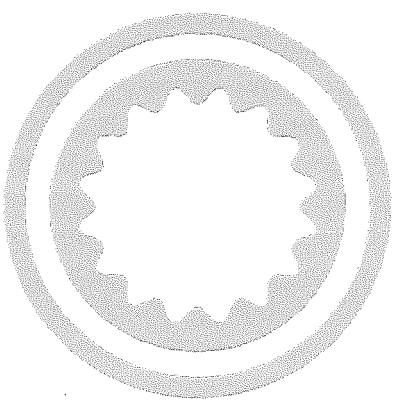
Affected by Abundant PLTP

The Atherogenic Role of a Lipid Transfer Protein in Transgenic Mice



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Aangetast door overmatig PLTP

De atherogene rol van een lipide-transfereiwit in transgene muizen

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus
Prof.dr. S.W.J. Lamberts
en volgens besluit van het College voor Promoties.

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Matthijs Moerland

geboren te Rotterdam

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Contents

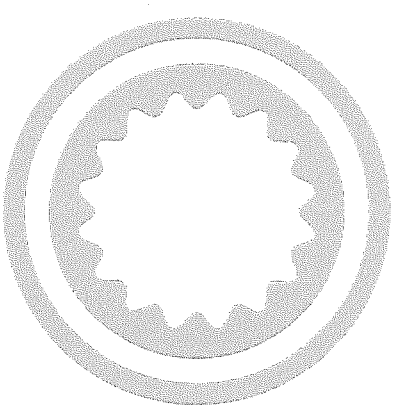
Abbreviations

ABC	adenosine triphosphate-binding cassette transporter
ACAT	Acyl-CoA:cholesterol acyl-transferase
AcLDL	acetylated low-density lipoprotein
AU	arbitrary unit
apo	apolipoprotein
bp	base pair
BPI	bactericidal permeability-increasing protein
BSA	bovine serum albumin
C	cholesterol
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CMV	cytomegalovirus
Ct	threshold cycle
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
dox	doxycycline
EDTA	ethylene diamine tetra-acetic acid
EGFP	enhanced green fluorescent protein
EL	endothelial lipase
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FPLC	fast-protein liquid chromatography
FXR	farnesoid X receptor
GFP	green fluorescent protein
h/Hu	human
HDL	high-density lipoprotein
HL	hepatic lipase
HFHC	high fat, high cholesterol
HPRT	hypoxanthine-guanine phosphoribosyl transferase
IAA	iodoacetic acid
IDL	intermediate-density lipoprotein
IFN	interferon
IL	interleukin
indPLTP	inducible PLTP
kb	kilobase
kDa	kilodalton
LBP	lipopolysaccharide binding protein
LCAT	lecithin:cholesterol acyl-transferase
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
lp(a)	lipoprotein a
LPL	lipoprotein lipase
LPS	lipopolysaccharide
LRP	LDLR-related protein
LTP	lipid transfer protein
LXR	liver X receptor
mAb	monoclonal antibody
MCS	monocyte chemotactic stimulation
MCSF	macrophage colony-stimulating factor

MTP	microsomal triglyceride transfer protein
mRNA	messenger ribonucleic acid
mutPLTP	mutant PLTP
PBS	phosphate-buffered saline
PL	phospholipid
PLTP	phospholipid transfer protein
PON	paraoxonase
PPAR	peroxisome proliferator-activated receptor
RCT	reverse cholesterol transport
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
rtTA	reverse tetracycline transactivator
RXR	retinoid X receptor
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SR-BI	scavenger receptor-class B type I
T1317	TO-901317 compound
TC	total cholesterol
tet	tetracycline
tg	transgenic
TG	triglyceride/triacylglycerol
TNF	tumor necrosis factor
TRE	tetracycline response element
VLDL	very low-density lipoprotein
VSMC	vascular smooth muscle cell
wt	wild type

Chapter 1

Introduction



1 Introduction

1.1 Atherosclerosis

Atherosclerosis is a progressive disease of the large and medium-sized arteries. The disease is characterised by endothelial dysfunction, inflammation and the accumulation of fatty and fibrous substances in the vessel wall, resulting in thickening and loss of elasticity of the arteries. The word atherosclerosis has been derived from the Greek words "athera", porridge or gruel, and "skleros", hard or stiff. These words describe the external features of the lipid-loaded lesions that characterize the disease. Although atherosclerosis has been discovered in blood vessels of people living more than 3000 years ago, until the end of the 18th century its prevalence was very rare. During the 20th century, mortality caused by atherosclerosis strongly increased. Nowadays, complications of atherosclerosis are the main cause of death in the developed world, and are predicted to be the leading cause of death worldwide by the year 2020 (Fonarow, 2007).
It is difficult to accurately determine the true frequency of atherosclerosis because it is a predominantly asymptomatic condition (Kavey *et al.*, 2006). Early atherosclerotic lesions can already be found in the aorta shortly after birth, increasing in number during childhood. More advanced lesions begin to develop at an age of approximately 25 years. Generally, the clinical manifestations of the disease become apparent in the sixth decade of life. Symptomatic atherosclerotic disease most often involves the arteries supplying the heart, brain, kidneys, and lower extremities, and becomes apparent when arterial blood flow is obstructed. Complete occlusion of an affected artery can result in myocardial infarction, cerebral infarction, or peripheral vascular disease.

Both genetic and environmental risk factors contribute to the susceptibility to develop atherosclerosis, such as male gender, family history of atherosclerotic disease, hypertension, diabetes mellitus, dyslipidemia, obesity, and high age, but also personal attributes like tobacco smoking, physical inactivity, high fat diet and stress or mental depression. The pathophysiology of atherosclerosis is complex, multifactorial and reflexes an interaction between vessel wall and plasma. Three main processes involved in the development of atherosclerosis are hypercholesterolemia (Steinberg, 2005), injury of the endothelial lining (Ross and Glomset, 1976), and inflammation of the vascular wall (Stoll and Bendszus, 2006) There is no need to consider these processes as three alternative hypotheses to explain. the pathogenesis of atherosclerosis; each of the processes may be involved. However, the 'classical' risk factor for atherosclerosis development is hypercholesterolemia. For already almost a hundred years scientists are focussing on the relationship between plasma lipid

metabolism and atherogenesis. With the discovery of the low-density lipoprotein (LDL) receptor, responsible for the clearance of plasma cholesterol (Brown and Goldstein, 1976), strong evidence was provided for a causal relationship between elevated LDL-cholesterol levels and atherosclerosis. Small dense LDL particles can infiltrate the arterial wall, where they undergo progressive oxidation. Oxidized cholesterol is highly toxic, and as part of a defense mechanism, it gets internalized by macrophages through the scavenger receptors, leading to the formation of lipid-rich foam cells (Han *et al.*, 1997; Morel *et al.*, 1983; Steinberg, 1997). Accumulation of these foam cells forms the earliest atherosclerotic lesions, called "fatty streaks". Besides the accumulation of macrophage foam cells, inflammatory cells such as T lymphocytes, and smooth muscle cells are observed in the lesions. By progressive lipid accumulation and the interplay between various immunologic and inflammatory processes, the fatty streak may further progress to eventually become a vulnerable plaque. After evidence was provided for a causal relationship between elevated LDL-cholesterol levels and the onset of atherosclerosis as described above, cardiovascular research focused on the relationship between lipoprotein metabolism and atherosclerotic disease. In the next paragraph, the different lipoprotein subclasses, their metabolism, and their relationship with cardiovascular disease will be described in detail.

1.2 Lipoprotein metabolism

1.2.1 Lipoproteins

Cholesterol, phospholipids and triglycerides are the main lipids in the circulation. Lipid molecules are relatively insoluble in water because they consist largely of long hydrophobic hydrocarbon tails. To transport plasma lipids through the body, they are packaged into water-soluble complexes, so-called lipoproteins. Plasma lipoproteins are complex, spherical particles that consist of a hydrophobic core of neutral lipids (cholesteryl esters and triglycerides) surrounded by a polar shell of free cholesterol, phospholipids and apolipoproteins (Figure 1). Apolipoproteins serve as enzyme co-factor, receptor ligand, and structural component regulating the intravascular metabolism of lipoproteins and their ultimate tissue uptake. Lipoproteins constitute a heterogeneous population of particles, which can be classified according to their densities. Five main categories are distinguished, listed with increasing density: chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) (Davis, 1996) (Table 1). The individual functions of these lipoprotein subclasses in lipid metabolism will be discussed in the following sections. Lipoprotein metabolism can be divided into two major pathways: the transport of dietary lipids taken

up from the intestine, described as the exogenous pathway, and the transport of liver-synthesized lipids, the endogenous pathway. The transport of cholesterol from peripheral tissues to the liver, where it is converted into bile acids and excreted, is called reverse cholesterol transport.

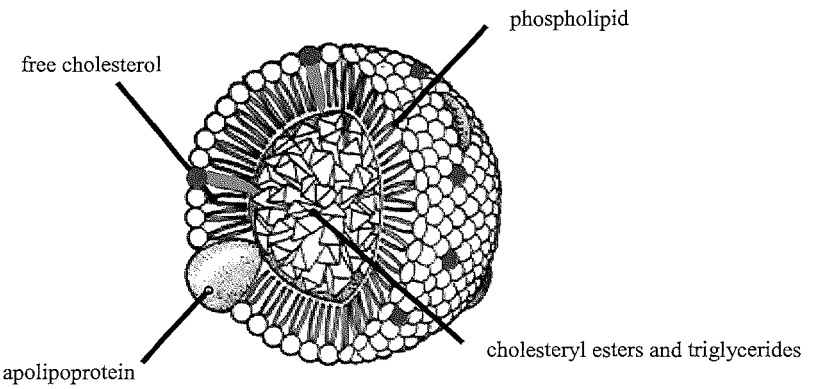


Figure 1. Schematic illustration of a lipoprotein particle. Modified from Oxford University Press.

1.2.2 Exogenous pathway

The exogenous pathway describes the process in which dietary lipids are taken up from the intestine and transported to the liver and peripheral tissues (Figure 2). Before transport over the intestinal membrane and uptake by enterocytes, dietary cholesterol, phospholipids and triglycerides are hydrolyzed in the intestinal lumen into monoacylglycerols and free fatty acids. Intracellularly, these compounds are transformed into triglycerides and packaged into chylomicrons. Nascent chylomicrons are primarily composed of triglycerides (86%) together with some free cholesterol and cholesteryl esters. The main apolipoprotein components are apoB48, apoAI and apoAIV (Hussain *et al.*, 1996). Chylomicrons are released by exocytosis from enterocytes and secreted into lymphatic vessels originating in the villi of the small intestine, after which they are secreted into the bloodstream. After entering the bloodstream, chylomicrons exchange apolipoproteins with HDL. Chylomicrons transport exogenous lipids to adipose tissue, and to a lesser extent to cardiac and skeletal muscle. In the capillary beds of these tissues, chylomicron triglycerides are hydrolyzed by the lipolytic action of lipoprotein lipase (LPL), present at the surface of endothelial cells (Redgrave, 2004). This results in the generation of free fatty acids, which can be used as energy source for muscles or stored in adipocytes. Once the triglyceride content of the chylomicrons has been distributed among tissues, remnant particles are left, enriched in

cholesteryl esters and apoE. ApoE is an important factor for facilitating the clearance of chylomicron remnants from the circulation by the liver via remnant receptors, i.e. the low-density lipoprotein receptor (LDLR) and the LDLR-related protein (LRP) (Cooper, 1997; Herz *et al.*, 1995; Mahley *et al.*, 1989; Rohlmann *et al.*, 1998). In addition, scavenger receptor-class B type I (SR-BI) might be involved in the hepatic uptake of chylomicron remnants (Out *et al.*, 2004).

Table 1. Physical properties and composition of human plasma lipoproteins. Based on (Alonzi *et al.*, 2008; Wasan and Cassidy, 1998).

	Chylomicrons	**VLDL**	**IDL**	**LDL**	**HDL**
Density (g/ml)	<0.95	0.95-1.006	1.006-1.019	1.019-1.063	1.063-1.21
Diameter (nm)	75-1200	30-80	25-35	18-25	5-12
Composition (% w/w)					
Triglycerides	85-88	50-55	25-30	10-15	3-15
Phospholipids	8	18-20	25-27	20-28	26-46
Cholesterol	4	20-25	40-45	45-58	17-40
Protein	1-2	7-10	10-12	20-22	33-57
Apolipoproteins					
apoA	AI, AII, AIV, AV	AV	–	–	AI, AII, AIV, AV
apoB	B48	B100	B100	B100	–
apoC	CI, CII, CIII	CI, CII, CIII	CI, CII, CIII	–	CI, CII, CIII
apoE	E	E	E	–	E

1.2.3 Endogenous pathway

The endogenous lipoprotein pathway describes the metabolic fate of lipoproteins that are synthesized by the liver (Figure 2). In the liver, VLDL is synthesized by a process consisting of two separate steps (Shelness and Sellers, 2001). In the first step, a partially lipidated apoB-containing VLDL precursor is formed by the action of the microsomal triglyceride transfer protein (MTP). In the second step, VLDL-sized triglyceride droplets (derived from the uptake of plasma remnants or free fatty acids) fuse with the apoB-containing precursor particles. Under the influence of the intracellular cholesterol-esterifying enzyme Acyl-CoA:cholesterol acyl-transferase (ACAT), the nascent VLDL is further lipidated with cholesterol (derived from remnant uptake or *de novo* synthesis) resulting in mature VLDL, which is subsequently secreted into the circulation. Upon entering the circulation, nascent

VLDL is enriched with apoAV, apoCs and apoE, mainly acquired from HDL. Triglycerides in the core of the VLDL particle are hydrolyzed by LPL, thereby generating free fatty acids

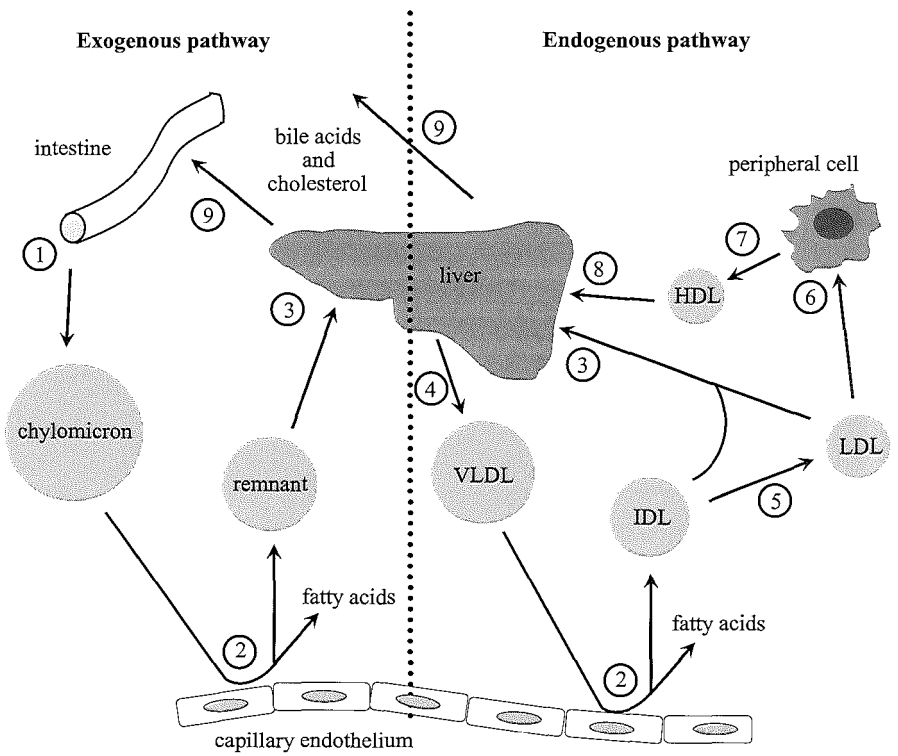


Figure 2. Schematic and simplified representation of human lipoprotein metabolism.
1) uptake of dietary lipids from the intestine and secretion of chylomicrons into the blood; 2) lipolysis of triglyceride-rich lipoproteins by endothelial LPL; 3) uptake of apoB-containing lipoproteins by liver receptors; 4) hepatic VLDL secretion into the blood; 5) lipolysis of IDL by LPL and HL, generating LDL; 6) uptake of LDL-cholesterol by peripheral cells; 7) cholesterol efflux from peripheral cells to lipid-poor apoAI and HDL; 8) selective uptake of HDL-cholesterol by the liver; 9) excretion of cholesterol into bile and faeces.

1.2.4 Reverse cholesterol transport

For processing, degradation and secretion into bile, cellular cholesterol must be transported from peripheral tissues via the blood back to the liver. This process is called reverse

cholesterol transport (Fielding and Fielding, 1995) (Figure 3). Because the liver is considered as the most important organ for cholesterol synthesis in the body, and the site from where cholesterol is transported to the peripheral tissues, cholesterol that moves from peripheral tissues to the liver is moved in the reverse direction. In relation to atherosclerosis, reverse cholesterol transport has been postulated to play an important atheroprotective role, because it stimulates the efflux of accumulated cholesterol from macrophage foam cells in the arterial wall, thereby preventing the formation of atherosclerotic lesions (Tall, 2008). The initial step in reverse cholesterol transport is the efflux of cellular free cholesterol and phospholipids to acceptor particles. ATP-binding Cassette transporter A1 (ABCA1) mediates lipid efflux to lipid-poor apoAI (Jessup *et al.*, 2006). This lipid-poor apoAI may be secreted by the liver or intestine, it may be released from triglyceride-rich lipoproteins during lipolysis, or it may be generated during remodelling of mature HDL particles (Rye and Barter, 2004). In this way, a nascent discoidal HDL is formed, so-called preβ-HDL. By the action of the enzyme lecithin:cholesterol acyl-transferase (LCAT), free cholesterol in preβ-HDL is converted into cholesteryl esters, resulting in the formation of mature α-migrating spherical HDL (Zannis *et al.*, 2006). In this way, LCAT maintains a concentration gradient of free cholesterol between peripheral cells and plasma HDL and has a key role in the maturation of HDL particles. Cholesterol efflux from macrophages is further enhanced by passive diffusion or active efflux through ATP-binding Cassette transporter G1 (ABCG1) or scavenger receptor BI (SR-BI) to mature α-HDL particles (Jessup *et al.*, 2006). In plasma, continuous extensive remodeling of HDL particles takes place. Apolipoproteins are exchanged between different plasma lipoprotein particles and lipids are transferred by a range of plasma factors, resulting in changes in size and composition of lipoprotein particles. In the process of HDL remodeling, mature HDL particles are fused to form larger HDL particles, with the release of lipid-poor apoAI, serving as initiator of reverse cholesterol transport. The main plasma lipoprotein remodeling proteins are LCAT, phospholipid transfer protein (PLTP), cholesteryl ester transfer protein (CETP), lipoprotein lipase (LPL) and hepatic lipase (HL) and endothelial lipase (EL).

The final steps of reverse cholesterol transport involve uptake of HDL-cholesterol by the liver, and subsequent excretion of cholesterol and bile acids in the bile and faeces. HDL-cholesterol may be removed from the circulation by different pathways. One of these is the selective uptake by hepatic SR-BI, which has been identified as an HDL receptor (Acton *et al.*, 1996). SR-BI removes cholesteryl esters from the HDL particle without the uptake of HDL proteins. In humans, a second pathway for removal of HDL-cholesterol from the circulation involves the action of CETP (Tall, 1995). By the action of this lipid transfer protein, cholesteryl esters, in exchange for triglycerides, can be transferred from HDL particles to apoB-containing lipoproteins, such as VLDL and LDL. Subsequently, cholesteryl esters in these particles can be taken up by the liver via hepatic LDL receptors and LRP.

Once cholesterol has been taken up by the liver it may be secreted into the bile either as bile acids or as free cholesterol, by a process in which the bile canalicular transporters ABCG5 and ABCG8 are involved, or it may be re-used for lipoprotein assembly. Cholesterol and bile acids can be either reabsorbed from the intestine, or excreted into the faeces.
Proteins with a central role in reverse cholesterol transport will be discussed in more detail in section 1.3.

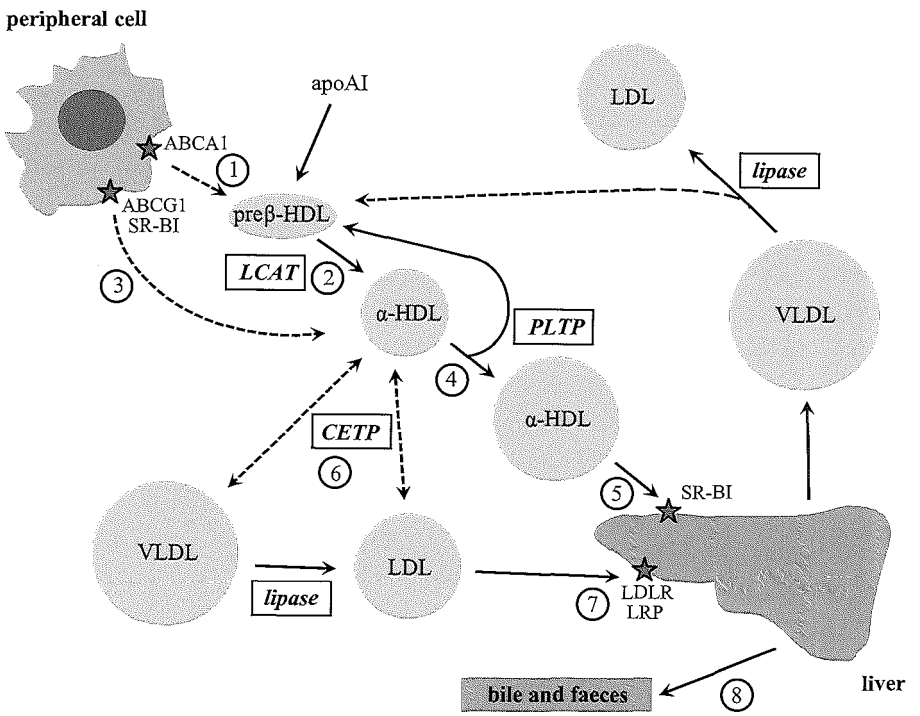


Figure 3. Schematic and simplified representation of reverse cholesterol transport.
1) ABCA1-dependent lipid efflux to lipid-poor apoAI with the formation of preβ-HDL; 2) maturation of HDL particles through esterification of free cholesterol by LCAT; 3) lipid efflux to mature α-HDL via ABCG1 or SR-BI; 4) PLTP-mediated HDL remodeling with the generation of large fused HDL and preβ-HDL; 5) selective uptake of HDL-cholesterol by hepatic SR-BI; 6) CETP-mediated transfer of cholesteryl esters from HDL to VLDL/LDL in exchange for triglycerides; 7) uptake of LDL by hepatic receptors; 8) excretion of lipids into bile and faeces.

