysaccharide
LT/LBP	lipid transfer/lipopolysaccharide-binding protein family
LXR	liver X receptor
MCP-1	monocyte chemotactic protein 1
mRNA	messenger ribonucleic acid
NO	nitric oxide
PAD	peripheral arterial disease
PAF-AH	platelet-activating factor acetyl hydrolase
PC	phosphatidylcholine
PL	phospholipid
PLTP	phospholipid transfer protein
PON	paraoxonase
PPAR	peroxisome proliferator-activated receptor
RCT	reverse cholesterol transport
rHDL	reconstituted HDL
RXR	retinoid X receptor
SAA	serum amyloid A
SMC	smooth muscle cells
SR-BI	scavenger receptor class B type I

SREBP	sterol regulatory element-binding protein
TC	total cholesterol
TG	triglyceride
TNF-α	tumour necrosis factor α
TRL	triglyceride-rich lipoprotein
VCAM-1	vascular cell adhesion molecule 1
VLDL	very low-density lipoprotein
WT	wild-type
WTD	Western-type diet

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Lee-Rueckert M*, Vikstedt R*, Metso J, Ehnholm C, Kovanen PT, Jauhiainen M. Absence of endogenous phospholipid transfer protein impairs ABCA1-dependent efflux of cholesterol from macrophage foam cells. *J Lipid Res.* 2006;47:1725-1732.

II Vikstedt R, Ye D, Metso J, Hildebrand RB, Van Berkel TJ, Ehnholm C, Jauhiainen M, Van Eck M. Macrophage phospholipid transfer protein contributes significantly to total plasma phospholipid transfer activity and its deficiency leads to diminished atherosclerotic lesion development. *Arterioscler Thromb Vasc Biol.* 2007;27:578-586.

III Vikstedt R, Metso J, Hakala J, Olkkonen VM, Ehnholm C, Jauhiainen M. Cholesterol efflux from macrophage foam cells is enhanced by active phospholipid transfer protein through generation of two types of acceptor particles. *Biochemistry* 2007;46:11979-11986.

IV Lee-Rueckert M*, Vikstedt R*, Metso J, Jauhiainen M, Kovanen PT. Association of cholesteryl ester transfer protein with HDL particles reduces its proteolytic inactivation by mast cell chymase. *J Lipid Res.* 2008;49:358-368.

V Nakanishi S*, Vikstedt R*, Söderlund S, Lee-Rueckert M, Hiukka A, Ehnholm C, Muilu M, Metso J, Naukkarinen J, Palotie L, Kovanen PT, Jauhiainen M, Taskinen MR. Serum, but not monocyte macrophage foam cells derived from low HDL-C subjects, displays reduced cholesterol efflux capacity. *J Lipid Res.* 2009;50:183-192.

* The authors contributed equally to the study.

1 INTRODUCTION

Cardiovascular diseases (CVD) have been the leading cause of mortality worldwide, and although death rates from CVDs have declined in recent years, their prevalence remains high (Murray and Lopez, 1997; Hennekens, 1998; Lloyd-Jones et al., 2009). CVDs are an entity of diseases of the heart and vasculature, mainly caused by atherosclerosis. High plasma cholesterol levels have been shown to be associated with the increased incidence of cardiovascular diseases (Castelli et al., 1986), and several lipid-modifying drugs that control cholesterol biosynthesis have been developed. Statins, which are inhibitors of 3-hydroxy-3-methylgrutaryl coenzyme A (HMG-CoA) reductase introduced in the late 1980s, were the first tool for efficient reduction of plasma cholesterol levels (Shepherd, 2004). Statins reduce the availability of cellular cholesterol by inhibiting cholesterol biosynthesis and increasing the clearance of low-density lipoprotein (LDL) cholesterol and its precursors, thereby decreasing plasma cholesterol levels. Plasma LDL levels are further decreased when statins are combined with ezetimibe, a cholesterol-lowering compound that inhibits absorption of cholesterol from the small intestine. In addition to lowering LDL cholesterol, statins have several pleiotropic effects involving improvement of endothelial function and stability of atherosclerotic plaques, and inhibition of oxidative stress and vascular inflammation (Takemoto and Liao, 2001).

High-density lipoprotein (HDL) protects against development of atherosclerosis through several mechanisms. The beneficial role of HDL was established already in the early 1950s (Nikkila, 1953), and after that the association between low levels of HDL and increased risk of CVDs has been well documented (Gordon et al., 1977; Kannel, 1983; Castelli et al., 1986; Jacobs et al., 1990; Assmann et al., 1996; Barter et al., 2007a). Furthermore, different subpopulations of HDL may differ in their capacity to protect against CVDs (Lamon-Fava et al., 2008). Due to the limited benefits obtained by LDL lowering alone and the beneficial effects of HDL, HDL has become an interesting target of drug development (Linsel-Nitschke and Tall, 2005). Despite various favorable effects of HDL on lipid metabolism, the role of HDL in the reverse cholesterol transport (RCT) process is nowadays regarded the most important antiatherogenic effect of HDL (Fielding and Fielding, 1995; Wang and Rader, 2007). Cholesterol accumulation in arterial macrophages is the first hallmark of atherosclerosis, and membrane transporters, such as ATP-binding cassette (ABC) transporters A1 and G1, have an essential role in cellular cholesterol homeostasis (Pennings et al., 2006). Promoting RCT by affecting these membrane lipid transporters has become a therapeutic topic of interest (Wang and Rader, 2007).

Phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP) are two important lipid transfer proteins affecting HDL metabolism. The roles of PLTP and CETP in RCT are not known in detail, and both pro- and antiatherogenic properties have been associated with these lipid transfer proteins. The aim of this thesis was to clarify the function of PLTP and CETP in the removal of cholesterol from macrophages, the first step of the RCT process. In addition, the properties of macrophages and sera recovered from subjects with low or high HDL levels in cholesterol efflux were investigated.

When the functions of PLTP and CETP in cellular lipid metabolism are known, plasma lipid levels may be pharmaceutically modified by inhibiting or activating the expression of these proteins in specific tissues. So far, inhibitors of CETP have been tested in clinical trials in an attempt to increase plasma HDL levels. However, in the case of PLTP, no pharmacological trials are in progress.

2 REVIEW OF THE LITERATURE

2.1 ATHEROSCLEROSIS

2.1.1 Risk factors of the disease

Atherosclerosis is a progressive disease of the large and medium-sized arteries and the most important cause of heart disease and stroke in humans. Epidemiological studies have revealed that atherosclerosis is a multifactorial disease with several environmental and genetic risk factors contributing to the total risk of the disease. Risk factors of atherosclerosis with a strong genetic component include family history of atherosclerotic disease, male gender, elevated blood pressure, elevated levels of circulating LDL or very low-density lipoprotein (VLDL) cholesterol, reduced levels of circulating HDL cholesterol, diabetes, and obesity (Lusis, 2000; Homma, 2004). Metabolic syndrome, a cluster of metabolic disturbances including hypertension, high plasma triglycerides, low plasma HDL cholesterol, insulin resistance, and obesity, is strongly associated with the risk of atherosclerotic cardiovascular disease. High plasma levels of Lp(a) may also increase the risk of atherosclerosis, especially when associated with elevated LDL levels, although its role in atherogenesis remains controversial (Berglund and Ramakrishnan, 2004). Environmental factors associated with an increased risk of atherosclerosis and coronary heart disease include a high-fat diet, smoking, and lack of exercise (Lusis, 2000). Moreover, recent reports have demonstrated that atherosclerosis is an inflammatory disease in which the immune system interacts with metabolic risk factors (Leinonen and Saikku, 2002; Esteve et al., 2005; Hansson, 2005).

2.1.2 Development of the disease

The vessel wall of an intact artery consists of three morphologically distinct layers, the intima, media, and adventitia. The intima, the innermost layer of the vessel wall and the site of lipid accumulation in atherosclerosis, consists of an extracellular connective tissue matrix covered by a monolayer of endothelial cells on the luminal side and separated from the media by the internal elastic lamina. The middle layer, the media, is a layer of smooth muscle cells (SMC) separated from the adventitia by the external elastic lamina, while the outer layer, the adventitia, consists of connective tissue, fibroblasts, and SMCs. An important event in the initiation of the development of an atherosclerotic lesion is endothelial dysfunction, i.e. injury of the endothelial cell layer at lesion-prone sites of the arteries, such as branches,

bifurcations, and curvatures, due to altered hemodynamic forces acting on the endothelial cells. The increased permeality of the damaged endothelium facilitates the entry of atherogenic lipoproteins such as LDL into the intima and their retention in the vessel wall as a result of interaction with matrix proteoglycans (Ross, 1993; Lusis, 2000; Rader and Daugherty, 2008). Native LDL is not taken up by macrophages efficiently, but oxidation, aggregation, proteolysis, or enzymatic modifications of LDL can increase cellular accumulation of cholesterol (Goldstein et al., 1979). Interaction of LDL with reactive oxygen species leads to formation of minimally oxidized LDL, which has proinflammatory activity. Minimally oxidized LDL stimulates the endothelial cells to express several proinflammatory molecules, including adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), and secrete chemotactic proteins such as monocyte chemotactic protein 1 (MCP-1), and growth factors such as macrophage colony-stimulating factor (M-CSF). These molecules are involved in the recruitment of monocytes and lymphocytes to the artery wall, their proliferation, and differentiation of monocytes into macrophages (Nakashima et al., 1998; Mertens and Holvoet, 2001). Based on the oxidation hypothesis of atherogenesis, it is the oxidized LDL phospholipids generated by lipoxygenase and myeloperoxidase pathways that are recognized by the innate immune system (Navab et al., 2004). Oxidized LDL is also able to inhibit production of vasodilatating nitric oxide (NO) (Lusis, 2000). Following further oxidation of LDL, highly oxidized LDL forms aggregates that are recognized by macrophage scavenger receptors such as SR-A, CD36, and CD68 (Kunjathoor et al., 2002). This leads to formation of lipid-laden foam cells, followed by generation of a fatty streak, an early atherosclerotic lesion, together with T lymphocytes and SMCs (Ross, 1993). Differentiation of monocytes into macrophages and uptake of oxidized LDL through the scavenger receptor CD36 are promoted by peroxisome proliferator-activated receptor γ (PPAR-γ) (Tontonoz et al., 1998).

In addition to scavenger receptors, macrophages bear Toll-like receptors (TLR) that bind molecules such as modified lipids or heat-shock proteins (Hansson, 2005). In contrast to scavenger receptors, Toll-like receptors can initiate activation of macrophages, followed by production of inflammatory cytokines, chemokines, proteases as well as oxygen and nitrogen radicals. Activated T cells in atherosclerotic lesions represent type 1 helper T (Th1) effector cells that secrete interferon-γ (INF-γ), which improves the efficiency of antigen presentation and stimulates synthesis of inflammatory cytokines tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1). Various cytokines and growth factors produced by macrophages and T lymphocytes induce migration of SMCs from the media and their proliferation and extracellular matrix production (Lusis, 2000). SMCs increase retention of atherogenic lipoproteins by secreting extracellular matrix proteins such

as collagen and they also give rise to a fibrous cap beneath the endothelium. The foam cells in the atherosclerotic lesions eventually die, which leads to formation of a necrotic core within the plaque. Destabilization and rupture of the fibrous cap covering the lipid-rich core is caused by degradation of the extracellular matrix by proteases such as matrix metalloproteinases (MMP) and cysteine proteases including cathepsins (Hansson, 2005; Newby, 2005). This induces the release of thrombogenic material and formation of a thrombus in the lumen of the vessel, which could lead to myocardial infarction if the thrombus prevents blood flow through the coronary artery.

2.1.3 Animal models of atherosclerosis

Several genetically engineered mouse models have been generated to define the mechanisms of atherosclerosis (Breslow, 1996; Getz and Reardon, 2006). The mouse strains most widely used in atherosclerotic research are C57BL mice deficient in apolipoprotein E (apoE$^{-/-}$) or low-density lipoprotein receptor (LDLr$^{-/-}$) (Plump et al., 1992; Ishibashi et al., 1993). ApoE$^{-/-}$ mice are extremely prone to developing atherosclerotic lesions of different phases throughout the arterial tree when fed a chow or a Western-type fat- and cholesterol-rich diet, whereas wild-type mice typically develop lesions only in the proximal aorta (Nakashima et al., 1994; Reddick et al., 1994). The plasma lipoprotein profile of apoE$^{-/-}$ mice differs from that of humans, since most of the plasma cholesterol is carried in VLDL, while LDL is the major carrier of plasma cholesterol in humans (Plump et al., 1992). A mouse is naturally a HDL animal with low plasma levels of LDL and VLDL.

LDLr$^{-/-}$ mice have been created to enhance accumulation of atherogenic lipoproteins in the circulation, and these mice display a lipoprotein phenotype similar to that seen in humans with familial hypercholesterolemia (Ishibashi et al., 1993; Ishibashi et al., 1994). Despite the increased level of plasma LDL, LDLr$^{-/-}$ mice on a chow diet develop vascular lesions very slowly. However, their lesion development is markedly increased when the content of fat and cholesterol in their diet is increased. Furthermore, transgenic mice expressing the human apolipoprotein B (apoB-Tg) as well as mouse models with modified expression of various inflammatory or metabolic markers have been generated for use as models of atherosclerosis (Breslow, 1996; Ohashi et al., 2004). Development of mouse models has made significant advances in cardiovascular research. However, generalizations of results obtained from mouse studies to human physiology have to be made carefully, as small murines such as the mouse may not presicely reflect human cardiovascular physiology.

2.2 LIPOPROTEIN METABOLISM

2.2.1 Lipoproteins

Lipoproteins are water-soluble particles typically composed of a core of neutral lipids, mostly triglycerides (TG) and cholesteryl esters (CE), surrounded by a surface monolayer of amphipathic molecules, phospholipids (PL), free cholesterol (FC), and apolipoproteins. Five major human plasma lipoproteins are chylomicrons (CM), VLDL, intermediate-density lipoprotein (IDL), LDL, and HDL (**Table 1**) (Gotto et al., 1986; Betteridge, 1999). Plasma lipoproteins have been traditionally separated from serum by sequential ultracentrifugation at increasing solvent density (Havel et al., 1955). Lipoprotein classes differ in their size, density, electrophoretic mobility as well as in their protein and lipid composition. Lipoproteins transport hydrophobic TGs and cholesterol through the aqueous plasma compartment of blood and deliver lipids to tissues in a controlled manner. Lipoproteins are dynamic particles which are constantly modified in the circulation by several processes, including enzymatic reactions, transfer of lipids, and exchange of apolipoproteins (Gotto et al., 1986; Betteridge, 1999).

The major routes involved in lipoprotein-mediated extracellular lipid transport, exogenous and endogenous lipid transport, and reverse cholesterol transport are discussed in more detail in the following chapters.

2.2.2 Exogenous lipid transport

The exogenous lipid transport pathway is comprised of the transport and processing of dietary fats and fat-soluble vitamins composed of TGs, PLs, and cholesterol. For absorption into enterocytes, dietary fats are first hydrolyzed into free fatty acids and monoacylglycerols by pancreatic lipase. Once in the enterocytes, TGs are resynthesized within the endoplasmic reticulum and assembled with other lipids and apolipoproteins into CMs for release into the lymphatic system and further into the circulation via the thoracic duct (Green and Glickman, 1981). CMs are large, heterogenous, TG-rich particles. The core of CMs consists of mainly TGs and CEs, while PLs, FC, apolipoproteins, and some saturated TGs are found on the surface of the particles (Zilversmit, 1965). The size of chylomicrons is determined by the rate of lipid absorption (Green and Glickman, 1981), while the fatty acid composition of TGs, but not the fatty acid composition of PLs in CMs, depends on the dietary fat composition (Kayden et al., 1963; Zilversmit, 1965; Hussain et al., 2001).

Apolipoprotein B-48 (apoB-48), found in CMs and CM remnants in humans, is synthesized by the intestine and is necessary for biosynthesis of CMs. ApoB-48 protein is translated from the mRNA of apoB-100, which is post-transcriptionally modified in the intestinal cells to incorporate a stop codon (Powell et al., 1987). In addition to apoB-48, lymph CMs consist of several other apolipoproteins, including apoA-I, apoA-II, apoA-IV, apoE, and apoC synthesized in the intestine and/or liver. Following entry into the circulation, the apoE and apoC contents of chylomicrons increase as these apolipoproteins are derived from the plasma HDL fraction (Havel et al., 1973; Green and Glickman, 1981; Gotto et al., 1986). ApoCs have an essential function in the metabolism of CMs, as apoC-II functions as a cofactor required for activation of lipoprotein lipase (LPL), a lipolytic enzyme bound to the luminal surface of the capillary endothelium (Kinnunen et al., 1977; Goldberg et al., 1990). Core TGs of CMs as well as some surface PLs are hydrolyzed by LPL in the peripheral tissues, mainly in the adipose tissue and in the striated muscles, accompanied by the transfer of PLs to HDL. Free fatty acids (FFA) released from TGs are bound to plasma albumin and transported to the tissues for use as energy or for storage in adipose tissue (Havel, 1997).

Hydrolysis of CMs leads to formation of CM remnants, particles depleted of TGs and enriched with CEs, which are taken up into hepatic parenchymal cells by receptor-mediated endocytosis through binding of apoE to a low-density lipoprotein receptor (LDLr) (Russell et al., 1983) or a LDL receptor-related protein (LRP) (Herz et al., 1988). ApoCs inhibit hepatic uptake of TG-rich lipoproteins, thus opposing the uptake promoted by apoE (Windler et al., 1980a; Windler et al., 1980b). ApoC, apoA-I, and apoA-IV as well as the PL contents of plasma CMs decrease during remnant formation, as these apolipoproteins are transferred to HDL (Havel et al., 1973; Mjos et al., 1975; Tall et al., 1979), and the loss of apoC enables hepatic clearance of remnants. In the absence of LPL, endothelial lipase (EL) may provide FFAs and contribute to the subsequent uptake of FFAs to adipose tissue (Kratky et al., 2005). Furthermore, the composition of CM remnants is also affected by two circulatory lipid transfer proteins, CETP and PLTP (Tall, 1986).

2.2.3 Endogenous lipid transport

In addition to the exogenous origin of lipids from diet, several tissues of the human body are able to synthesize cholesterol and TGs. In humans, the liver is the main lipid-synthesizing organ, regulating plasma lipid levels and lipid homeostasis (Dietschy et al., 1993). Furthermore, the liver synthesizes various apolipoproteins, including apoA-I, apoA-II, apoB, apoC-II, apoC-III, and apoE (Zannis et al., 1981), which can assemble with lipids, generating lipoproteins. The endogenous lipid

transport pathway involves the transport of lipids from the liver to peripheral tissues. The liver synthesizes VLDL particles, which consist of cholesterol and TGs derived from both *de novo* synthesis and CM remnants taken up to the liver (Gibbons, 1990). The core of VLDL particles is rich in TGs and CEs, while the PL surface of the particles mainly carries apoB-100, small amounts of apoCs, apoE, and recently identified apoA-V (Gotto et al., 1986; O'Brien et al., 2005). ApoB-100, synthesized by the liver, is essential for the assembly of VLDL particles and their secretion into the circulation (Borchardt and Davis, 1987; Olofsson and Boren, 2005). ApoB-100 contains hydrophobic and amphipathic sequences that probably interact with lipids (Olofsson et al., 1987) as well as a domain for cellular uptake of cholesterol by the LDLr-mediated pathway (Knott et al., 1986; Yang et al., 1986). Hepatic secretion of apoB was shown to be increased by cholesterol and CEs (Fuki et al., 1989; Cianflone et al., 1990), whereas ω-3 fatty acids decreased secretion of apoB due to enhanced intracellular degradation of apoB (Wang et al., 1993).