1.2.5 The role of HDL in macrophage reverse cholesterol transport

The fact that elevated levels of HDL have been associated with a reduced risk for cardiovascular disease might be explained at least partly by the key role of HDL in reverse cholesterol transport. In patients with heterozygous familial hypercholesterolemia, infusion of human pro-apoAI, a precursor of apoAI, resulted in a transient rise in HDL levels followed by an increase in faecal neutral sterol and bile acid excretion, suggesting a potential stimulation of reverse cholesterol transport with HDL level as an important determinant (Eriksson *et al.*, 1999). Rader and colleagues developed a method to quantify macrophage reverse cholesterol transport in animal models. By injecting radiolabeled lipid-loaded macrophages into the peritoneal cavity and subsequently tracing cholesterol radioactivity in plasma and faeces, macrophage lipid efflux to the plasma and overall macrophage reverse cholesterol transport can be quantified. Using this approach, overexpression of human apoAI in mice (Zhang *et al.*, 2003b) or treatment of mice with a synthetic LXR agonist (Naik *et al.*, 2006) has shown to result in the increase of macrophage reverse cholesterol transport, likely attributable to increased plasma HDL levels. However, mice lacking SR-BI had a reduced macrophage reverse cholesterol transport despite higher plasma HDL levels (Zhang *et al.*, 2005), and expression of CETP in mice reduced HDL levels but promoted macrophage reverse cholesterol transport (Tanigawa *et al.*, 2007). Moreover, wild type mice with ABCA1 or ABCG1 deficient macrophages had an impaired macrophage reverse cholesterol transport, whereas plasma lipid levels were not different (Wang *et al.*, 2007c). These findings indicate that reverse cholesterol transport rate is not simply a function of HDL plasma concentration. A complex network of cellular and plasma proteins is involved in the process of reverse cholesterol transport, and the interplay of these factors may determine the efficiency of reverse cholesterol transport.

The pool of HDL particles is structurally and functionally diverse, consisting of many highly dynamic particle subpopulations that differ in their capacity to mediate reverse cholesterol transport and protect against atherosclerosis (Rye *et al.*, 2008). In the first step of reverse cholesterol transport, small preβ-migrating lipid-free/lipid-poor apoAI could be a more efficient acceptor of cellular cholesterol than large α-migrating HDL, implying that smaller particles may be more cardioprotective than larger particles (Oram and Vaughan, 2006). However, recently it was demonstrated that mature α-HDL is more important than preβ-HDL in controlling reverse cholesterol transport *in vivo* (Wang *et al.*, 2007b). Importantly, epidemiological evidence supporting a cardioprotective role for preβ-HDL is lacking. On the contrary, a recent analysis of data collected in a large intervention trial demonstrated that subjects with new cardiovascular events had significantly lower levels of α-HDL, and significantly higher levels of preβ-HDL compared to event-free subjects (Asztalos *et al.*, 2008). These studies demonstrate that although the importance of HDL for

reverse cholesterol transport is commonly accepted, the exact physiological involvement of different HDL subpopulations in reverse cholesterol transport and atherosclerotic disease should be clarified. Importantly, reverse cholesterol transport is only one mechanism by which HDL may protect against atherosclerosis. HDL displays a variety of other properties that could contribute to its anti-atherogenicity, such as anti-oxidative, anti-inflammatory and anti-thrombotic effects. HDL protects against harmful effects of oxidized LDL. These anti-oxidative properties may be in part related to its content of apoAI and paraoxonases (PON), enzymes that remove the lipid hydroperoxides that oxidize LDL phospholipids (Banka, 1996). The anti-inflammatory properties of HDL are related to its ability to remove cholesterol and oxysterols from cells in the arterial wall, thereby inhibiting the formation of foam cells and subsequent increase in expression of inflammatory proteins (Tall, 2008). HDL can also repress the induction of cell-adhesion molecules and inhibit monocyte accumulation (Barter *et al.*, 2002). In addition, several anti-thrombotic properties of HDL have been described, including inhibition of platelet aggregation and activation, downregulation of cell-adhesion factors, inhibition of the activation of coagulation factors, and stimulation of the upregulation of anticoagulant factors (Hausenloy and Yellon, 2008; Sviridov *et al.*, 2008).

1.2.6 Lipoprotein metabolism and cardiovascular research in mice

During the last twenty years, the ability to create transgenic animals has largely increased the scientific opportunities to perform functional studies of a gene of interest. A frequently used organism in transgenic research is the mouse. There is a strong genetic resemblance between mice and men, and physiology and metabolic processes are comparable. When performing functional studies with mice, the genetic variability and inter-individual differences regarding life style and environmental factors can be reduced to a minimum, because inbred strains are used and living conditions can be strictly controlled. The genetic map of the mouse is well described, and special techniques have become available to manipulate mouse genes by transgenesis and gene targeting. Also in the field of cardiovascular research the mouse is a frequently used model. Atherosclerosis is a complex process with an onset decades before the acute manifestation of cardiovascular disease. Studying atherogenic mechanisms in humans is hindered by the complexity of the disease process, combined with the difficulty to characterize lesions in patients. These practical problems can be overcome by using the mouse as a research model. However, in general, mice are highly resistant to develop atherosclerosis. Only the C57BL/6 strain, when fed a very high cholesterol diet containing cholic acid, develops atherosclerotic lesions (Jawien *et al.*, 2004; Paigen *et al.*, 1985). These lesions are small, largely confined to the aortic root, and generally do not develop beyond the stage of fatty streak (Paigen *et al.*, 1987).

The inability to induce pronounced atherosclerosis in most mouse strains is caused by the atheroprotective murine lipoprotein profile, with the main plasma lipoprotein class being HDL. In contrast, in humans the predominant lipoprotein classes are VLDL and LDL, which are considered atherogenic (Ishida *et al.*, 1991). These major differences in plasma lipoprotein distribution could be explained by marked differences in lipoprotein metabolism between mice and man, which are summarized in Table 2.

Table 2. Lipoprotein metabolism in mice and men.

Characteristic	Mouse	Human	Reference
apoB editing	liver and intestine	intestine	(Anant and Davidson, 2001)
VLDL synthesis	lipidation of apoB100 and apoB48	lipidation of apoB100	(Greeve *et al.*, 1993)
apoE gene	one isoform	polymorphic (apoE2, -E3, -E4)	(Knouff *et al.*, 1999)
hepatic lipase	circulating in plasma	bound to vascular endothelium	(Dallinga-Thie *et al.*, 2007; Peterson *et al.*, 1986)
CETP	absent	present	(Guyard-Dangremont *et al.*, 1998; Speijer *et al.*, 1991)
lp(a)	absent	present	(Berglund and Ramakrishnan, 2004)

Different targeted mouse models have been developed to overcome the discrepancy between the murine and the human lipoprotein profile (Jawien *et al.*, 2004). Of these genetically engineered mouse models, the apoE deficient model is the only one that develops extensive atherosclerotic lesions on a chow diet (Plump *et al.*, 1992; Zhang *et al.*, 1992). The LDLR deficient model has elevated LDL levels, but no lesions form on a chow diet (Ishibashi *et al.*, 1993). In this model, advanced atherosclerotic lesions only develop when a high fat, high cholesterol diet is provided to the animals (Ishibashi *et al.*, 1994). When mice with only one functional LDLR allele ($LDLR^{+/-}$) are used, administration of a cholate-enriched high fat, high cholesterol diet is required. In addition to these most commonly used mouse models, a wide variety of other mouse models with affected lipoprotein metabolism has been developed to study cardiovascular disease (Jawien *et al.*, 2004).

1.3 Proteins involved in reverse cholesterol transport

Reverse cholesterol transport involves a series of steps, beginning with the efflux of cholesterol from the peripheral cell and ending with its uptake by the liver, degradation into bile acids and excretion into the faeces. A wide variety of cellular and plasma proteins is involved in this pathway. In this section, the key players in reverse cholesterol transport are discussed, divided by their role in the pathway: mediators of cholesterol efflux, lipoprotein remodeling proteins, and mediators of hepatic cholesterol uptake and biliary excretion.

1.3.1 Mediators of cellular cholesterol efflux

ABCA1

ATP-binding cassette (ABC) transporters constitute a large family of transmembrane proteins that use ATP as an energy source to translocate a wide variety of substrates across cellular membranes. One of the best characterized ABC transporters is the ubiquitously expressed ABCA1. ABCA1 is highly expressed in macrophages, and transcription of ABCA1 is markedly induced by overloading cells with cholesterol (Van Eck *et al.*, 2005). In addition, ABCA1 is also highly expressed in the liver, where it plays a role in the lipidation of apoAI (Oram and Heinecke, 2005). ABCA1 mediates the unidirectional transport of phospholipids and cholesterol to lipid-free and lipid-poor apolipoproteins, preferentially apoAI (Jessup *et al.*, 2006). ApoAI can bind directly to ABCA1, thereby stabilizing ABCA1 and enhancing lipid efflux (Arakawa and Yokoyama, 2002; Wang *et al.*, 2001).

Mutations in ABCA1 were discovered as the genetic defect in Tangier disease, characterized by an almost complete deficiency of plasma HDL, accumulation of cholesterol in peripheral macrophages, and premature cardiovascular disease (Bodzioch *et al.*, 1999). In mouse models, absence of ABCA1 strongly reduces plasma HDL levels (McNeish *et al.*, 2000; Orso *et al.*, 2000), while overexpression of ABCA1 increases plasma HDL levels (Cavelier *et al.*, 2001; Singaraja *et al.*, 2001; Vaisman *et al.*, 2001). Studies determining the effect of ABCA1 overexpression on atherosclerosis development provided conflicting results (Joyce *et al.*, 2002; Singaraja *et al.*, 2002).

The role of macrophage ABCA1 in lipoprotein metabolism and reverse cholesterol transport is well-described. Macrophage-specific deletion of ABCA1 results in an impaired cholesterol efflux *in vitro* (Haghpassand *et al.*, 2001) and a decreased reverse cholesterol transport *in vivo* (Wang et al., 2007a; Wang *et al.*, 2007c). Furthermore, macrophage-specific ABCA1 deficiency resulted in accelerated atherosclerosis in mice (Aiello *et al.*, 2002; van Eck *et al.*, 2002). In accordance with these findings, overexpression of macrophage ABCA1 stimulated lipid efflux *in vitro* (Singaraja et al., 2002; Van Eck *et al.*, 2006).

ABCG1

Another member of the superfamily of ABC transporters is ABCG1, which is found throughout the whole body, but particularly in macrophage-rich tissues (Jessup *et al.*, 2006). Like for ABCA1, expression of macrophage ABCG1 is also strongly responsive to cellular cholesterol status (Venkateswaran *et al.*, 2000). Whereas ABCA1 exports cholesterol and phospholipids to lipid-poor apolipoproteins, ABCG1 mediates unidirectional efflux of cholesterol to mature HDL particles (Kennedy *et al.*, 2001; Klucken *et al.*, 2000). ABCA1 and ABCG1 might act together in exporting cellular lipids: by loading lipid-poor apoAI with cellular lipids, ABCA1 generates particles which serve as acceptors for ABCG1-mediated cholesterol efflux (Gelissen *et al.*, 2006).

ABCG1 deficient mice accumulate cholesterol in macrophages within multiple tissues, whereas mice overexpressing ABCG1 have diminished cholesterol accumulation (Kennedy *et al.*, 2005). This can be explained by the importance of ABCG1 for an efficient reverse cholesterol transport. ABCG1 deficiency in macrophages resulted in an impaired macrophage reverse cholesterol transport, whereas ABCG1 overexpression in macrophages promoted reverse cholesterol transport *in vivo*. Double knockdown of ABCA1 and ABCG1 in macrophages greatly impaired macrophage reverse cholesterol transport compared to macrophage-ABCG1 deficiency alone, indicating that ABCA1 and ABCG1 cooperatively contribute to macrophage reverse cholesterol transport (Wang *et al.*, 2007c). The exact physiologic role of macrophage ABCG1 in atherogenesis remains uncertain: as investigated by three independent research groups, mice with ABCG1 deficient macrophages had either decreased (Baldan *et al.*, 2006; Ranalletta *et al.*, 2006) or modestly increased (Out *et al.*, 2006) atherosclerosis. A dramatic increase in atherosclerosis was found in mice with macrophages lacking both ABCA1 and ABCG1 (Yvan-Charvet *et al.*, 2007).

SR-BI

Scavenger Receptor-BI (SR-BI) or CLA-1 (its human analogue) is a multiple transmembrane receptor that can bind modified forms of LDL and native HDL, LDL, and VLDL, with the highest affinity for HDL (Acton *et al.*, 1996). SR-BI was initially identified as a protein taking up cholesterol esters, phospholipids and triglycerides from lipoproteins. Now it is clear that SR-BI also mediates the bidirectional exchange of free cholesterol between cells and lipoprotein particles, in which the overall direction of cholesterol movement depends on its concentration gradient. SR-BI is highly expressed in the liver and macrophages, and in steroidogenic tissues such as the adrenal glands, ovaries, and testes (Van Eck *et al.*, 2005).

Expression of SR-BI in macrophages is considered to have an atheroprotective effect, as mice with SR-BI deficient macrophages develop more atherosclerosis compared to mice with SR-BI-expressing macrophages (Covey *et al.*, 2003; Zhang *et al.*, 2003a).

This atheroprotective effect was explained by the fact that macrophage SR-BI could promote cholesterol efflux to mature HDL particles, as demonstrated *in vitro* (Ji *et al.*, 1997). However, in another study, cholesterol efflux from SR-BI deficient macrophages to HDL_3 or whole mouse serum was found to be similar compared to efflux from SR-BI expressing cells, and reverse cholesterol transport *in vivo* was not impaired in mice after transfer of bone marrow-derived SR-BI deficient macrophages (Wang *et al.*, 2007c). These findings suggest that the SR-BI might have anti-atherogenic effects that are independent of cholesterol efflux from macrophages.

1.3.2 Lipoprotein remodeling proteins

CETP and PLTP

The exchange of lipids between lipoprotein subclasses is performed by two closely related proteins, named CETP and PLTP. PLTP and CETP belong to the lipid transfer/ lipopolysaccharide binding protein (LT/LBP) family, which also includes lipopolysaccharide binding protein (LBP) and bactericidal permeability-increasing protein (BPI). LBP and BPI are able to bind and transfer bacterial endotoxins and lipopolysaccharide (LPS), thereby modulating the response to bacterial infection (Schumann *et al.*, 1990). CETP is a plasma protein that is able to transfer neutral lipids, triglycerides and cholesteryl esters between lipoprotein particles, while PLTP transfers phospholipids, free cholesterol and vitamin E. The molecular characteristics and function of PLTP will be discussed in detail in the next chapter.

CETP

CETP was discovered in the late 1970s (Pattnaik *et al.*, 1978; Zilversmit *et al.*, 1975). In contrast to human plasma, mouse, pig and rat plasma do not display CETP activity, which demonstrates that CETP activity varies largely between mammalian species (Guyard-Dangremont *et al.*, 1998; Speijer *et al.*, 1991). CETP mediates the transfer of HDL-cholesteryl esters to apoB-containing lipoproteins in exchange for triglycerides (Ohta *et al.*, 1995). The resulting HDL particles have a decreased cholesterol content and particle size, and are enriched with triglycerides. Cholesteryl esters in apoB-containing lipoproteins can be removed from plasma through receptor-mediated uptake by the liver.

In humans, CETP deficiency results in elevated plasma HDL levels (Inazu *et al.*, 1990; Koizumi *et al.*, 1985; Kurasawa *et al.*, 1985). Clinical studies showed that elevated plasma CETP activity or concentration levels are associated with increased cardiovascular risk (Dullaart *et al.*, 2007). In general, expression of CETP in mice resulted in the reduction of HDL levels, but also in the stimulation of macrophage reverse cholesterol transport (Tanigawa *et al.*, 2007). The effect of CETP expression on atherosclerosis susceptibility

in animal models has been studied extensively and appears highly dependent on the level of CETP expression and on the genetic and metabolic context (Parini and Rudel, 2003). Dependent on the background of the mice, and efficacy of clearance of apoB-containing lipoproteins, CETP might be either pro- or anti-atherogenic (Tanigawa *et al.*, 2007). Studies in human subjects showed that an elevated plasma CETP activity or concentration is associated with increased cardiovascular risk (Dullaart *et al.*, 2007). As plasma HDL levels are elevated in CETP deficiency (Inazu *et al.*, 1990; Koizumi *et al.*, 1985; Kurasawa *et al.*, 1985), much effort is being paid to therapeutic CETP inhibition. CETP inhibition by monoclonal antibodies or synthetic compounds in animal models and preclinical trials demonstrated clinical efficacy on plasma lipid levels (Vourvouhaki and Dedoussis, 2008). However, a study with CETP inhibitor torcetrapib in patients with coronary artery disease was recently stopped because of increased mortality, despite a significant increase in HDL level and decrease in LDL level (Kontush *et al.*, 2008).

LCAT

Another protein with an important function in HDL metabolism is lecithin cholesterol acyl-transferase (LCAT). After secretion by the liver, LCAT circulates in the blood, bound to HDL. The main activator of LCAT is apoAI. LCAT mediates the esterification of free cholesterol, mainly derived from cellular membranes, primarily at the surface of HDL. After esterification, the formed cholesteryl esters migrate to the inner core of the lipoprotein particle. In this way, LCAT converts preβ-HDL into α-HDL, thereby contributing to the maturation of HDL particles. LCAT has been suggested to play a role in reverse cholesterol transport by maintaining a free cholesterol gradient between peripheral cells and plasma HDL and by maintaining HDL acceptor particle shape and structure (Glomset, 1968).
Animal studies elucidating the role of LCAT in reverse cholesterol transport (Alam *et al.*, 2001; Berard *et al.*, 1997; Francone *et al.*, 1997) and in the development of atherosclerosis have provided conflicting results (Berard *et al.*, 1997; Föger *et al.*, 1999; Furbee and Parks, 2002; Mehlum *et al.*, 1997). Modest plasma levels of LCAT protect against atherosclerosis, but when plasma LCAT levels exceed a certain level, this could result in the development of atherosclerosis (Hovingh *et al.*, 2005). Coexpression of both human LCAT and CETP in mice resulted in reduced atherosclerosis development compared to overexpression of LCAT alone (Föger *et al.*, 1999; Rigotti *et al.*, 1997).

HL

Lipases are water-soluble proteins that hydrolyze ester bonds of water-insoluble substrates such as triglycerides, phospholipids and cholesteryl esters, thereby generating free fatty acids. Lipases are involved in a diversity of biological processes ranging from metabolism of dietary triglycerides to cell signalling and inflammation. The lipase gene family consists

of multiple members that originated from a common ancestral precursor and evolved into proteins with distinct organ-specific expression and functions. One of these is hepatic lipase (HL), a glycoprotein that is synthesized mainly by hepatocytes. After secretion it binds to heparan sulfate proteoglycans on the surface of sinusoidal endothelial cells and parenchymal cells in the space of Disse (Hasham and Pillarisetti, 2006). The main function of HL is to hydrolyze triglycerides and phospholipids in VLDL remnants, IDL, LDL and HDL. During HDL remodeling, triglyceride-enriched HDL particles formed by CETP activity are substrates for HL. By mediating the hydrolysis of HDL-triglyceride, HL reduces the size of the HDL core. In addition to its role as a lipolytic enzyme, lipoprotein-bound HL serves as a ligand for the binding of lipoproteins to the surface of the hepatocytes, thereby facilitating the uptake of lipids via cell surface receptors (Krapp *et al.*, 1996). The expression of macrophage-derived HL in apoE deficient and LCAT overexpressing mice enhanced atherosclerotic lesion formation (Nong *et al.*, 2003). Although the role of HL in cardiovascular disease has been studied extensively, the question whether HL is a pro-or anti-atherogenic enzyme has still not been answered satisfactory (Gonzalez-Navarro *et al.*, 2002; Jansen *et al.*, 2002; Lambert *et al.*, 2000; Santamarina-Fojo *et al.*, 2004).

LPL

Lipoprotein lipase (LPL) is synthesized in parenchymal cells of tissues that use free fatty acids as energy source (heart, skeletal muscle) or for storage purposes (adipose tissue). In addition, LPL is expressed by macrophages in the vascular wall (Hasham and Pillarisetti, 2006). LPL is secreted and attached to the luminal side of the vascular endothelium via charged heparan sulfate proteoglycans. Lipolysis is initiated when circulating triglyceride-rich lipoproteins interact with endothelial cell-associated dimeric LPL. The enzyme is activated by apoCII on the surface of these lipoprotein particles. LPL is the rate-limiting enzyme in the hydrolysis of triglycerides in chylomicrons and VLDL, thereby generating non-esterified fatty acids (Santamarina-Fojo and Brewer, 1994). In addition to its role as an enzyme, LPL has been proposed to be a bridging factor that facilitates the uptake of HDL-cholesterol esters by the liver and by macrophages (Beisiegel, 1996; Mulder *et al.*, 1993; Rinninger *et al.*, 2001).

The tissue site of LPL expression appears to be a key determinant of its effects on atherosclerosis (Tontonoz P, 2003). While the main effect of LPL, lipolysis, is per se atheroprotective by lowering plasma triglycerides and generating material for HDL formation, macrophage expression of LPL in the artery wall could also have a pro-atherogenic effect due to prolonged exposure of the endothelium to lipolysis products. Systemic overproduction of human LPL in mice resulted in decreased plasma triglyceride and cholesterol levels, and protected against atherosclerosis (Shimada *et al.*, 1996; Yagyu *et al.*, 1999). However,

macrophage-specific overexpression of LPL accelerated atherosclerosis in atherosclerosis-prone mice (Babaev *et al.*, 2000; Wilson *et al.*, 2001). Recently, spontaneous atherosclerosis in aged LPL-deficient mice was reported, mediated by the oxidation of chylomicrons and the activation of vascular endothelial cells (Zhang *et al.*, 2008). The option of gene therapy for LPL deficiency has been studied in mouse models. Intramuscular injection of a natural variant of LPL resulted in an efficient transgene expression and reduction of fasting plasma triglycerides in wild type, LPL deficient and LDLR deficient mice (Rip *et al.*, 2005; Ross *et al.*, 2004). However, this local overexpression of LPL in skeletal muscle did not influence plasma cholesterol levels or atherosclerosis development, confirming the importance of extent and site of LPL expression in these processes (Rip *et al.*, 2007).

EL

As its name implies, endothelial lipase (EL) is mainly synthesized by endothelial cells. In contrast to HL and LPL, the triglyceride hydrolase activity of EL is low. EL primarily has phospholipase activity, thereby promoting the turnover of HDL components and increasing the catabolism of apoAI (Jaye and Krawiec, 2004; McCoy *et al.*, 2002). Hepatic overexpression of EL in mice resulted in strongly reduced HDL levels and increased HDL catabolism (Jaye *et al.*, 1999). Expression of EL at the atherosclerotic lesion site may have direct pro-atherogenic effects (Lamarche and Paradis, 2007). Inhibition of EL in mice, either by antibody injection or by targeted gene deletion, results in a strong increase in HDL-cholesterol levels (Ishida *et al.*, 2003; Jin *et al.*, 2003).