Like CMs, VLDL particles are hydrolyzed in the circulation by LPL, resulting in formation of IDL particles, which can be further modified into LDL particles by the action of hepatic lipase (HL) (Fielding et al., 1978; Demant et al., 1988; Demant et al., 1991). In addition to LPL, HL can cause hydrolysis of TGs in VLDL particles (Nicoll and Lewis, 1980). Large $VLDL_1$ particles are catabolized slowly and are less efficiently converted to IDL and LDL particles than are smaller-sized $VLDL_2$ particles (Packard et al., 1984). It appears that apolipoproteins of VLDL have an important effect on VLDL metabolism, as apoC inhibits hepatic uptake of VLDL particles as well as small CMs (Windler and Havel, 1985). Furthermore, apoE polymorphisms influence the metabolism of apoB-containing lipoproteins, since $apoE_2$ subjects have more highly active hepatic LDL receptors than $apoE_4$ subjects and decreased conversion of VLDL to LDL (Demant et al., 1991). During lipolysis of VLDL particles, PLTP transfers PLs to HDL particles (Huuskonen et al., 2001), whereas CETP mediates the exchange of TGs with CEs (Tall, 1993). LDL particles are transferred from the circulation to the liver and peripheral tissues via LDLr (Brown and Goldstein, 1986). LDLr interacts with apoB, which leads to internalization of the receptor-ligand complexes. Following that, the receptor is recycled back to the cell surface, while the LDL particle is degraded in the lysosomes. Released cholesterol can be re-esterified via acyl-CoA:cholesterol acyltransferase 2 (ACAT2) or converted into bile acids or vitamin D. The expression and the number of LDL receptors are regulated by the balance between the intracellular cholesterol content and the need for cholesterol.

Table 1. ***Properties of lipoprotein classes present in human plasma.***

	CM	VLDL	IDL	LDL	HDL_2	HDL_3
Density (g/ml)	0.93	0.95-1.006	1.006-1.019	1.019-1.063	1.063-1.125	1.125-1.210
Diameter (nm)	75-1200	30-80	25-35	18-25	9-12	5-9
Electrophoretic mobility	at origin	pre-β	slow pre-β	β	α	α
Core composition (mass%)						
TG	86	55	23	6	5	3
CE	3	12	29	42	17	13
Surface composition (mass%)						
FC	2	7	9	8	5	4
PL	7	18	19	22	33	25
Prot[a]	2	8	19	22	40	55
Apolipoproteins	AI, AII, AV B-48 CI, CII, CIII E	AV B-100 CI, CII, CIII E	B-100 CI, CII, CIII E	B-100	AI, AII CI, CII, CIII E	AI, AII, AV CI, CII, CIII E
Source	Intestine	Liver	VLDL	VLDL/IDL	Liver/ intestine	Liver/ intestine
Main function	Transport of exogenous TG and C	Transport of endogenous TG	Transport of endogenous TG	Transport of endogenous C	Reverse cholesterol transport	Reverse cholesterol transport

TG, triglycerides; CE, cholesteryl ester; FC, free cholesterol; PL, phospholipids; Prot, protein; C, cholesterol.
[a]Protein does not include bound carbohydrate.

Adapted from Gotto et al., 1986; Wasan and Cassidy, 1998; Betteridge, 1999; O'Brien et al., 2005.

2.2.4 Function of ATP-binding cassette transporters

ABC transporters represent one of the largest membrane transporter families, and most of them are conserved in all vertebrae. ABC transporters bind and hydrolyze adenosine triphosphate (ATP) to generate energy for transportation of various molecules across the plasma membrane as well as intracellular membranes. Based on the sequence homology and organization of the structural domains, human ABC transporters are divided into seven subclasses designated from A to G. ABC transporters can be classified as either full transporters consisting of two

transmembrane domains (TM) and two cytoplasmic nucleotide-binding folds (NBF) or half-transporters consisting of only one TM domain and one NBF, respectively. To gain functionality, half-transporters must form homodimers or heterodimers with other half-transporters. In 2005, 48 ABC transporters were identified in humans and they are involved in several metabolic processes (Dean and Annilo, 2005).

Four members of the ABC transporter family, ABCA1, ABCG1, ABCG5, and ABCG8, modulate lipid levels in the circulation and tissues in humans (Oram and Vaughan, 2006). ABCA1 mRNA is present in various human tissues, the most abundant expression being detected in the liver, lung, placenta, and small intestine (Langmann et al., 1999; Kielar et al., 2001). ABCA1 mediates the removal of PLs, cholesterol and other lipophilic molecules from peripheral cells to lipid-poor HDL. Late endosomes and lysosomes are preferential sources of cholesterol for ABCA1-mediated cholesterol removal (Chen et al., 2001). ABCA1 has a broad substrate specificity for apolipoproteins of lipid-poor HDL, including apoA-I, apoA-II, apoA-IV, apoC-I, apoC-II, apoC-III, and apoE (Remaley et al., 2001). Of note, lipid-free helical apolipoproteins can stabilize ABCA1 protein and prevent its degradation by calpain (Arakawa and Yokoyama, 2002; Wang et al., 2003a). In addition to helical apolipoproteins, PLTP is suggested to stabilize ABCA1 protein (Oram et al., 2003; Oram et al., 2008). The expression of ABCA1 is upregulated by cholesterol loading as well as during monocyte and adipocyte differentiation (Langmann et al., 1999; Kielar et al., 2001). The expression of ABCA1 induced by cholesterol loading is mediated through activation of the liver X receptors (LXRα and β) and the retinoid X receptors (RXR) (Costet et al., 2000; Schwartz et al., 2000; Venkateswaran et al., 2000a). PPARα and PPARγ are also indirectly involved in the upregulation of ABCA1 expression via enhanced transcription of LXRα (Chawla et al., 2001a; Chinetti et al., 2001). Furthermore, factors independent of LXR/RXR, including membrane-permeable analogs of cyclic adenosine monophosphate (cAMP), can stimulate ABCA1 expression in macrophages (Oram et al., 2000). Proinflammatory cytokines, such as INF-γ, as well as C-reactive protein (CRP) have been reported to inhibit ABCA1 expression, whereas transforming growth factor-β (TGF-β) had the opposite effect (Panousis et al., 2001; Wang et al., 2008).

Mutations in the gene that encodes ABCA1 lead to Tangier disease, an autosomal recessive disorder of lipid metabolism characterized by HDL deficiency syndrome, hepatosplenomegaly, and prevalent atherosclerosis (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Rust et al., 1999). Pathophysiological hallmarks similar to those of Tangier disease are observed in mice with ABCA1 deficiency, whereas elevated plasma HDL levels are observed in mice that overexpress ABCA1, confirming the importance of ABCA1 in lipoprotein metabolism and HDL biogenesis (McNeish et al., 2000; Orso et al., 2000; Joyce et al., 2002; Wellington et

al., 2003). Both hepatic and extrahepatic ABCA1, especially in the intestine, are involved in the production of plasma HDL (Timmins et al., 2005; Brunham et al., 2006; Singaraja et al., 2006b). Hepatic ABCA1 is also essential for catabolism of plasma HDL (Singaraja et al., 2006a). ABCA1 is a prerequisite for adequate lipidation of newly synthesized apoA-I, and lipidation of apoA-I by ABCA1 in hepatocytes occurs in the Golgi compartments and at the plasma membrane (Francone et al., 2003; Maric et al., 2005). ABCA1 also affects glucose homeostasis, as pancreatic ABCA1 is involved in insulin secretion from β-cells and in their cholesterol homeostasis (Brunham et al., 2007). Results from bone marrow transplantation studies have demonstrated that macrophage ABCA1 prevents accumulation of cholesterol into arterial macrophages, but it has only a minor contribution to plasma HDL levels (Haghpassand et al., 2001; van Eck et al., 2002; Van Eck et al., 2006).

The ABCG family includes five characterized half-transporters (ABCG1, ABCG2, ABCG4, ABCG5, ABCG8), of which ABCG1 and ABCG4 are involved in RCT (Kusuhara and Sugiyama, 2007; Velamakanni et al., 2007). ABCG1 mRNA is ubiquitously expressed in human tissues, the highest expression being observed in the adrenal glands, lung, heart, and spleen (Klucken et al., 2000). The regulation of ABCG1 gene expression resembles that of ABCA1, as the expression of ABCG1 is induced by LXRs and PPARγ (Venkateswaran et al., 2000b; Kennedy et al., 2001; Li et al., 2004; Sabol et al., 2005). LXR activation induces a redistribution of ABCG1 from intracellular sites to the plasma membrane, which leads to enhanced availability and transfer of cholesterol to acceptor particles (Wang et al., 2006). Like ABCA1, ABCG1 is upregulated during the differentiation of monocytes into macrophages and during the conversion of these macrophages into foam cells (Klucken et al., 2000; Venkateswaran et al., 2000b). Statins reportedly down-regulate the gene expression of both ABCA1 and ABCG1, although the clinical relevance of this finding is not currently known (Wong et al., 2008). In contrast to ABCA1, cAMP analogs do not increase the expression of ABCG1 (Suzuki et al., 2004). Furthermore, increased expression of ABCG1 was observed in macrophages from patients with Tangier disease, suggesting a compensatory response in macrophages to the absence of the normal functioning of ABCA1 (Lorkowski et al., 2001).

ABCG1 and ABCG4 mediate cholesterol efflux to mature HDL_2 and HDL_3 particles, but not to lipid-poor apoA-I. However, ABCG4 is mainly expressed in the brain and may thus play a role in brain lipid metabolism (Wang et al., 2004). According to the results from experiments carried out in mice with a deficiency of ABCG1 or mice that overexpress ABCG1, ABCG1 is essential for tissue lipid homeostasis (Kennedy et al., 2005). Targeted disruption of ABCG1 led to massive

accumulation of neutral lipids and PLs in hepatocytes and macrophages on a high-fat diet, whereas overexpression of ABCG1 protected tissues from diet-induced lipid accumulation. The participation of ABCG1 in RCT and the development of atherosclerosis have been studied by several research groups using the bone marrow transplantation technique. So far, the results have been inconsistent, as bone marrow transplantations of ABCG1$^{-/-}$ bone marrow into LDLr$^{-/-}$ mice have indicated only a slightly increased or even reduced incidence of atherosclerotic lesions (Baldan et al., 2006; Out et al., 2006; Ranalletta et al., 2006), while increased atherosclerosis was observed when ABCG1$^{+/+}$ bone marrow was used (Basso et al., 2006). Studies on ABCA1/ABCG1 double knockout mice have demonstrated that the combined actions of ABCA1 and ABCG1 are essential to maintain lipid homeostasis in macrophages and to prevent foam cell formation (Out et al., 2008).

In addition to ABCA1, another member of the ABCA family, ABCA7, mediates cellular PL efflux to apolipoproteins, but unlike ABCA1 it does not promote cellular cholesterol efflux (Wang et al., 2003b). ABCG5 and ABCG8, half-transporters highly expressed in the liver and small intestine, control intestinal absorption as well as excretion of sterols from the liver to bile (Berge et al., 2000; Lee et al., 2001). ABCG5/ABCG8 genes are defective in sitosterolemia, a genetic disorder characterized by increased levels of plasma and tissue cholesterol and other neutral sterols. ABCG5/ABCG8 heterodimers are required for LXR-mediated stimulation of RCT from macrophages to feces *in vivo* (Calpe-Berdiel et al., 2008). The dual function of ABCG5/ABCG8 in the regulation of intestinal cholesterol absorption and biliary sterol excretion is essential for protection against atherosclerosis, as decreased apoB-containing lipoproteins and atherosclerosis were observed in mice with hepatic overexpression of ABCG5/ABCG8 only when cholesterol absorption from the intestine was also limited (Basso et al., 2007).

2.2.5 Reverse cholesterol transport

Reverse cholesterol transport is a pathway by which accumulated cholesterol is transported from peripheral cells to the liver and eliminated via bile into the feces (Fielding and Fielding, 1995; Ohashi et al., 2005). By increasing the removal of cholesterol from the vessel wall, RCT can prevent the development of atherosclerosis. The role of RCT in the active transport of cholesterol was first recognized by Glomset et al., who recognized that cellular FC was converted to CE in extracellular space by lecithin:cholesterol acyltransferace (LCAT) (Glomset, 1968). The principal constituents of RCT are cholesterol acceptors α-HDL, preβ-HDL, and apoA-I, enzymes LCAT and HL, lipid transfer proteins PLTP and CETP, transmembrane proteins ABCA1, ABCG1, and scavenger receptor BI (SR-BI)

(Fielding and Fielding, 2001; Ohashi et al., 2005; Tall, 2008). ApoA-I is synthesized by the liver and intestine and secreted into the circulation (Wu and Windmueller, 1979). The liver and intestine are the principal sites of HDL biosynthesis, and hepatic and intestinal ABCA1 mediate the transfer of PLs and cholesterol to lipid-free apoA-I, generating nascent HDL that further matures into spherical HDL (Timmins et al., 2005; Tsujita et al., 2005; Brunham et al., 2006). Moreover, HDL particles can be formed during lipolysis of TG-rich lipoproteins, as these particles can provide lipids and apolipoproteins for HDL (Patsch et al., 1978). HDL is present in several discrete subpopulations of which spherical, α-mobile HDL_2 and HDL_3 present the major HDL subclasses in human plasma (Eisenberg, 1984; Barter et al., 2003a). HDLs are the smallest, densest, and most protein-rich lipoproteins among the major lipoprotein classes in humans containing various apolipoproteins, including apoA (I, II, IV, V), apoCs, apoD, apoE, apoJ, apoL, and apoM (Xu and Dahlback, 1999). ApoA-I and apoA-II are most abundant in HDL. In addition, HDL carries several other proteins, including CETP, LCAT, PLTP, platelet-activating factor acetyl hydrolase (PAF-AH), serum paraoxonase-1 (PON-1), and serum amyloid A (SAA) (Navab et al., 1998; Van Lenten et al., 2001; Vaisar et al., 2007).

The specific transporter proteins, ABCA1, ABCG1, and SR-BI, as well as aqueous diffusion, can remove cholesterol from peripheral tissues (**Figure 1**) (Yancey et al., 2003; Brewer et al., 2004; Tall, 2008). ABCA1 promotes unidirectional removal of PLs and cholesterol from peripheral cells onto lipid-poor apoA-I, generating nascent preβ-HDL besides its function in HDL formation in the liver and small intestine (Wang et al., 2001; Wang et al., 2004). Preβ-HDL, a small, discoidal, preβ-migrating lipoprotein with apoA-I as its only protein component, has been shown to act as a plasma recipient of cellular FC (Castro and Fielding, 1988). Externalized FC on the surface of nascent $preβ_1$-HDL particles is subsequently esterified by LCAT, leading to generation of mature, spherical HDL_3 and HDL_2 particles with CEs in their hydrophobic core (Barrans et al., 1996; Nakamura et al., 2004; Zannis et al., 2006). In contrast to ABCA1, ABCG1 (Wang et al., 2004; Kennedy et al., 2005) and SR-BI (Trigatti et al., 2003) can mediate cholesterol efflux to mature HDL particles, but not to lipid-poor apoA-I. The relative importance of different efflux pathways is not known in detail and it may depend on cellular cholesterol status as well as the gradient of cholesterol between the cell and the acceptor. However, ABCA1 and ABCG1 have been shown to have an essential role in the removal of cholesterol from cholesterol-enriched macrophages, while SR-BI had only a minor role in that process (Adorni et al., 2007). The essential roles of ABCA1 and ABCG1 in RCT have also been supported by other studies (Wang et al., 2007b; Yvan-Charvet et al., 2007b). Furthermore, ABCA1 and ABCG1 may act sequentially with ABCA1 in initiating the formation of nascent HDL particles, which then acquire additional lipids via the action of ABCG1 (Gelissen et al., 2006;

Vaughan and Oram, 2006). Cholesterol efflux via the ABCG1-mediated pathway is modulated by apoE and LCAT associated with HDL_2 (Matsuura et al., 2006). Besides its role in cholesterol efflux and clearance of TG-rich lipoproteins, apoE participates in the biogenesis of apoE-containing HDL particles, which may contribute to atheroprotection (Mahley et al., 2006; Kypreos and Zannis, 2007).

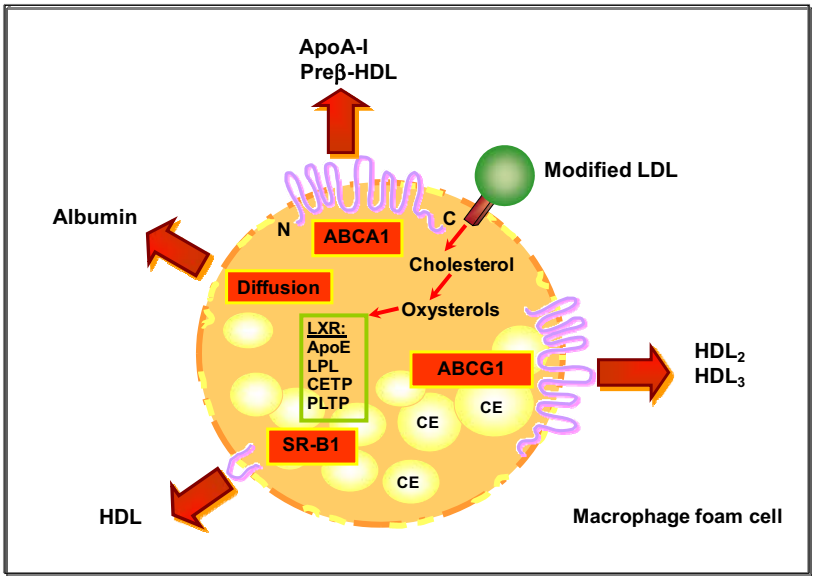

Figure 1. ***Cholesterol efflux from macrophage foam cells.***

HDL particles are continuously modified by several factors, including CETP, PLTP, HL, and EL, in the circulation (Wang and Briggs, 2004). In humans, CETP transports CEs from HDL to VLDL and LDL, and TGs in the opposite direction, while PLTP transfers surface PLs acquired from VLDL and CM hydrolysis by LPL to HDL. EL is mainly involved in PL hydrolysis, whereas HL causes hydrolysis of TGs and PLs of large TG-rich HDL_2 particles, generating smaller HDL_3 particles and preβ-HDL. In addition to HL, also CETP and PLTP are able to generate preβ-HDL during remodeling of HDL particles (Barter et al., 2003a). Eventually, CEs from HDL are transported to the liver via SR-BI, especially in HDL mammals such as mice (Acton et al., 1996). In humans, CEs are removed mainly via apoB-containing lipoproteins taken up by hepatic LDL receptors (Schwartz et al., 2004). In addition, a mechanism independent of hepatic SR-BI and LDLr has been postulated as a mediator of this uptake (Zhou et al., 2006b). Following uptake into the liver, cholesterol can be utilized by the cells for VLDL or HDL formation or

transported into bile via ABCG5 and ABCG8 (Yu et al., 2002). Lipid-poor apoA-I can be also catabolized via cubilin in the kidney (Kozyraki et al., 1999).

2.2.6 Antiatherogenic properties of HDL

Besides the essential role of HDL in RCT, it has been proposed to have anti-inflammatory, antioxidant, antiapoptotic, antithrombotic, and vasodilatating properties (Barter et al., 2003a; deGoma et al., 2008; Tall, 2008). HDL is able to inhibit cytokine-induced expression of endothelial adhesion molecules, including VCAM-1, ICAM-1, and E-selectin, resulting in decreased binding of monocytes onto the vascular endothelium (Barter et al., 2002). Importantly, HDL can prevent oxidative modifications of LDL, thus reducing its atherogenicity (Van Lenten et al., 2001). The antioxidative properties of HDL are conferred by apoA-I as well as the presence of other HDL-associated proteins such as PON and PAF-AH. HDL can provide additional antiatherogenic effects by inhibiting platelet activity and by inactivating coagulation factors within the coagulation cascade (deGoma et al., 2008). The antithrombotic potential of HDL is at least partially conferred by the neutralizing effect of apoA-I on the procoagulant properties of anionic PLs (Oslakovic et al., 2009). Furthermore, upon binding to the surface of endothelial cells, HDL increases the activation of endothelial nitric oxide synthase (eNOS), leading to the release of NO, which can promote vasodilatation and reduce apoptosis (Tall, 2008). Finally, HDL seems to enhance endothelial repair by inducing endothelial progenitor cell entry into the arterial wall (Seetharam et al., 2006).