1.3.3 Mediators of hepatic cholesterol uptake and biliary excretion

LDLR

The removal of large cholesterol-rich lipoproteins from the circulation is mainly mediated by the hepatic LDL receptor (LDLR), binding both apoB100 and apoE. A genetic defect in this receptor is the underlying pathological mechanism of familial hypercholesterolemia, a disease in which plasma concentrations of apoB100-containing lipoprotein particles are dramatically increased (Brown and Goldstein, 1986). The LDLR binds circulating cholesteryl ester-rich lipoprotein particles, with apoB100 or apoE as protein component. Up to 70% of the circulating LDL particles are taken up by the LDLR via apoB100-mediated endocytosis (Kong *et al.*, 2006). After internalization, lipoprotein particles are degraded in the lysosomes and cholesterol is either degraded to bile acids or excreted as free cholesterol in the bile, or it is re-used for lipoprotein assembly. The LDLR deficient mouse is a frequently used model in cardiovascular research. This mouse model has elevated LDL levels, but no atherosclerotic lesions develop on a chow diet. A humanized lipoprotein profile can be mimicked in these animals when a high fat high cholesterol diet is administered, resulting in advanced atherosclerotic lesion formation (Ishibashi *et al.*, 1994).

LRP

LDLR-related protein (LRP), present in numerous cell types, is a membrane receptor with various cellular functions. It has multiple extracellular ligands, including apoE and LPL (Overton *et al.*, 2007). In the liver, LRP plays a important role in the clearance of chylomicron remnants, suggesting an atheroprotective role of hepatic LRP via reduction of plasma lipid levels (Field and Gibbons, 2000; Rohlmann *et al.*, 1998). In addition, LRP plays an important atheroprotective role in macrophages, attributable to an inhibition of the autocrine cellular inflammatory response (Gaultier *et al.*, 2008).

SR-BI

Besides expression in peripheral tissues, SR-BI is highly expressed in the liver. SR-BI binds HDL with high affinity, thereby facilitating the selective delivery of cholesterol to the liver. In contrast to the endocytic cholesterol uptake by the LDL receptor, uptake of HDL-cholesterol by SR-BI occurs via selective transfer of HDL-derived cholesteryl esters into cells without degradation of HDL particles. Studies in SR-BI deficient and SR-BI overexpressing animal models demonstrated the physiological importance of the protein in HDL metabolism. SR-BI knockout mice suffer from hypercholesterolemia primarily because of increased HDL levels and reduced biliary cholesterol secretion (Rigotti *et al.*, 1997; Varban *et al.*, 1998), whereas SR-BI overexpressing mice have decreased HDL levels due to an accelerated hepatic HDL clearance (Kozarsky *et al.*, 1997; Mardones *et al.*, 2001; Ueda *et al.*, 1999; Wang *et al.*, 1998). Importantly, hepatic overexpression of SR-BI results in an increased macrophage reverse cholesterol transport, despite reduced plasma HDL levels (Zhang *et al.*, 2005). In line with this finding, reverse cholesterol transport is impaired in SR-BI deficient mice. The role of SR-BI in reverse cholesterol transport may explain the results of atherosclerosis studies in mice. SR-BI overexpression in mice resulted in a reduced susceptibility to atherosclerosis (Arai *et al.*, 1999; Kozarsky *et al.*, 2000; Ueda *et al.*, 2000), while SR-BI deficiency increased atherogenesis (Braun *et al.*, 2002; Covey *et al.*, 2003; Trigatti *et al.*, 1999). CETP expression in SR-BI deficient mice increased macrophage reverse cholesterol transport and attenuated the development of atherosclerosis (Harder *et al.*, 2007; Tanigawa *et al.*, 2007), which suggests that clearance of plasma lipids via CETP can act as an alternative pathway of SR-BI-mediated lipid excretion.

ABCG5 and ABCG8

In addition to ABCA1 and ABCG1, ABCG5 and ABCG8 are two other members of the superfamily of ABC transporters. ABCG5 and ABCG8 are highly expressed in the intestine and the liver, where they have a key role in the excretion of cholesterol and other sterols from the body. Both transporters limit cholesterol and plant-sterol absorption in the gut, and facilitate biliary excretion by hepatocytes (Graf *et al.*, 2002; Yu *et al.*, 2002a). Mutations

in either one of them cause accumulation of plant sterols in the body (Berge *et al.*, 2000; Lee *et al.*, 2001). Deficiency of both genes simultaneously strongly inhibits hepatobiliary cholesterol excretion in mice (Yu *et al.*, 2002a), whereas overexpression of promotes lipid excretion and reduces fractional absorption of cholesterol in the intestine (Yu *et al.*, 2002b). In a recent report, it was demonstrated that liver X receptor -mediated activation of macrophage reverse cholesterol transport requires ABCG5 and ABCG8 (Calpe-Berdiel *et al.*, 2008).

1.4 Phospholipid transfer protein

In the early eighties, in addition to CETP a second lipid transfer protein was discovered in human plasma. This protein was named lipid transfer protein-II (LTP-II). LTP-II directly facilitated the transfer of phospholipids from apoB-containing lipoproteins to HDL, but it was incapable of transferring cholesteryl esters or triglycerides (Albers *et al.*, 1984; Tollefson *et al.*, 1988). The term "LTP-II" is not used anymore; nowadays the protein is referred to as phospholipid transfer protein (PLTP) (van Tol, 2002).

1.4.1 Molecular characteristics

The human PLTP gene is located on chromosome 20 (Whitmore *et al.*, 1995). It has a size of approximately 13.3 kb, containing sixteen exons (Tu *et al.*, 1995). The functional promoter region of the human PLTP gene consists of a TATA box, a high GC region, and multiple consensus sequences for the potential binding of transcription factors (Tu *et al.*, 1997; Tu *et al.*, 1995). mRNA expression analysis shows a single transcript of 1.8 kb. The PLTP complementary DNA (cDNA) encodes a hydrophobic signal peptide of 17 amino acids and a mature protein of 476 amino acids. The calculated molecular mass of PLTP is 55 kDa, but due to glycosylation the mature plasma protein weights approximately 81 kDa (Day *et al.*, 1994; Huuskonen *et al.*, 1998). PLTP has a two-domain structure with conserved lipid-binding pockets consisting of apolar residues in each domain (Huuskonen *et al.*, 1999). The N-terminal pocket plays an essential role in the transfer of phospholipids, the C-terminal pocket is important for the association of PLTP with HDL (Desrumaux *et al.*, 2001; Huuskonen *et al.*, 1999; Ponsin *et al.*, 2003). It has been demonstrated that this association takes place via binding of PLTP to apoAI and apoAII, the two main apolipoprotein classes in HDL particles (Pussinen *et al.*, 1998). A disulfide bridge between cysteine residues 146 and 185 is necessary for the correct folding of PLTP, resulting in its specific boomerang-shaped appearance.

The presence of PLTP has been demonstrated in plasma from a variety of species (Albers *et al.*, 1995; Day *et al.*, 1994; Jiang and Bruce, 1995; Speijer *et al.*, 1991). In contrast to CETP, the absence of PLTP has not been described in any species. The organization of the mouse gene encoding PLTP, located on chromosome 2, is strikingly similar to that encoding the human PLTP, as the exon-intron junctions of the PLTP gene in these two species are completely conserved (Tu *et al.*, 1997). Mouse and human PLTP genes share an 81% nucleotide sequence identity in the promoter region (LeBoeuf *et al.*, 1996; Tu *et al.*, 1997). The mature mouse and human PLTP protein both contain 476 amino acids. Mouse PLTP shares 83% amino acid sequence identity with human PLTP, and has a computed mass of 52.7 kDa. Most sequence differences between mouse and human PLTP are conserved substitutions (Albers *et al.*, 1995).

1.4.2 Transcriptional regulation of PLTP by nuclear receptors

Transcription of PLTP is known to be regulated by the nuclear receptors farnesoid X receptor (FXR), peroxisome proliferator-activated receptor α (PPARα), and liver X receptor (LXR). These transcription factors belong to the nuclear receptor superfamily comprising metabolite-activated transcription factors that form permissive heterodimers with retinoid X receptor (RXR). In the absence of ligands, most RXR heterodimers are bound to specific response elements in the promoter regions of the target genes in association with co-repressors, maintaining active repression of these genes. Upon ligand binding, a conformational change in the receptor takes place, resulting in the exchange of co-repressors for co-activators, and the subsequent initiation of target gene transcription (Glass and Rosenfeld, 2000; Zelcer and Tontonoz, 2006). Ligand binding can also inhibit the transcription of certain genes via process named trans-repression.
FXR forms a heterodimer with RXR upon binding to bile acids, the natural ligand of FXR. FXR might protect the liver against the toxic effects of bile salts by reducing their synthesis and uptake, and increasing their excretion (Goodwin *et al.*, 2000; Lu *et al.*, 2000; Plass *et al.*, 2002). In mice, bile salt synthesis is repressed upon binding of cholate to FXR (Li-Hawkins *et al.*, 2002). Hepatic PLTP expression has been demonstrated to be regulated by FXR *in vitro* and *in vivo* (Kast *et al.*, 2001; Tu and Albers, 2001; Urizar *et al.*, 2000).
PPARα is mainly expressed in tissues where fatty acids are catabolised, such as the liver, brown adipose tissue, heart and muscle. PPARα is involved in mitochondrial and peroxisomal β-oxidation of fatty acids, thereby regulating cellular energy metabolism, as well as in systemic regulation of cholesterol and fatty acid homeostasis, and apoptosis upon inflammation. Polyunsaturated fatty acids serve as endogenous PPARα ligands. Well-characterized synthetic ligands for PPARα are fibrates, a class of drugs that efficiently decrease triglycerides and increase plasma HDL levels (Heller and Harvengt, 1983; Staels

et al., 1998). Regulation of PLTP expression by PPARα is species-specific, as fenofibrate treatment of human PLTP transgenic mice increased the hepatic expression of endogenous PLTP, but not human PLTP (Lie *et al.*, 2005). Furthermore, fenofibrate treatment significantly reduced promoter activity of the human PLTP gene (Tu and Albers, 1999), but significantly increased transcriptional activity of PLTP and plasma PLTP activity levels in mice, accounting for a marked enlargement of HDL (Bouly *et al.*, 2001; Tu and Albers, 2001).

In addition to FXR and PPARα response elements, the PLTP promoter region also contains LXR-binding sequences. Two LXR subtypes are known: LXRα, which is highly expressed in the liver, and LXRß, which is expressed ubiquitously (Repa and Mangelsdorf, 2000). LXRs are activated by oxysterols, formed as by-products during cholesterol synthesis as well as by oxidation of dietary cholesterol (Lehmann *et al.*, 1997). LXR functions as a nuclear cholesterol sensor that is activated in response to elevated intracellular cholesterol levels in multiple cell types. It induces the transcription of genes involved in cholesterol absorption, efflux, transport, and excretion. In addition to its ability to modulate cholesterol metabolism, LXR stimulates hepatic lipogenesis. Furthermore, LXRs have also been found to modulate immune and inflammatory responses in macrophages (Castrillo and Tontonoz, 2004). Upon LXR activation, PLTP expression is increased in human and murine macrophages and in primary human hepatocytes (Laffitte *et al.*, 2003; Mak *et al.*, 2002). Pharmacological activation of LXR in mice resulted in an increased hepatic PLTP expression, a dose-dependent increase of plasma PLTP activity, increased HDL levels, and a PLTP-dependent enlargement of HDL particles (Cao *et al.*, 2002). Coadministration of a synthetic LXR and PPARα ligand to mice resulted in elevated HDL levels and enlarged HDL particles, and increased hepatic PLTP expression and plasma PLTP activity levels (Beyer *et al.*, 2004).

1.4.3 Tissue expression of PLTP

PLTP is widely expressed in human tissues, pointing at a crucial cellular role or multifunctional role of the protein. Relatively high mRNA levels are found in ovary, thymus, and placenta, suggesting a role for PLTP in reproduction and foetal development (Albers *et al.*, 1995). PLTP mRNA is also relatively high in pancreas, lung, as well as in subcutaneous and visceral adipose tissue (Dusserre *et al.*, 2000). Furthermore, PLTP is expressed at moderate levels in, testes, and prostate, and at low levels in kidney, liver, spleen, heart, colon, skeletal muscle, leukocytes and brain (Albers *et al.*, 1995). PLTP activity in the cerebrospinal fluid represents 15% of the plasma activity, indicating active PLTP synthesis in the brain (Vuletic *et al.*, 2003). In addition, human seminal plasma displays significant phospholipid transfer activity due to the presence of active PLTP, possibly for maintenance

of the lipid composition of the cell membranes of spermatozoa (Masson *et al.*, 2003). Furthermore, human tear fluid contains catalytically active PLTP, suggesting that PLTP plays a role in the formation of the tear film (Jauhiainen *et al.*, 2005).

PLTP was detected in human atherosclerotic lesions, where it colocalised with macrophages, indicating that this cell type is an important source of PLTP (Desrumaux *et al.*, 2003; Laffitte *et al.*, 2003; O'Brien *et al.*, 2003). In addition to macrophages, PLTP can also be detected in smooth muscle foam cells in human atherosclerotic lesions, suggesting that cellular cholesterol accumulation increases PLTP expression in both cell types (O'Brien *et al.*, 2003). The distribution of PLTP within human atherosclerotic lesions suggests that PLTP plays a role in cellular cholesterol metabolism and lipoprotein retention on the extracellular matrix.

Like in humans, also murine PLTP expression is widely distributed among tissues (Albers *et al.*, 1995). In mice, relatively high PLTP mRNA levels are detected in lung, brain and heart. The lung is the major site expressing PLTP, suggesting an important role for PLTP in maintaining normal function of this organ. In human collagenase transgenic mice, an emphysematous animal model, lung PLTP expression is increased. Remarkably, there is no effect of PLTP deficiency on lung function in PLTP knockout mice (Jiang *et al.*, 1998). Furthermore, PLTP is widely expressed in the brain where it transfers alpha-tocopherol, the main isomer of vitamin E. This is a key process in preventing oxidative damage in the brain (Desrumaux *et al.*, 2005). The ability of PLTP to transfer alpha-tocopherol also plays a role in fertility, both in male and female mice. PLTP is highly expressed in epididymis and in the embryo-containing oviduct, implicating a role for PLTP in the motility of spermatozoa and preimplantation embryonal development (Drouineaud *et al.*, 2006; Lee *et al.*, 2005).

1.4.4 Functions of plasma PLTP: data from *in vitro* studies

Phospholipid transfer

In the early eighties, Tall and Green showed that egg phosphatidylcholine is transferred to spherical and discoidal HDL particles when it is incubated with isolated human HDL_3 or with plasma (Damen *et al.*, 1982; Tall and Green, 1981). At this time, the mechanism behind the incorporation of phospholipids into HDL particles was not known. Although phospholipids exchange between plasma lipoproteins spontaneously, the transfer rate would be too slow to be a physiologically important mechanism (Massey, 1984). Several studies showed that PLTP is responsible for the specific transfer of phospholipids between lipoprotein subclasses (Albers *et al.*, 1984; Tall *et al.*, 1983; Tu *et al.*, 1993). During lipolysis, human PLTP is able to enhance the net transfer of VLDL phospholipids into HDL and the exchange of phospholipids between VLDL and HDL (Tall *et al.*, 1985). PLTP is able to transfer all common phospholipid classes non-specifically (Huuskonen *et al.*, 1996).

However, lipoprotein class and composition determines the rate of phosphatidylcholine transfer by PLTP (Rao *et al.*, 1997). The exact mechanism of PLTP-mediated phospholipid transfer is still not completely understood. It is unlikely that PLTP forms a tight complex with its substrate as no PLTP-phospholipid intermediates were detected during an incubation of phospholipid vesicles with human HDL in the presence of purified PLTP (Huuskonen *et al.*, 1996). Possibly, PLTP functions as a bridging molecule between donor and acceptor particle to facilitate the transfer of phospholipids. However, it has also been suggested that active PLTP is able to transfer lipids by a shuttle mechanism. In that case, the formation of a complex between PLTP, acceptor, and donor particle should not be required for phospholipid transfer (Setala *et al.*, 2007).
In addition to facilitate the transfer of phospholipids, PLTP is also able to transfer other hydrophobic compounds, such as alpha-tocopherol, cholesterol and lipopolysaccharide (LPS). Alpha-tocopherol is an important anti-oxidant of plasma lipoproteins and cell membranes. It is secreted by the liver into the blood stream, together with VLDL. PLTP catalyzes the exchange and transfer of alpha-tocopherol between plasma lipoprotein classes and cells (Kostner *et al.*, 1995). Furthermore, purified human PLTP is able to enhance the transfer of cholesterol from vesicles containing phosphatidylcholine and cholesterol to HDL_3. However, PLTP possesses a considerably higher affinity and binding capacity for phosphatidylcholine than for cholesterol (Nishida and Nishida, 1997). In addition, PLTP is able to transfer LPS, the major outer membrane component of gram-negative bacteria, and a potent endotoxin that triggers cytokine-mediated systemic inflammatory responses in the host. Incubation of LPS with purified recombinant PLTP resulted in the inhibition LPS-mediated inflammatory responses (Hailman *et al.*, 1996). In human whole blood, PLTP induced the transfer of LPS from HDL to LDL, resulting in the remodeling of HDL into two subpopulations (Levels *et al.*, 2005).

HDL conversion

PLTP acts as an HDL conversion factor (Albers *et al.*, 1995; Jauhiainen *et al.*, 1993; Pussinen *et al.*, 1995; Settasatian *et al.*, 2008; Tu *et al.*, 1993; von Eckardstein *et al.*, 1996). The two main activities of PLTP, phospholipid transfer and HDL conversion, are closely interrelated. Two recombinant PLTP mutant proteins, defective in phospholipid transfer activity due to a mutation in the N-terminal lipid-binding pocket, were unable to convert human HDL_3, demonstrating that phospholipid transfer is a prerequisite for HDL conversion (Huuskonen *et al.*, 2000b). Particle fusion is the main process involved in PLTP-mediated HDL remodeling (Korhonen *et al.*, 1998; Lusa *et al.*, 1996; Settasatian *et al.*, 2001). After fusion of HDL particles, which requires the presence of apoAI, the fusion product is rearranged into small and large particles (Lusa *et al.*, 1996; Settasatian *et al.*, 2001). The small particles that are generated during HDL conversion only contain some

phospholipid and apoAI. By generating small HDL particles during HDL conversion, PLTP may increase reverse cholesterol transport, as these particles are the initial acceptor for efflux of cellular cholesterol in peripheral tissues.

Cholesterol efflux

The role of PLTP in HDL remodeling may be directly connected to an indirect role of PLTP in cellular cholesterol efflux. Plasma PLTP stimulates the formation of preβ-HDL, which can serve as cholesterol acceptor particles *in vitro* (Jessup *et al.*, 2006). Cholesterol efflux from fibroblasts to plasma isolated from subjects with increased plasma PLTP activity levels, as in diabetes mellitus or metabolic syndrome, was increased, or at least not defective despite low HDL levels (de Vries *et al.*, 2007; de Vries *et al.*, 2005; Dullaart *et al.*, 2008b). The hypothesis that PLTP may play a role in cholesterol efflux is supported by the finding that cholesterol efflux from cholesterol-loaded fibroblasts to total plasma increased by 40% when PLTP was added during the incubation (von Eckardstein *et al.*, 1996). In a more recent study, it was shown that macrophage cholesterol efflux to HDL was increased after preincubation of HDL with purified active PLTP, through the formation of preβ-HDL and large fused HDL particles, both efficient cholesterol acceptors *in vitro* (Vikstedt *et al.*, 2007a).

In addition to the generation of lipid acceptor particles, PLTP might have a direct role in lipid efflux by increasing the cellular binding of HDL. Exposure of cholesterol-loaded fibroblasts to PLTP increased specific HDL binding to the cells and enhanced cholesterol and phospholipid efflux to small HDL particles (Wolfbauer *et al.*, 1999). This effect was not seen when fibroblasts of Tangier disease patients, lacking functional ABCA1 expression, were used, which led to the hypothesis that cellular PLTP might play a role in ABCA1-dependent lipid efflux. This was confirmed in a later study, in which purified PLTP, added to cultured macrophages, enhanced cellular cholesterol efflux to HDL particles, by promoting cell-surface binding, interacting with and stabilizing ABCA1 (Oram, 2003). Recently, an amphipathic helical region of the N-terminal barrel of PLTP was shown to be critical for ABCA1-dependent cholesterol efflux (Oram *et al.*, 2008).

In addition to investigation of the role of exogenously added PLTP, also the role of macrophage-derived PLTP in lipid efflux has been studied. Cholesterol efflux to apoAI and HDL from cholesterol-loaded peritoneal macrophages isolated from PLTP deficient mice was decreased compared to wild type macrophages (Lee-Rueckert *et al.*, 2006). However, in three other studies, no effect on cholesterol efflux was found towards apoAI (Cao *et al.*, 2002; Ogier *et al.*, 2007) or towards HDL (Valenta *et al.*, 2006). The importance of peripheral PLTP for macrophage cholesterol efflux is still poorly understood.

1.4.5 Human studies

Despite a relatively low PLTP expression level, the liver and intestine are major contributors to plasma PLTP concentration due to their large organ mass (Albers *et al.*, 1995). In human plasma, mean PLTP concentrations are found in a range of 4-15 mg/l (Desrumaux *et al.*, 1999; Huuskonen *et al.*, 2000a; Oka *et al.*, 2000b). PLTP activity level can be determined by different methods, resulting in varying values (Cheung *et al.*, 1996; Colhoun *et al.*, 2001; Desrumaux *et al.*, 1999). In a study in a Finnish population, mean plasma activity was either 6.6 μmol/mL/h or 1.4 μmol/mL/h, quantified with an exogenous, lipoprotein-independent radiometric assay or an endogenous, lipoprotein-dependent radiometric assay, respectively (Janis *et al.*, 2004). The exogenous assay measures phospholipid transfer from phospholipid-liposomes to exogenously added HDL (Damen *et al.*, 1982), while the endogenous assay measures transfer to endogenous serum HDL (Lagrost *et al.*, 1999a). PLTP activity levels are comparable between men and women (Cheung *et al.*, 2002; Colhoun *et al.*, 2001; Janis *et al.*, 2004). The absence of a correlation between human plasma PLTP mass and phospholipid transfer activity led to the identification of a low-active form of plasma PLTP besides the catalytically active form (Huuskonen *et al.*, 2000a; Oka *et al.*, 2000b). Both forms were found associated with different types of lipoprotein particles. Complexes with active PLTP had an average molecular mass of 160 kDa, whereas complexes with low-active PLTP had an average mass of 520 kDa, indicating that low-active PLTP is associated with larger particles. The low-active form of PLTP has been reported to be 46 to 70% of total PLTP mass (Janis *et al.*, 2004; Oka *et al.*, 2000a). Contradictory results have been reported in literature regarding the association of active and inactive PLTP fractions with different apolipoprotein classes. One research group suggested that PLTP with low activity forms a complex with apoAI, and highly active PLTP with apoE (Janis *et al.*, 2005; Karkkainen *et al.*, 2002; Siggins *et al.*, 2003). In contrast, another group reported that active plasma PLTP is associated primarily with apoAI- but not with apoE-containing lipoproteins (Cheung and Albers, 2006). Until now, no function of inactive PLTP has been described.
Although there are no reports of human PLTP deficiency until today, genetic variation of the human PLTP gene has been reported, in most cases not affecting plasma PLTP activity (Aouizerat *et al.*, 2006; Bosse *et al.*, 2005; Engler *et al.*, 2008; Palmen *et al.*, 2005; Tahvanainen *et al.*, 1999). In a population study in subjects with hypoalphalipoproteinemia, four missense mutations in the PLTP gene were discovered, of which one was associated with a low binding capacity for HDL and strongly decreased phospholipid transfer activity (Aouizerat *et al.*, 2006).