2.3 PHOSPHOLIPID TRANSFER PROTEIN (PLTP)

2.3.1 Characteristics of PLTP

PLTP gene and protein

PLTP is a member of the plasma lipid transfer/lipopolysaccharide-binding protein (LT/LBP) family, together with CETP, lipopolysaccharide-binding protein (LBP), and bactericidal permeability-increasing protein (BPI). The human PLTP gene is located in chromosome 20q12-q13.1 (Day et al., 1994; Whitmore et al., 1995),

whereas the mouse PLTP gene is localized in chromosome 2, which corresponds to human chromosome 20 (LeBoeuf et al., 1996). The genes for human LBP and BPI are encoded in close proximity to the PLTP gene in chromosome 20q11.23-q12 (Gray et al., 1993), while the gene for CETP is located in chromosome 16q12-q21 (Lusis et al., 1987). The genomic organization of human PLTP, LBP, and BPI is highly conserved and a comparison of these genes has revealed high homology with almost identical exon/intron junctions and exon sizes, suggesting that LBP, BPI, and PLTP originate from a common ancestral gene (Hubacek et al., 1997; Kirschning et al., 1997).

The promoter region of the human PLTP gene consists of a TATA box, two GC-rich regions, and several consensus sequences for potential binding of transcription factors including AP-2, Sp1, CCAAT/enhancer-binding protein (C/EBP), IRE, NF-κB, XRE, MRE, AP-3, and ERE. The binding sites of transcription factors Sp1 (-114 to -108) and AP-2 (-94 to -85) are located within the area responsible for full promoter activity, and these two factors seem to be essential for transcription of the PLTP gene (Tu et al., 1995). The structural organizations of the mouse and human PLTP genes are identical. The mouse and human PLTP genes share an 81.1% identity within the promoter area and the consensus sequences for transcription factors AP-2 and Sp1 are located at the same positions. Similarities in the structures of the mouse and human PLTP genes suggest similar transcriptional regulation mechanisms of gene expression (Tu et al., 1997a). Both mouse and human mature PLTP contain 476 amino acids, of which 42% are hydrophobic. Mouse and human PLTP share an 83% amino acid sequence identity (Albers et al., 1995). Pig PLTP protein is 93% identical with human PLTP and 81% identical with mouse PLTP (Pussinen et al., 1997b).

In human plasma, PLTP and CETP are the two key lipid transfer proteins responsible for the transfer of lipids between different lipoproteins. PLTP activity has been detected in several species, while large variation has been observed in CETP activities among vertebrate species (Ha and Barter, 1982; Guyard-Dangremont et al., 1998). The homology of PLTP to CETP, LBP, and BPI has been shown to be 20%, 24%, and 26%, respectively (Day et al., 1994). Based on the structure of BPI, molecular models for LBP (Beamer et al., 1998) and PLTP have been constructed (**Figure 2**) (Huuskonen et al., 1999). The molecular model of PLTP suggests a boomerang-shaped, two-domain structure containing barrel-type structural units at each end of the protein and central β-sheets forming an interface between the barrels. Each domain of the PLTP molecule contains a hydrophobic lipid-binding pocket (Huuskonen et al., 1999). Both lipid-binding pockets are important in PLTP-mediated PL transfer, but the N-terminal pocket may have a more important role in this process. The C-terminal lipid-binding pocket may be

envisioned to be important for the interaction of PLTP with HDL molecules (Huuskonen et al., 1999; Ponsin et al., 2003). The mechanism of PLTP-mediated PL transfer is not known in detail. One hypothesis is that both lipid-binding pockets are involved in binding PL molecules to be transferred, while another hypothesis suggests that one lipid-binding pocket could accommodate PLs and the other mediates the interaction of PLTP with HDL. However, a recent report suggested that PLTP facilitates lipid transfer by a shuttle mechanism, and formation of a complex between PLTP, acceptor, and donor particles is not necessary for PL transfer (Setala et al., 2007).

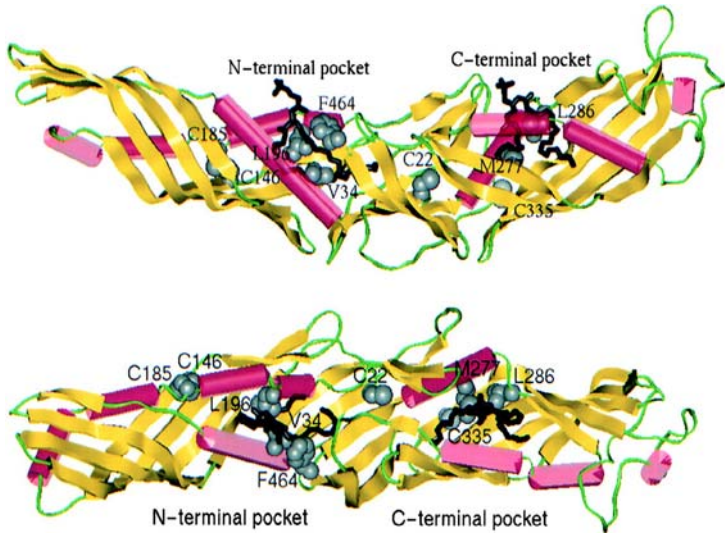

Figure 2. ***The structural model of human PLTP. The α-helixes are shown in red, the β-strands in yellow, and the phosphatidylcholine molecules in black (Huuskonen et al., 1999).***

The computed molecular mass of mature human PLTP protein is 55 kDa and of mouse PLTP protein, 52.7 kDa. However, the mass of human PLTP estimated by sodium duodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is 81 kDa. The discrepancy between the computed mass and the mass estimated by SDS-PAGE may be due to N-glycosylation, since human and pig PLTP contain 6 potential N-glycosylation sites and mouse PLTP, 7 potential N-glycosylation sites (Day et al., 1994; Albers et al., 1995; Pussinen et al., 1997b). N-glycosylation may be important for PLTP trafficking from the endoplasmic reticulum to the Golgi compartment and for PLTP secretion (Huuskonen et al., 1998a; Qu et al., 1999; Qu et al., 2006). However, when PLTP was expressed in Sf-9 insect cells synthesizing only high-

mannose type N-glycans, efficient secretion of fully active PLTP was observed, indicating that complex N-glycans are not necessary for efficient PLTP secretion or activity (Huuskonen et al., 1998b). The 30 C-terminal amino acids of PLTP are dispensable for PLTP secretion, however they are necessary for PL transfer activity (Huuskonen et al., 1998a). The sequence of human PLTP also contains several potential O-glycosylation sites (Day et al., 1994). Of the four cysteines, Cys_{129} and Cys_{168} are completely conserved among the members of the LT/LBP family, whereas Cys_5 and Cys_{318} are found only in PLTP (Beamer et al., 1997). The disulphide bridge between Cys_{129} and Cys_{168} is essential for secretion of human PLTP, while the other cysteine residues neither participate in disulphide link formation nor have a dramatic effect on human PLTP synthesis and secretion (Huuskonen et al., 1999; Qu et al., 1999). However, Cys_5 is a prerequisite for correct folding of pig PLTP and its secretion (Pussinen et al., 1997b). In addition to hydrophobic interactions, the electrostatic charge of lipoprotein particles affects the lipid transfer activities of PLTP and CETP (Desrumaux et al., 1998; Desrumaux et al., 2001)

Regulation of PLTP gene expression

Nuclear receptors are transcription factors that function in a ligand-activated state and regulate the expression of genes affecting several processes such as reproduction, development, and general metabolism. Adopted orphan nuclear receptors are one member of the nuclear receptor superfamily, and the members of the adopted orphan nuclear receptors include receptors of retinoic acid (RXRα,β,γ), oxysterols (LXRα,β), bile acids (FXR), fatty acids (PPARα,γ,δ), and xenobiotics (SXR/PXR and CAR). The RXR subfamily consists of three members, RXRα, RXRβ, and RXRγ, which usually function as heterodimers with other nuclear receptors. The natural ligand of RXRs is a vitamin A derivative, 9-cis retinoic acid. LXRs exist in LXRα and LXRβ isoform, of which LXRα is mainly expressed in tissues associated with lipid metabolism, such as the liver, kidney, intestine, lung, adrenals, adipose tissue, and macrophages, whereas LXRβ is expressed ubiquitously. LXRs act as cholesterol sensors, which regulate many genes involved in cholesterol homeostasis and lipogenesis (Chawla et al., 2001b; Edwards et al., 2002; Tontonoz and Mangelsdorf, 2003). Oxysterols can serve as regulators of cholesterol homeostasis by activating LXRs (Gill et al., 2008). Expression of the farnesoid X receptor (FXR) is restricted to the liver, kidney, intestine and adrenal gland, and bile acids, especially chenodeoxycholic acid, are physiological ligands of FXR (Chawla et al., 2001b; Edwards et al., 2002). PPARs exist in three isoforms, PPARα, PPARγ, and PPARδ. They are activated by fatty acids and eicosanoids, and

PPARα is regarded as a global regulator of fatty acid metabolism, while PPARγ as a key regulator of adipogenesis (Schoonjans et al., 1996). Lipid-lowering drugs, called fibrates, are ligands of PPARα (Forman et al., 1997), and antidiabetic drugs, thiazolidinediones, are ligands of PPARγ (Lehmann et al., 1995; Staels et al., 1997).

PLTP expression is regulated transcriptionally by LXRs (Cao et al., 2002; Mak et al., 2002a; Laffitte et al., 2003), FXR (Urizar et al., 2000; Kast et al., 2001; Tu and Albers, 2001; Mak et al., 2002a), and PPAR (Tu and Albers, 1999; Tu and Albers, 2001). The PLTP gene contains two potential LXR response elements, DR4A and DR4B. DR4A is unable to bind LXR/RXR, but DR4B effectively binds LXR/RXR heterodimers. The requirement of LXR in the induction of PLTP expression was verified by using mice lacking both LXRα and LXRβ; the induction of PLTP expression by LXR and RXR ligands was completely lost in these mice (Laffitte et al., 2003). The DNA sequences of the human and mouse PLTP genes contain motifs homologous to the potential binding motifs of PPAR and FXR (Tu and Albers, 1999; Tu and Albers, 2001). Chenodeoxycholic acid can increase human PLTP promoter activity approximately 8-10-fold in the presence of the FXR-RXRα heterodimer. This requires an IR-1 sequence in the PLTP gene promoter to which FXR-RXRα heterodimers specifically bind (Urizar et al., 2000). Similarly, chenodeoxycholic acid clearly enhances the promoter activity of the mouse PLTP gene (Tu and Albers, 2001). Regulation of PLTP expression differs in humans and mice, as fenofibrate reduces transcription of the human PLTP gene, whereas in mice, expression is increased (Tu and Albers, 1999; Tu and Albers, 2001).

Simultaneous activation of LXR and PPARα causes a synergistic increase in liver PLTP mRNA and plasma PLTP activity levels in mice (Beyer et al., 2004). In addition, the PLTP gene contains potential binding sites for sterol regulatory element-binding protein (SREBP), C/EBP, and Sp1. SREBP proteins are inefficient transcriptional activators, and the presence of both SREBP and Sp1 binding sites close to each other in the human PLTP gene suggests that Sp1 might enhance the transcriptional activity of the PLTP gene, regulated by SREBP (Tu and Albers, 1999). Earlier reports demonstrate that Sp1 and AP-2 are essential for transcription of the human and mouse PLTP genes (Tu et al., 1997b; Tu and Albers, 2001). C/EBP and PPAR have the same binding region on the PLTP gene, thus C/EBP can limit binding of PPAR and subsequently have an effect on the action of fibrates on PLTP gene expression (Tu and Albers, 1999).

In addition to PLTP, LXR upregulates the expression of many genes involved in lipid and lipoprotein metabolism, including apoE (Laffitte et al., 2001; Mak et al., 2002b), LPL (Zhang et al., 2001a), ABCG1 (Venkateswaran et al., 2000b; Kennedy et al., 2001), ABCG5, ABCG8 (Repa et al., 2002), ABCA1 (Costet et al., 2000; Schwartz et al., 2000; Venkateswaran et al., 2000a), CETP (Luo and Tall, 2000),

SREBP1c (Repa et al., 2000), fatty acid synthase (Joseph et al., 2002), and CYP7A (Lehmann et al., 1997).

PLTP expression in tissues

The PLTP mRNA transcript has a wide tissue distribution, suggesting important and various functions for the PLTP protein. In humans, PLTP expression is highest in the ovary, thymus, and placenta, with moderate levels in the pancreas, small intestine, testes, lung, and prostate. PLTP expression is moderately low in the kidney, liver, and spleen, and very low in the heart, colon, skeletal muscle, leukocytes, and brain in humans. Even though only moderate PLTP expression was detected in the liver, the liver as a large organ could still be a major contributor to plasma PLTP concentration (Day et al., 1994; Albers et al., 1995). Mouse PLTP expression differs clearly from that observed in humans. The highest expression of mouse PLTP mRNA is detected in the lung, brain, and heart, with relatively low levels in the liver, skeletal muscle, and testes and very low levels in the spleen and kidney. Expression of PLTP is very low in the mouse kidney, while the human kidney has moderate PLTP transcript levels (Albers et al., 1995). The tissue distribution of pig PLTP mRNA differs from PLTP expression in humans and mice, the highest levels of expression being observed in the pancreas, brain, lung, and liver (Pussinen et al., 1997b). PLTP mRNA is expressed in alveolar type II epithelial cells of the lungs of humans and mice, and its expression is induced during hypoxia and emphysema (Jiang et al., 1998). PLTP is also expressed in the epididymis of male mice and it is one factor that determines sperm motility (Drouineaud et al., 2006). Expression of PLTP in the ovaries, testes, prostate, and epididymis suggests that PLTP is important for reproduction and fertility. PLTP is also expressed in the cells of the central nervous system, in neurons, astrocytes, microglia, and oligodendroglia (Vuletic et al., 2003). Recently, PLTP protein was detected in human tear fluid and it may facilitate tear PL trafficking and protect the corneal epithelium against drying (Jauhiainen et al., 2005).

HA-PLTP and LA-PLTP

PLTP exists in human plasma in two forms, one catalytically active (HA-PLTP) and the other inactive (LA-PLTP). The molecular mass of the active form of PLTP is approximately 160 kDa, while for the inactive form of PLTP, the molecular mass of the complex is approximately 520 kDa (Oka et al., 2000a; Karkkainen et al., 2002). Two forms of PLTP were also observed when human PLTP was transiently

expressed in mice by using the adenoviral vector (Jaari et al., 2001). In a normal Finnish population sample, approximately 46% of the serum PLTP concentration was in the catalytically active form and 54% in the inactive form (Janis et al., 2004). Partial characterization of PLTP particles demonstrated that HA-PLTP and LA-PLTP are associated with lipoproteins, HA-PLTP with apoE and LA-PLTP with apoA-I (Karkkainen et al., 2002). PLTP secreted by HepG2 cells also associated with apoE but not with apoA-I, thus resembling the highly active form of human PLTP (Siggins et al., 2003). However, association of plasma active form of PLTP mainly with apoA-I-containing lipoproteins and only a little with apoE-containing lipoproteins has also been suggested (Cheung and Albers, 2006). The first hypothesis is supported by findings that PLTP is capable of interacting with apoE, apoA-I, and apoA-IV, of which apoE-containing proteoliposomes can activate LA-PLTP, whereas apoA-I proteoliposomes are unable to cause activation. In addition to apoE proteoliposomes, also apoA-IV proteoliposomes can induce concentration-dependent activation of LA-PLTP (Janis et al., 2005). The role of different apolipoproteins in the regulation of PLTP activity *in vivo* was further supported by the observation that apoE was the main determinant of circulating PLTP activity in type II diabetes mellitus patients (Tan et al., 2006).

Several methods have been developed for measuring plasma PLTP activity (Damen et al., 1982; Jauhiainen et al., 1993; Lagrost et al., 1999) and mass (Desrumaux et al., 1999a; Huuskonen et al., 2000a; Oka et al., 2000b; Siggins et al., 2004; Jauhiainen and Ehnholm, 2005). Exogenous, lipoprotein-independent PLTP activity ($PLTP_{exo}$) is measured by the transfer of radioactively labeled PLs from donor liposomes to exogenously added HDL particles (Damen et al., 1982; Jauhiainen et al., 1993). Endogenous, lipoprotein-dependent PLTP activity ($PLTP_{endo}$) is measured by the transfer of PLs to endogenous serum HDL (Lagrost et al., 1999). However, the results have been conflicting, and PLTP mass and activity in human plasma samples do not correlate. Only in one study was a correlation of plasma PLTP activity and PLTP mass demonstrated among normolipidemic study subjects (Desrumaux et al., 1999a). The lack of correlation might be due to the use of different antibodies in the mass assays and differences in the reactivities of the antibodies to LA-PLTP and HA-PLTP (Murdoch et al., 2002). To resolve this problem and to quantitate both forms of PLTP, plasma samples were first treated with anionic detergent SDS and after this treatment, dextran sulphate-$CaCl_2$ precipitation was used to separate LA-PLTP and HA-PLTP forms (Siggins et al., 2004). In a subsample (n = 250) of the Finnish Health 2000 Examination Survey, considerable individual variation was observed between the relative amounts of HA-PLTP and LA-PLTP in normolipidemic individuals (Janis et al., 2004). The distribution of HA-PLTP and LA-PLTP in subjects with dyslipidemia is presently unknown. However, based on another immunoassay, no difference in PLTP mass

was observed between normolipidemic subjects, type IIa hyperlipidemic patients, and type IIb hyperlipidemic patients (Desrumaux et al., 1999a).

2.3.2 Functions of PLTP

Phospholipid transfer

The physiological function of PLTP and its role in lipid metabolism was discussed in three reviews (Huuskonen and Ehnholm, 2000; Huuskonen et al., 2001; Albers and Cheung, 2004). PLTP has two major functions, PL transfer and HDL remodeling. PLTP mediates the transfer of PLs from VLDL and CMs into HDL during lipolysis and exchanges PLs of VLDL and HDL (Tall et al., 1985). PLTP also transfers PLs from unilamellar vesicles of egg phosphatidylcholine (PC) into HDL (Tall et al., 1983b). PLTP transfers a number of lipids, thus PLTP is a nonspecific lipid transfer protein. Studies using pyrene-labeled phospholipids have demonstrated that PLTP is able to transfer phosphatidic acid (PA), PC, phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SM), but for phosphatidylethanolamine (PE) the transfer was slower (Huuskonen et al., 1996; Rao et al., 1997). The rate of PL transfer by PLTP is enhanced by unsaturation of the PC acyl chain (Rao et al., 1997), whereas an increase in acyl chain length decreased the rate of PL transfer. PLTP is unable to distinguish between positional isomers, as the position of the acyl chain, sn-1 or sn-2, had no effect on the transfer rate (Huuskonen et al., 1996; Rao et al., 1997). Increased FC content of reconstituted HDL (rHDL) particles as well as decreased HDL particle size were shown to decrease PL transfer (Rao et al., 1997). The exact mechanism by which PLTP mediates PL transfer is not known in detail. PLTP does not form a tight complex with lipids, since no PLTP-PL intermediates were detected when purified PLTP was incubated with PC vesicles (Huuskonen et al., 1996). These findings suggest that PLTP does not act as a true lipid carrier, but instead it might facilitate PL transfer by forming a ternary complex between donor and acceptor lipoprotein particles. However, a recent report suggested that formation of a ternary complex between PLTP, acceptor, and donor particles is not necessary for PL transfer (Setala et al., 2007). The N-terminal tip of the human PLTP molecule contains a cluster of hydrophobic amino acids, of which Trp_{91}, Phe_{92}, and Phe_{93} are critical for PL transfer to HDL (Desrumaux et al., 2001).

Separation of PLTP and CETP has shown that the functions of these two lipid transfer proteins are different (Tall et al., 1983a). However, PLTP is able to increase CETP-mediated CE transfer from HDL_3 to LDL even though PLTP itself has no intrinsic CE transfer activity (Lagrost et al., 1994). In addition to PLs, PLTP is able to transfer cholesterol from vesicles containing both PC and cholesterol to HDL_3 particles. The key function of PLTP in PL transfer is supported by the finding that the affinity and binding capacity of PLTP for PC are substantially higher than they are for cholesterol (Nishida and Nishida, 1997).

HDL remodeling

In addition to PL transfer, the other major function of PLTP is HDL remodeling. PLTP-mediated HDL conversion remodels small HDL_3 particles into larger HDL_{2b}-like particles, concomitantly releasing small apoA-I-containing particles, called preβ_1-HDL (Jauhiainen et al., 1993; Tu et al., 1993). PLTP and CETP have opposite effects on the size distribution of plasma HDL, since PLTP can promote the formation of HDL_{2b} particles at the expense of HDL_{3a}, while CETP can increase formation of HDL_{3b} particles at the expense of HDL_{2a} (Lagrost et al., 1996). PLTP-generated preβ-HDL particles functioned as efficient cholesterol acceptors from human skin fibroblasts, thus suggesting the physiological significance of PLTP-mediated HDL remodeling (von Eckardstein et al., 1996). In addition to human PLTP, mouse and pig PLTP are able to convert HDL into populations of large and small HDL particles (Albers et al., 1995; Pussinen et al., 1995). Several mechanisms for PLTP-mediated HDL conversion have been suggested, but particle fusion was shown to be responsible for the enlargement of HDL particles observed upon incubation with PLTP (Lusa et al., 1996; Korhonen et al., 1998). Further studies have suggested that the initial event of HDL remodeling by PLTP involves a large, unstable fusion product with six apoA-I molecules. Then, the fusion product rearranges into three small particles, each containing two apoA-I molecules, or two molecules of apoA-I dissociate from the fusion product, concomitantly forming a large particle with four molecules of apoA-I (Settasatian et al., 2001). Mutations of hydrophobic residues at the surface of the human PLTP molecule were shown to impair HDL remodeling (Desrumaux et al., 2001). Furthermore, PLTP can also remodel HDL_2 particles, simultaneously generating preβ-HDL particles (Marques-Vidal et al., 1997).

The two main functions of PLTP have a causal relationship, since efficient PLTP-mediated HDL conversion is dependent on efficient PL transfer (Huuskonen et al., 2000b). Only a small increase in HDL particle size can occur in the absence of

apoA-I release, but continuation of the process with the release of apoA-I requires efficient PL transfer by PLTP. PLTP-mediated HDL conversion requires the presence of apoA-I, as demonstrated by the inability of human plasma PLTP to remodel rHDL particles containing apoA-II as a sole protein component (Lusa et al., 1996). Furthermore, the increased apoA-II/apoA-I molar ratio in HDL particles inhibits PLTP-mediated HDL interconversion (Pussinen et al., 1997a). PLTP also remodels spherical, apoE-containing rHDL particles into large and small particles via a series of particle fusions and structural rearrangements without concomitant dissociation of lipid-free or lipid-poor apoE (Settasatian et al., 2008). The steps of remodeling of apoE-containing particles differ from those reported for apoA-I-containing particles, which involve a single particle fusion and a rearrangement of the fusion product or release of lipid-free or lipid-poor apoA-I (Lusa et al., 1996; Korhonen et al., 1998; Settasatian et al., 2001; Settasatian et al., 2008). PLTP-mediated remodeling of apoE-containing rHDL is more extensive than that of apoA-I-containing rHDL, but apoE reduces the capacity of PL transfer by PLTP compared with apoA-I in HDL particles (Settasatian et al., 2008). Increased TG content of HDL particles promotes PLTP-mediated remodeling (Rye et al., 1998; Settasatian et al., 2001), which may be due to destabilization of apoA-I in TG-rich HDL particles (Settasatian et al., 2001).

PLTP is also able to remodel HDL during an acute-phase reaction, as PLTP-facilitated conversion of HDL enriched with SAA was more effective than conversion of native HDL_3, despite decreased apoA-I concentration (Pussinen et al., 2001). However, PLTP-mediated degradation of apoA-I in SAA-enriched HDL was accelerated compared with that of native HDL, which might be associated with the decreased levels of HDL seen during inflammation. Oxidative modifications of HDL_3 and TG-rich HDL_3 impair the substrate properties of the particles, decreasing PLTP-mediated HDL conversion (Pussinen et al., 2003).

Other functions of PLTP

In addition to PLs, PLTP is able to transfer LPS (Hailman et al., 1996) and α-tocopherol (Kostner et al., 1995). PLTP was found to be able to bind and neutralize LPS, exchange LPS between micelles, and transfer LPS to rHDL particles (Hailman et al., 1996) and from HDL particles to LDL particles (Levels et al., 2005). The transfer of LPS from HDL to LDL particles causes a generation of two subpopulations of HDL particles, which may contribute to the dyslipidemia seen during the acute-phase response to infection (Levels et al., 2005). PLTP may also extract LPS from bacterial membranes before its transfer to HDL (Vesy et al., 2000).

A high concentration of PLTP in the blood may diminish inflammatory responses to LPS, since addition of recombinant PLTP inhibited the ability of LPS to stimulate cytokine production in blood (Hailman et al., 1996). Whether the neutralizing effect of recombinant PLTP was due to neutralization by binding LPS or by transfer of LPS to lipoproteins was not clarified. LPS and dietary cholesterol regulate PLTP expression in opposite directions in mice, as LPS injection decreased both plasma PLTP activity and mRNA expression, whereas a high-fat, high-cholesterol diet increased them (Jiang and Bruce, 1995). However, PLTP was unable to transfer LPS to CD14, thus it does not mediate cellular responses to LPS (Hailman et al., 1996). Just recently, the role of PLTP in the prevention of endotoxic shock was demonstrated by a study showing that LPS-induced mortality was increased in mice with PLTP deficiency (Gautier et al., 2008).

α-tocopherol, a potent antioxidant form of vitamin E, is transported by plasma lipoproteins, and besides its antioxidant function, it plays an important role in several cell functions such as cell signaling and gene expression (Traber and Packer, 1995). PLTP catalyses the transfer of α-tocopherol to HDL particles and the exchange between different lipoprotein classes (Kostner et al., 1995). PLTP promotes net movement of α-tocopherol from HDL particles to oxidized LDL particles and to endothelial cells (Desrumaux et al., 1999b). Thus, PLTP-mediated α-tocopherol transfer may have at least two antiatherogenic functions by providing LDL with antioxidant and by preserving the normal relaxing function of endothelial cells in the vessel wall. Recent results indicate that α-tocopherol transfer is mediated by multiple mechanisms, since not only PLTP, but also CETP transfers α-tocopherol to lipoproteins (Hacquebard et al., 2008). Macrophage PLTP deficiency was shown to cause a significant decrease in α-tocopherol content together with increased oxidative stress in bone marrow cells (Valenta et al., 2006b), while dietary α-tocopherol supplementation led to a significant decrease in cholesterol accumulation in macrophages from PLTP-deficient mice (Ogier et al., 2007).

In addition, PLTP is capable of causing proteolytic cleavage of apoA-I (Jauhiainen et al., 1999). Cleavage of apoA-I occurs in the C-terminal portion of apoA-I between the amino acids Ala_{196} and Thr_{197}. Furthermore, plasma PLTP activity was shown to be the major determinant of LpA-I, but not LpA-I:A-II, kinetics in men, and it may contribute to maintenance of the plasma concentrations of these lipoproteins in different hypercatabolic conditions of HDL particles (Ooi et al., 2006). PLTP may also affect the plasma distribution of amphotericin B, a polyene macrolide antibiotic used in the treatment of systemic fungal infections (Patankar and Wasan, 2006). Recently, the involvement of PLTP in apoptosis of macrophages was reported (Wehinger et al., 2007).

Cholesterol efflux

Besides its functions in PL transfer and HDL remodeling, increasing evidence points to a function for PLTP in the removal of cholesterol and PLs from peripheral cells. PLTP treatment of a plasma sample was shown to cause a significant increase in preβ-HDL formation and cholesterol efflux from human skin fibroblasts (von Eckardstein et al., 1996). Furthermore, plasma PLTP activity was positively correlated to cholesterol efflux from Fu5AH hepatoma cells (Syvanne et al., 1996). The role of PLTP in cholesterol efflux was also supported by findings that efflux from Fu5AH cells to plasma from insulin-resistant subjects was not impaired, which may be related to higher plasma PLTP activity and enhanced preβ-HDL formation (Dullaart and van Tol, 2001). The capability of plasma PLTP and CETP to generate preβ-HDL was evaluated using plasma samples from human PLTP transgenic mice (huPLTPtg), human CETP transgenic mice (huCETPtg), and mice transgenic for both (huPLTPtg/huCETPtg) (Lie et al., 2001). Based on the results, the ability of PLTP to generate preβ-HDL is more efficient than that of CETP, thus supporting the role of PLTP in RCT. Mast cells in human atherosclerotic lesions contain a neutral protease chymase, which degrades PLTP and preβ-HDL generated by PLTP, and impairs cholesterol efflux from foam cells (Lee et al., 2003).

PLTP increased PL and cholesterol efflux from cholesterol-loaded human skin fibroblasts to HDL, but not to bovine serum albumin or apoA-I (Wolfbauer et al., 1999). The enhanced lipid efflux was shown to be due to the ability of PLTP to enhance binding of HDL to the cell surfaces, which may improve remodeling of HDL particles, thus increasing their ability to remove PLs and cholesterol by the apolipoprotein-mediated pathway. PLTP-stimulated lipid efflux was absent in fibroblasts derived from Tangier disease patients, suggesting that PLTP enhanced lipid efflux by an ABCA1-dependent mechanism. The connection between ABCA1-mediated lipid removal and PLTP was confirmed using murine macrophages with induced ABCA1 expression and ABCA1-transfected baby hamster kidney (BHK) cells (Oram et al., 2003). The results demonstrated that when ABCA1 was induced, PLTP alone increased removal of cholesterol and promoted cholesterol and PL efflux to HDL. In addition, both PLTP and apoA-I covalently cross-linked to ABCA1 at the same or closely related sites and stabilized ABCA1 protein. Further studies have demonstrated that amino acids 144-163, located at the tip of the N-terminal barrel of the PLTP molecule, are critical for ABCA1-mediated cholesterol efflux, stabilization of ABCA1 protein, and interaction with PLs and PL transfer (Oram et al., 2008). In contrast, PLTP showed no effect on cholesterol efflux in three studies (Cao et al., 2002; Valenta et al., 2006b; van Haperen et al., 2008) in which thioglycollate-elicited peritoneal macrophages from PLTP-deficient mice,

mice deficient in macrophage PLTP, and huPLTPtg/tg mice were used. However, thioglycollate treatment of mice induces an inflammatory reaction, which may modulate the results (Cook et al., 2003).

Recently, enhanced cholesterol efflux from cultured fibroblasts to plasma from hypertriglyceridemic type II diabetic patients was observed, and it was associated with increased PLTP activity and cholesterol esterification (de Vries et al., 2008). Preβ-HDL levels and preβ-HDL formation were unaltered in diabetic hypertriglyceridemia, which could contribute to maintenance of functional cholesterol efflux. A recent report demonstrated that heavy alcohol drinkers had increased plasma PLTP activity and decreased CETP activity, which may partly explain the increased amount of HDL_{2b} particles and increased HDL_2 PLs as well as enhanced cholesterol efflux to HDL_2 compared with controls (Makela et al., 2008). However, in one study, increased plasma PLTP activity and decreased cholesterol efflux were observed in type II diabetic patients with or without coronary artery disease (Attia et al., 2007).

2.3.3 PLTP and atherosclerosis

The function of PLTP in the accumulation or removal of cholesterol to/from the arterial wall was supported by the presence of PLTP protein in macrophages of human atherosclerotic lesions (Desrumaux et al., 2003; Laffitte et al., 2003; O'Brien et al., 2003), whereas only a minimal amount of PLTP was detected in nonatherosclerotic coronary arteries (O'Brien et al., 2003). PLTP gene expression gradually increased during macrophage differentiation, and differentiated macrophages were able to produce and secrete PLTP (Desrumaux et al., 2003). Cholesterol loading of macrophages increased PLTP mRNA levels, PLTP protein expression, and its activity (Desrumaux et al., 2003; O'Brien et al., 2003). Observations that extracellular PLTP and apolipoproteins colocalized with the extracellular matrix proteoglycan biglycan, and that active and inactive PLTP increased HDL binding to biglycan, suggest that PLTP may mediate lipoprotein binding to proteoglycans independent of its PL transfer activity (O'Brien et al., 2003). PLTP protein was also present in the necrotic cores of atherosclerotic lesions, while biglycans were not, which may be due to degradation of extracellular matrix biglycan by specific matrix metalloproteinases in the necrotic cores.

Plasma PLTP activity was found to be an independent risk factor of coronary artery disease (CAD) in patients with stable or unstable angina pectoris or recent acute myocardial infarction (Schlitt et al., 2003). Patients within the highest quintile

of PLTP activity had a 1.9-fold increased risk of CAD compared with patients in the lowest quintile. The authors suggested that high plasma PLTP activity may decrease HDL concentration and increase the concentration of TG-rich lipoproteins, both of which are well-known risk factors of CAD. Furthermore, high PLTP activity may provide a pro-oxidant stage, and it may transfer more oxidized PLs from TG-rich lipoproteins to HDL. In a recent study, high plasma PLTP activity was associated with fatal and nonfatal cardiovascular events in CAD patients under statin treatment, although the underlying mechanism was not presented by the authors (Schlitt et al., 2009). The atherogenic role of PLTP was further supported by the observation that high plasma PLTP activity is associated with increased intima-media thickness (IMT) in type II diabetic patients (de Vries et al., 2006). Another study demonstrated that PLTP activity was significantly decreased in patients with peripheral arterial disease (PAD), while PLTP mass did not differ between PAD patients and controls (Schgoer et al., 2008). These results indicate that in non-diabetic, non-smoking subjects, low rather than high PLTP activity is a marker for the presence of PAD, and the distribution of PLTP between low-activity and high-activity forms is disturbed. PLTP activity was shown to associate positively with plasma CRP and SAA levels in patients with low HDL and CVD, a state of chronic inflammation (Cheung et al., 2006). On the other hand, serum PLTP mass was shown to be a possible protective factor of coronary heart disease in a cohort of Japanese men, although no association between serum PLTP concentration and risk of stroke was observed (Yatsuya et al., 2004).

2.3.4 Mouse models of atherosclerosis with modified PLTP expression

PLTP knockout mice

The physiological importance of PLTP *in vivo* has been demonstrated in PLTP knockout (PLTP-KO or PLTP$^{-/-}$) mice (Jiang et al., 1999). Complete loss of PL transfer activity *in vitro* and *in vivo* as well as partial loss of FC transfer activity were observed in the plasma of PLTP-KO mice. HDL PL, cholesterol, and apoA-I were significantly decreased in PLTP-KO animals on a chow diet. On a high-fat diet, HDL levels were similarly decreased, while VLDL and LDL PLs, FC, and CEs were increased without changes in apoB levels. These results suggest that the surface components of TG-rich lipoproteins accumulate in the absence of PLTP-mediated transfer to HDL particles. The lamellar lipoproteins accumulating in the PLTP-KO mice were found to be enriched in PLs, FC, apoA-IV, and apoE (Qin et

al., 2000). In these animals, increased catabolism of HDL protein as well as CEs was observed, a condition that can lead to hypoalphalipoproteinemia. When CETP-transgenic mice were crossed into a PLTP-KO background, the expression of CETP could not compensate the low-HDL phenotype, and in fact the phenotypes were additive, leading to markedly decreased HDL (Kawano et al., 2000). Further studies have demonstrated that SR-BI and HL are involved in the clearance of PL- and FC-rich particles in PLTP-KO mice, although dietary factors can influence clearance (Kawano et al., 2002).

To study the effect of PLTP deficiency on dyslipidemia and atherosclerosis, PLTP-KO mice were crossed into apoB-Tg, apoE$^{-/-}$, or LDLr$^{-/-}$ backgrounds (Jiang et al., 2001). In all three mouse models, PLTP deficiency resulted in markedly decreased atherosclerosis. Decreased levels of apoB-containing lipoproteins were observed in apoB-Tg/PLTP$^{-/-}$ and apoE/PLTP$^{-/-}$ mice, with reduced levels of CEs, FC, PLs, and TGs in VLDL and LDL, suggesting the involvement of PLTP in the regulation of plasma levels of apoB-containing lipoproteins in these animals. This was accompanied by diminished secretion of apoB-100 and apoB-48 from the hepatocytes of the apoB-Tg/PLTP$^{-/-}$ mice compared with the apoB-Tg controls. Instead, PLTP deficiency showed no significant effects on the levels of apoB-containing lipoproteins in LDLr$^{-/-}$ mice, indicating the role of LDLr in reducing plasma VLDL and LDL levels in PLTP-KO mice. PLTP deficiency was shown to increase the α-tocopherol content of VLDL and LDL, thus decreasing the susceptibility of these lipoproteins to oxidation (Jiang et al., 2002). Further studies have demonstrated that the α-tocopherol content in the liver was diminished, the content of lipid peroxides in hepatocytes was increased, and ROS-dependent destruction of newly synthesized apoB-100 was enhanced in PLTP-KO mice (Jiang et al., 2005). Vitamin E supplementation decreased the cellular lipid peroxide content and ROS activation and normalized the production of apoB.

PLTP deficiency was shown to improve the anti-inflammatory properties of HDL and reduce LDL-induced monocyte chemotactic activity (Yan et al., 2004). The anti-inflammatory role of PLTP deficiency was further supported by findings that PLTP-KO mice had significantly lower IL-6 levels compared with control animals (Schlitt et al., 2005). PLTP deficiency was also associated with decreased cholesterol uptake and secretion by enterocytes without any significant effect on the uptake and secretion of fatty acids (Liu et al., 2007b). Niemann-Pick C1-like 1 mRNA levels were decreased in the small intestine of PLTP-KO mice, which could decrease the uptake of cholesterol. Additionally, the mRNA level of ABCA1 and microsomal triglyceride transfer protein (MTP) activity were decreased in the small intestine of PLTP-KO mice, possibly contributing to lower secretion of cholesterol. Thus, decreased cholesterol absorption could be one mechanism causing low

cholesterol levels and decreased atherosclerosis in PLTP-KO mice. PLTP deficiency was shown to alter hepatic lipid status, decreasing PL species containing long-chain fatty acids and increasing PL species containing medium-chain mono- or di-unsaturated fatty acids (Siggins et al., 2007). This was associated with secretion of apoA-I with a lower PL content and reduced stability from hepatocytes.