Several factors affect PLTP activity and concentration. Plasma PLTP activity increases with age and correlates with body mass index and subcutaneous fat area, fasting blood

glucose, plasma triglycerides, and nonesterified fatty acids (Dullaart *et al.*, 1994b; Kaser *et al.*, 2001; Murdoch *et al.*, 2003; Riemens *et al.*, 1998). Plasma PLTP activity can be affected by life style. Increased PLTP activity levels have been reported in subjects after consumption of French-press coffee (De Roos *et al.*, 2000). Moderate alcohol consumption does not affect plasma PLTP activity (van Tol and Hendriks, 2001), but increased levels were reported in alcohol abusers (Liinamaa *et al.*, 1997), and after a large intravenous fat load or by increasing the fatty acid content of the diet (Lagrost *et al.*, 1999b; Riemens *et al.*, 1999). Smoking has been associated with reduced plasma PLTP activity levels (Mero *et al.*, 1998; Zaratin *et al.*, 2004), although there is also one report of increased plasma PLTP activity levels in smokers (Dullaart *et al.*, 1994a).

Various pathophysiological conditions associated with altered plasma lipid levels and lipoprotein subclass distribution, have also been associated with changes in phospholipid transfer activity of PLTP. High PLTP activity levels are frequently found in plasma of subjects suffering from familial combined hyperlipidemia (Soro *et al.*, 2003), obese subjects (Dullaart *et al.*, 1994b; Kaser *et al.*, 2001; Riemens *et al.*, 1998), and patients with diabetes mellitus type 1 and type 2 (Colhoun *et al.*, 2001; de Vries *et al.*, 2007; de Vries *et al.*, 2008; Dullaart *et al.*, 2008a). In diabetic patients, an independent association between C-reactive protein and PLTP activity has been reported, suggesting that subclinical inflammation may influence PLTP activity (Tan *et al.*, 2005). Also in patients with systemic inflammation or severe sepsis, plasma PLTP activity levels are increased (Barlage *et al.*, 2001; Levels *et al.*, 2007). Probably, the decrease of HDL levels observed during a severe acute phase inflammation response is caused by a combination of reduced LCAT and increased PLTP activities (Pussinen *et al.*, 2001a; Pussinen *et al.*, 2001b). In patients with Alzheimer's disease, PLTP levels in brain tissue are significantly increased, whereas in cerebrospinal fluid PLTP activity is reduced compared to control subjects (Vuletic *et al.*, 2003). A role of PLTP-mediated apoE secretion is suggested in the pathogenesis of the disease (Vuletic *et al.*, 2005).

The relationship between plasma PLTP activity levels and cardiovascular disease is not unequivocal. Increased plasma PLTP activity has been demonstrated in patients with increased risk for cardiovascular disease, implying that the protein could play a role in the development of the disease (Ruhling *et al.*, 1999; Schlitt *et al.*, 2003; Schlitt *et al.*, 2007). However, another study indicated that low rather than high PLTP activity is a marker for peripheral atherosclerosis and that the distribution of PLTP between high-activity and low-activity forms may be disturbed in peripheral artery disease (Schgoer *et al.*, 2008). A prospective study in a cohort of Japanese men indicated that an elevated concentration of PLTP may be a protective marker of cardiovascular heart disease (Yatsuya *et al.*, 2004). Taken together, literature demonstrates that changes in PLTP activity can be associated

with pathophysiological conditions such as hyperlipidemia, inflammation, and diabetes, but there is no consensus about the relationship between PLTP and cardiovascular disease.

1.4.6 Mouse studies

PLTP deficient mice

The physiological role of PLTP in lipoprotein metabolism has been studied extensively using PLTP-deficient mouse models, as summarized in Table 3. To evaluate the role of PLTP in lipoprotein metabolism, Jiang *et al.* created a PLTP knockout mouse model (Jiang *et al.*, 1999). In chow-fed mice, targeted mutation of the PLTP gene resulted in a strong decrease in HDL phospholipid (60%), cholesterol (65%) and apoAI (85%), but no changes in non-HDL lipid or apoB levels. Low HDL levels in PLTP deficient mice arise from a marked increase in HDL catabolism, both of the protein and cholesteryl ester content of the particles (Qin *et al.*, 2000). The increased HDL catabolism is possibly related to a decrease in phospholipid content of the HDL particles. In addition, PLTP deficiency might lead to destabilization of liver-secreted apoAI, due to defective phospholipidation. This indicates a possible intracellular role for PLTP in the formation of nascent HDL particles (Siggins *et al.*, 2007). On a high fat diet, next to decreased HDL levels, also non-HDL levels are significantly altered in PLTP deficient mice (Jiang *et al.*, 1999). VLDL and LDL phospholipids, free cholesterol and cholesteryl esters levels are largely increased without changes in apoB levels, suggesting accumulation of surface components of triglyceride-rich lipoproteins. These findings show the importance of PLTP activity for the transfer of surface components of triglyceride-rich lipoproteins to HDL. Although non-HDL levels are significantly increased in PLTP knockout mice on a high fat diet, when crossed into an apoB transgenic or an apoE deficient background, PLTP deficiency reduced plasma levels of apoB-rich lipoproteins (Jiang *et al.*, 2001). Isolated hepatocytes from these mice revealed a decreased secretion rate of apoB-rich lipoproteins, probably explained by the fact that newly synthesized apoB is destructed by reactive oxygen species, generated due to decreased vitamin E content (Jiang *et al.*, 2005). The decreased plasma levels and production rate of apoB-containing lipoproteins could explain the finding that PLTP deficient mice have a decreased susceptibility to develop atherosclerosis compared to mice expressing PLTP (Jiang *et al.*, 2001). An additional explanation for the atheroprotective effect of PLTP deficiency is the fact that circulating apoB-containing lipoproteins are protected from oxidation due to an increase in the bioavailability of plasma vitamin E (Jiang *et al.*, 2002; Yan *et al.*, 2004). Isolated LDL from PLTP deficient mice induced significantly less monocyte chemotactic stimulation (MCS) than LDL isolated from wild type mice. Furthermore, the ability of isolated HDL from PLTP deficient mice to remove oxidized phospholipids from LDL was significantly increased. In addition, Schlitt *et al.* stated that PLTP deficiency has an anti-

Table 3. Studies in PLTP deficient mouse models.

Background	Pro-atherogenic characteristics	Anti-atherogenic characteristics	Atherogenicity	Reference
wild type	non-HDL ↑ (diet), HDL ↓	–	N/D	(Jiang *et al.*, 1999)
wild type	HDL ↓, HDL catabolism ↑	–	N/D	(Qin *et al.*, 2000)
wild type	–	plasma vitamin E ↑, oxidation VLDL/LDL ↓	N/D	(Jiang *et al.*, 2002)
wild type	–	IL-6 ↓	N/D	(Schlitt *et al.*, 2005)
wild type	HDL, apoAI ↓, non-HDL ↑, hepatic lipids ↑	–	N/D	(Siggins *et al.*, 2007)
wild type	–	intestinal chol uptake ↓, chol secretion from enterocytes ↓	N/D	(Liu *et al.*, 2007b)
LDLR -/-	HDL ↓	–	decreased	(Jiang *et al.*, 2001)
LDLR -/-	–	plasma vitamin E ↑, oxidation VLDL/LDL ↓	N/D	(Jiang *et al.*, 2002)
LDLR -/-	HDL ↓, HDL catabolism ↑	anti-inflamm properties of HDL ↑, LDL-induced MCS ↓	N/D	(Yan *et al.*, 2004)
apoE -/-	–	apoB secretion ↓, apoB levels ↓, non-HDL ↓	decreased	(Jiang *et al.*, 2001)
apoE -/-	–	plasma vitamin E ↑, oxidation VLDL/LDL ↓	N/D	(Jiang *et al.*, 2002)
apoB tg	HDL ↓	apoB secretion ↓, apoB levels ↓, non-HDL ↓	decreased	(Jiang *et al.*, 2001)
apoB tg	HDL ↓, HDL catabolism ↑	anti-inflamm properties of HDL ↑, LDL-induced MCS ↓	N/D	(Yan *et al.*, 2004)
CETP tg	HDL, apoAI ↓	–	N/D	(Kawano *et al.*, 2000)
apoB*CETP tg	–	plasma vitamin E ↑, oxidation VLDL/LDL ↓	N/D	(Jiang *et al.*, 2002)

MCS: monocyte chemotactic stimulation

inflammatory effect per se, as mice lacking PLTP have significantly lower IL-6 levels than wild type mice, and a reduced inflammatory response to IL-6 treatment (Schlitt *et al.*, 2005). Liu *et al.* demonstrated that PLTP deficient mice have a diminished cholesterol absorption, attributable to a decreased cholesterol uptake by enterocytes, and subsequently a reduced cholesterol secretion from these cells (Liu *et al.*, 2007b).

PLTP overexpressing mice

Also experimental work in different mouse models overexpressing PLTP has greatly contributed to the clarification of the role of PLTP in lipoprotein metabolism and the development of atherosclerosis. Table 4 summarizes the different studies that have been performed in PLTP overexpressing mouse models. The first transgenic mice expressing human PLTP were generated by Albers *et al.* (Albers *et al.*, 1996). In these transgenic mice expression levels of human PLTP were minimal, and transgenic mice and control mice had similar plasma PLTP activity levels. Not surprisingly, no marked differences in lipoprotein levels were observed. Shortly after, Jiang *et al.* generated another human PLTP-transgenic mouse (Jiang *et al.*, 1996). This transgenic mouse showed a broad human PLTP mRNA expression. Plasma PLTP activity was increased by 29% in transgenic mice, but plasma lipoprotein analysis did not reveal significant changes in plasma lipids or apolipoprotein levels. When these human PLTP-transgenic mice were crossbred with human apoAI transgenic mice, PLTP activity levels were increased by 47% and. A 56% increase in preβ-HDL was observed, indicating a potential anti-atherogenic effect of increased plasma PLTP activity levels. Föger *et al.* used a recombinant adenoviral vector containing human PLTP cDNA to establish the role of PLTP in lipoprotein metabolism. In line with the findings of Jiang *et al.*, PLTP induced the generation of preβ-HDL. However, induction of human PLTP expression also strongly lowered total plasma HDL levels in a dose-dependent way, due to an accelerated fractional catabolism of HDL (Föger *et al.*, 1997). These findings demonstrated the involvement of PLTP in HDL remodeling *in vivo*. Also the group of Ehnholm used an adenoviral gene transfer system, resulting in the liver-specific expression of human PLTP in wild type mice (C57/Bl) and human apoAI transgenic mice (Ehnholm *et al.*, 1998). Overexpression of human PLTP, resulting in 4-fold increased plasma PLTP activity levels, strongly reduced total plasma cholesterol and phospholipid levels, due to a dramatic reduction of HDL levels. Overexpression of PLTP in apoAI transgenic mice redistributed HDL particles, with the formation of larger HDL species.

To further elucidate the involvement of PLTP in lipoprotein metabolism, our group generated human PLTP transgenic mice with a 2.5- to 4.5-fold increase in plasma PLTP activity, resulting in a 30% to 40% decreased HDL-cholesterol level (van Haperen *et al.*, 2000). Absolute preβ-HDL levels were not increased in PLTP overexpressing mice compared to

wild type mice, as was the case in the mouse models of Jiang *et al.* and Föger *et al.*, but the formation of preβ-HDL in plasma of human PLTP expressing mice was 2- to 3-fold increased. On the absolute level, preβ-HDL is only a minor subfraction of the total HDL level, but as a very efficient acceptor of cellular cholesterol it is a key mediator in the reverse cholesterol transport. Therefore, it was suggested that although PLTP lowers absolute HDL levels, it might have an atheroprotective potential by induction of preβ-HDL generation, in this way preventing cellular cholesterol overload and foam cell formation. An increased faecal bile acid excretion in human PLTP transgenic mice supported this hypothesis (Post *et al.*, 2003). In order to provide more insight into the relationship between plasma PLTP activity, lipoprotein levels, and atherogenesis, different human PLTP transgenic mouse lines were generated with varying levels of PLTP activity (van Haperen *et al.*, 2002). These mouse lines were crossed into a hemizygous LDL receptor knockout background and fed a high fat/ high cholesterol diet. Overexpression of human PLTP dose-dependently deceased plasma HDL levels. In addition, increased plasma PLTP activity resulted in a moderate stimulation of hepatic VLDL secretion. Importantly, elevation of PLTP activity levels increased the susceptibility to develop atherosclerosis in a dose-responsive manner, regardless of the site of PLTP production (ubiquitous expression versus liver-specific expression). In accordance with this study, long-term adenoviral overexpression of PLTP in apoE deficient mice resulted in an increase in atherosclerotic lesions in the proximal aorta (Yang *et al.*, 2003). The increased PLTP activity in these mice resulted in a decrease in plasma vitamin E levels and vitamin E content of individual lipoprotein fractions, thereby increasing lipoprotein oxidizability. Also human PLTP transgenic mice in a CETP transgenic/LDLR deficient background had an increased atherosclerosis susceptibility, even despite lower levels of apoB-containing lipoproteins (Lie *et al.*, 2004).

Macrophage-specific targeting of PLTP in mice

All of the above mentioned studies focused on the effects of systemic PLTP on lipoprotein metabolism and atherogenesis. However, during the last three years, much attention has been paid specifically to the functionality of macrophage-derived PLTP. Macrophages, being a key component of atherosclerotic lesions, have been demonstrated to highly express PLTP (Desrumaux *et al.*, 2003; Laffitte *et al.*, 2003; O'Brien *et al.*, 2003). Cholesterol loading of macrophages results in an increase in PLTP mRNA, protein expression, and activity (Desrumaux *et al.*, 2003; O'Brien *et al.*, 2003). Plasma PLTP has been shown to enhance preß-HDL formation, thereby generating efficient acceptor particles for cholesterol efflux from macrophages. However, it is uncertain whether macrophage-derived PLTP within the artery wall influences the development of atherosclerosis. In order to elucidate the role of macrophage-derived PLTP in atherogenesis, a series of bone marrow transplantation studies

Table 4. Human PLTP overexpressing mouse models.

PLTP construct	Background	PLTP act	Phenotype	Atherogenicity	Reference
endogenous promoter +human PLTP gene	wild type	no effect	HDL ↑ non-HDL ↓	N/D	(Albers *et al.*, 1996)
endogenous promoter +human PLTP gene	wild type	+ 29%	no effect	N/D	(Jiang *et al.*, 1996)
endogenous promoter +human PLTP gene	apoAI tg	+ 47%	HDL, apoAI ↑ preβ-HDL ↑	N/D	(Jiang *et al.*, 1996)
endogenous promoter +human PLTP gene	wild type	no effect	no effect	N/D	(Tu *et al.*, 1999)
endogenous promoter +human PLTP gene	wild type	+ 250-450%	TC ↓ HDL, apoAI ↓ preβ-HDL formation ↑	N/D	(van Haperen *et al.*, 2000)
endogenous promoter +human PLTP gene	CETP tg	+ 400%	HDL ↓ preβ-HDL formation ↑	N/D	(Lie *et al.*, 2001)
endogenous promoter +human PLTP gene	wild type	+150-350%	TC ↓ apoAI ↓	N/D	(van Haperen *et al.*, 2002)
endogenous promoter +human PLTP gene	LDLR +/-	+200-250%	HDL ↓ VLDL secretion ↑	increased	(van Haperen *et al.*, 2002)
endogenous promoter +human PLTP gene	CETP tg	+200%	TC ↓ HDL ↓ VLDL secretion ↑	N/D	(Lie *et al.*, 2002)
endogenous promoter +human PLTP gene	CETP tg*LDLR +/-	+150%	TC ↓ HDL ↓ non-HDL ↓	increased	(Lie *et al.*, 2004)

(continued) **PLTP construct**	**Background**	**PLTP act**	**Phenotype**	**Atherogenicity**	**Reference**
albumin promoter +human PLTP gene	wild type	+250-900%	TC ↓ apoAI ↓	N/D	(van Haperen *et al.*, 2002)
albumin promoter +human PLTP gene	LDLR +/-	+300-800%	HDL ↓ VLDL secretion ↑	increased	(van Haperen *et al.*, 2002)
albumin promoter +human PLTP gene	CETP tg*LDLR +/-	+600%	TC ↓ HDL ↓ non-HDL ↓	increased	(Lie *et al.*, 2004)
albumin promoter +human PLTP gene	wild type	+1500%	TC ↓ HDL ↓ faecal bile acids excr ↑	N/D	(Post *et al.*, 2003)
CMV +human cDNA[#]	wild type	+1300-4000%	HDL, apoAI ↓ HDL catabolism ↑ preβ-HDL formation ↑	N/D	(Föger *et al.*, 1997)
CMV + human cDNA[#]	wild type	+350%	HDL ↓ preβ-HDL formation ↑	N/D	(Jaari *et al.*, 2001)
CMV +human cDNA[#]	wild type	+300%	TC ↓ HDL ↓	N/D	(Ehnholm *et al.*, 1998)
CMV +human cDNA[#]	apoAI tg	+165%	TC ↓ HDL ↓ redistribution of HDL	N/D	(Ehnholm *et al.*, 1998)
albumin promoter +mouse cNDA[#]	apoE -/-	+130-200%	HDL, apoAI ↓ plasma vitamin E ↓ lipoprotein oxidation ↑	increased	(Yang *et al.*, 2003)

[#] adenovirus-mediated

has been performed in which bone marrow of mice either lacking or expressing PLTP was transplanted into acceptor mice. An overview of these studies is provided in Table 5. Both in LDLR knockout and in apoE knockout mice, macrophage-specific PLTP deficiency resulted in an increase in atherosclerosis development (Liu *et al.*, 2007a; Valenta *et al.*, 2006). The transplantation of either PLTP deficient or wild type bone marrow into PLTP deficient mice in an LDLR knockout background confirmed the atheroprotective potential of macrophage-derived PLTP (Valenta *et al.*, 2008). Different mechanisms have been suggested to explain a possible atheroprotective role of macrophage-derived PLTP. Macrophage-derived PLTP may have an indirect role in cellular cholesterol efflux by the generation of preβ-HDL particles. However, this atheroprotective effect could be neutralized by the presence of abundant apoAI (Valenta *et al.*, 2006). In addition, PLTP might stimulate ABCA1-dependent lipid efflux from macrophages (Lee-Rueckert *et al.*, 2006). Another explanation for the possible anti-atherogenic properties of macrophage-derived PLTP might be the ability of macrophage-derived PLTP to reduce cholesterol accumulation in macrophages through changes in the alpha-tocopherol content and oxidative status of the cells. Ogier *et al.* demonstrated that macrophages isolated from PLTP deficient mice have an increased basal cholesterol content and an increased cholesterol accumulation in the presence of LDL (Ogier *et al.*, 2007). Furthermore, macrophage-PLTP might play a role in apoE secretion and the clearance of lipids from the circulation. PLTP-deficient macrophages were shown to secrete significantly less apoE than wild type macrophages, which was associated with the accumulation of cholesterol in the circulation (Liu *et al.*, 2007a).

In contrast, there is also evidence for a possible pro-atherogenic role for macrophage-derived PLTP. Vikstedt *et al.* used a comparable experimental setup as Valenta *et al.* to study the effect of macrophage-specific PLTP deficiency. Macrophage-specific PLTP contributed significantly to plasma PLTP activity level (Vikstedt *et al.*, 2007b). In contrast to Valenta's finding, in this study PLTP deficiency in macrophages resulted in a decreased atherosclerotic lesion development, increased apoAI levels and lower VLDL/LDL levels, suggesting a pro-atherogenic role for PLTP in the vessel wall. The contribution of PLTP to atherosclerosis development might be determined by the balance between the pro-atherogenic properties of plasma PLTP activity and the anti-atherogenic properties of local PLTP activity in the vessel wall. The fact that the net result of this balance is opposite in the studies of Valenta and Vikstedt might be due to differences in experimental setup, such as recovery time after irradiation, diet composition, and gender of recipient mice. As a consequence, the extent to which macrophage-derived PLTP contributed to systemic PLTP levels differed significantly between these studies. Overall, the contradictory results of these bone-marrow transplantation studies indicate that the role of macrophage-PLTP in lipoprotein metabolism and the development of atherosclerosis is far from resolved.

Table 5. Macrophage-specific targeting of PLTP in mice.

Donor[#]	Recipient	PLTP act	Phenotype	Athero-genicity	Reference
PLTP -/- wild type	LDLR -/-	-32%	minimal	increased	(Valenta *et al.*, 2006)
PLTP -/- wild type	apoAI-/-*LDLR -/-*hapoAI tg	-12%	minimal	not affected	(Valenta *et al.*, 2006)
PLTP -/- wild type	apoE -/-	-20%	plasma TC, PL ↑ plasma apoE ↓	increased	(Liu *et al.*, 2007a)
PLTP -/- wild type	LDLR -/-	-50%	plasma TC, PL ↓ plasma TG ↓ apoAI ↑	decreased	(Vikstedt *et al.*, 2007b)
PLTP -/- wild type	LDLR -/-*PLTP -/-	N/D	plasma TC, TG ↑ plasma HDL ↓ apoAI ↓	increased	(Valenta *et al.*, 2008)

[#] Note: all comparisons in the table are PLTP -/- versus wild type bone marrow

1.5 Outline of the thesis

The exact role of PLTP in lipoprotein metabolism and cardiovascular disease is far from resolved. On the one hand, PLTP clearly possesses several properties that can be considered pro-atherogenic. Increased plasma PLTP activity levels result in decreased plasma HDL levels. In addition, PLTP stimulates the production of apoB-containing lipoproteins. Furthermore, PLTP might directly influence the inflammatory response and oxidation of lipoproteins. On the other hand, the ability of PLTP to enhance the net transfer of phospholipids from VLDL to HDL during lipolysis might provide a gradient that maintains the flux of lipids from peripheral cells to the liver for breakdown and excretion. In addition, during HDL conversion PLTP generates preß-HDL, a quantitatively minor HDL subclass that is an efficient cholesterol acceptor *in vitro*. PLTP might stimulate the efflux of cholesterol and phospholipids from cells to lipoprotein particles, clearly an anti-atherogenic effect. By the experiments described in this thesis, we tried to further elucidate the exact role of PLTP in lipoprotein metabolism and atherogenesis. For this purpose, we focussed on the systemic and cellular effects of abundant PLTP expression, using the mouse as research model.