Overexpression of PLTP

Human PLTP transgenic (huPLTPtg) mice were established in 1996 (Albers et al., 1996; Jiang et al., 1996). PLTP activity levels as well as their effects on lipoprotein and apolipoprotein levels have been variable. When huPLTPtg mice were cross-bred with human apoA-I transgenic (huApoA-Itg) mice, increased PLTP activity (~50%) was accompanied by a remarkable increase in preβ-HDL and a small increase in α-HDL (Jiang et al., 1996). Further studies of huPLTPtg mice showing a 2.5-4.5-fold increase in PLTP activity have demonstrated a significant reduction in plasma HDL levels (van Haperen et al., 2000). However, formation of preβ-HDL was increased 2-3-fold in huPLTPtg mice. Thus, despite lower total HDL levels, PLTP can act as an antiatherogenic factor that prevents cholesterol accumulation. Adenovirus-mediated overexpression of PLTP in wild-type C57BL mice and in huApoA-Itg mice led to a 2.5-40-fold increase in plasma PLTP activity levels as well as a significant reduction in serum cholesterol, PLs, and TGs as well as increased preβ-HDL levels (Foger et al., 1997; Ehnholm et al., 1998). Plasma apoA-I concentration was clearly reduced in these animals due to the accelerated rate of fractional catabolism of HDL particles, which caused a substantial decrease in HDL levels. Similar changes in plasma lipids and lipoproteins were observed in a study using mice with transient adenovirus-mediated overexpression of human PLTP (Jaari et al., 2001). The distributions of PLTP activity and mass in serum were shown to be different, which suggested the presence of two forms of PLTP, one with high and the other with low PL transfer activity. PLTP overexpression enhanced hepatic uptake of CEs and PLs from HDL (Foger et al., 1997). In another study, a high overexpression of PLTP (15-fold) caused rapid clearance of HDL from the circulation and a 1.8-fold increase in hepatic CEs (Post et al., 2003). This led to a 2-fold increase in bile acid secretion and a 1.4-fold increase in the amount of excreted fecal bile acids, demonstrating the beneficial effect of PLTP on cholesterol removal from the body. To further characterize the role of PLTP in lipid metabolism, mice with inducible but reversible overexpression of PLTP were generated (Moerland et al., 2007a). An inducible mouse model provides stable and easily controlled PLTP overexpression, which differs from the transient induction obtained with an adenoviral expression system. Upon induction of PLTP expression, decreased

plasma cholesterol and PLs as well as decreased HDL cholesterol and PLs were observed.

The effect of PLTP overexpression on VLDL secretion was studied by overexpressing PLTP in mice heterozygous for LDLr ($LDLr^{+/-}$ mice) (van Haperen et al., 2002). Multiorgan and hepatic overexpression of PLTP caused a ~1.5-fold increase in VLDL secretion as well as a dose-dependent increase in susceptibility to atherosclerosis. When VLDL secretion was studied in huCETPtg and huCETPtg/huPLTPtg mice in a C57BL background, a similar 1.5-fold increase in VLDL secretion caused by PLTP overexpression without changes in the composition of VLDL particles was observed (Lie et al., 2002). PLTP overexpression in $apoE^{-/-}$ mice using an adenovirus-associated virus-mediated system caused an increase in plasma PLTP activity and decreased α-tocopherol content in plasma and lipoproteins (Yang et al., 2003). The results indicate that PLTP represents a factor that determines vitamin E content and oxidizability of lipoproteins, thus suggesting a proatherogenic role for PLTP. The proatherogenic role of systemic PLTP activity was further supported in several mice studies (Lie et al., 2004; Moerland et al., 2007b; Moerland et al., 2008).

Bone marrow transplantation studies

Recently, the effect of macrophage-derived PLTP on the development of atherosclerosis was studied using the bone marrow transplantation technique (Valenta et al., 2006b; Valenta et al., 2008). In both studies, macrophage-derived PLTP was shown to be atheroprotective, as increased atherosclerosis was observed in mice deficient in macrophage PLTP, whereas atherosclerotic lesions were significantly smaller in mice expressing PLTP only in hematopoietic cells. PLTP deficiency also decreased α-tocopherol content and increased oxidative stress in bone marrow cells (Valenta et al., 2006b). However, overexpression of human apoA-I was shown to provide substantial and sustained atheroprotection in $LDLr^{-/-}$ mice (Valenta et al., 2006a; Valenta et al., 2006b). Macrophage PLTP deficiency can reduce apoE secretion from macrophages, which may promote accumulation of cholesterol in the circulation and eventually the vessel wall (Liu et al., 2007a). A proatherogenic role for macrophage PLTP has also been suggested (van Haperen et al., 2008).

The results from bone marrow transplantation studies suggest that the effects of systemic PLTP on lipid metabolism and development of atherosclerosis are considerably different from those of PLTP expressed locally in tissues (Valenta et al., 2006b; Valenta et al., 2008). PLTP produced locally by macrophages within the

vessel intima may accelerate RCT by improving local generation of preβ-HDL particles without affecting plasma preβ-HDL levels, or it can stabilize ABCA1 and increase ABCA1-mediated cholesterol efflux, whereas the effect of systemic PLTP is mainly proatherogenic. The hypothesis of the differential role of systemic and macrophage PLTP was also supported by another research group using PLTP transgenic mice, which demonstrated that higher systemic PLTP levels may promote development of atherosclerosis by decreasing the rate of macrophage RCT (Samyn et al., 2008).

Mouse models have increased our knowledge about the function of PLTP in lipid and lipoprotein metabolism as well as in atherogenesis. However, PLTP levels in mice that overexpress PLTP and in PLTP-KO mice are nonphysiological and very different from those observed in humans. PLTP deficiency does not exist in humans, but increased PLTP levels are seen in humans with type I or type II diabetes and obesity, although the increase in PLTP activity in these conditions is moderate. Thus, dramatic alterations in PLTP activity may modulate the results and complicate generalization of the results to human pathophysiology (Tall and Lalanne, 2003).

2.4 CHOLESTERYL ESTER TRANSFER PROTEIN (CETP)

2.4.1 Characteristics of CETP

CETP gene and its regulation

The human CETP gene exists as a single copy in chromosome 16q12-16q21 (Lusis et al., 1987) and consists of 16 exons, encompassing about 25 kb of genomic DNA (Agellon et al., 1990). CETP, originally called lipid transfer protein I (LTP-I), was isolated from human plasma and separated from PLTP, originally called LTP-II (Tall et al., 1983a; Albers et al., 1984; Tollefson et al., 1988), in the 1980s. CETP activity is observed in various vertebrate species. However, considerable variation in activities is reported among different vertebrate species (Ha and Barter, 1982; Guyard-Dangremont et al., 1998). No CETP activity has been observed in mice, which are widely used in atherosclerotic research. Rabbit and hamster CETP share an overall sequence homology of approximately 80% with human CETP at the amino acid level (Drayna et al., 1987; Nagashima et al., 1988; Jiang et al., 1991).

The CETP gene is expressed at high levels in adipose tissue, the heart, and skeletal muscle and at lower levels in many other tissues in several mammalian species, while in humans, high levels of CETP expression are found in adipose tissue and the liver (Jiang et al., 1991). CETP is capable of enhancing selective uptake of CEs from HDL by human adipocytes, suggesting that this pathway may have an effect on HDL remodeling and adipocyte cholesterol accumulation (Benoist et al., 1997). In agreement with these findings and hypothesis, the expression of CETP in adipose tissue was shown to have a significant contribution to circulatory CETP mass and activity and to cause a reduction in HDL and an increase in non-HDL cholesterol levels (Zhou et al., 2006a). A high-cholesterol diet and increased cellular cholesterol content upregulate CETP mRNA levels and increase plasma CETP activity (Quinet et al., 1990; Jiang et al., 1991; Jiang et al., 1992; Oliveira et al., 1996). Regulation of CETP promoter activity involves LXRs and SREBPs. Both LXRα/RXRα and LXRβ/RXRα activate the CETP promoter via its DR4 element in a sterol-responsive fashion, while SREBP-1a and SREBP-2 activate the CETP promoter via interaction with its cholesterol response element (CRE) (Gauthier et al., 1999; Luo and Tall, 2000). Additionally, the human CETP gene promoter contains binding sites for several transcription factors that regulate its activity, such as ARP-1, YY-1, C/EBP, and Sp1 (Bruce et al., 1998b).

Structure of CETP protein

Mature human CETP protein comprises 476 amino acids. The translated molecular mass of CETP is 53 kDa, but the mass estimated by SDS-PAGE is 74 kDa (Drayna et al., 1987). CETP protein contains four potential asparagine-linked glycosylation sites at residues 88, 240, 341 and 396, and glycosylation of the protein can cause the discrepancy between the translated molecular mass and the mass estimated by SDS-PAGE. Based on sequence analysis, CETP is an extremely hydrophobic protein. The previous model of CETP was based on the crystal structure of BPI. However, as the amino acid identity of human CETP and BPI is only 22%, the accuracy of the model was not comprehensive (Bruce et al., 1998a).

The crystal structure of CETP was solved in 2007 (**Figure 3**) (Qiu et al., 2007). Based on that model, CETP is an elongated boomerang-shaped molecule. The structure of CETP is divided into four structural units, barrel C at the C-terminal end of the protein, barrel N at the N-terminal end, a central β-sheet between the two barrels and a C-terminal extension. The N- and C-terminal barrels as well as the central β-sheet are also found in BPI, but the C-terminal extension is not present in BPI (Beamer et al., 1997; Beamer, 2003). The terminal barrels of the CETP and BPI

molecules include a highly twisted β-sheet and two helices (Beamer et al., 1997; Beamer, 2003; Qiu et al., 2007). The central β-sheet of CETP was shown to contain six antiparallel strands, including residues from both the N- and C-terminal domains (Qiu et al., 2007). The structural unit unique to CETP, helix X, consisting of residues 465-476 at the C-terminal end of the CETP molecule, forms a distorted amphipathic helix that unwinds slightly at the end. The three structural units of CETP overlay well with the homologous units of BPI, and the crystal structure of CETP confirms the folds of the structural units of the molecule predicted earlier. However, the relative orientations of the structural units of CETP differ, resulting in a dissimilar overall structure for CETP (Bruce et al., 1998a; Qiu et al., 2007).

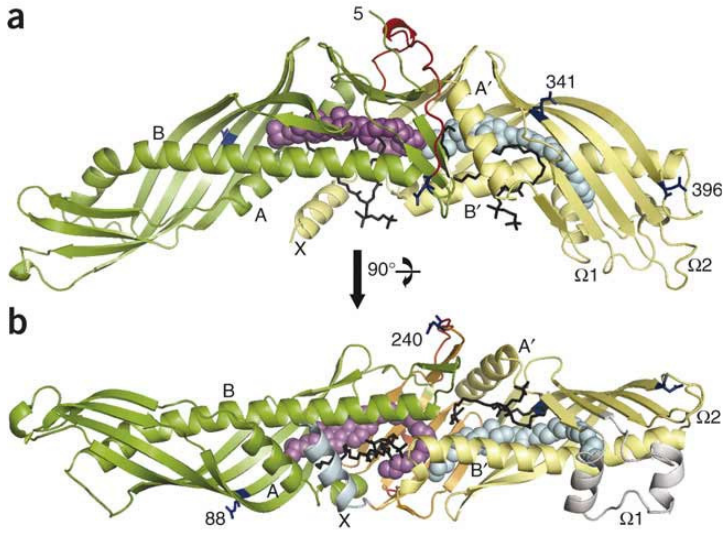

Figure 3. ***Overall structure of CETP (Qiu et al., 2007). a) The N-terminal domain is shown in green and the C-terminal domain in yellow, with the linker in red. CE1 (magenta) and CE2 (cyan) are shown as space fills and PLs as black bonds. N-glycosylation sites at residues 341 and 396 are labeled. '5' represents the N-terminus of the molecule. Helices A, B, A', B' and X are labeled. Helix X belongs to the C-terminal domain but interacts with the N-terminal domain. b) The structure after a 90º rotation. The four structural units are shown; the N-terminal barrel in green, the central β-sheet in orange, the C-terminal barrel in yellow, and the helix X in cyan. N-glycosylation sites at residues 88 and 240 are labeled.***

The earlier hypothesis was that the CETP molecule contains one neutral lipid-binding site and one PL-binding site (Bruce et al., 1998a). This was based on

quantitative analyses, which had indicated that 1.0 mol of CETP is able to bind 1.0 mol of cholesterol, 0.5 mol of TGs, and 1.3 mol of PLs (Connolly et al., 1996). However, the detailed crystal structure of CETP demonstrates that CETP can bind two neutral lipids and two PLs (Qiu et al., 2007). The binding sites for neutral lipids can accommodate two CEs, one CE and one TG, or possibly two TGs. The CETP molecule contains a 60-Å-long continuous tunnel with a large volume (2560 $Å^3$).The tunnel traverses the core of the molecule and has two distinct openings, the N- and C-terminal openings. The cross-sectional areas of both openings and the dimensions of the tunnel are large enough to allow lipid access and passage of neutral lipids. The observation that CE transfer is faster than TG transfer may be due to the fact that smaller CE molecules can pass through the tunnel with less resistance than larger TG molecules (Ohnishi et al., 1995; Connolly et al., 1996). The C-terminal end of the CETP molecule, including the helix X, is essential for proper functioning of CETP, as deletion mutants lacking amino acids 470-475 showed defective binding of CEs and TGs, suggesting the importance of helix X in the transfer of neutral lipids (Wang et al., 1995). However, deletion of the C-terminus had no effect on the binding of PLs by CETP or the association of CETP with HDL. Amino acids Lys_{233} and Arg_{259}, highly conserved residues within the LT/LBP family, are necessary for the CE transfer activity of CETP, possibly by mediating the binding of CETP to lipoproteins (Jiang et al., 1995).

The concave surface of the CETP molecule is the most probable site for lipoprotein binding, as it contains the N-terminal and C-terminal openings of the tunnel, helix X and the Ω1 flap (Qiu et al., 2007). The binding of lipoproteins to the other surfaces of CETP is unlikely, since they lack proper curvatures to bind spherical lipoproteins and direct access to the tunnel, and they are highly glycosylated. CETP has a higher binding affinity towards 10-nm-diameter nascent discoidal HDL particles than towards particles with a smaller diameter (Bruce et al., 1995), and this corresponds to the diameter of the concave surface of CETP. Thus, the authors hypothesized that CETP can bind a single HDL particle on the concave face of the CETP molecule (Qiu et al., 2007). The binding of HDL may cause slight movements of helix X and the Ω1 flap, and helix X may also be partially inserted into the bound lipoprotein. The overall HDL particle size and shape as well as lipid composition have a significant effect on the binding of CETP to HDL (Bruce et al., 1995). Moreover, these results indicated that binding of CETP to cholesterol- and CE-containing discs was increased, which suggests that CETP has a higher affinity for nascent HDL than for mature HDL.

2.4.2 Functions of CETP

Lipid transfer

CETP enhances the redistribution of CEs, TGs, and to lesser extent, PLs between plasma lipoproteins (Tall, 1993; Barter et al., 2003b). CETP mediates the heteroexchange of CEs in HDL for TGs in triglyceride-rich lipoproteins (TRL). Thus, the overall effect of CETP is a net mass transfer of CEs from HDLs into TRLs and LDL particles and of TGs from TRLs to HDL and LDL particles. The functions of CETP and PLTP *in vivo* are nonoverlapping (Kawano et al., 2000). Both CETP and PLTP are able to mediate the transfer of PLs from PC-containing HDL particles to LDL, but unlike PLTP, CETP is unable to transfer PLs from PC-containing liposomes to HDL (Lagrost et al., 1994). CETP was shown to induce redistribution of HDL_3 particles containing both apoA-I and apoA-II towards two particle subpopulations, one with a larger and the other with a smaller diameter compared with original particles (Lagrost, 1992).

Based on the crystal structure of CETP, a mechanism for CETP-mediated lipid transfer was suggested (**Figure 4**) (Qiu et al., 2007). The concave surface of the CETP molecule and the curvature of a 10-nm HDL particle are well suited to each other. Nevertheless, when CETP binds larger VLDL particles (30-100 nm), it must adopt a straighter conformation. It is unlikely that CETP can affect the surface curvature of VLDL particles. Instead, CETP can adopt a more gently curved conformation by twisting its barrels around the central β-sheet without significantly affecting the shape of the tunnel. Structural units of CETP may facilitate binding of lipoprotein to CETP, as access to the N-terminal opening is aided by helix X being inserting into the PL monolayer and facilitating neutral lipid exchange, whereas the Ω1 flap may help lipids access to the C-terminal opening. The neutral lipid transfer between lipoproteins in plasma occurs mainly by heteroexchange, as TG transfer activity was shown to be essential for CE transfer (Ko et al., 1994).

As long as it remains at a moderate level, plasma VLDL concentration is the driving force for the transfer of CEs (Mann et al., 1991). In hypertriglyceridemia, VLDL concentration increases and CETP becomes a rate-limiting factor that inhibits the net transfer of CEs from HDL. At normal VLDL levels, CEs are directed from HDL particles mainly to LDL, whereas at higher VLDL levels, large $VLDL_1$ particles are the preferential acceptors of CEs from HDL (Guerin et al., 2001). The total transfer of CE mass from HDL to total TRLs, including CMs, $VLDL_1$, $VLDL_2$ and IDL, was shown to be increased in the postprandial state, thus causing accumulation of CE-rich remnant particles of elevated atherogenicity (Guerin et al.,

2002). However, despite increased CETP activity, no changes in PLTP activity were observed in the postprandial state, indicating that plasma CETP and PLTP activities are differentially regulated (Syeda et al., 2003).

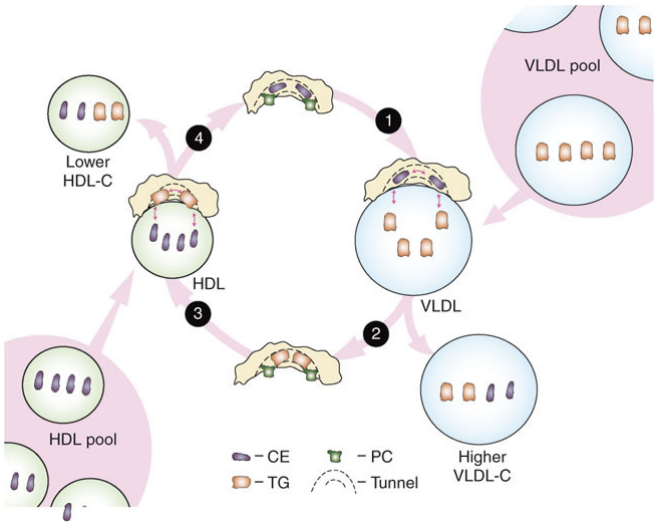

Figure 4. ***Proposed mechanism for CETP-mediated lipid transfer (Qiu et al., 2007). TG-rich VLDL is shown in blue and CE-rich HDL in green. Step 1, CETP filled with CE binds VLDL and releases the bound phospholipids (PC). Then, one or two TGs can enter the tunnel and an equal amount of CE is transferred into VLDL. Step 2, the TG-bound CETP dissociates from VLDL and leaves the VLDL particle with a higher CE content. Step 3, the TG-bound CETP binds HDL and releases the PCs. Next, one or two new CEs enter the tunnel and an equal amount of TG is transferred into HDL. Step 4, the CE-filled CETP dissociates from HDL and completes a full cycle of heteroexchange. The openings of the tunnel are refilled with PCs before dissociation of CETP from the lipoproteins to allow the return to the aqueous environment.***

Elevated plasma CETP levels accompanied by increased peripheral CE transfer to TRLs and LDL have been observed in patients with various atherogenic dyslipidemias (McPherson et al., 1991). CETP was shown to contribute to an atherogenic lipid phenotype in several ways. By increasing the CE content of VLDL particles, it increases the atherogenicity of VLDLs (Chung et al., 1998). Moreover, CETP increases the formation of small, dense LDL particles (Chung et al., 1998; Guerin et al., 2001) as well as very small HDL particles (Newnham and Barter, 1990). CETP-mediated lipid transfer was shown to cause a reduction in HDL particle size (Liang et al., 1994; Rye et al., 1995; Rye et al., 1997), which was associated with a dissociation of lipid-poor preβ-migrating apoA-I from HDL (Liang

et al., 1994; Rye et al., 1995). However, the capability of CETP to generate preβ-HDL is essentially lower than that of PLTP (Lie et al., 2001). Characterization of plasma CETP activity in the early phase of acute myocardial infarction revealed that high CE transfer rates are related to the presence of high non-HDL cholesterol, high CETP mass, and low HDL_{2b} concentration in young patients, suggesting the contribution of CETP to the accelerated progression of the disease among subjects without clinical symptoms (Zeller et al., 2007). In addition, plasma CETP levels might be related to risks of thrombotic events, as coagulation via both intrinsic and extrinsic pathways were positively correlated with plasma levels of CETP, increasing the activation of prothrombin (Deguchi et al., 2007).

Reverse cholesterol transport

As early as the 1980s it was suggested that CETP may promote cholesterol removal from cells of the human aortic wall (Stein et al., 1986). Later on, the presence of CETP in the foam cells of human aortic and coronary atherosclerotic lesions, but not in normal arteries, was demonstrated, suggesting that CETP might have a function in the removal of cholesterol from cells in the arterial wall (Zhang et al., 2001b). HDL-mediated efflux of FC from the COS-7 cells that express CETP was significantly increased, while the uptake of HDL lipids was unaltered compared with control mock-transfected cells. Furthermore, decreased cholesterol efflux was observed from macrophages obtained from CETP-deficient subjects compared with macrophages obtained from subjects with normal CETP expression. The ability of HDL derived from CETP-deficient subjects to promote removal of cholesterol from LXR-activated macrophages was studied recently (Matsuura et al., 2006). HDL_2 from CETP-deficient subjects showed 2-3-fold stimulation in ABCG1-mediated net cholesterol efflux compared with control HDL_2. The enhanced ability of HDL_2 to promote cholesterol efflux was associated with increased LCAT and apoE contents of HDL_2 particles. Besides ABCG1, efflux pathways mediated by SR-BI and ABCA1 were shown to be functional when sera from CETP-deficient subjects were used as cholesterol acceptors (Miwa et al., 2009). Moreover, increased plasma CETP activity and increased cholesterol efflux from Fu5AH cells to plasma samples were observed during the postprandial state in normolipidemic men (Syeda et al., 2003). However, no correlation of plasma CETP activity with plasma cholesterol efflux capacity was observed in this study. Recently, a decrease in plasma CETP activity accompanied by decreased cholesterol efflux from THP-1 macrophages to plasma samples obtained from subjects treated with rosuvastatin was observed (Sviridov et al., 2008).

2.4.3 CETP deficiency as a predictor of atherosclerosis

CETP deficiency reportedly exists in humans (Brown et al., 1989; Inazu et al., 1990). It is associated with a potentially antiatherogenic lipoprotein profile, as subjects homozygous for CETP deficiency exhibited substantially elevated HDL and apoA-I levels and decreased LDL and apoB levels (Inazu et al., 1990). Subjects heterozygous for CETP deficiency had lowered plasma CETP levels accompanied by moderately increased levels of HDL and apoA-I and an increased HDL_2/HDL_3 ratio. CETP deficiency was shown to result in the formation of large HDL particles containing all the major apolipoproteins, apoA-I, apoA-II, apoC (I, II, III), and apoE, except for apoA-IV (Asztalos et al., 2004). Subjects heterozygous for CETP deficiency had increases in large α-1 and preα-1 particles, whereas preβ_1-HDL was lower. The increased HDL levels in CETP-deficient subjects have been shown to be due to delayed catabolism of apoA-I and apoA-II without changes in the production rates of apolipoproteins (Ikewaki et al., 1993). The HDL fraction from subjects deficient in CETP was especially enriched in large apoE- and CE-rich HDL particles (Yamashita et al., 1990). When the effect of HDL isolated from patients homozygous for CETP deficiency on the accumulation and removal of cholesterol was studied, the large apoE-free, CE-rich HDL_2 particles from CETP-deficient subjects had decreased ability to prevent cholesterol accumulation into macrophages and remove cholesterol from lipid-laden macrophages while HDL_3 particles were functional (Ishigami et al., 1994).

2.4.4 Animal models of atherosclerosis with modified CETP expression

Mice, among several other species, are naturally deficient in CETP, and therefore introduction of the human CETP transgene into mice has provided an opportunity to study the effect of CETP on lipoprotein metabolism and RCT. Expression of the CETP transgene in mice caused a significant decrease in plasma HDL level (Agellon et al., 1991; Jiang et al., 1993) as well as increased VLDL and LDL cholesterol and apoB levels (Jiang et al., 1993). With increasing CETP concentration, hepatic LDLr mRNA abundance was progressively decreased, leading to accumulation of apoB-containing lipoprotein particles. Introduction of the simian CETP transgene into mice was shown to result in more severe atherosclerosis compared with nontransgenic controls, probably due to CETP-induced changes in lipoprotein distribution (Marotti et al., 1993). The effect of bone marrow-derived CETP on atherosclerosis was studied by transplanting bone marrow from huCETPtg mice into $LDL^{-/-}$ mice (Van Eck et al., 2007). Bone marrow-derived CETP increased plasma

VLDL and LDL cholesterol and decreased HDL cholesterol, and these changes in CE distribution were accompanied with enhanced formation of atherosclerotic lesions. However, macrophage CETP production showed no effect on cholesterol efflux. Furthermore, the results of this study demonstrated that bone marrow-derived cells, including macrophages, are important contributors to total serum CETP activity and mass.

The effect of CETP on the RCT process is unclear, as both promotion and inhibition of RCT have been reported in animal studies. Targeted expression of CETP in the livers of different mouse models demonstrated that CETP expression promoted macrophage RCT despite a reduction in plasma HDL cholesterol levels, and this effect of CETP required the presence of LDLr (Tanigawa et al., 2007). Previous results have shown that CETP-mediated remodeling of HDL leads to enrichment of HDL with TGs, and subsequent lipolysis of TG-rich HDL by HL can promote the uptake of CEs from HDL by hepatic SR-BI (Collet et al., 1999). Moreover, later results have indicated that CETP is able to restore to normal the rate of RCT in mice deficient in SR-BI (Tanigawa et al., 2007). This observation is in agreement with the results demonstrating that CETP is able to mediate selective uptake of CEs from HDL by hepatocytes via a mechanism independent of SR-BI and LDL receptors (Gauthier et al., 2005), suggesting that CETP is involved in a physiological system that facilitates functional RCT. Moreover, recent results demonstrated that both CETP overexpression and inhibition of CETP activity by torcetrapib may result in enhancement of the RCT process (Tchoua et al., 2008). In contrast, no enhancement of RCT was observed when simian (Stein et al., 2002) or human CETP (Rotllan et al., 2008) was expressed in mice. CETP overexpression was also unable to improve the protection conferred by HDL against oxidative modification of LDL, and the result was consistent with the absence of effects of CETP expression on plasma activities of antioxidative HDL enzymes PON-1 and PAF-AH (Rotllan et al., 2008).

In certain circumstances, CETP expression may have antiatherogenic rather than proatherogenic effects in mice. In hypertriglyceridemic human apoC-III transgenic mice, decreased development of atherosclerotic lesions due to CETP expression was observed (Hayek et al., 1995). Also another study demonstrated that the human CETP transgene was antiatherogenic in hypertriglyceridemic mice produced by streptozotocin-induced diabetes and LPL deficiency (Kako et al., 2002). In a diabetic mouse model, db/db mice, CETP expression led to lower plasma total cholesterol, VLDL and LDL cholesterol, and attenuated the development of atherosclerotic lesions, which suggests that in the context of diabetic obesity, CETP might be antiatherogenic (MacLean et al., 2003). In ovariectomized mice, CETP expression was accompanied by lower plasma levels of total cholesterol, VLDL,

LDL, and HDL cholesterol, as well as lower titers of antibodies against oxidized LDL and smaller mean aortic lesion areas (Cazita et al., 2003). These results suggested that CETP expression can compensate for the lack of estrogen.

2.4.5 Therapeutic interventions affecting CETP activity

Animal studies

As rabbits have a naturally high plasma level of CETP and extremely high susceptibility to atherosclerosis, rabbits have been widely used to study the effects of CETP inhibition on the development of atherosclerosis. Several compounds have been generated for inhibition of CETP activity. One of these is JTT-705, which is a synthetic compound that inhibits CETP activity by forming a disulphide bond with CETP. When CETP activity was inhibited in mildly hypercholesterolaemic rabbits by administering JTT-705 for six months, a 90% elevation in plasma HDL cholesterol, a 40-50% reduction in non-HDL cholesterol and a 70% decrease in lesion area were observed (Okamoto et al., 2000). JTT-705 increased both HDL_2 and HDL_3, while the increase of HDL_2 was more pronounced. However, in rabbits with more severe hypercholesterolaemia, JTT-705 was unable to inhibit the extent of atherosclerotic lesions, despite a significant increase in plasma HDL (Huang et al., 2002). In a second study, non-HDL cholesterol decreased less compared with the first study (25% vs. 40-50%), and the results demonstrated that the extent of the lesion area correlated with non-HDL cholesterol but not with HDL cholesterol. The increase in HDL levels in JTT-705-treated Japanese White rabbits was shown to be due to increased apoA-I synthesis without changes in the rate of fractional catabolism of apoA-I (Shimoji et al., 2004). In addition to increased HDL levels and an increased HDL_2/HDL_3 ratio, JTT-705 treatment of rabbits increased the apoE content of HDL as well as the PL content of apoE-rich HDL (Zhang et al., 2004). The activities of HDL-associated enzymes, PON-1 and PAF-AH, were increased, indicating that CETP inhibition by JTT-705 has several beneficial effects on plasma HDL.

Torcetrapib is a potent inhibitor of CETP activity, which by enhancing the association between CETP and HDL leads to the formation of complexes that inhibit lipid transfer between HDL and other lipoproteins (Barter and Kastelein, 2006). Administration of torcetrapib to hamsters elevated plasma HDL levels and enhanced RCT *in vivo* (Tchoua et al., 2008). In hyperlipidemic *ApoE*3-Leiden.CETP*

transgenic (*E3L.CETP*) mice, torcetrapib increased plasma HDL levels by 30% and reduced atherosclerotic lesion size by 43% (de Haan et al., 2008a). However, atorvastatin caused a similar reduction in lesion size, and a combination of torcetrapib and atorvastatin failed to cause a further decrease in lesion size compared with atorvastatin alone. Furthermore, torcetrapib changed the phenotype of lesions towards a more unstable and proinflammatory state characterized by an increased macrophage and a decreased collagen content. Also statins are able to inhibit CETP activity, as increased plasma HDL levels in *E3L.CETP* mice were accompanied by decreased circulating CETP activity caused by down-regulation of hepatic CETP mRNA expression by atorvastatin (de Haan et al., 2008b). Besides beneficial effects of torcetrapib on HDL levels, elevated blood pressure associated with increased circulating levels of aldosterone and corticosterone were found in several animal species, including mice, rats, dogs, and rhesus monkeys, following torcetrapib treatment (Forrest et al., 2008). However, the observed changes are probably independent of CETP inhibition, as no increase in blood pressure or adrenal steroids was observed with another CETP inhibitor, anacetrapib.

Vaccines against CETP are one approach to inhibiting plasma CETP activity that is under investigation. Intramuscular immunization of rabbits with a chimeric peptide containing a region of CETP necessary for neutral lipid transfer decreased plasma CETP activity significantly and resulted in 42% higher HDL, 24% lower LDL, and ~40% smaller aortic lesion area compared with control-vaccinated rabbits (Rittershaus et al., 2000). A search for non-invasive routes for immunization against atherosclerosis has led to development of intranasal vaccinations (Yuan et al., 2008). Intranasal and intramuscular immunizations using a DNA vaccine against CETP resulted in a similar reduction in aortic lesion area (~60% and ~70%, respectively) in rabbits, suggesting that non-invasive intranasal vaccination might be used for therapeutic purposes.

Human studies

After studies conducted in animals, the function and effectiveness of CETP inhibitors have been tested in carefully controlled trials in humans. When CETP inhibitor JTT-705 was tested in a phase II, randomized, double-blind, placebo-controlled study of healthy subjects with mild dyslipidemia, a dose-dependent decrease in CETP activity and an increase in HDL cholesterol were observed, accompanied by a minimal decrease in LDL cholesterol (de Grooth et al., 2002). A combination of pravastatin and JTT-705 was assessed in a randomized, double-blind, placebo-controlled trial in subjects with type II dyslipidemia using pravastatin

(Kuivenhoven et al., 2005). Four weeks of treatment with JTT-705 led to a 30% decrease in CETP activity and a 28% increase in HDL cholesterol, while LDL cholesterol decreased by ~5%. In both trials, JTT-705 was shown to be safe and well tolerated.

Torcetrapib was tested in 40 healthy individuals randomized to receive either a placebo or torcetrapib in doses increasing from 10 to 240 mg daily for 14 days (Clark et al., 2004). The mean inhibition of CETP activity was from 12% to 80%, depending on the dose of torcetrapib. HDL cholesterol concentration increased by 16% to 91%, whereas that of LDL decreased by 21% to 42%. These changes were accompanied with increased apoA-I and apoE and decreased apoB concentrations. Torcetrapib reduced VLDL, IDL, and LDL apoB-100 levels by increasing the rate of apoB-100 clearance (Millar et al., 2006), and VLDL apoE levels by increasing VLDL apoE clearance (Millar et al., 2008). In another study conducted in 19 subjects with low HDL levels, torcetrapib at a daily dose of 120 mg increased plasma HDL concentration by 61% or 46% in the presence or absence of concomitant therapy with atorvastatin, whereas treatment with torcetrapib alone at a dose of 120 mg twice daily increased HDL cholesterol by 106% (Brousseau et al., 2004). The mean size of HDL particles increased following treatment with torcetrapib, which is in good agreement with the previous results of large HDL particles observed in individuals with a genetic deficiency in CETP (Matsuura et al., 2006). Torcetrapib increased plasma apoA-I in HDL by delaying apoA-I catabolism (Brousseau et al., 2005). Recently, enhanced cholesterol efflux from macrophage foam cells due to increased HDL levels and improved particle functionality was observed following torcetrapib administration in humans (Yvan-Charvet et al., 2007a). Torcetrapib may modulate the biological activities of HDL_2 and HDL_3 particles and thus affect their function in RCT (Catalano et al., 2009). However, in another study, no increase in overall RCT in humans, measured by fecal sterol, was found by torcetrapib, regardless of increased HDL levels (Brousseau et al., 2005).

Torcetrapib has already been tested in humans in large, long-term trials (Barter et al., 2007b; Bots et al., 2007; Kastelein et al., 2007; Nissen et al., 2007). At the end of 2006, all torcetrapib clinical trials were terminated in the interest of patient safety. Torcetrapib increased HDL cholesterol and decreased LDL cholesterol in each study, although significantly more major cardiovascular events were observed in the group receiving torcetrapib in combination with atorvastatin than in the group receiving atorvastatin monotherapy (Barter et al., 2007b). Furthermore, torcetrapib failed to attenuate the progression of atherosclerosis in the common carotic arteries (Bots et al., 2007; Kastelein et al., 2007) and coronary arteries (Nissen et al., 2007), and it increased systolic blood pressure in all the trials. Whether the lack of efficacy and the observed adverse effects are related to the

mechanism of drug class, i.e. CETP inhibition, or to molecule-specific properties, needs to be determined. In two double-blind, randomized, placebo-controlled phase I studies in healthy individuals and in patients with dyslipidemia, anacetrapib, a CETP inhibitor of the same structural class as torcetrapib, increased HDL cholesterol and decreased LDL cholesterol without any effects on blood pressure, suggesting that CETP inhibition itself might not lead to increased blood pressure (Krishna et al., 2007). It has been postulated that torcetrapib can lead to the formation of HDL particles that function improperly or are proinflammatory (Singh et al., 2007).

CETP vaccine has also been tested in humans. However, in a phase I trial only one patient out of a total of 36 patients at the highest dose of a single injection of CETP vaccine developed auto-antibodies against CETP (Davidson et al., 2003). Following a second injection of vaccine, 8 patients out of a total of 23 patients had a measurable antibody response, whereas no response was observed in a placebo group.

3 AIMS OF THE STUDY

I) To study the role of endogenous PLTP in the removal of cholesterol from macrophage foam cells.

II) To determine the effects of macrophage-derived PLTP on the development of atherosclerosis.

III) To clarify the role of HA-PLTP and LA-PLTP in the removal of cholesterol from macrophage foam cells.

IV) To determine the effect of CETP on cholesterol efflux from macrophage foam cells and whether chymase affects degradation of CETP and its function in the efflux process.

V) To investigate whether monocyte macrophages derived from low- and high-HDL subjects differ in cholesterol efflux and whether their sera differ as cholesterol acceptors.

4 MATERIALS AND METHODS

4.1 LIST OF PUBLISHED METHODS

The methods used in this thesis project are listed in the table below in alphabetical order. The original publications in which the methods appear are indicated by the Roman numerals.

Method	Original publication
Acetylation of LDL	I, III, IV, V
Bone marrow isolation and transplantation	II
Cell culture	I, II, III, IV, V
CETP activity assay	IV, V
Cholesterol efflux assays	I, III, IV, V
Crossed immunoelectrophoresis	I, II, III, IV, V
Enhanced chemiluminescence detection	IV, V
Enzymatic lipid analysis	II, III, V
Hepatic cell separation	II
Hepatic lipase activity assay	II
Histological analysis of the aortic root	II
Immunohistochemistry	II
Immunoprecipitation of preβ-HDL	III
Isolation of leukocytes from buffy coat	III, V
Isolation of peritoneal leukocytes	I
LCAT activity assay	II, V
Lipid extraction	I, III, V
Lowry protein determination	I, III, IV, V
Non-denaturing gradient gel electrophoresis	III, V
Plasma apolipoprotein and lipoprotein analysis	I, II, III, IV, V
Plasma CRP and glucose analysis	V
PLTP activity assay	I, II, III, IV, V
PLTP ELISA	II, III, V
Purification of CETP	IV
Purification of PLTP	III, IV
Radiolabeling of acetylated LDL	I, III, IV, V
Real-time quantitative PCR	II, V
SDS-PAGE and Western blotting	IV, V
Size-exclusion chromatography	II, III
Statistical analysis	I, II, III, IV, V
Thin layer chromatography	I, III, V

5 RESULTS AND DISCUSSION

5.1 Absence of endogenous PLTP impairs ABCA1-dependent efflux of cholesterol from macrophage foam cells *(I)*

Data on the participation of exogenously added PLTP in cholesterol efflux from cells are available (von Eckardstein et al., 1996; Wolfbauer et al., 1999; van Haperen et al., 2000; Lee et al., 2003; Oram et al., 2003), whereas the role of endogenous PLTP in the efflux process is unclear. As foam cells present in human atherosclerotic plaques contain an abundant amount of PLTP (Desrumaux et al., 2003; Laffitte et al., 2003; O'Brien et al., 2003), it raises the possibility that endogenous PLTP may also participate in RCT. To address this question, ABCA1-dependent efflux of cholesterol from peritoneal macrophages derived from PLTP-KO mice was compared with that from macrophages derived from wild-type (WT) mice.

5.1.1 Cholesterol efflux to HDL_3, apoA-I, and plasma

First, the morphological features of PLTP-KO and WT macrophages loaded in the presence of [^{3}H]CE-acetyl-LDL were compared. The morphological features of cholesterol-loaded PLTP-KO and WT macrophages were similar when evaluated by light microscopy (***I, Fig. 1***). The viabilities of the PLTP-KO and WT macrophages with or without acetyl-LDL loading did not differ.