Despite potential anti-atherogenic effects of PLTP, different studies in atherosclerosis-prone mouse models revealed that overexpression of PLTP increases the susceptibility to develop atherosclerosis. This implies that the pro-atherogenic properties of PLTP outweigh its anti-atherogenic properties, suggesting that an increase in PLTP activity might be a

long-term risk factor for atherosclerosis development in humans. However, the effect of PLTP overexpression on lipoprotein metabolism and atherogenesis might be strongly influenced by metabolic status and experimental setup. A different and more controlled experimental approach could further explain the impact of PLTP on lipoprotein metabolism and atherogenesis. The effects of acute changes in plasma PLTP expression have not been addressed before, and could provide important insights into the physiological role of PLTP. For this purpose, we generated a new mouse model with conditional expression of the human PLTP gene, using the tetracycline-responsive gene system. In **chapter 2** we describe the development and characteristics of transgenic mice with conditional PLTP overexpression. Using this model we studied the effects of acute changes in PLTP activity on lipoprotein metabolism. In a consecutive study, we used the model to investigate the effects of PLTP expression on lipoprotein metabolism under diet-induced lipidemic conditions, and to determine the effects of PLTP expression on pre-existing atherosclerosis **(chapter 3)**.
The role of PLTP in atherosclerosis development has been studied extensively in many different mouse models, focussing either on the complete absence of plasma PLTP, or on the overexpression of PLTP, which results in increased plasma phospholipid transfer activity levels. The importance of this lipid transfer activity *in vivo* is not completely understood. In human plasma, two forms of PLTP have been described; the catalytically active form, and an inactive form. In order to provide more insight into the role of PLTP transfer activity and PLTP mass, we generated a new mouse model with overexpression of mutant human PLTP, deficient in phospholipid transfer activity but still capable of binding to HDL (**chapter 4**). Using this mouse model we investigated the importance of PLTP transfer activity in lipoprotein levels, preβ-HDL formation, hepatic VLDL secretion and the development of atherosclerosis.

It is important to note that PLTP is a lipid transfer protein and thus very sensitive to environmental changes in the plasma compartment. The absence or presence of certain proteins that act in close relationship with PLTP might strongly influence the action of human PLTP. Therefore, we assessed the effects of increased PLTP expression in two 'humanized' mouse models: the human apoAI transgenic and the human CETP transgenic mouse. In **chapter 5**, we studied the effect of increased human PLTP expression on different HDL properties (size, density, functionality) in a human apoAI transgenic mouse model. In addition, the effect of the introduction of the human PLTP gene in a human apoAI transgenic mouse on atherosclerotic lesion development was determined. In **chapter 6** we describe the effects of PLTP in another humanized mouse model, the human CETP transgenic mouse. In this mouse model, we studied which of two main pro-atherogenic effects of PLTP, either lowering of plasma HDL levels or stimulation of apoB-containing lipoproteins production, might be of more importance for the increased atherosclerosis development in PLTP

transgenic mice. For this purpose, we studied sex differences in lipoprotein metabolism and linked these differences to atherosclerosis development in human PLTP transgenic mice. Combining transgenic traits by cross-breeding is not only a good approach to generate a 'humanized' mouse model in which the role of PLTP can be studied, but it is also an interesting way to study a possible functional interaction between PLTP and another protein. Hepatic lipase is such a protein that might affect the function of PLTP in plasma. Hepatic lipase is a lipolytic enzyme that is involved in the conversion of VLDL into LDL, and in HDL remodeling, two pathways that the enzyme shares with PLTP. We investigated the possibility of a functional relationship between PLTP and hepatic lipase, as described in **chapter 7**. We assessed the effect of PLTP overexpression on hepatic lipase activity, and the effect of hepatic lipase deficiency on PLTP activity. Furthermore, we investigated whether the presence of hepatic lipase is required for the PLTP-dependent increase in hepatic triglyceride secretion or for PLTP-mediated HDL catabolism.

In another set of experiments, described in **chapter 8**, we focussed on the relationship between PLTP expression and plasma HDL levels. In Tangier disease patients, extremely low HDL levels are found due to a defective ABCA1 transporter. In these patients, plasma PLTP activity levels are strongly reduced. A comparable phenotype is observed in ABCA1 knockout mice. We investigated how ABCA1 deficiency affects PLTP expression in mice by studying PLTP synthesis, secretion and plasma distribution. In addition, we treated different mouse models with varying plasma HDL and PLTP levels with a synthetic ligand of the liver X receptor (LXR), thereby inducing changes in lipoprotein metabolism and PLTP expression. We investigated whether induced PLTP levels and PLTP distribution in plasma were influenced by plasma lipoprotein levels and distribution.

In the last part of this thesis, we focussed on the functionality of cellular, macrophage-PLTP besides its systemic role in lipid metabolism and atherogenesis. For decennia, atherosclerosis was defined as a disease of the liver. Liver and plasma lipoprotein metabolism took the main role in investigation of atherogenesis. However, during the last 10 years, increasing attention has been paid to inflammatory and local processes in the vessel wall. In earlier chapters, we extensively clarified the effect of elevated systemic PLTP levels on lipoprotein metabolism and atherosclerosis development in mice. However, the macrophage is a key component of the atherosclerotic lesion and PLTP has been shown to colocalise with foam cells in atherosclerotic lesions. In **chapter 9,** we demonstrated the contribution of macrophage-derived PLTP to plasma phospholipid transfer activity levels and atherosclerosis development. Therefore, bone marrow transplantations from PLTP transgenic mice or wild type mice to LDLR-deficient mice were performed, and the development of diet-induced atherosclerosis was studied. Different research groups

suggest that macrophage-derived PLTP could have an atheroprotective role by enhancing cholesterol efflux and reverse cholesterol transport through the generation of preß-HDL. PLTP has been shown to play a key role in HDL metabolism and reverse cholesterol transport *in vitro*. However, until recently, quantification of reverse cholesterol transport *in vivo* was performed using techniques determining the transport rate of cholesterol derived from the whole body. The recent development of an assay in which lipid-loaded macrophages are injected intraperitoneally in mice, with subsequent monitoring of the efflux of cholesterol from these cells to the plasma compartment and subsequent faecal excretion, allowed us to measure reverse cholesterol transport specifically from macrophages. In **chapter 10**, we studied the effect of high systemic PLTP levels on macrophage reverse cholesterol transport, as well as the effect of elevated macrophage-derived PLTP expression on reverse cholesterol transport in mice.

In **chapter 11**, the results of all these studies are reviewed. The new insights into the role of PLTP in lipoprotein metabolism and atherogenesis, and perspectives for future research are discussed.

1.6 References

Acton, S., Rigotti, A., Landschulz, K.T., Xu, S., Hobbs, H.H., and Krieger, M. (1996). Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science *271*, 518-520.

Aiello, R.J., Brees, D., Bourassa, P.A., Royer, L., Lindsey, S., Coskran, T., Haghpassand, M., and Francone, O.L. (2002). Increased atherosclerosis in hyperlipidemic mice with inactivation of ABCA1 in macrophages. Arterioscler Thromb Vasc Biol *22*, 630-637.

Alam, K., Meidell, R.S., and Spady, D.K. (2001). Effect of up-regulating individual steps in the reverse cholesterol transport pathway on reverse cholesterol transport in normolipidemic mice. J Biol Chem *276*, 15641-15649.

Albers, J.J., Tollefson, J.H., Chen, C.H., and Steinmetz, A. (1984). Isolation and characterization of human plasma lipid transfer proteins. Arteriosclerosis *4*, 49-58.

Albers, J.J., Tu, A. Y., Paigen, B., Chen, H., Cheung, M.C., and Marcovina, S.M. (1996). Transgenic mice expressing human phospholipid transfer protein have increased HDL/non-HDL cholesterol ratio. Int J Clin Lab Res *26*, 262-267.

Albers, J.J., Wolfbauer, G., Cheung, M. C., Day, J.R., Ching, A.F., Lok, S., and Tu, A.Y. (1995). Functional expression of human and mouse plasma phospholipid transfer protein: effect of recombinant and plasma PLTP on HDL subspecies. Biochim Biophys Acta *1258*, 27-34.

Alonzi, T., Mancone, C., Amicone, L., and Tripodi, M. (2008). Elucidation of lipoprotein particles structure by proteomic analysis. Expert Rev Proteomics *5*, 91-104.

Anant, S., and Davidson, N.O. (2001). Molecular mechanisms of apolipoprotein B mRNA editing. Curr Opin Lipidol *12*, 159-165.

Aouizerat, B.E., Engler, M.B., Natanzon, Y., Kulkarni, M., Song, J., Eng, C., Huuskonen, J., Rivera, C., Poon, A., Bensley, M., *et al.* (2006). Genetic variation of PLTP modulates lipoprotein profiles in hypoalphalipoproteinemia. J Lipid Res *47*, 787-793.

Arai, T., Wang, N., Bezouevski, M., Welch, C., and Tall, A.R. (1999). Decreased atherosclerosis in heterozygous low density lipoprotein receptor-deficient mice expressing the scavenger receptor BI transgene. J Biol Chem *274*, 2366-2371.

Arakawa, R., and Yokoyama, S. (2002). Helical apolipoproteins stabilize ATP-binding cassette transporter A1 by protecting it from thiol protease-mediated degradation. J Biol Chem *277*, 22426-22429.

Asztalos, B.F., Collins, D., Horvath, K.V., Bloomfield, H.E., Robins, S.J., and Schaefer, E.J. (2008). Relation of gemfibrozil treatment and high-density lipoprotein subpopulation profile with cardiovascular events in the Veterans Affairs High-Density Lipoprotein Intervention Trial. Metabolism *57*, 77-83.

Babaev, V. R., Patel, M.B., Semenkovich, C.F., Fazio, S., and Linton, M.F. (2000). Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in low density lipoprotein receptor-deficient mice. J Biol Chem *275*, 26293-26299.

Baldan, A., Pei, L., Lee, R., Tarr, P., Tangirala, R.K., Weinstein, M.M., Frank, J., Li, A.C., Tontonoz, P., and Edwards, P.A. (2006). Impaired development of atherosclerosis in hyperlipidemic Ldlr-/- and ApoE-/- mice transplanted with Abcg1-/- bone marrow. Arterioscler Thromb Vasc Biol *26*, 2301-2307.

Banka, C.L. (1996). High density lipoprotein and lipoprotein oxidation. Curr Opin Lipidol *7*, 139-142.

Barlage, S., Frohlich, D., Bottcher, A., Jauhiainen, M., Muller, H.P., Noetzel, F., Rothe, G., Schutt, C., Linke, R.P., Lackner, K.J., *et al.* (2001). ApoE-containing high density lipoproteins and phospholipid transfer protein activity increase in patients with a systemic inflammatory response. J Lipid Res *42*, 281-290.

Barter, P.J., Baker, P.W., and Rye, K.A. (2002). Effect of high-density lipoproteins on the expression of adhesion molecules in endothelial cells. Curr Opin Lipidol *13*, 285-288.

Beisiegel, U. (1996). New aspects on the role of plasma lipases in lipoprotein catabolism and atherosclerosis. Atherosclerosis *124*, 1-8.

Berard, A.M., Föger, B., Remaley, A., Shamburek, R., Vaisman, B.L., Talley, G., Paigen, B., Hoyt, R.F., Jr., Marcovina, S., Brewer, H.B., Jr., and Santamarina-Fojo, S. (1997). High plasma HDL concentrations associated with enhanced atherosclerosis in transgenic mice overexpressing lecithin-cholesteryl acyltransferase. Nat Med *3*, 744-749.

Berge, K.E., Tian, H., Graf, G. A., Yu, L., Grishin, N.V., Schultz, J., Kwiterovich, P., Shan, B., Barnes, R., and Hobbs, H.H. (2000). Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. Science *290*, 1771-1775.

Berglund, L., and Ramakrishnan, R. (2004). Lipoprotein(a): an elusive cardiovascular risk factor. Arterioscler Thromb Vasc Biol *24*, 2219-2226.

Beyer, T.P., Schmidt, R. J., Foxworthy, P., Zhang, Y., Dai, J., Bensch, W.R., Kauffman, R.F., Gao, H., Ryan, T.P., Jiang, X.C., *et al.* (2004). Coadministration of a liver X receptor agonist and a peroxisome proliferator activator receptor-alpha agonist in Mice: effects of nuclear receptor interplay on high-density lipoprotein and triglyceride metabolism in vivo. J Pharmacol Exp Ther *309*, 861-868.

Bodzioch, M., Orso, E., Klucken, J., Langmann, T., Bottcher, A., Diederich, W., Drobnik, W., Barlage, S., Buchler, C., Porsch-Ozcurumez, M., *et al.* (1999). The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. Nat Genet *22*, 347-351.

Bosse, Y., Bouchard, L., Despres, J.P., Bouchard, C., Perusse, L., and Vohl, M.C. (2005). Haplotypes in the phospholipid transfer protein gene are associated with obesity-related phenotypes: the Quebec Family Study. Int J Obes (Lond) *29*, 1338-1345.

Bouly, M., Masson, D., Gross, B., Jiang, X.C., Fievet, C., Castro, G., Tall, A.R., Fruchart, J.C., Staels, B., Lagrost, L., and Luc, G. (2001). Induction of the phospholipid transfer protein gene accounts for the high density lipoprotein enlargement in mice treated with fenofibrate. J Biol Chem *276*, 25841-25847.

Braun, A., Trigatti, B.L., Post, M.J., Sato, K., Simons, M., Edelberg, J.M., Rosenberg, R.D., Schrenzel, M., and Krieger, M. (2002). Loss of SR-BI expression leads to the early onset of occlusive atherosclerotic coronary artery disease, spontaneous myocardial infarctions, severe cardiac dysfunction, and premature death in apolipoprotein E-deficient mice. Circ Res *90*, 270-276.

Brown, M.S., and Goldstein, J.L. (1976). Receptor-mediated control of cholesterol metabolism. Science *191*, 150-154.

Brown, M.S., and Goldstein, J.L. (1986). A receptor-mediated pathway for cholesterol homeostasis. Science *232*, 34-47.

Calpe-Berdiel, L., Rotllan, N., Fievet, C., Roig, R., Blanco-Vaca, F., and Escola-Gil, J.C. (2008). Liver X receptor-mediated activation of reverse cholesterol transport from macrophages to feces in vivo requires ABCG5/G8. J Lipid Res *49*, 1904-1911.

Cao, G., Beyer, T.P., Yang, X.P., Schmidt, R.J., Zhang, Y., Bensch, W.R., Kauffman, R.F., Gao, H., Ryan, T. P., Liang, Y., *et al.* (2002). Phospholipid transfer protein is regulated by liver X receptors in vivo. J Biol Chem *277*, 39561-39565.

Castrillo, A., and Tontonoz, P. (2004). Nuclear receptors in macrophage biology: at the crossroads of lipid metabolism and inflammation. Annu Rev Cell Dev Biol *20*, 455-480.

Cavelier, L.B., Qiu, Y., Bielicki, J.K., Afzal, V., Cheng, J.F., and Rubin, E.M. (2001). Regulation and activity of the human ABCA1 gene in transgenic mice. J Biol Chem *276*, 18046-18051.

Cheung, M.C., and Albers, J.J. (2006). Active plasma phospholipid transfer protein is associated with apoA-I- but not apoE-containing lipoproteins. J Lipid Res *47*, 1315-1321.

Cheung, M.C., Knopp, R.H., Retzlaff, B., Kennedy, H., Wolfbauer, G., and Albers, J.J. (2002). Association of plasma phospholipid transfer protein activity with IDL and buoyant LDL: impact of gender and adiposity. Biochim Biophys Acta *1587*, 53-59.

Cheung, M.C., Wolfbauer, G., and Albers, J.J. (1996). Plasma phospholipid mass transfer rate: relationship to plasma phospholipid and cholesteryl ester transfer activities and lipid parameters. Biochim Biophys Acta *1303*, 103-110.

Colhoun, H.M., Scheek, L.M., Rubens, M.B., Van Gent, T., Underwood, S.R., Fuller, J.H., and Van Tol, A. (2001). Lipid transfer protein activities in type 1 diabetic patients without renal failure and nondiabetic control subjects and their association with coronary artery calcification. Diabetes *50*, 652-659.

Cooper, A.D. (1997). Hepatic uptake of chylomicron remnants. J Lipid Res *38*, 2173-2192.

Covey, S.D., Krieger, M., Wang, W., Penman, M., and Trigatti, B.L. (2003). Scavenger receptor class B type I-mediated protection against atherosclerosis in LDL receptor-negative mice involves its expression in bone marrow-derived cells. Arterioscler Thromb Vasc Biol *23*, 1589-1594.

Dallinga-Thie, G.M., Zonneveld-de Boer, A.J., van Vark-van der Zee, L.C., van Haperen, R., van Gent, T., Jansen, H., De Crom, R., and van Tol, A. (2007). Appraisal of hepatic lipase and lipoprotein lipase activities in mice. J Lipid Res *48*, 2788-2791.

Damen, J., Regts, J., and Scherphof, G. (1982). Transfer of [14C]phosphatidylcholine between liposomes and human plasma high density lipoprotein. Partial purification of a transfer-stimulating plasma factor using a rapid transfer assay. Biochim Biophys Acta *712*, 444-452.

Davis, R.A. (1996). Structure, assembly and secretion of lipoproteins. Biochemistry of lipids, lipoproteins and membranes Elsevier, New York, NY *Edited by Vance, D.E. and Vance, J.E.*, 473–493.

Day, J.R., Albers, J.J., Lofton-Day, C.E., Gilbert, T.L., Ching, A.F., Grant, F.J., O'Hara, P.J., Marcovina, S.M., and Adolphson, J.L. (1994). Complete cDNA encoding human phospholipid transfer protein from human endothelial cells. J Biol Chem *269*, 9388-9391.

De Roos, B., Van Tol, A., Urgert, R., Scheek, L.M., Van Gent, T., Buytenhek, R., Princen, H.M., and Katan, M.B. (2000). Consumption of French-press coffee raises cholesteryl ester transfer protein activity levels before LDL cholesterol in normolipidaemic subjects. J Intern Med *248*, 211-216.

de Vries, R., Groen, A.K., Perton, F.G., Dallinga-Thie, G.M., van Wijland, M.J., Dikkeschei, L.D., Wolffenbuttel, B.H., van Tol, A., and Dullaart, R.P. (2007). Increased cholesterol efflux from cultured fibroblasts to plasma from hypertriglyceridemic type 2 diabetic patients: Roles of pre beta-HDL, phospholipid transfer protein and cholesterol esterification. Atherosclerosis.

de Vries, R., Groen, A.K., Perton, F.G., Dallinga-Thie, G.M., van Wijland, M.J., Dikkeschei, L.D., Wolffenbuttel, B.H., van Tol, A., and Dullaart, R.P. (2008). Increased cholesterol efflux from cultured fibroblasts to plasma from hypertriglyceridemic type 2 diabetic patients: roles of pre beta-HDL, phospholipid transfer protein and cholesterol esterification. Atherosclerosis *196*, 733-741.

de Vries, R., Kerstens, M.N., Sluiter, W.J., Groen, A.K., van Tol, A., and Dullaart, R.P. (2005). Cellular cholesterol efflux to plasma from moderately hypercholesterolaemic type 1 diabetic patients is enhanced, and is unaffected by simvastatin treatment. Diabetologia *48*, 1105-1113.

Desrumaux, C., Athias, A., Bessede, G., Verges, B., Farnier, M., Persegol, L., Gambert, P., and Lagrost, L. (1999). Mass concentration of plasma phospholipid transfer protein in normolipidemic, type IIa hyperlipidemic, type IIb hyperlipidemic, and non-insulin-dependent diabetic subjects as measured by a specific ELISA. Arterioscler Thromb Vasc Biol *19*, 266-275.

Desrumaux, C., Labeur, C., Verhee, A., Tavernier, J., Vandekerckhove, J., Rosseneu, M., and Peelman, F. (2001). A hydrophobic cluster at the surface of the human plasma phospholipid transfer protein is critical for activity on high density lipoproteins. J Biol Chem *276*, 5908-5915.

Desrumaux, C., Risold, P.Y., Schroeder, H., Deckert, V., Masson, D., Athias, A., Laplanche, H., Le Guern, N., Blache, D., Jiang, X.C., *et al.* (2005). Phospholipid transfer protein (PLTP) deficiency reduces brain vitamin E content and increases anxiety in mice. Faseb J *19*, 296-297.

Desrumaux, C.M., Mak, P.A., Boisvert, W.A., Masson, D., Stupack, D., Jauhiainen, M., Ehnholm, C., and Curtiss, L.K. (2003). Phospholipid transfer protein is present in human atherosclerotic lesions and is expressed by macrophages and foam cells. J Lipid Res *44*, 1453-1461.

Drouineaud, V., Lagrost, L., Klein, A., Desrumaux, C., Le Guern, N., Athias, A., Menetrier, F., Moiroux, P., Sagot, P., Jimenez, C., *et al.* (2006). Phospholipid transfer protein deficiency reduces sperm motility and impairs fertility of mouse males. Faseb J *20*, 794-796.

Dullaart, R.P., Dallinga-Thie, G.M., Wolffenbuttel, B.H., and van Tol, A. (2007). CETP inhibition in cardiovascular risk management: a critical appraisal. Eur J Clin Invest *37*, 90-98.

Dullaart, R.P., de Vries, R., Dallinga-Thie, G.M., Sluiter, W.J., and van Tol, A. (2008a). Phospholipid transfer protein activity is determined by type 2 diabetes mellitus and metabolic syndrome, and is positively associated with serum transaminases. Clin Endocrinol (Oxf) *68*, 375-381.

Dullaart, R.P., Groen, A.K., Dallinga-Thie, G.M., de Vries, R., Sluiter, W.J., and van Tol, A. (2008b). Fibroblast cholesterol efflux to plasma from metabolic syndrome subjects is not defective despite low high-density lipoprotein cholesterol. Eur J Endocrinol *158*, 53-60.

Dullaart, R.P., Hoogenberg, K., Dikkeschei, B.D., and van Tol, A. (1994a). Higher plasma lipid transfer protein activities and unfavorable lipoprotein changes in cigarette-smoking men. Arterioscler Thromb *14*, 1581-1585.

Dullaart, R.P., Sluiter, W.J., Dikkeschei, L.D., Hoogenberg, K., and Van Tol, A. (1994b). Effect of adiposity on plasma lipid transfer protein activities: a possible link between insulin resistance and high density lipoprotein metabolism. Eur J Clin Invest *24*, 188-194.

Dusserre, E., Moulin, P., and Vidal, H. (2000). Differences in mRNA expression of the proteins secreted by the adipocytes in human subcutaneous and visceral adipose tissues. Biochim Biophys Acta *1500*, 88-96.

Ehnholm, S., van Dijk, K.W., van 't Hof, B., van der Zee, A., Olkkonen, V.M., Jauhiainen, M., Hofker, M., Havekes, L., and Ehnholm, C. (1998). Adenovirus mediated overexpression of human phospholipid transfer protein alters plasma HDL levels in mice. J Lipid Res *39*, 1248-1253.