Next, the efflux of cholesterol from PLTP-KO and WT macrophages, both in their basal state and after their conversion to foam cells, to HDL_3 was investigated. For the efflux studies, peritoneal macrophages were labeled by exposing the cells to [^{3}H]free cholesterol for 24 h in the presence of an ACAT inhibitor, Sandoz 58-035 compound (Ross et al., 1984), or cholesterol-loaded by incubating them with [^{3}H]CE-acetyl-LDL for 18 h. Cholesterol efflux from the nonloaded macrophages was low, and no significant effect of PLTP was evident. However, when the macrophages were loaded with acetyl-LDL, high-affinity and saturable efflux of cholesterol to HDL_3 was observed (**Figure 5A**). Cholesterol efflux from PLTP-KO macrophages loaded with acetyl-LDL was significantly reduced ($P < 0.05$) compared with cholesterol-loaded WT macrophages. The K_m value was 2-fold higher for PLTP-KO macrophages than for WT macrophages (15.6 vs. 8.2 µg/ml, respectively). These results suggest that PLTP deficiency disturbed ABCA1-mediated cholesterol efflux involving cholesterol mobilization from late endocytic compartments (Chen et al., 2001), rather than efflux of FC from cell surfaces.

To characterize the role of PLTP in cholesterol efflux, cholesterol efflux from cholesterol-loaded macrophages to human lipid-free apoA-I was studied. The expression of ABCA1 is very low in nonloaded macrophages, while cholesterol loading leads to the formation of macrophage foam cells with subsequent overexpression of ABCA1 (Langmann et al., 1999). The rate of cholesterol efflux to apoA-I was higher from WT than from PLTP-KO macrophage foam cells at all apoA-I concentrations tested (**Figure 5B**). As ABCA1 mediates cholesterol efflux to apoA-I, the results demonstrate that the absence of endogenous PLTP impairs ABCA1-mediated cholesterol efflux.

In addition to HDL and apoA-I, plasma was used as a cholesterol acceptor. Plasma has been widely used as a cholesterol acceptor in efflux studies, as it represents a more physiological acceptor (Fielding and Fielding, 1995). When cholesterol efflux from WT macrophages was studied, plasma from WT mice promoted higher cholesterol efflux than plasma from PLTP-KO mice (***I, Fig. 4***). Moreover, WT plasma promoted less efficient cholesterol efflux from PLTP-KO macrophages than from WT macrophages. Normalization of efflux values for the plasma apoA-I concentration did not change the results markedly, which confirms that endogenous PLTP has an essential role in the cholesterol efflux process from macrophages.

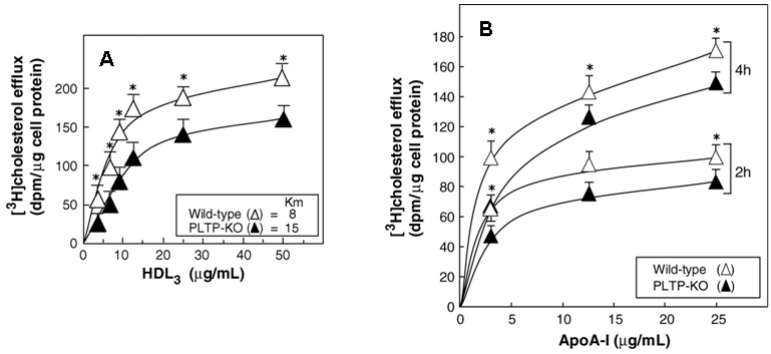

Figure 5. ***Cholesterol efflux from PLTP-KO and WT macrophages to HDL_3 (A) and apoA-I (B). Each value is the mean of triplicate wells ± SD. * $P < 0.05$. K_m values are shown in the insets.***

5.1.2 Cholesterol loading of macrophages

Following the observed differences in cholesterol efflux, the ability of PLTP-KO and WT macrophages to take up acetyl-LDL was compared. Loading of

macrophages with acetyl-LDL increased their cellular lipid content and stimulated formation of foam cells in both PLTP-KO and WT macrophages (***I, Table 1***). However, the intracellular FC and CE contents of PLTP-KO and WT macrophages loaded with various concentrations of acetyl-LDL did not differ, verifying that genetic deficiency in PLTP does not prevent foam cell formation and thereby affect the efflux process.

5.1.3 Cholesterol efflux under the stimulation of ABCA1

Stimulation with cAMP leads to upregulation of the expression and activity of ABCA1 in murine macrophages (Oram et al., 2000; Kiss et al., 2005). It was therefore of interest to study the effect of 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (cAMP) on cholesterol efflux from nonloaded and cholesterol-loaded PLTP-KO and WT macrophages to apoA-I. Treatment of mouse macrophages with cAMP increases the expression of ABCA1 without any effects on the expression of ABCG1 (Khovidhunkit et al., 2003). In contrast to nonloaded cells, the co-stimulatory effect of cholesterol loading and cAMP treatment on ABCA1-mediated cholesterol efflux caused a clear enhancement of cholesterol efflux from PLTP-KO macrophages and resulted in similar levels of cholesterol efflux from PLTP-KO and WT macrophages (***I, Fig. 5***). These results suggest that PLTP deficiency can be compensated by stimulation of ABCA1 expression and ABCA1-mediated cholesterol efflux. In a previous report (Cao et al., 2002), only minimal reduction was found in the efflux capacity of PLTP-KO macrophage foam cells compared with WT macrophage foam cells before or after LXR stimulation. A possible explanation for this observed difference between the results may be due to responses induced by an inflammatory challenge (Cook et al., 2003) brought about by thioglycollate exposure of the elicited macrophages in the previous study, whereas in this study the macrophages were collected without thioglycollate treatment.

5.2 Macrophage PLTP contributes significantly to total plasma phospholipid transfer activity and its deficiency leads to diminished atherosclerotic lesion development *(II)*

To evaluate the role of macrophage PLTP in lipoprotein metabolism and in the development of atherosclerotic lesions, PLTP gene expression was specifically disrupted in cells of hematopoietic origin by bone marrow transplantation. Development of atherosclerotic lesions was induced by feeding the mice with a

Western-type diet (WTD) containing 15% (w/w) total fat and 0.25% (w/w) cholesterol for 9 weeks.

5.2.1 Effect of macrophage PLTP deficiency on atherosclerosis

Lesion development was analyzed in the aortic root of the $PLTP^{+M/+M}$ and $PLTP^{-M/-M}$ mice after 9 weeks of WTD feeding. Macrophage PLTP deficiency led to a 29% ($P < 0.01$) decrease in mean atherosclerotic lesion area (**Figure 6**). Macrophage PLTP deficiency showed no significant effect on the relative macrophage content of the lesions. Instead, macrophage PLTP deficiency led to slightly reduced collagen content ($P = 0.07$) and significantly smaller cap thickness ($P < 0.01$) in the lesions compared with $PLTP^{+M/+M}$ mice, which could be related to the smaller lesion size observed in $PLTP^{-M/-M}$ animals.

5.2.2 Measurement of serum lipid levels and lipoprotein distribution

The decreased lesion size in the $PLTP^{-M/-M}$ group coincided with significantly lower serum total cholesterol (TC), FC ($P < 0.05$ for both TC and FC), and TG ($P < 0.01$) levels in these mice compared with $PLTP^{+M/+M}$ mice. All the other details are shown in ***Table 1*** (***II***). Macrophage PLTP deficiency showed no significant effect on circulating preβ-HDL levels at 8 weeks posttransplantation, whereas 24% lower preβ-HDL levels were observed in $PLTP^{-M/-M}$ mice compared with $PLTP^{+M/+M}$ mice after 9 weeks of WTD feeding ($P < 0.05$). Lesion development was reduced in $PLTP^{-M/-M}$ mice in spite of the decreased levels of preβ-HDL, which implicates that the proatherogenic effects of lower preβ-HDL levels were overruled by other factors. Higher plasma apoA-I levels and HDL PLs observed in $PLTP^{-M/-M}$ mice may lead to formation of HDL subclasses with a higher acceptor potential in the efflux process, leading to decreased lesion size.

PLTP activity (Damen et al., 1982; Jauhiainen et al., 1993) was almost exclusively associated with HDL lipoproteins in both groups. Plasma PLTP activity was 1.4-fold lower in the $PLTP^{-M/-M}$ group compared with the $PLTP^{+M/+M}$ group on chow diet ($P < 0.01$) and 2-fold lower during WTD ($P < 0.001$). These data demonstrate that macrophage PLTP is an important contributor to plasma PLTP activity.

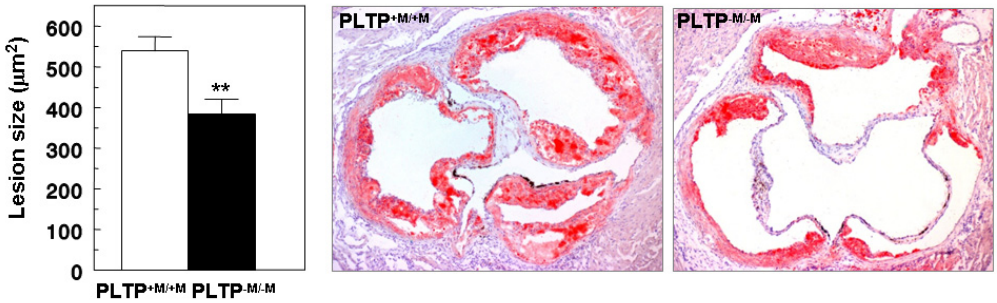

Figure 6. ***Atherosclerotic lesion formation in LDLr$^{-/-}$ mice with macrophage PLTP deficiency. Values represent the means ± SEM calculated from 14 PLTP$^{+M/+M}$ mice and 10 PLTP$^{-M/-M}$ mice. **P < 0.01. Original magnification x50.***

5.2.3 PLTP activity and expression in the liver and lung

PLTP is a ubiquitously expressed protein with a moderate level of expression in the liver (Albers et al., 1995). Despite only moderate hepatic PLTP expression, the liver as a large organ can significantly contribute to circulating PLTP levels. PLTP mRNA expression in whole liver tissue samples taken from PLTP$^{+M/+M}$ and PLTP$^{-M/-M}$ mice at 17 weeks posttransplantation was studied. PLTP expression was slightly reduced in livers from the PLTP$^{-M/-M}$ mice compared with livers from the PLTP$^{+M/+M}$ animals. In accordance with the mRNA data, PLTP activity levels in the liver homogenates were 26% lower in the PLTP$^{-M/-M}$ mice ($P = 0.0019$) (***II, Fig. 3***). These results confirm the importance of bone marrow cells in hepatic PLTP production.

The liver contains several different types of cells, of which the majority are parenchymal cells, which contribute 92.5% of the total mass of liver protein. Other cell types, endothelial cells and Kupffer cells, account for 3.3% and 2.5% of the mass of liver protein, respectively (Blouin et al., 1977; Nagelkerke et al., 1983). Of the hepatic cells, Kupffer cells are resident macrophages of the liver and of hematopoietic origin. In the present study, expression of PLTP mRNA was 6-fold ($P < 0.01$) higher in Kupffer cells compared with parenchymal cells, whereas endothelial cells produced only a minority of PLTP mRNA. As a consequence, Kupffer cells contribute approximately 14% of the total expression of liver PLTP, despite the low contribution to total liver protein. Parenchymal cells and endothelial cells contributed 85.7% and 0.5% of the total liver PLTP mRNA, respectively. As plasma PLTP activity reportedly correlates positively with TG levels (Janis et al., 2004), and macrophage PLTP deficiency resulted in substantially lower plasma

VLDL levels, it is conceivable that PLTP from Kupffer cells could also directly or indirectly influence VLDL biosynthesis or secretion.

In addition to liver, PLTP is expressed in the lungs, where the expression level is very high (Albers et al., 1995). Localizations of PLTP and macrophages were analyzed in liver and lung samples from $PLTP^{+M/+M}$ and $PLTP^{-M/-M}$ mice. Immunohistochemical localization of PLTP protein in the livers and lungs of $PLTP^{+M/+M}$ and $PLTP^{-M/-M}$ mice clearly demonstrated a reduction in PLTP protein expression due to disruption of PLTP in bone marrow-derived cells (***II, Fig. 4***). Moreover, macrophage PLTP deficiency resulted in a clear decrease in PLTP expression in the bronchioles of the lung. The results demonstrate that the significant contribution of Kupffer cells to PLTP expression and production, combined with that of other resident macrophages in the lung, adipose tissue, and spleen, may explain the observed effects of PLTP deficiency in hematopoietic cells on lipoprotein metabolism and atherosclerosis, as well as plasma PLTP activity.

A proatherogenic role for macrophage-derived PLTP was suggested in a recent report (van Haperen et al., 2008). However, opposite results have also been published showing that macrophage-derived PLTP is atheroprotective against diet-induced atherosclerosis in $LDLr^{-/-}$ mice (Valenta et al., 2006b; Valenta et al., 2008). The discrepancies between the results may be due to several factors: 1) the degree of hypercholesterolemia achieved by the atherogenic diets; 2) the time-dependency of the progression of atherosclerosis; 3) the genetic background and sex of the mice; and 4) other factors, including environmental factors as well as the time of recovery from irradiation (Curtiss, 2006). Thus, the differences in the experimental setups may have caused the opposite results and conclusions reported by us and Valenta et al. Furthermore, Yang et al (Yang et al., 2006) recently reported that, in mice, many hepatic genes show sexual dimorphism, and the largest changes (> 3-fold) in gene expression between females and males are observed in genes involved in steroid and lipid metabolism.

5.3 Cholesterol efflux from macrophage foam cells is enhanced by active PLTP via generation of two types of acceptors *(III)*

Exogenous PLTP has been implicated in lipid efflux (von Eckardstein et al., 1996; Lee et al., 2003; Oram et al., 2003), although the exact function of different forms of PLTP, HA-PLTP and LA-PLTP (Oka et al., 2000a; Karkkainen et al., 2002), in the

RCT pathway is not known. The aim of this study was to clarify the role of the two forms of PLTP in cholesterol efflux from cholesterol-loaded macrophages.

5.3.1 Effect of HA-PLTP and LA-PLTP on cholesterol efflux

For the efflux experiments, the monocytes were treated with phorbol 12-myristate-13-acetate (PMA) to differentiate the cells into macrophages, followed by loading of the cells in the presence of [^{3}H]CE-acetyl-LDL. Reportedly, acetyl-LDL-delivered cholesterol is preferentially transported to late endosomes and lysosomes, and this pool is readily accessible to apoA-I-facilitated efflux by the ABCA1-dependent pathway (Wang et al., 2007a). To study the effect of PLTP on cholesterol efflux from macrophages to HDL and apoA-I, apoE-free HDL_3 and lipid-free apoA-I were incubated prior to efflux for 24 h at +37°C with i) HA-PLTP, ii) LA-PLTP or with iii) PBS only serving as a control. The results demonstrate that HA-PLTP increased preβ-HDL formation and caused a 42% increase in cholesterol efflux to HDL ($P < 0.001$), while LA-PLTP neither formed preβ-HDL nor increased cholesterol efflux, demonstrating that the PL transfer activity of PLTP is a prerequisite for enhanced cholesterol efflux from macrophages to HDL (***III, Fig. 1***). When lipid-free apoA-I was used as a cholesterol acceptor, neither HA- nor LA-PLTP were able to enhance cholesterol efflux to lipid-free apoA-I. PLTP alone did not induce cholesterol efflux, an observation that is discrepant with the observation by Oram et al (Oram et al., 2003). The reason for this discrepancy may be caused by the difference in cells used in the experiments (macrophages vs. non-macrophage cells).

In addition to THP-1 cells, primary monocyte-derived macrophages isolated from human blood were used for the efflux experiments. In accordance with the results from THP-1 cells, increased cholesterol efflux to HDL preincubated with HA-PLTP was observed ($P < 0.01$), whereas LA-PLTP was unable to improve cholesterol efflux.

5.3.2 Isolation of preβ-HDL and large fused HDL particles and their functionality in cholesterol efflux

To analyze cholesterol efflux to particles generated by HA-PLTP, the HDL conversion products, small preβ-HDL and large fused HDL particles, were isolated by size-exclusion chromatography and analyzed with native gradient gel electrophoresis (GGE) (Pussinen et al., 1995). During HA-PLTP-facilitated conversion, large fused HDL particles with an apparent size of 250 kDa were formed. LA-PLTP had no effect on the size of HDL particles. Isolated preβ-HDL acted as a functional cholesterol acceptor in the efflux of cholesterol from THP-1

cells. Furthermore, removal of preβ-HDL by immunoprecipitation caused a 47% decrease in cholesterol efflux compared with the control ($P < 0.001$) (***III, Fig. 4***). These results demonstrate that the effect of HA-PLTP on cholesterol efflux is partly mediated by its ability to enhance preβ-HDL formation.

Besides preβ-HDL, also spherical HDL particles have been shown to function as cholesterol acceptors (Wang et al., 2004). The large fused HDL particles generated by HA-PLTP were more potent cholesterol acceptors than the control HDL particles not treated with PLTP ($P < 0.01$). This observation agreed with previous results demonstrating that large spherical HDL particles as well as large rHDL particles are more potent cholesterol acceptors than small-sized HDL particles (Davidson et al., 1995; Gelissen et al., 2006). The large fused HDL particles generated by HA-PLTP released cellular cholesterol more efficiently compared with the control HDL particles. Compositional analyses demonstrated that the PL and TG contents of these particles were slightly increased, whereas the protein content was lower compared with the particles not treated with HA-PLTP. The total cholesterol content of the particles was similar. Moreover, the relative apoE content of these large fused HDL particles was about 40% higher than that of the HDL particles not treated with PLTP. Increased HDL particle size and increased PL (Davidson et al., 1995; Fournier et al., 1996; Fournier et al., 1997; Gelissen et al., 2006) as well as apoE (Matsuura et al., 2006) contents can improve the acceptor properties of the particles. HDL enriched with apoE can accept free cholesterol from macrophages, after which the cholesterol can be esterified by LCAT and delivered to the liver for excretion (Matsuura et al., 2006). The mechanism with which PLTP-remodeled HDL particles participate in the removal of cholesterol from macrophage foam cells is illustrated in **Figure 7**.

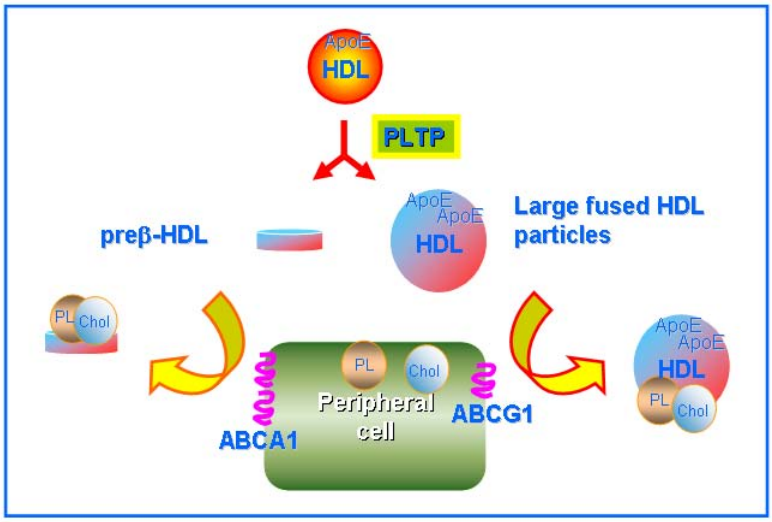

Figure 7. ***Function of PLTP-remodeled HDL particles in the removal of cholesterol from peripheral cells.***

5.4 Association of CETP with HDL particles reduces its proteolytic inactivation by mast cell chymase *(IV)*

The human atherosclerotic intima contains several types of inflammatory cells, including mast cells that secrete the neutral serine protease chymase into the intimal fluid (Kaartinen et al., 1994; Lindstedt et al., 2004). The intimal fluid also contains nascent discoidal HDL particles as well as proteins that are able to modify the structure of HDL, such as CETP. Most of the CETP in plasma is bound to α-HDL particles (Marcel et al., 1990), and CETP can enter the intimal fluid from the plasma compartment in association with HDL particles or it can be expressed and secreted locally by cholesterol-loaded macrophages (Faust et al., 1990). Chymase has been shown to degrade PLTP, leading to reduced PL transfer activity (Lee et al., 2003). However, it is not known whether chymase is able to degrade CETP, its structurally closely related lipid transfer protein. Thus, chymase-dependent degradation of human CETP and CETP-HDL complexes *in vitro* was investigated. Furthermore, the effect of CETP on HDL-facilitated cholesterol efflux was studied.