Engler, M.B., Pullinger, C.R., Malloy, M.J., Natanzon, Y., Kulkarni, M.V., Song, J., Eng, C., Huuskonen, J., Rivera, C., Poon, A., *et al.* (2008). Genetic variation in phospholipid transfer protein modulates lipoprotein profiles in hyperalphalipoproteinemia. Metabolism *57*, 1719-1724.

Eriksson, M., Carlson, L.A., Miettinen, T.A., and Angelin, B. (1999). Stimulation of fecal steroid excretion after infusion of recombinant proapolipoprotein A-I. Potential reverse cholesterol transport in humans. Circulation *100*, 594-598.

Field, P.A., and Gibbons, G.F. (2000). Decreased hepatic expression of the low-density lipoprotein (LDL) receptor and LDL receptor-related protein in aging rats is associated with delayed clearance of chylomicrons from the circulation. Metabolism *49*, 492-498.

Fielding, C.J., and Fielding, P.E. (1995). Molecular physiology of reverse cholesterol transport. J Lipid Res *36*, 211-228.

Föger, B., Chase, M., Amar, M.J., Vaisman, B.L., Shamburek, R.D., Paigen, B., Fruchart-Najib, J., Paiz, J.A., Koch, C.A., Hoyt, R.F., *et al.* (1999). Cholesteryl ester transfer protein corrects dysfunctional high density lipoproteins and reduces aortic atherosclerosis in lecithin cholesterol acyltransferase transgenic mice. J Biol Chem *274*, 36912-36920.

Föger, B., Santamarina-Fojo, S., Shamburek, R.D., Parrot, C.L., Talley, G.D., and Brewer, H.B., Jr. (1997). Plasma phospholipid transfer protein. Adenovirus-mediated overexpression in mice leads to decreased plasma high density lipoprotein (HDL) and enhanced hepatic uptake of phospholipids and cholesteryl esters from HDL. J Biol Chem *272*, 27393-27400.

Fonarow, G.C. (2007). The global burden of atherosclerotic vascular disease. Nat Clin Pract Cardiovasc Med *4*, 530-531.

Francone, O.L., Haghpassand, M., Bennett, J.A., Royer, L., and McNeish, J. (1997). Expression of human lecithin:cholesterol acyltransferase in transgenic mice: effects on cholesterol efflux, esterification, and transport. J Lipid Res *38*, 813-822.

Furbee, J.W., Jr., and Parks, J.S. (2002). Transgenic overexpression of human lecithin: cholesterol acyltransferase (LCAT) in mice does not increase aortic cholesterol deposition. Atherosclerosis *165*, 89-100.

Gaultier, A., Arandjelovic, S., Niessen, S., Overton, C.D., Linton, M.F., Fazio, S., Campana, W.M., Cravatt, B.F., 3rd, and Gonias, S.L. (2008). Regulation of tumor necrosis factor receptor-1 and the IKK-NF-kappaB pathway by LDL receptor-related protein explains the antiinflammatory activity of this receptor. Blood *111*, 5316-5325.

Gelissen, I.C., Harris, M., Rye, K.A., Quinn, C., Brown, A.J., Kockx, M., Cartland, S., Packianathan, M., Kritharides, L., and Jessup, W. (2006). ABCA1 and ABCG1 synergize to mediate cholesterol export to apoA-I. Arterioscler Thromb Vasc Biol *26*, 534-540.

Glass, C.K., and Rosenfeld, M.G. (2000). The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev *14*, 121-141.

Glomset, J.A. (1968). The plasma lecithins:cholesterol acyltransferase reaction. J Lipid Res *9*, 155-167.

Goldberg, I.J. (1996). Lipoprotein lipase and lipolysis: central roles in lipoprotein metabolism and atherogenesis. J Lipid Res *37*, 693-707.

Gonzalez-Navarro, H., Nong, Z., Freeman, L., Bensadoun, A., Peterson, K., and Santamarina-Fojo, S. (2002). Identification of mouse and human macrophages as a site of synthesis of hepatic lipase. J Lipid Res *43*, 671-675.

Goodwin, B., Jones, S.A., Price, R.R., Watson, M.A., McKee, D.D., Moore, L.B., Galardi, C., Wilson, J.G., Lewis, M.C., Roth, M.E., *et al.* (2000). A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. Mol Cell *6*, 517-526.

Graf, G.A., Li, W.P., Gerard, R.D., Gelissen, I., White, A., Cohen, J.C., and Hobbs, H.H. (2002). Coexpression of ATP-binding cassette proteins ABCG5 and ABCG8 permits their transport to the apical surface. J Clin Invest *110*, 659-669.

Greeve, J., Altkemper, I., Dieterich, J.H., Greten, H., and Windler, E. (1993). Apolipoprotein B mRNA editing in 12 different mammalian species: hepatic expression is reflected in low concentrations of apoB-containing plasma lipoproteins. J Lipid Res *34*, 1367-1383.

Guyard-Dangremont, V., Desrumaux, C., Gambert, P., Lallemant, C., and Lagrost, L. (1998). Phospholipid and cholesteryl ester transfer activities in plasma from 14 vertebrate species. Relation to atherogenesis susceptibility. Comp Biochem Physiol B Biochem Mol Biol *120*, 517-525.

Haghpassand, M., Bourassa, P.A., Francone, O.L., and Aiello, R.J. (2001). Monocyte/macrophage expression of ABCA1 has minimal contribution to plasma HDL levels. J Clin Invest *108*, 1315-1320.

Hailman, E., Albers, J.J., Wolfbauer, G., Tu, A.Y., and Wright, S.D. (1996). Neutralization and transfer of lipopolysaccharide by phospholipid transfer protein. J Biol Chem *271*, 12172-12178.

Han, J., Hajjar, D.P., Febbraio, M., and Nicholson, A.C. (1997). Native and modified low density lipoproteins increase the functional expression of the macrophage class B scavenger receptor, CD36. J Biol Chem *272*, 21654-21659.

Harder, C., Lau, P., Meng, A., Whitman, S.C., and McPherson, R. (2007). Cholesteryl ester transfer protein (CETP) expression protects against diet induced atherosclerosis in SR-BI deficient mice. Arterioscler Thromb Vasc Biol *27*, 858-864.

Hasham, S.N., and Pillarisetti, S. (2006). Vascular lipases, inflammation and atherosclerosis. Clin Chim Acta *372*, 179-183.

Hausenloy, D.J., and Yellon, D.M. (2008). Targeting residual cardiovascular risk: raising high-density lipoprotein cholesterol levels. Heart *94*, 706-714.

Heller, F., and Harvengt, C. (1983). Effects of clofibrate, bezafibrate, fenofibrate and probucol on plasma lipolytic enzymes in normolipaemic subjects. Eur J Clin Pharmacol *25*, 57-63.

Herz, J., Qiu, S.Q., Oesterle, A., DeSilva, H.V., Shafi, S., and Havel, R.J. (1995). Initial hepatic removal of chylomicron remnants is unaffected but endocytosis is delayed in mice lacking the low density lipoprotein receptor. Proc Natl Acad Sci U S A *92*, 4611-4615.

Hovingh, G.K., Hutten, B.A., Holleboom, A.G., Petersen, W., Rol, P., Stalenhoef, A., Zwinderman, A.H., de Groot, E., Kastelein, J.J., and Kuivenhoven, J.A. (2005). Compromised LCAT function is associated with increased atherosclerosis. Circulation *112*, 879-884.

Hussain, M.M., Kancha, R.K., Zhou, Z., Luchoomun, J., Zu, H., and Bakillah, A. (1996). Chylomicron assembly and catabolism: role of apolipoproteins and receptors. Biochim Biophys Acta *1300*, 151-170.

Huuskonen, J., Ekstrom, M., Tahvanainen, E., Vainio, A., Metso, J., Pussinen, P., Ehnholm, C., Olkkonen, V.M., and Jauhiainen, M. (2000a). Quantification of human plasma phospholipid transfer protein (PLTP): relationship between PLTP mass and phospholipid transfer activity. Atherosclerosis *151*, 451-461.

Huuskonen, J., Jauhiainen, M., Ehnholm, C., and Olkkonen, V.M. (1998). Biosynthesis and secretion of human plasma phospholipid transfer protein. J Lipid Res *39*, 2021-2030.

Huuskonen, J., Olkkonen, V.M., Ehnholm, C., Metso, J., Julkunen, I., and Jauhiainen, M. (2000b). Phospholipid transfer is a prerequisite for PLTP-mediated HDL conversion. Biochemistry *39*, 16092-16098.

Huuskonen, J., Olkkonen, V.M., Jauhiainen, M., Metso, J., Somerharju, P., and Ehnholm, C. (1996). Acyl chain and headgroup specificity of human plasma phospholipid transfer protein. Biochim Biophys Acta *1303*, 207-214.

Huuskonen, J., Wohlfahrt, G., Jauhiainen, M., Ehnholm, C., Teleman, O., and Olkkonen, V.M. (1999). Structure and phospholipid transfer activity of human PLTP: analysis by molecular modeling and site-directed mutagenesis. J Lipid Res *40*, 1123-1130.

Inazu, A., Brown, M.L., Hesler, C.B., Agellon, L.B., Koizumi, J., Takata, K., Maruhama, Y., Mabuchi, H., and Tall, A.R. (1990). Increased high-density lipoprotein levels caused by a common cholesteryl-ester transfer protein gene mutation. N Engl J Med *323*, 1234-1238.

Ishibashi, S., Brown, M.S., Goldstein, J.L., Gerard, R.D., Hammer, R.E., and Herz, J. (1993). Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. J Clin Invest *92*, 883-893.

Ishibashi, S., Goldstein, J.L., Brown, M.S., Herz, J., and Burns, D.K. (1994). Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. J Clin Invest *93*, 1885-1893.

Ishida, B.Y., Blanche, P.J., Nichols, A.V., Yashar, M., and Paigen, B. (1991). Effects of atherogenic diet consumption on lipoproteins in mouse strains C57BL/6 and C3H. J Lipid Res *32*, 559-568.

Ishida, T., Choi, S., Kundu, R.K., Hirata, K., Rubin, E.M., Cooper, A.D., and Quertermous, T. (2003). Endothelial lipase is a major determinant of HDL level. J Clin Invest *111*, 347-355.

Jaari, S., van Dijk, K.W., Olkkonen, V.M., van der Zee, A., Metso, J., Havekes, L., Jauhiainen, M., and Ehnholm, C. (2001). Dynamic changes in mouse lipoproteins induced by transiently expressed human phospholipid transfer protein (PLTP): importance of PLTP in prebeta-HDL generation. Comp Biochem Physiol B Biochem Mol Biol *128*, 781-792.

Janis, M.T., Metso, J., Lankinen, H., Strandin, T., Olkkonen, V.M., Rye, K. ., Jauhiainen, M., and Ehnholm, C. (2005). Apolipoprotein E activates the low-activity form of human phospholipid transfer protein. Biochem Biophys Res Commun *331*, 333-340.

Janis, M.T., Siggins, S., Tahvanainen, E., Vikstedt, R., Silander, K., Metso, J., Aromaa, A., Taskinen, M. R., Olkkonen, V.M., Jauhiainen, M., and Ehnholm, C. (2004). Active and low-active forms of serum phospholipid transfer protein in a normal Finnish population sample. J Lipid Res *45*, 2303-2309.

Jansen, H. (2004). Hepatic lipase: friend or foe and under what circumstances? Curr Atheroscler Rep *6*, 343-347.

Jansen, H., Verhoeven, A.J., and Sijbrands, E.J. (2002). Hepatic lipase: a pro- or anti-atherogenic protein? J Lipid Res *43*, 1352-1362.

Jauhiainen, M., Metso, J., Pahlman, R., Blomqvist, S., van Tol, A., and Ehnholm, C. (1993). Human plasma phospholipid transfer protein causes high density lipoprotein conversion. J Biol Chem *268*, 4032-4036.

Jauhiainen, M., Setala, N. L., Ehnholm, C., Metso, J., Tervo, T. M., Eriksson, O., and Holopainen, J.M. (2005). Phospholipid transfer protein is present in human tear fluid. Biochemistry *44*, 8111-8116.

Jawien, J., Nastalek, P., and Korbut, R. (2004). Mouse models of experimental atherosclerosis. J Physiol Pharmacol *55*, 503-517.

Jaye, M., and Krawiec, J. (2004). Endothelial lipase and HDL metabolism. Curr Opin Lipidol *15*, 183-189.

Jaye, M., Lynch, K.J., Krawiec, J., Marchadier, D., Maugeais, C., Doan, K., South, V., Amin, D., Perrone, M., and Rader, D.J. (1999). A novel endothelial-derived lipase that modulates HDL metabolism. Nat Genet *21*, 424-428.

Jessup, W., Gelissen, I.C., Gaus, K., and Kritharides, L. (2006). Roles of ATP binding cassette transporters A1 and G1, scavenger receptor BI and membrane lipid domains in cholesterol export from macrophages. Curr Opin Lipidol *17*, 247-257.

Ji, Y., Jian, B., Wang, N., Sun, Y., Moya, M.L., Phillips, M.C., Rothblat, G.H., Swaney, J.B., and Tall, A.R. (1997). Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. J Biol Chem *272*, 20982-20985.

Jiang, X., Francone, O.L., Bruce, C., Milne, R., Mar, J., Walsh, A., Breslow, J.L., and Tall, A.R. (1996). Increased prebeta-high density lipoprotein, apolipoprotein AI, and phospholipid in mice expressing the human phospholipid transfer protein and human apolipoprotein AI transgenes. J Clin Invest *98*, 2373-2380.

Jiang, X.C., and Bruce, C. (1995). Regulation of murine plasma phospholipid transfer protein activity and mRNA levels by lipopolysaccharide and high cholesterol diet. J Biol Chem *270*, 17133-17138.

Jiang, X.C., Bruce, C., Mar, J., Lin, M., Ji, Y., Francone, O.L., and Tall, A.R. (1999). Targeted mutation of plasma phospholipid transfer protein gene markedly reduces high-density lipoprotein levels. J Clin Invest *103*, 907-914.

Jiang, X.C., D'Armiento, J., Mallampalli, R.K., Mar, J., Yan, S.F., and Lin, M. (1998). Expression of plasma phospholipid transfer protein mRNA in normal and emphysematous lungs and regulation by hypoxia. J Biol Chem *273*, 15714-15718.

Jiang, X.C., Li, Z., Liu, R., Yang, X. P., Pan, M., Lagrost, L., Fisher, E.A., and Williams, K.J. (2005). Phospholipid transfer protein deficiency impairs apolipoprotein-B secretion from hepatocytes by stimulating a proteolytic pathway through a relative deficiency of vitamin E and an increase in intracellular oxidants. J Biol Chem *280*, 18336-18340.

Jiang, X.C., Qin, S., Qiao, C., Kawano, K., Lin, M., Skold, A., Xiao, X., and Tall, A.R. (2001). Apolipoprotein B secretion and atherosclerosis are decreased in mice with phospholipid-transfer protein deficiency. Nat Med *7*, 847-852.

Jiang, X.C., Tall, A.R., Qin, S., Lin, M., Schneider, M., Lalanne, F., Deckert, V., Desrumaux, C., Athias, A., Witztum, J.L., and Lagrost, L. (2002). Phospholipid transfer protein deficiency protects circulating lipoproteins from oxidation due to the enhanced accumulation of vitamin E. J Biol Chem *277*, 31850-31856.

Jin, W., Millar, J. S., Broedl, U., Glick, J.M., and Rader, D.J. (2003). Inhibition of endothelial lipase causes increased HDL cholesterol levels in vivo. J Clin Invest *111*, 357-362.

Joyce, C.W., Amar, M.J., Lambert, G., Vaisman, B.L., Paigen, B., Najib-Fruchart, J., Hoyt, R.F., Jr., Neufeld, E.D., Remaley, A.T., Fredrickson, D.S., *et al.* (2002). The ATP binding cassette transporter A1 (ABCA1) modulates the development of aortic atherosclerosis in C57BL/6 and apoE-knockout mice. Proc Natl Acad Sci U S A *99*, 407-412.

Karkkainen, M., Oka, T., Olkkonen, V.M., Metso, J., Hattori, H., Jauhiainen, M., and Ehnholm, C. (2002). Isolation and partial characterization of the inactive and active forms of human plasma phospholipid transfer protein (PLTP). J Biol Chem *277*, 15413-15418.

Kaser, S., Sandhofer, A., Föger, B., Ebenbichler, C.F., Igelseder, B., Malaimare, L., Paulweber, B., and Patsch, J.R. (2001). Influence of obesity and insulin sensitivity on phospholipid transfer protein activity. Diabetologia *44*, 1111-1117.

Kast, H.R., Nguyen, C.M., Sinal, C.J., Jones, S.A., Laffitte, B.A., Reue, K., Gonzalez, F.J., Willson, T.M., and Edwards, P.A. (2001). Farnesoid X-activated receptor induces apolipoprotein C-II transcription: a molecular mechanism linking plasma triglyceride levels to bile acids. Mol Endocrinol *15*, 1720-1728.

Kavey, R.E., Allada, V., Daniels, S.R., Hayman, L.L., McCrindle, B.W., Newburger, J.W., Parekh, R.S., and Steinberger, J. (2006). Cardiovascular risk reduction in high-risk pediatric patients: a scientific statement from the American Heart Association Expert Panel on Population and Prevention Science; the Councils on Cardiovascular Disease in the Young, Epidemiology and Prevention, Nutrition, Physical Activity and Metabolism, High Blood Pressure Research, Cardiovascular Nursing, and the Kidney in Heart Disease; and the Interdisciplinary Working Group on Quality of Care and Outcomes Research: endorsed by the American Academy of Pediatrics. Circulation *114*, 2710-2738.

Kawano, K., Qin, S.C., Lin, M., Tall, A.R., and Jiang, X.C. (2000). Cholesteryl ester transfer protein and phospholipid transfer protein have nonoverlapping functions in vivo. J Biol Chem *275*, 29477-29481.

Kennedy, M.A., Barrera, G.C., Nakamura, K., Baldan, A., Tarr, P., Fishbein, M.C., Frank, J., Francone, O. L., and Edwards, P.A. (2005). ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. Cell Metab *1*, 121-131.

Kennedy, M.A., Venkateswaran, A., Tarr, P.T., Xenarios, I., Kudoh, J., Shimizu, N., and Edwards, P.A. (2001). Characterization of the human ABCG1 gene: liver X receptor activates an internal promoter that produces a novel transcript encoding an alternative form of the protein. J Biol Chem *276*, 39438-39447.

Klucken, J., Buchler, C., Orso, E., Kaminski, W.E., Porsch-Ozcurumez, M., Liebisch, G., Kapinsky, M., Diederich, W., Drobnik, W., Dean, M., *et al.* (2000). ABCG1 (ABC8), the human homolog of the Drosophila white gene, is a regulator of macrophage cholesterol and phospholipid transport. Proc Natl Acad Sci U S A *97*, 817-822.

Knouff, C., Hinsdale, M.E., Mezdour, H., Altenburg, M.K., Watanabe, M., Quarfordt, S.H., Sullivan, P.M., and Maeda, N. (1999). Apo E structure determines VLDL clearance and atherosclerosis risk in mice. J Clin Invest *103*, 1579-1586.

Koizumi, J., Mabuchi, H., Yoshimura, A., Michishita, I., Takeda, M., Itoh, H., Sakai, Y., Sakai, T., Ueda, K., and Takeda, R. (1985). Deficiency of serum cholesteryl-ester transfer activity in patients with familial hyperalphalipoproteinaemia. Atherosclerosis *58*, 175-186.

Kong, W.J., Liu, J., and Jiang, J.D. (2006). Human low-density lipoprotein receptor gene and its regulation. J Mol Med *84*, 29-36.

Kontush, A., Guerin, M., and Chapman, M.J. (2008). Spotlight on HDL-raising therapies: insights from the torcetrapib trials. Nat Clin Pract Cardiovasc Med *5*, 329-336.

Korhonen, A., Jauhiainen, M., Ehnholm, C., Kovanen, P.T., and Ala-Korpela, M. (1998). Remodeling of HDL by phospholipid transfer protein: demonstration of particle fusion by 1H NMR spectroscopy. Biochem Biophys Res Commun *249*, 910-916.

Kostner, G.M., Oettl, K., Jauhiainen, M., Ehnholm, C., Esterbauer, H., and Dieplinger, H. (1995). Human plasma phospholipid transfer protein accelerates exchange/transfer of alpha-tocopherol between lipoproteins and cells. Biochem J *305 (Pt 2)*, 659-667.

Kozarsky, K.F., Donahee, M.H., Glick, J.M., Krieger, M., and Rader, D.J. (2000). Gene transfer and hepatic overexpression of the HDL receptor SR-BI reduces atherosclerosis in the cholesterol-fed LDL receptor-deficient mouse. Arterioscler Thromb Vasc Biol *20*, 721-727.

Kozarsky, K.F., Donahee, M.H., Rigotti, A., Iqbal, S.N., Edelman, E.R., and Krieger, M. (1997). Overexpression of the HDL receptor SR-BI alters plasma HDL and bile cholesterol levels. Nature *387*, 414-417.

Krapp, A., Ahle, S., Kersting, S., Hua, Y., Kneser, K., Nielsen, M., Gliemann, J., and Beisiegel, U. (1996). Hepatic lipase mediates the uptake of chylomicrons and beta-VLDL into cells via the LDL receptor-related protein (LRP). J Lipid Res *37*, 926-936.

Kurasawa, T., Yokoyama, S., Miyake, Y., Yamamura, T., and Yamamoto, A. (1985). Rate of cholesteryl ester transfer between high and low density lipoproteins in human serum and a case with decreased transfer rate in association with hyperalphalipoproteinemia. J Biochem *98*, 1499-1508.

Laffitte, B.A., Joseph, S.B., Chen, M., Castrillo, A., Repa, J., Wilpitz, D., Mangelsdorf, D., and Tontonoz, P. (2003). The phospholipid transfer protein gene is a liver X receptor target expressed by macrophages in atherosclerotic lesions. Mol Cell Biol *23*, 2182-2191.

Lagrost, L., Athias, A., Lemort, N., Richard, J.L., Desrumaux, C., Chatenet-Duchene, L., Courtois, M., Farnier, M., Jacotot, B., Braschi, S., and Gambert, P. (1999a). Plasma lipoprotein distribution and lipid transfer activities in patients with type IIb hyperlipidemia treated with simvastatin. Atherosclerosis *143*, 415-425.

Lagrost, L., Mensink, R.P., Guyard-Dangremont, V., Temme, E.H., Desrumaux, C., Athias, A., Hornstra, G., and Gambert, P. (1999b). Variations in serum cholesteryl ester transfer and phospholipid transfer activities in healthy women and men consuming diets enriched in lauric, palmitic or oleic acids. Atherosclerosis *142*, 395-402.

Lamarche, B., and Paradis, M.E. (2007). Endothelial lipase and the metabolic syndrome. Curr Opin Lipidol *18*, 298-303.

Lambert, G., Amar, M.J., Martin, P., Fruchart-Najib, J., Föger, B., Shamburek, R.D., Brewer, H.B., Jr., and Santamarina-Fojo, S. (2000). Hepatic lipase deficiency decreases the selective uptake of HDL-cholesteryl esters in vivo. J Lipid Res *41*, 667-672.

LeBoeuf, R. C., Caldwell, M., Tu, A., and Albers, J.J. (1996). Phospholipid transfer protein maps to distal mouse chromosome 2. Genomics *34*, 259-260.

Lee, K.F., Kwok, K.L., Chung, M.K., Lee, Y.L., Chow, J.F., and Yeung, W.S. (2005). Phospholipid transfer protein (PLTP) mRNA expression is stimulated by developing embryos in the oviduct. J Cell Biochem *95*, 740-749.

Lee, M.H., Lu, K., Hazard, S., Yu, H., Shulenin, S., Hidaka, H., Kojima, H., Allikmets, R., Sakuma, N., Pegoraro, R., *et al.* (2001). Identification of a gene, ABCG5, important in the regulation of dietary cholesterol absorption. Nat Genet *27*, 79-83.

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

Lehmann, J.M., Kliewer, S.A., Moore, L.B., Smith-Oliver, T.A., Oliver, B.B., Su, J.L., Sundseth, S.S., Winegar, D.A., Blanchard, D.E., Spencer, T.A., and Willson, T.M. (1997). Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. J Biol Chem *272*, 3137-3140.

Levels, J.H., Marquart, J.A., Abraham, P.R., van den Ende, A.E., Molhuizen, H.O., van Deventer, S.J., and Meijers, J.C. (2005). Lipopolysaccharide is transferred from high-density to low-density lipoproteins by lipopolysaccharide-binding protein and phospholipid transfer protein. Infect Immun *73*, 2321-2326.

Levels, J.H., Pajkrt, D., Schultz, M., Hoek, F.J., van Tol, A., Meijers, J.C., and van Deventer, S.J. (2007). Alterations in lipoprotein homeostasis during human experimental endotoxemia and clinical sepsis. Biochim Biophys Acta.

Lie, J., de Crom, R., Jauhiainen, M., van Gent, T., van Haperen, R., Scheek, L., Jansen, H., Ehnholm, C., and van Tol, A. (2001). Evaluation of phospholipid transfer protein and cholesteryl ester transfer protein as contributors to the generation of pre beta-high-density lipoproteins. Biochem J *360*, 379-385.

Lie, J., de Crom, R., van Gent, T., van Haperen, R., Scheek, L., Lankhuizen, I., and van Tol, A. (2002). Elevation of plasma phospholipid transfer protein in transgenic mice increases VLDL secretion. J Lipid Res *43*, 1875-1880.

Lie, J., de Crom, R., van Gent, T., van Haperen, R., Scheek, L., Sadeghi-Niaraki, F., and van Tol, A. (2004). Elevation of plasma phospholipid transfer protein increases the risk of atherosclerosis despite lower apolipoprotein B-containing lipoproteins. J Lipid Res *45*, 805-811.

Lie, J., Lankhuizen, I.M., Gross, B., van Gent, T., van Haperen, R., Scheek, L., Staels, B., de Crom, R., and van Tol, A. (2005). Fenofibrate reverses the decline in HDL cholesterol in mice overexpressing human phospholipid transfer protein. Biochim Biophys Acta *1738*, 48-53.

Li-Hawkins, J., Gafvels, M., Olin, M., Lund, E.G., Andersson, U., Schuster, G., Bjorkhem, I., Russell, D.W., and Eggertsen, G. (2002). Cholic acid mediates negative feedback regulation of bile acid synthesis in mice. J Clin Invest *110*, 1191-1200.

Liinamaa, M.J., Hannuksela, M.L., Kesaniemi, Y.A., and Savolainen, M.J. (1997). Altered transfer of cholesteryl esters and phospholipids in plasma from alcohol abusers. Arterioscler Thromb Vasc Biol *17*, 2940-2947.

Liu, R., Hojjati, M.R., Devlin, C.M., Hansen, I.H., and Jiang, X.C. (2007a). Macrophage phospholipid transfer protein deficiency and ApoE secretion: impact on mouse plasma cholesterol levels and atherosclerosis. Arterioscler Thromb Vasc Biol *27*, 190-196.

Liu, R., Iqbal, J., Yeang, C., Wang, D.Q., Hussain, M.M., and Jiang, X.C. (2007b). Phospholipid transfer protein-deficient mice absorb less cholesterol. Arterioscler Thromb Vasc Biol *27*, 2014-2021.

Lu, T.T., Makishima, M., Repa, J.J., Schoonjans, K., Kerr, T. A., Auwerx, J., and Mangelsdorf, D.J. (2000). Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. Mol Cell *6*, 507-515.

Lusa, S., Jauhiainen, M., Metso, J., Somerharju, P., and Ehnholm, C. (1996). The mechanism of human plasma phospholipid transfer protein-induced enlargement of high-density lipoprotein particles: evidence for particle fusion. Biochem J *313 (Pt 1)*, 275-282.

Mahley, R.W., Hui, D.Y., Innerarity, T.L., and Beisiegel, U. (1989). Chylomicron remnant metabolism. Role of hepatic lipoprotein receptors in mediating uptake. Arteriosclerosis *9*, I14-18.

Mak, P.A., Kast-Woelbern, H.R., Anisfeld, A.M., and Edwards, P.A. (2002). Identification of PLTP as an LXR target gene and apoE as an FXR target gene reveals overlapping targets for the two nuclear receptors. J Lipid Res *43*, 2037-2041.

Mardones, P., Quinones, V., Amigo, L., Moreno, M., Miquel, J.F., Schwarz, M., Miettinen, H.E., Trigatti, B., Krieger, M., VanPatten, S., *et al.* (2001). Hepatic cholesterol and bile acid metabolism and intestinal cholesterol absorption in scavenger receptor class B type I-deficient mice. J Lipid Res *42*, 170-180.

Massey, J.B. (1984). Kinetics of transfer of alpha-tocopherol between model and native plasma lipoproteins. Biochim Biophys Acta *793*, 387-392.

Masson, D., Drouineaud, V., Moiroux, P., Gautier, T., Dautin, G., Schneider, M., Fruchart-Najib, J., Jauhiainen, M., Ehnholm, C., Sagot, P., *et al.* (2003). Human seminal plasma displays significant phospholipid transfer activity due to the presence of active phospholipid transfer protein. Mol Hum Reprod *9*, 457-464.

McCoy, M.G., Sun, G.S., Marchadier, D., Maugeais, C., Glick, J. M., and Rader, D.J. (2002). Characterization of the lipolytic activity of endothelial lipase. J Lipid Res *43*, 921-929.

McNeish, J., Aiello, R.J., Guyot, D., Turi, T., Gabel, C., Aldinger, C., Hoppe, K.L., Roach, M.L., Royer, L. ., de Wet, J., *et al.* (2000). High density lipoprotein deficiency and foam cell accumulation in mice with targeted disruption of ATP-binding cassette transporter-1. Proc Natl Acad Sci U S A *97*, 4245-4250.

Mehlum, A., Muri, M., Hagve, T.A., Solberg, L.A., and Prydz, H. (1997). Mice overexpressing human lecithin: cholesterol acyltransferase are not protected against diet-induced atherosclerosis. Apmis *105*, 861-868.

Mero, N., Van Tol, A., Scheek, L.M., Van Gent, T., Labeur, C., Rosseneu, M., and Taskinen, M.R. (1998). Decreased postprandial high density lipoprotein cholesterol and apolipoproteins A-I and E in normolipidemic smoking men: relations with lipid transfer proteins and LCAT activities. J Lipid Res *39*, 1493-1502.

Morel, D.W., Hessler, J.R., and Chisolm, G.M. (1983). Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. J Lipid Res *24*, 1070-1076.

Mulder, M., Lombardi, P., Jansen, H., van Berkel, T.J., Frants, R.R., and Havekes, L.M. (1993). Low density lipoprotein receptor internalizes low density and very low density lipoproteins that are bound to heparan sulfate proteoglycans via lipoprotein lipase. J Biol Chem *268*, 9369-9375.

Murdoch, S.J., Kahn, S.E., Albers, J.J., Brunzell, J.D., and Purnell, J.Q. (2003). PLTP activity decreases with weight loss: changes in PLTP are associated with changes in subcutaneous fat and FFA but not IAF or insulin sensitivity. J Lipid Res *44*, 1705-1712.

Naik, S.U., Wang, X., Da Silva, J.S., Jaye, M., Macphee, C.H., Reilly, M.P., Billheimer, J.T., Rothblat, G.H., and Rader, D.J. (2006). Pharmacological activation of liver X receptors promotes reverse cholesterol transport in vivo. Circulation *113*, 90-97.

Nishida, H.I., and Nishida, T. (1997). Phospholipid transfer protein mediates transfer of not only phosphatidylcholine but also cholesterol from phosphatidylcholine-cholesterol vesicles to high density lipoproteins. J Biol Chem *272*, 6959-6964.

Nong, Z., Gonzalez-Navarro, H., Amar, M., Freeman, L., Knapper, C., Neufeld, E.B., Paigen, B.J., Hoyt, R.F., Fruchart-Najib, J., and Santamarina-Fojo, S. (2003). Hepatic lipase expression in macrophages contributes to atherosclerosis in apoE-deficient and LCAT-transgenic mice. J Clin Invest *112*, 367-378.

O'Brien, K.D., Vuletic, S., McDonald, T.O., Wolfbauer, G., Lewis, K., Tu, A.Y., Marcovina, S., Wight, T.N., Chait, A., and Albers, J.J. (2003). Cell-associated and extracellular phospholipid transfer protein in human coronary atherosclerosis. Circulation *108*, 270-274.

Ogier, N., Klein, A., Deckert, V., Athias, A., Bessede, G., Le Guern, N., Lagrost, L., and Desrumaux, C. (2007). Cholesterol accumulation is increased in macrophages of phospholipid transfer protein-deficient mice: normalization by dietary alpha-tocopherol supplementation. Arterioscler Thromb Vasc Biol *27*, 2407-2412.

Ohta, T., Nakamura, R., Takata, K., Saito, Y., Yamashita, S., Horiuchi, S., and Matsuda, I. (1995). Structural and functional differences of subspecies of apoA-I-containing lipoprotein in patients with plasma cholesteryl ester transfer protein deficiency. J Lipid Res *36*, 696-704.

Oka, T., Kujiraoka, T., Ito, M., Egashira, T., Takahashi, S., Nanjee, M.N., Miller, N.E., Metso, J., Olkkonen, V.M., Ehnholm, C., *et al.* (2000a). Distribution of phospholipid transfer protein in human plasma: presence of two forms of phospholipid transfer protein, one catalytically active and the other inactive. J Lipid Res *41*, 1651-1657.

Oka, T., Kujiraoka, T., Ito, M., Nagano, M., Ishihara, M., Iwasaki, T., Egashira, T., Miller, N.E., and Hattori, H. (2000b). Measurement of human plasma phospholipid transfer protein by sandwich ELISA. Clin Chem *46*, 1357-1364.

Oram, J.F. (2003). HDL apolipoproteins and ABCA1: partners in the removal of excess cellular cholesterol. Arterioscler Thromb Vasc Biol *23*, 720-727.

Oram, J.F., and Heinecke, J.W. (2005). ATP-binding cassette transporter A1: a cell cholesterol exporter that protects against cardiovascular disease. Physiol Rev *85*, 1343-1372.

Oram, J.F., and Vaughan, A.M. (2006). ATP-Binding cassette cholesterol transporters and cardiovascular disease. Circ Res *99*, 1031-1043.

Oram, J.F., Wolfbauer, G., Tang, C., Davidson, W.S., and Albers, J.J. (2008). An amphipathic helical region of the N-terminal barrel of phospholipid transfer protein is critical for ABCA1-dependent cholesterol efflux. J Biol Chem *283*, 11541-11549.

Orso, E., Broccardo, C., Kaminski, W.E., Bottcher, A., Liebisch, G., Drobnik, W., Gotz, A., Chambenoit, O., Diederich, W., Langmann, T., *et al.* (2000). Transport of lipids from golgi to plasma membrane is defective in tangier disease patients and Abc1-deficient mice. Nat Genet *24*, 192-196.

Out, R., Hoekstra, M., Hildebrand, R.B., Kruit, J.K., Meurs, I., Li, Z., Kuipers, F., Van Berkel, T.J., and Van Eck, M. (2006). Macrophage ABCG1 deletion disrupts lipid homeostasis in alveolar macrophages and moderately influences atherosclerotic lesion development in LDL receptor-deficient mice. Arterioscler Thromb Vasc Biol *26*, 2295-2300.

Out, R., Kruijt, J.K., Rensen, P.C., Hildebrand, R.B., de Vos, P., Van Eck, M., and Van Berkel, T.J. (2004). Scavenger receptor BI plays a role in facilitating chylomicron metabolism. J Biol Chem *279*, 18401-18406.

Overton, C.D., Yancey, P.G., Major, A.S., Linton, M.F., and Fazio, S. (2007). Deletion of macrophage LDL receptor-related protein increases atherogenesis in the mouse. Circ Res *100*, 670-677.

Paigen, B., Morrow, A., Brandon, C., Mitchell, D., and Holmes, P. (1985). Variation in susceptibility to atherosclerosis among inbred strains of mice. Atherosclerosis *57*, 65-73.

Paigen, B., Morrow, A., Holmes, P.A., Mitchell, D., and Williams, R.A. (1987). Quantitative assessment of atherosclerotic lesions in mice. Atherosclerosis *68*, 231-240.

Palmen, J., Colhoun, H.M., Van Tol, A., Hattori, H., and Humphries, S.E. (2005). A novel common variant -181 G insertion in the promoter of the gene for phospholipid transfer protein. Atherosclerosis *180*, 211-213.

Parini, P., and Rudel, L.L. (2003). Is there a need for cholesteryl ester transfer protein inhibition? Arterioscler Thromb Vasc Biol *23*, 374-375.

Pattnaik, N.M., Montes, A., Hughes, L.B., and Zilversmit, D.B. (1978). Cholesteryl ester exchange protein in human plasma isolation and characterization. Biochim Biophys Acta *530*, 428-438.

Peterson, J., Bengtsson-Olivecrona, G., and Olivecrona, T. (1986). Mouse preheparin plasma contains high levels of hepatic lipase with low affinity for heparin. Biochim Biophys Acta *878*, 65-70.

Plass, J.R., Mol, O., Heegsma, J., Geuken, M., Faber, K. N., Jansen, P. L., and Muller, M. (2002). Farnesoid X receptor and bile salts are involved in transcriptional regulation of the gene encoding the human bile salt export pump. Hepatology *35*, 589-596.

Plump, A.S., Smith, J.D., Hayek, T., Aalto-Setala, K., Walsh, A., Verstuyft, J.G., Rubin, E.M., and Breslow, J.L. (1992). Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. Cell *71*, 343-353.

Ponsin, G., Qu, S.J., Fan, H.Z., and Pownall, H.J. (2003). Structural and functional determinants of human plasma phospholipid transfer protein activity as revealed by site-directed mutagenesis of charged amino acids. Biochemistry *42*, 4444-4451.

Post, S.M., de Crom, R., van Haperen, R., van Tol, A., and Princen, H.M. (2003). Increased fecal bile acid excretion in transgenic mice with elevated expression of human phospholipid transfer protein. Arterioscler Thromb Vasc Biol *23*, 892-897.

Pussinen, P., Jauhianinen, M., Metso, J., Tyynela, J., and Ehnholm, C. (1995). Pig plasma phospholipid transfer protein facilitates HDL interconversion. J Lipid Res *36*, 975-985.

Pussinen, P.J., Jauhiainen, M., Metso, J., Pyle, L.E., Marcel, Y.L., Fidge, N.H., and Ehnholm, C. (1998). Binding of phospholipid transfer protein (PLTP) to apolipoproteins A-I and A-II: location of a PLTP binding domain in the amino terminal region of apoA-I. J Lipid Res *39*, 152-161.

Pussinen, P.J., Malle, E., Metso, J., Sattler, W., Raynes, J.G., and Jauhiainen, M. (2001a). Acute-phase HDL in phospholipid transfer protein (PLTP)-mediated HDL conversion. Atherosclerosis *155*, 297-305.

Pussinen, P.J., Metso, J., Malle, E., Barlage, S., Palosuo, T., Sattler, W., Schmitz, G., and Jauhiainen, M. (2001b). The role of plasma phospholipid transfer protein (PLTP) in HDL remodeling in acute-phase patients. Biochim Biophys Acta *1533*, 153-163.

Qin, S., Kawano, K., Bruce, C., Lin, M., Bisgaier, C., Tall, A.R., and Jiang, X. (2000). Phospholipid transfer protein gene knock-out mice have low high density lipoprotein levels, due to hypercatabolism, and accumulate apoA-IV-rich lamellar lipoproteins. J Lipid Res *41*, 269-276.

Ranalletta, M., Wang, N., Han, S., Yvan-Charvet, L., Welch, C., and Tall, A. R. (2006). Decreased atherosclerosis in low-density lipoprotein receptor knockout mice transplanted with Abcg1-/- bone marrow. Arterioscler Thromb Vasc Biol *26*, 2308-2315.

Rao, R., Albers, J.J., Wolfbauer, G., and Pownall, H.J. (1997). Molecular and macromolecular specificity of human plasma phospholipid transfer protein. Biochemistry *36*, 3645-3653.

Redgrave, T.G. (2004). Chylomicron metabolism. Biochem Soc Trans *32*, 79-82.

Repa, J.J., and Mangelsdorf, D.J. (2000). The role of orphan nuclear receptors in the regulation of cholesterol homeostasis. Annu Rev Cell Dev Biol *16*, 459-481.

Riemens, S.C., van Tol, A., Sluiter, W.J., and Dullaart, R.P. (1998). Plasma phospholipid transfer protein activity is related to insulin resistance: impaired acute lowering by insulin in obese Type II diabetic patients. Diabetologia *41*, 929-934.

Riemens, S.C., Van Tol, A., Sluiter, W.J., and Dullaart, R.P. (1999). Acute and chronic effects of a 24-hour intravenous triglyceride emulsion challenge on plasma lecithin: cholesterol acyltransferase, phospholipid transfer protein, and cholesteryl ester transfer protein activities. J Lipid Res *40*, 1459-1466.

Rigotti, A., Trigatti, B.L., Penman, M., Rayburn, H., Herz, J., and Krieger, M. (1997). A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. Proc Natl Acad Sci U S A *94*, 12610-12615.

Rinninger, F., Brundert, M., Brosch, I., Donarski, N., Budzinski, R.M., and Greten, H. (2001). Lipoprotein lipase mediates an increase in selective uptake of HDL-associated cholesteryl esters by cells in culture independent of scavenger receptor BI. J Lipid Res *42*, 1740-1751.

Rip, J., Nierman, M.C., Sierts, J.A., Petersen, W., Van den Oever, K., Van Raalte, D., Ross, C.J., Hayden, M.R., Bakker, A.C., Dijkhuizen, P., *et al.* (2005). Gene therapy for lipoprotein lipase deficiency: working toward clinical application. Hum Gene Ther *16*, 1276-1286.

Rip, J., Sierts, J.A., Vaessen, S.F., Kastelein, J.J., Twisk, J., and Kuivenhoven, J.A. (2007). Adeno-associated virus LPL(S447X) gene therapy in LDL receptor knockout mice. Atherosclerosis *194*, 55-61.

Rohlmann, A., Gotthardt, M., Hammer, R.E., and Herz, J. (1998). Inducible inactivation of hepatic LRP gene by cre-mediated recombination confirms role of LRP in clearance of chylomicron remnants. J Clin Invest *101*, 689-695.

Ross, C.J., Twisk, J., Meulenberg, J.M., Liu, G., van den Oever, K., Moraal, E., Hermens, W.T., Rip, J., Kastelein, J.J., Kuivenhoven, J.A., and Hayden, M.R. (2004). Long-term correction of murine lipoprotein lipase deficiency with AAV1-mediated gene transfer of the naturally occurring LPL(S447X) beneficial mutation. Hum Gene Ther *15*, 906-919.

Ross, R., and Glomset, J.A. (1976). The pathogenesis of atherosclerosis (second of two parts). N Engl J Med *295*, 420-425.

Ruhling, K., Lang, A., Richard, F., Van Tol, A., Eisele, B., Herzberg, V., and Till, U. (1999). Net mass transfer of plasma cholesteryl esters and lipid transfer proteins in normolipidemic patients with peripheral vascular disease. Metabolism *48*, 1361-1366.

Rye, K.A., and Barter, P.J. (2004). Formation and metabolism of prebeta-migrating, lipid-poor apolipoprotein A-I. Arterioscler Thromb Vasc Biol *24*, 421-428.

Rye, K. A., Bursill, C.A., Lambert, G., Tabet, F., and Barter, P.J. (2008). The metabolism and anti-atherogenic properties of HDL. J Lipid Res.

Santamarina-Fojo, S., and Brewer, H.B., Jr. (1994). Lipoprotein lipase: structure, function and mechanism of action. Int J Clin Lab Res *24*, 143-147.

Santamarina-Fojo, S., Gonzalez-Navarro, H., Freeman, L., Wagner, E., and Nong, Z. (2004). Hepatic lipase, lipoprotein metabolism, and atherogenesis. Arterioscler Thromb Vasc Biol *24*, 1750-1754.

Schgoer, W., Mueller, T., Jauhiainen, M., Wehinger, A., Gander, R., Tancevski, I., Salzmann, K., Eller, P., Ritsch, A., Haltmayer, M., *et al.* (2008). Low phospholipid transfer protein (PLTP) is a risk factor for peripheral atherosclerosis. Atherosclerosis *196*, 219-226.

Schlitt, A., Bickel, C., Thumma, P., Blankenberg, S., Rupprecht, H.J., Meyer, J., and Jiang, X.C. (2003). High plasma phospholipid transfer protein levels as a risk factor for coronary artery disease. Arterioscler Thromb Vasc Biol *23*, 1857-1862.

Schlitt, A., Heine, G.H., Jiang, X.C., Messow, M., Blankenberg, S., Rupprecht, H.J., Ulrich, C., Buerke, M., Werdan, K., Lackner, K.J., *et al.* (2007). Phospholipid transfer protein in hemodialysis patients. Am J Nephrol *27*, 138-143.

Schlitt, A., Liu, J., Yan, D., Mondragon-Escorpizo, M., Norin, A.J., and Jiang, X.C. (2005). Anti-inflammatory effects of phospholipid transfer protein (PLTP) deficiency in mice. Biochim Biophys Acta *1733*, 187-191.

Schumann, R.R., Leong, S.R., Flaggs, G.W., Gray, P.W., Wright, S.D., Mathison, J.C., Tobias, P.S., and Ulevitch, R.J. (1990). Structure and function of lipopolysaccharide binding protein. Science *249*, 1429-1431.

Setala, N.L., Holopainen, J.M., Metso, J., Wiedmer, S.K., Yohannes, G., Kinnunen, P.K., Ehnholm, C., and Jauhiainen, M. (2007). Interfacial and lipid transfer properties of human phospholipid transfer protein: implications for the transfer mechanism of phospholipids. Biochemistry *46*, 1312-1319.