5.4.1 Effect of chymase on CETP

To study the effect of chymase on the proteolytic degradation and lipid transfer activity of CETP, CETP was incubated with recombinant human chymase, either as such or after complexing CETP with human lipid-free apoA-I, discoidal rHDL particles, HDL_2, and HDL_3. Incubation of lipid-free apoA-I or apoA-I-containing HDL with CETP leads to formation of CETP-containing complexes (Guyard-Dangremont et al., 1994; Bruce et al., 1995). Analysis of the CETP-containing complexes by SDS-PAGE and immunoblotting using the CETP-specific monoclonal antibody (MAb) TP2 (Swenson et al., 1989) demonstrated that chymase rapidly and progressively degraded CETP, generating a specific proteolytic pattern of two major bands with approximate molecular masses of 45 and 34 kDa with a concomitant reduction in its CE transfer activity (***IV, Fig. 1, Fig. 3***). Complexation of CETP with lipid-free apoA-I or HDL_2 rendered CETP more resistant against the proteolytic action of chymase. Complexes of CETP with HDL_3 were also partially protected from degradation. In contrast, complexation with rHDL conferred on CETP the least resistance against chymase, and the degree of proteolysis was similar to that of free CETP. Furthermore, CETP activity was partially preserved when it was complexed with different HDL ligands. ApoA-I and HDL_2 conferred the highest protection of CETP activity, HDL_3 partially protected CETP activity, and rHDL conferred the lowest protection. A previous study demonstrated that two other proteases, trypsin and α-chymotrypsin, caused significant fragmentation of human CETP, but rendered its CE transfer activity (Hesler et al., 1989). As CETP activity was lowered by chymase, it suggests that chymase, contrary to trypsin and α-chymotrypsin, is able to unfold the tertiary structure of CETP, necessary for CE transfer activity. The crystal structure of CETP has implicated that CETP can bind a single HDL particle on the concave face of the CETP molecule (Qiu et al., 2007). Our observation that incubation of CETP with apoA-I-containing particles of various sizes and shapes led to the formation of complexes in which CETP was less exposed to degradation by chymase may indicate a deeper surface insertion of CETP in complexes with spherical rather than discoidal HDL particles, thus rendering the domains required for CE transfer less accessible to chymase.

5.4.2 Effect of chymase on apoA-I in CETP-HDL complexes

Since complexation of CETP with lipid-free apoA-I or apoA-I-containing particles generated CETP-containing complexes that protected CETP against proteolysis by chymase, the integrity of apoA-I in those complexes was analyzed next. Previously, lipid-poor apoA-I particles have been shown to serve as substrates for chymase (Lee-Rueckert and Kovanen, 2006). In addition to CETP, the ability of PLTP to

protect lipid-free apoA-I from being proteolyzed by chymase was studied. The results demonstrated that the protective effect against extensive apoA-I fragmentation by chymase was attributed specifically to CETP, and the presence of PLTP did not abolish this CETP-associated protective effect. Moreover, the complexation with CETP efficiently protected apoA-I in rHDL particles against proteolysis (***IV, Fig. 5***). ApoA-I in the spherical HDL_3 and HDL_2 particles was also poorly degraded by chymase, and the formation of complexes with CETP had no further effect on such a minor degree of apoA-I degradation. Our results are in agreement with the previous findings that discoidal, rather than spherical HDL particles are the most sensitive targets of mast cell chymase (Lee et al., 1999; Lee et al., 2000). Here, only the effect of chymase was studied. However, apoA-I in discoidal HDL is susceptible to several proteases present in the intimal fluid (Lee-Rueckert and Kovanen, 2006), and their role in apoA-I degradation needs to be studied.

5.4.3 Effect of CETP on cholesterol efflux

The role of CETP in the removal of cellular cholesterol is controversial (Zhang et al., 2001b; Van Eck et al., 2007). Thus, to gain more insight into the potential effect of CETP in this process, functional experiments with cholesterol-loaded THP-1 macrophages were performed. Lipid-free apoA-I strongly increased cholesterol efflux (up to 27%), reflecting stimulation of the ABCA1 pathway, while formation of CETP-apoA-I complexes had no further effect. Discoidal rHDL, HDL_3, and HDL_2 all promoted similar efflux (~15%), and complexation of CETP with only HDL_3 enhanced efflux ($P = 0.0287$) (***IV, Fig. 6***). Chymase decreased cholesterol efflux to all acceptors used. Indeed, the inhibitory effect of chymase was associated with extensive degradation of apoE, both in HDL_3 and HDL_2, whether complexed with CETP or not. Even though CETP did not modify the efflux induced by rHDL, it fully restored the efflux-promoting ability of the chymase-treated CETP-rHDL complexes. CETP alone did not affect cholesterol efflux. Our *in vitro* data are supported by recent *in vivo* data showing that expression of human CETP in the mouse liver promotes macrophage RCT by increasing cholesterol uptake by the liver (Tanigawa et al., 2007), confirming the importance of macrophage-derived CETP in protecting nascent discoidal HDL against proteolysis.

The effect of chymase on the functional domains of apoA-I was further studied by Western blot analysis by using specific MAb targeted at either the N-terminus or the C-terminal region of apoA-I. According to earlier reports, the C-terminal sequence of apoA-I is essential for ABCA1-mediated efflux (Burgess et al., 2002; Vedhachalam et al., 2004). Analysis of different cleavage sites in the

functional domains of lipid-free apoA-I and rHDL as well as their complexes with CETP revealed that 26-kDa polypeptide derived from proteolysis of lipid-free apoA-I and its CETP complexes was immunoreactive against the N-terminus, whereas the C-terminal region of apoA-I had been cleaved off (***IV, Fig. 7***). In contrast, 26-kDa polypeptide in proteolyzed rHDL and CETP-rHDL complexes was immunoreactive against the C-terminal MAbs only, suggesting that cleavage had occurred at the N-terminus.

5.5 Serum, but not monocyte macrophage foam cells from low HDL-C subjects, displays reduced cholesterol efflux capacity *(V)*

The aim here was to investigate whether monocyte-derived macrophages from low- and high-HDL subjects would display different capacities to efflux cholesterol to apoA-I, HDL_2, or serum, and whether sera from low- and high-HDL subjects differ as acceptors of cholesterol from human THP-1 macrophages.

5.5.1 Characteristics of the study subjects

Details of the study subjects with low and high HDL-C are described in ***Table 1*** and ***Table 2 (V).*** As expected, HDL-C, TC, apoA-I, and apoA-II were significantly higher in the high-HDL-C subjects, while the low-HDL-C subjects had significantly higher levels of TGs, and a bigger waist circumference and body mass index (BMI) than the high-HDL-C subjects. LDL-C and apoB-100 were similar in the two groups. In the low-HDL-C subjects, ~30% of their HDL-associated cholesterol was recovered in the HDL_2 fraction, while in the high-HDL-C subjects, ~50% of the total HDL cholesterol represented HDL_2. In the low-HDL-C subjects the large-sized particles were significantly decreased, while in the high-HDL-C subjects the large HDL_{2b} particles represented the majority of HDL particles. Together, HDL_{2b} and HDL_{2a} represented ~70% of all HDL particles in the high-HDL-C subjects. Furthermore, the low-HDL-C subjects had smaller mean HDL particle size than did the high-HDL subjects. The observations regarding smaller mean HDL particle size and a decreased proportion of large HDL particles in low-HDL-C subjects are in accordance with the previous reports (Soderlund et al., 2005; Watanabe et al., 2006). Analysis of percent mass composition revealed that both HDL_2 and HDL_3 displayed significant enrichment of TGs in the low-HDL-C subjects. The basal level of preβ-HDL particles in the two study groups was similar. However, upon incubation in the presence of the LCAT inhibitor iodoacetamide, sera from the low-HDL-C subjects

generated higher levels of preβ-HDL (as a percentage of total HDL) compared with the high-HDL-C group ($P = 0.028$).

5.5.2 Cholesterol efflux from monocyte-derived macrophage foam cells isolated from the study subjects

Human monocyte-derived macrophages were obtained from human whole blood by cell culturing using a granulocyte-macrophage colony-stimulating factor (GM-CSF) to differentiate monocytes into macrophages. The criterion used to define a macrophage is based on the morphology of the differentiated cells, as reported previously (Waldo et al., 2008). Macrophages were loaded with acetyl-LDL and after loading, the majority of the cholesterol was in an esterified form (average, 76 ± 5.2% of the total cholesterol mass). Cholesterol efflux from macrophages obtained from low- or high- HDL-C subjects to apoA-I, HDL_2, and serum was similar (***V, Fig. 2***). The present data extend the previous finding, which demonstrated similar cholesterol efflux to lipid-free apoA-I from macrophages derived from either low-HDL-C subjects or control subjects (Soro-Paavonen et al., 2007).

5.5.3 Expression of ABCA1 and ABCG1 mRNA and protein

Transcripts of *ABCA1* and *ABCG1* were analyzed in cultured unloaded monocyte-derived macrophages. The relative levels of *ABCA1* and *ABCG1* mRNA expression in monocyte macrophages did not differ between the low- and high-HDL-C subjects. Expression of ABCA1 and ABCG1 protein in macrophages from the low- and high-HDL-C subjects, after the macrophages were loaded with acetyl-LDL, was also similar when measured by Western blot analysis (***V, Fig. 3***). These data imply that neither the ABCA1 pathway nor the ABCG1 pathway was significantly impaired, which is in accordance with similar efflux values observed between macrophages derived from low-HDL-C and high-HDL-C subjects.

As a proinflammatory state in monocytes and monocyte-derived macrophages from low-HDL-C subjects was recently reported (Sarov-Blat et al., 2007), the inflammation status of the isolated macrophages was evaluated by analyzing the levels of expression of the major inflammatory genes *TNF-α*, *IL-6*, and *MCP-1*. However, the levels of expression of these inflammation markers were similar in the low-HDL-C and high-HDL-C subjects. No significant difference was observed in inflammation markers between low-HDL-C subjects with or without coronary heart disease (CHD). Although the inflammatory status of unloaded macrophages derived from the low-HDL-C and high-HDL-C subjects was similar, the monocytes used in the present

study are differentiated into macrophages under cell culture conditions, and thus they may not phenotypically fully represent those present in the vessel wall *in vivo*.

5.5.4 Cholesterol efflux to sera from the study subjects

Cholesterol efflux from THP-1 foam cells to sera from the high-HDL-C subjects was slightly higher than that to sera from the low-HDL-C subjects ($P = 0.046$) (**Figure 8**). Cholesterol efflux from foam cells to individual sera showed a positive association with the serum percentage of preβ-HDL ($r = 0.626$, $P = 0.0001$), preβ-HDL concentration ($r = 0.754$, $P < 0.0001$), preβ-HDL concentration after incubation at +37°C ($r = 0.486$, $P = 0.005$), and HDL-C ($r = 0.359$, $P = 0.040$). The data are in accordance with the findings that ABCA1-mediated efflux is highly dependent on the availability of preβ-HDL (Lee et al., 2003; Mweva et al., 2006), and the PL content of HDL is an important determinant of cholesterol efflux (Fournier et al., 1997; Wang et al., 2004; Gelissen et al., 2006). Preβ-HDL may be the preferred acceptor for the concerted action of the ABCA1 and ABCG1 pathways, leading to high efflux from THP-1 macrophages to serum with a high preβ-HDL content. A recent report suggested that cellular cholesterol efflux pathways mediated by ABCA1 and SR-BI are gender-specific (Catalano et al., 2008). This was shown to be due to the increased levels of large CE-rich HDL_2 particles that facilitate SR-BI-mediated efflux in women, and the increased preβ-HDL levels that facilitate efflux via ABCA1 in men.

The effect of HDL particle size on efflux capacity and the impact of large HDL particles on cholesterol removal were recently demonstrated (Davidson et al., 1995; Matsuura et al., 2006; Makela et al., 2008). As high-HDL-C subjects demonstrated a higher proportion of large HDL_{2b} particles, the higher efflux to the sera of these subjects suggests a role of the ABCG1 pathway in cholesterol removal. Of note, HDL_{2b} particles have been shown to be important determinants of carotid atherosclerosis, evaluated as increased IMT (Watanabe et al., 2006). In the present study, cholesterol efflux was similar in low-HDL-C subjects with or without CHD.

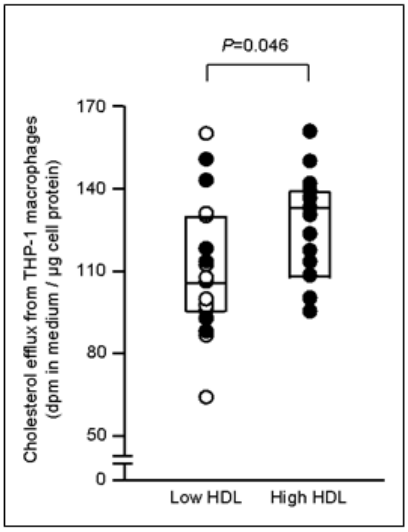

Figure 8. ***Cholesterol efflux from human THP-1 macrophage foam cells to serum (0.5%) from low- (n = 18) and high-HDL-C (n = 15) subjects. Box plots display the median and the lower and upper quartiles. The open and closed circles indicate the data from low-HDL-C subjects with and without CHD.***

LCAT, CETP, and PLTP are all involved in the remodeling of HDL particles (Rye et al., 1999) and affect RCT. CETP and PLTP activities were similar between the two groups. Interestingly, the low-HDL-C subjects displayed significantly higher LCAT activity and lower PLTP mass. HA-PLTP mass differed slightly between the two groups, whereas the high-HDL-C subjects had significantly higher levels of LA-PLTP, leading to significantly increased PLTP mass. An inverse relationship between serum total PLTP mass and CHD risk has been reported (Yatsuya et al., 2004), although the physiological role of LA-PLTP remains unknown. ApoE is important in HDL formation and maturation, in hepatic uptake of HDL (Mahley and Rall, 2000), and in RCT (Matsuura et al., 2006). Particularly, macrophage apoE was shown to promote cholesterol efflux and reduce atherosclerosis (Yu et al., 2006). However, *apoE* mRNA expression levels in unloaded monocyte-derived macrophages, as well as serum apoE concentration, were similar between the low-HDL-C and high-HDL-C subjects.

6 SUMMARY AND CONCLUSIONS

The aim of this thesis was to clarify the importance of PLTP and CETP in the RCT process, i.e the transport of cholesterol from peripheral cells to the liver for excretion.

First, the importance of endogenous PLTP in ABCA1-mediated cholesterol efflux from macrophage foam cells was demonstrated using peritoneal macrophages from WT and PLTP-KO mice. The uptake of cholesterol and the ability of macrophages to be converted into foam cells were not impaired by the genetic absence of PLTP. Reportedly, ABCA1 and apoA-I may interact along an intracellular recycling pathway. PLTP may aid apoA-I in the stabilization of ABCA1, and it may participate in intracellular recycling of ABCA1 by increasing the transfer of PLs to ABCA1, followed by phospholipidation of acceptor particles, such as apoA-I. This hypothesis could also explain the defective cholesterol efflux found in PLTP-KO foam cells. The role of PLTP in ABCA1-mediated cholesterol efflux was further supported by restoration of cholesterol efflux from the PLTP-KO foam cells after upregulation of ABCA1 by cAMP. Based on these results, endogenous PLTP produced by macrophages contributes to the optimal function of the ABCA1-mediated cholesterol efflux process.

To further characterize the role of PLTP in the RCT process, the effect of externally added PLTP on cholesterol efflux from human THP-1 macrophage foam cells and from human monocyte-derived macrophage foam cells was investigated. This study demonstrated that incubation of HDL with HA-PLTP, but not LA-PLTP, caused a 30-40% increase in cholesterol efflux. The observed increase was due in part to the formation of preβ-HDL, as preβ-HDL particles generated by HA-PLTP served as cholesterol acceptors for the ABCA1-mediated pathway. These results, obtained using acetyl-LDL-loaded macrophages, are in accordance with the observation that the acetyl-LDL and cholesterol esters formed are stored in a pool, readily accessible for efflux by the ABCA1-dependent pathway. In addition to preβ-HDL particles, large fused HDL particles enriched with PL and apoE formed by HA-PLTP also behaved as efficient acceptors of cholesterol, suggesting the involvement of the ABCG1-promoted efflux pathway. The data demonstrate that only HA-PLTP can enhance cholesterol efflux from macrophage foam cells, via formation of preβ-HDL and large fused HDL particles.

To reveal whether the results obtained *in vitro* bear any relevance *in vivo*, a $LDLr^{-/-}$ mouse model with a selective deficiency in PLTP in macrophages was generated. The selective deficiency in macrophage PLTP in $LDLr^{-/-}$ mice i)

decreased the size of atherosclerotic lesions, ii) decreased the relative levels of preβ-HDL, iii) decreased serum cholesterol and (iiii) lowered plasma PLTP activity. Thus, macrophage PLTP displayed a proatherogenic role in this mouse model. As both exogenous and endogenous PLTP *in vitro* increase cholesterol removal from macrophage foam cells, *in vivo* other proatherogenic properties of PLTP, probably related to the observed changes in plasma lipoproteins, override the antiatherogenic function of macrophage PLTP. Hepatic Kupffer cells express higher PLTP mRNA levels than do the other hepatic cell types that contribute to plasma PLTP activity. The results from bone marrow transplantation studies suggest that the contribution of PLTP to atherosclerosis is determined by a balance between lesion PLTP activity, which is antiatherogenic, and plasma PLTP activity, which is proatherogenic.

To study whether PLTP also regulates cholesterol efflux from macrophage foam cells in humans, cholesterol efflux from monocyte macrophages derived from low- and high-HDL-C subjects and the ability of the sera derived from these subjects to function as cholesterol acceptors were determined. Monocyte macrophages isolated from either low- or high-HDL-C subjects displayed similar cholesterol efflux to apoA-I, HDL_2, and whole serum. However, sera from high-HDL-C subjects promoted higher cholesterol efflux from THP-1 macrophage foam cells than did sera from low-HDL-C subjects. This was most probably due to the higher proportion of both HDL_{2b} and preβ-HDL particles. Thus, low levels of large HDL particles and preβ-HDL particles may limit the serum efflux capacity and increase the susceptibility of low-HDL-C subjects to lesion development. No difference in plasma PLTP activity between the two groups was evident.

As such, CETP, like PLTP, plays a crucial role in HDL metabolism, and a direct role for macrophage-derived CETP in cellular cholesterol efflux has been suggested. However, CETP-HDL functional interaction has not been studied in this process, and therefore the role of CETP in the cholesterol efflux process was analyzed further. Association of CETP with HDL particles protected the integrity and CE transfer function of CETP as well as the structure of associated apoA-I against the proteolytic effects of chymase. Spherical HDL particles conferred the highest protection against chymase to CETP, whereas CETP protected discoidal HDL particles against chymase-mediated proteolysis. Formation of CETP-HDL complexes was not able to prevent a decrease in cholesterol efflux to mature HDL particles, whereas CETP-rHDL complexes remained functional in cholesterol efflux in spite of chymase treatment. This suggests that the association of CETP with rHDL prevents chymase-dependent functional inactivation of discoidal rHDL by preserving apoA-I intactness. The protective function of CETP against chymase could be of significance, as the relative concentration of nascent discoidal HDL particles in the intimal fluid is relatively high.

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