Settasatian, N., Barter, P.J., and Rye, K.A. (2008). Remodeling of apolipoprotein E-containing spherical reconstituted high density lipoproteins by phospholipid transfer protein. J Lipid Res *49*, 115-126.

Settasatian, N., Duong, M., Curtiss, L.K., Ehnholm, C., Jauhiainen, M., Huuskonen, J., and Rye, K.A. (2001). The mechanism of the remodeling of high density lipoproteins by phospholipid transfer protein. J Biol Chem *276*, 26898-26905.

Shelness, G.S., and Sellers, J.A. (2001). Very-low-density lipoprotein assembly and secretion. Curr Opin Lipidol *12*, 151-157.

Shimada, M., Ishibashi, S., Inaba, T., Yagyu, H., Harada, K., Osuga, J.I., Ohashi, K., Yazaki, Y., and Yamada, N. (1996). Suppression of diet-induced atherosclerosis in low density lipoprotein receptor knockout mice overexpressing lipoprotein lipase. Proc Natl Acad Sci U S A *93*, 7242-7246.

Siggins, S., Bykov, I., Hermansson, M., Somerharju, P., Lindros, K., Miettinen, T.A., Jauhiainen, M., Olkkonen, V.M., and Ehnholm, C. (2007). Altered hepatic lipid status and apolipoprotein A-I metabolism in mice lacking phospholipid transfer protein. Atherosclerosis *190*, 114-123.

Siggins, S., Jauhiainen, M., Olkkonen, V.M., Tenhunen, J., and Ehnholm, C. (2003). PLTP secreted by HepG2 cells resembles the high-activity PLTP form in human plasma. J Lipid Res *44*, 1698-1704.

Singaraja, R.R., Bocher, V., James, E.R., Clee, S.M., Zhang, L.H., Leavitt, B.R., Tan, B., Brooks-Wilson, A., Kwok, A., Bissada, N., *et al.* (2001). Human ABCA1 BAC transgenic mice show increased high density lipoprotein cholesterol and ApoAI-dependent efflux stimulated by an internal promoter containing liver X receptor response elements in intron 1. J Biol Chem *276*, 33969-33979.

Singaraja, R.R., Fievet, C., Castro, G., James, E.R., Hennuyer, N., Clee, S.M., Bissada, N., Choy, J.C., Fruchart, J.C., McManus, B.M., *et al.* (2002). Increased ABCA1 activity protects against atherosclerosis. J Clin Invest *110*, 35-42.

Soro, A., Jauhiainen, M., Ehnholm, C., and Taskinen, M.R. (2003). Determinants of low HDL levels in familial combined hyperlipidemia. J Lipid Res *44*, 1536-1544.

Speijer, H., Groener, J.E., van Ramshorst, E., and van Tol, A. (1991). Different locations of cholesteryl ester transfer protein and phospholipid transfer protein activities in plasma. Atherosclerosis *90*, 159-168.

Staels, B., Dallongeville, J., Auwerx, J., Schoonjans, K., Leitersdorf, E., and Fruchart, J.C. (1998). Mechanism of action of fibrates on lipid and lipoprotein metabolism. Circulation *98*, 2088-2093.

Steinberg, D. (1997). Low density lipoprotein oxidation and its pathobiological significance. J Biol Chem *272*, 20963-20966.

Steinberg, D. (2005). Hypercholesterolemia and inflammation in atherogenesis: two sides of the same coin. Mol Nutr Food Res *49*, 995-998.

Stoll, G., and Bendszus, M. (2006). Inflammation and atherosclerosis: novel insights into plaque formation and destabilization. Stroke *37*, 1923-1932.

Sviridov, D., Mukhamedova, N., Remaley, A.T., Chin-Dusting, J., and Nestel, P. (2008). Antiatherogenic functionality of high density lipoprotein: how much versus how good. J Atheroscler Thromb *15*, 52-62.

Tahvanainen, E., Jauhiainen, M., Funke, H., Vartiainen, E., Sundvall, J., and Ehnholm, C. (1999). Serum phospholipid transfer protein activity and genetic variation of the PLTP gene. Atherosclerosis *146*, 107-115.

Tall, A.R. (1995). Plasma cholesteryl ester transfer protein and high-density lipoproteins: new insights from molecular genetic studies. J Intern Med *237*, 5-12.

Tall, A.R. (2008). Cholesterol efflux pathways and other potential mechanisms involved in the athero-protective effect of high density lipoproteins. J Intern Med *263*, 256-273.

Tall, A. ., Blum, C.B., and Grundy, S.M. (1983). Incorporation of radioactive phospholipid into subclasses of high-density lipoproteins. Am J Physiol *244*, E513-516.

Tall, A.R., and Green, P.H. (1981). Incorporation of phosphatidylcholine into spherical and discoidal lipoproteins during incubation of egg phosphatidylcholine vesicles with isolated high density lipoproteins or with plasma. J Biol Chem *256*, 2035-2044.

Tall, A.R., Krumholz, S., Olivecrona, T., and Deckelbaum, R.J. (1985). Plasma phospholipid transfer protein enhances transfer and exchange of phospholipids between very low density lipoproteins and high density lipoproteins during lipolysis. J Lipid Res *26*, 842-851.

Tan, K.C., Shiu, S.W., Wong, Y., and Tam, S. (2005). Plasma phospholipid transfer protein activity and subclinical inflammation in type 2 diabetes mellitus. Atherosclerosis *178*, 365-370.

Tanigawa, H., Billheimer, J.T., Tohyama, J., Zhang, Y., Rothblat, G., and Rader, D.J. (2007). Expression of cholesteryl ester transfer protein in mice promotes macrophage reverse cholesterol transport. Circulation *116*, 1267-1273.

Tollefson, J.H., Ravnik, S., and Albers, J.J. (1988). Isolation and characterization of a phospholipid transfer protein (LTP-II) from human plasma. J Lipid Res *29*, 1593-1602.

Tontonoz P,M.D. (2003). Liver X receptor signaling pathway in cardiovascular disease. Mol Endocrinol *17*, 985-993.

Trigatti, B., Rayburn, H., Vinals, M., Braun, A., Miettinen, H., Penman, M., Hertz, M., Schrenzel, M., Amigo, L., Rigotti, A., and Krieger, M. (1999). Influence of the high density lipoprotein receptor SR-BI on reproductive and cardiovascular pathophysiology. Proc Natl Acad Sci U S A *96*, 9322-9327.

Tu, A.Y., and Albers, J.J. (1999). DNA sequences responsible for reduced promoter activity of human phospholipid transfer protein by fibrate. Biochem Biophys Res Commun *264*, 802-807.

Tu, A.Y., and Albers, J.J. (2001). Functional analysis of the transcriptional activity of the mouse phospholipid transfer protein gene. Biochem Biophys Res Commun *287*, 921-926.

Tu, A.Y., Chen, H., Johnson, K.A., Paigen, B., and Albers, J.J. (1997). Characterization of the mouse gene encoding phospholipid transfer protein. Gene *188*, 115-118.

Tu, A.Y., Nishida, H.I., and Nishida, T. (1993). High density lipoprotein conversion mediated by human plasma phospholipid transfer protein. J Biol Chem *268*, 23098-23105.

Tu, A.Y., Paigen, B., Wolfbauer, G., Cheung, M.C., Kennedy, H., Chen, H., and Albers, J.J. (1999). Introduction of the human PLTP transgene suppresses the atherogenic diet-induced increase in plasma phospholipid transfer activity in C57BL/6 mice. Int J Clin Lab Res *29*, 14-21.

Tu, A.Y., Wolfbauer, G., and Albers, J.J. (1995). Functional characterization of the promoter region of the human phospholipid transfer protein gene. Biochem Biophys Res Commun *217*, 705-711.

Ueda, Y., Gong, E., Royer, L., Cooper, P.N., Francone, O.L., and Rubin, E.M. (2000). Relationship between expression levels and atherogenesis in scavenger receptor class B, type I transgenics. J Biol Chem *275*, 20368-20373.

Ueda, Y., Royer, L., Gong, E., Zhang, J., Cooper, P.N., Francone, O., and Rubin, E.M. (1999). Lower plasma levels and accelerated clearance of high density lipoprotein (HDL) and non-HDL cholesterol in scavenger receptor class B type I transgenic mice. J Biol Chem *274*, 7165-7171.

Urizar, N.L., Dowhan, D.H., and Moore, D.D. (2000). The farnesoid X-activated receptor mediates bile acid activation of phospholipid transfer protein gene expression. J Biol Chem *275*, 39313-39317.

Vaisman, B.L., Lambert, G., Amar, M., Joyce, C., Ito, T., Shamburek, R.D., Cain, W.J., Fruchart-Najib, J., Neufeld, E.D., Remaley, A.T., *et al.* (2001). ABCA1 overexpression leads to hyperalphalipoproteinemia and increased biliary cholesterol excretion in transgenic mice. J Clin Invest *108*, 303-309.

Valenta, D. T., Bulgrien, J.J., Bonnet, D.J., and Curtiss, L.K. (2008). Macrophage PLTP is atheroprotective in LDLr-deficient mice with systemic PLTP deficiency. J Lipid Res *49*, 24-32.

Valenta, D.T., Ogier, N., Bradshaw, G., Black, A.S., Bonnet, D.J., Lagrost, L., Curtiss, L. ., and Desrumaux, C.M. (2006). Atheroprotective potential of macrophage-derived phospholipid transfer protein in low-density lipoprotein receptor-deficient mice is overcome by apolipoprotein AI overexpression. Arterioscler Thromb Vasc Biol *26*, 1572-1578.

van Eck, M., Bos, I.S., Kaminski, W.E., Orso, E., Rothe, G., Twisk, J., Bottcher, A., Van Amersfoort, E.S., Christiansen-Weber, T.A., Fung-Leung, W.P., *et al.* (2002). Leukocyte ABCA1 controls susceptibility to atherosclerosis and macrophage recruitment into tissues. Proc Natl Acad Sci U S A *99*, 6298-6303.

Van Eck, M., Pennings, M., Hoekstra, M., Out, R., and Van Berkel, T.J. (2005). Scavenger receptor BI and ATP-binding cassette transporter A1 in reverse cholesterol transport and atherosclerosis. Curr Opin Lipidol *16*, 307-315.

Van Eck, M., Singaraja, R.R., Ye, D., Hildebrand, R.B., James, E.R., Hayden, M.R., and Van Berkel, T.J. (2006). Macrophage ATP-binding cassette transporter A1 overexpression inhibits atherosclerotic lesion progression in low-density lipoprotein receptor knockout mice. Arterioscler Thromb Vasc Biol *26*, 929-934.

van Haperen, R., van Tol, A., van Gent, T., Scheek, L., Visser, P., van der Kamp, A., Grosveld, F., and de Crom, R. (2002). Increased risk of atherosclerosis by elevated plasma levels of phospholipid transfer protein. J Biol Chem *277*, 48938-48943.

van Haperen, R., van Tol, A., Vermeulen, P., Jauhiainen, M., van Gent, T., van den Berg, P., Ehnholm, S., Grosveld, F., van der Kamp, A., and de Crom, R. (2000). Human plasma phospholipid transfer protein increases the antiatherogenic potential of high density lipoproteins in transgenic mice. Arterioscler Thromb Vasc Biol *20*, 1082-1088.

van Tol, A. (2002). Phospholipid transfer protein. Curr Opin Lipidol *13*, 135-139.

van Tol, A., and Hendriks, H. F. (2001). Moderate alcohol consumption: effects on lipids and cardiovascular disease risk. Curr Opin Lipidol *12*, 19-23.

Varban, M.L., Rinninger, F., Wang, N., Fairchild-Huntress, V., Dunmore, J.H., Fang, Q., Gosselin, M.L., Dixon, K.L., Deeds, J.D., Acton, S.L., *et al.* (1998). Targeted mutation reveals a central role for SR-BI in hepatic selective uptake of high density lipoprotein cholesterol. Proc Natl Acad Sci U S A *95*, 4619-4624.

Venkateswaran, A., Repa, J.J., Lobaccaro, J.M., Bronson, A., Mangelsdorf, D.J., and Edwards, P.A. (2000). Human white/murine ABC8 mRNA levels are highly induced in lipid-loaded macrophages. A transcriptional role for specific oxysterols. J Biol Chem *275*, 14700-14707.

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

Vikstedt, R., Ye, D., Metso, J., Hildebrand, R.B., Van Berkel, T.J., Ehnholm, C., Jauhiainen, M., and Van Eck, M. (2007b). 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 *27*, 578-586.

von Eckardstein, A., Jauhiainen, M., Huang, Y., Metso, J., Langer, C., Pussinen, P., Wu, S., Ehnholm, C., and Assmann, G. (1996). Phospholipid transfer protein mediated conversion of high density lipoproteins generates pre beta 1-HDL. Biochim Biophys Acta *1301*, 255-262.

Vourvouhaki, E., and Dedoussis, G.V. (2008). Cholesterol ester transfer protein: a therapeutic target in atherosclerosis? Expert Opin Ther Targets *12*, 937-948.

Vuletic, S., Jin, L. W., Marcovina, S.M., Peskind, E.R., Moller, T., and Albers, J.J. (2003). Widespread distribution of PLTP in human CNS: evidence for PLTP synthesis by glia and neurons, and increased levels in Alzheimer's disease. J Lipid Res *44*, 1113-1123.

Vuletic, S., Peskind, E.R., Marcovina, S.M., Quinn, J.F., Cheung, M.C., Kennedy, H., Kaye, J.A., Jin, L.W., and Albers, J.J. (2005). Reduced CSF PLTP activity in Alzheimer's disease and other neurologic diseases; PLTP induces ApoE secretion in primary human astrocytes in vitro. J Neurosci Res *80*, 406-413.

Wang, M.D., Franklin, V., and Marcel, Y.L. (2007a). *In vivo* reverse cholesterol transport from macrophages lacking ABCA1 expression is impaired. Arterioscler Thromb Vasc Biol *27*, 1837-1842.

Wang, M.D., Kiss, R.S., Franklin, V., McBride, H.M., Whitman, S.C., and Marcel, Y.L. (2007b). Different cellular traffic of LDL-cholesterol and acetylated LDL-cholesterol leads to distinct reverse cholesterol transport pathways. J Lipid Res *48*, 633-645.

Wang, N., Arai, T., Ji, Y., Rinninger, F., and Tall, A.R. (1998). Liver-specific overexpression of scavenger receptor BI decreases levels of very low density lipoprotein ApoB, low density lipoprotein ApoB, and high density lipoprotein in transgenic mice. J Biol Chem *273*, 32920-32926.

Wang, N., Silver, D.L., Thiele, C., and Tall, A.R. (2001). ATP-binding cassette transporter A1 (ABCA1) functions as a cholesterol efflux regulatory protein. J Biol Chem *276*, 23742-23747.

Wang, X., Collins, H.L., Ranalletta, M., Fuki, I.V., Billheimer, J.T., Rothblat, G.H., Tall, A.R., and Rader, D.J. (2007c). Macrophage ABCA1 and ABCG1, but not SR-BI, promote macrophage reverse cholesterol transport in vivo. J Clin Invest *117*, 2216-2224.

Wasan, K.M., and Cassidy, S.M. (1998). Role of plasma lipoproteins in modifying the biological activity of hydrophobic drugs. J Pharm Sci *87*, 411-424.

Whitmore, T.E., Day, J.R., and Albers, J.J. (1995). Localization of the human phospholipid transfer protein gene to chromosome 20q12-q13.1. Genomics *28*, 599-600.

Wilson, K., Fry, G.L., Chappell, D.A., Sigmund, C.D., and Medh, J.D. (2001). Macrophage-specific expression of human lipoprotein lipase accelerates atherosclerosis in transgenic apolipoprotein e knockout mice but not in C57BL/6 mice. Arterioscler Thromb Vasc Biol *21*, 1809-1815.

Wolfbauer, G., Albers, J.J., and Oram, J.F. (1999). Phospholipid transfer protein enhances removal of cellular cholesterol and phospholipids by high-density lipoprotein apolipoproteins. Biochim Biophys Acta *1439*, 65-76.

Yagyu, H., Ishibashi, S., Chen, Z., Osuga, J., Okazaki, M., Perrey, S., Kitamine, T., Shimada, M., Ohashi, K., Harada, K., *et al.* (1999). Overexpressed lipoprotein lipase protects against atherosclerosis in apolipoprotein E knockout mice. J Lipid Res *40*, 1677-1685.

Yan, D., Navab, M., Bruce, C., Fogelman, A.M., and Jiang, X.C. (2004). PLTP deficiency improves the anti-inflammatory properties of HDL and reduces the ability of LDL to induce monocyte chemotactic activity. J Lipid Res *45*, 1852-1858.

Yang, X.P., Yan, D., Qiao, C., Liu, R.J., Chen, J.G., Li, J., Schneider, M., Lagrost, L., Xiao, X., and Jiang, X.C. (2003). Increased atherosclerotic lesions in apoE mice with plasma phospholipid transfer protein overexpression. Arterioscler Thromb Vasc Biol *23*, 1601-1607.

Yatsuya, H., Tamakoshi, K., Hattori, H., Otsuka, R., Wada, K., Zhang, H., Mabuchi, T., Ishikawa, M., Murata, C., Yoshida, T., *et al.* (2004). Serum phospholipid transfer protein mass as a possible protective factor for coronary heart diseases. Circ J *68*, 11-16.

Yu, L., Hammer, R.E., Li-Hawkins, J., Von Bergmann, K., Lutjohann, D., Cohen, J.C., and Hobbs, H.H. (2002a). Disruption of Abcg5 and Abcg8 in mice reveals their crucial role in biliary cholesterol secretion. Proc Natl Acad Sci U S A *99*, 16237-16242.

Yu, L., Li-Hawkins, J., Hammer, R.E., Berge, K.E., Horton, J.D., Cohen, J.C., and Hobbs, H.H. (2002b). Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol. J Clin Invest *110*, 671-680.

Yvan-Charvet, L., Ranalletta, M., Wang, N., Han, S., Terasaka, N., Li, R., Welch, C., and Tall, A.R. (2007). Combined deficiency of ABCA1 and ABCG1 promotes foam cell accumulation and accelerates atherosclerosis in mice. J Clin Invest *117*, 3900-3908.

Zannis, V. I., Chroni, A., and Krieger, M. (2006). Role of apoA-I, ABCA1, LCAT, and SR-BI in the biogenesis of HDL. J Mol Med *84*, 276-294.

Zaratin, A.C., Quintao, E.C., Sposito, A.C., Nunes, V.S., Lottenberg, A.M., Morton, R.E., and de Faria, E.C. (2004). Smoking prevents the intravascular remodeling of high-density lipoprotein particles: implications for reverse cholesterol transport. Metabolism *53*, 858-862.

Zelcer, N., and Tontonoz, P. (2006). Liver X receptors as integrators of metabolic and inflammatory signaling. J Clin Invest *116*, 607-614.

Zhang, S.H., Reddick, R.L., Piedrahita, J.A., and Maeda, N. (1992). Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. Science *258*, 468-471.

Zhang, W., Yancey, P.G., Su, Y.R., Babaev, V.R., Zhang, Y., Fazio, S., and Linton, M.F. (2003a). Inactivation of macrophage scavenger receptor class B type I promotes atherosclerotic lesion development in apolipoprotein E-deficient mice. Circulation *108*, 2258-2263.

Zhang, X., Qi, R., Xian, X., Yang, F., Blackstein, M., Deng, X., Fan, J., Ross, C., Karasinska, J., Hayden, M.R., and Liu, G. (2008). Spontaneous atherosclerosis in aged lipoprotein lipase-deficient mice with severe hypertriglyceridemia on a normal chow diet. Circ Res *102*, 250-256.

Zhang, Y., Da Silva, J.R., Reilly, M., Billheimer, J. T., Rothblat, G.H., and Rader, D.J. (2005). Hepatic expression of scavenger receptor class B type I (SR-BI) is a positive regulator of macrophage reverse cholesterol transport *in vivo*. J Clin Invest *115*, 2870-2874.

Zhang, Y., Zanotti, I., Reilly, M.P., Glick, J.M., Rothblat, G.H., and Rader, D.J. (2003b). Overexpression of apolipoprotein A-I promotes reverse transport of cholesterol from macrophages to feces in vivo. Circulation *108*, 661-663.

Zilversmit, D.B., Hughes, L.B., and Balmer, J. (1975). Stimulation of cholesterol ester exchange by lipoprotein-free rabbit plasma. Biochim Biophys Acta *409*, 393-398.

Chapter 2

Inducible expression of phospholipid transfer protein (PLTP) in transgenic mice: acute effects of PLTP on lipoprotein metabolism

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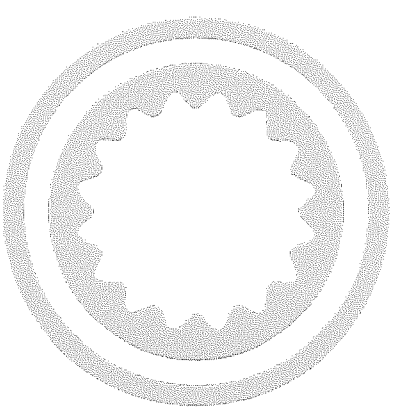

Inducible expression of phospholipid transfer protein (PLTP) in transgenic mice: acute effects of PLTP on lipoprotein metabolism

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Abstract One main determinant in high-density lipoprotein (HDL) metabolism is phospholipid transfer protein (PLTP), a plasma protein that is associated with HDL. In transgenic mice overexpressing human PLTP we found that elevated plasma PLTP levels dose-dependently increased the susceptibility to diet-induced atherosclerosis. This could be mainly due to the fact that most functions of PLTP are potentially atherogenic, such as decreasing plasma HDL levels. To further elucidate the role of PLTP in lipoprotein metabolism and atherosclerosis we generated a novel transgenic mouse model that allows conditional expression of human PLTP. In this mouse model a human PLTP encoding sequence is controlled by a Tet-On system. Upon induction of PLTP expression, our mouse model showed a strongly increased PLTP activity (from 3.0 ± 0.6 to 11.4 ± 2.8 AU, $p < 0.001$). The increase in PLTP activity resulted in an acute decrease in plasma cholesterol of 33% and a comparable decrease in phospholipids. The decrease in total plasma cholesterol and phospholipids was caused by a 35% decrease in HDL-cholesterol level and a 41% decrease in HDL-phospholipid level. These results demonstrate the feasibility of our mouse model to induce an acute elevation of PLTP activity, which is easily reversible. As a direct consequence of an increase in PLTP activity, HDL-cholesterol and HDL-phospholipid levels strongly decrease. Using this mouse model, it will be possible to study the effects of acute elevation of PLTP activity on lipoprotein metabolism and pre-existing atherosclerosis.

Keywords PLTP · Inducible expression · HDL · Cholesterol · Atherosclerosis · Lipoprotein metabolism

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Introduction

Over the last years, in vitro studies and studies in mice showed that phospholipid transfer protein (PLTP) plays several key roles in lipoprotein metabolism. These include the transfer of phospholipids from triglyceride-rich lipoproteins to high-density lipoproteins (HDL) during lipolysis (Tall et al. 1985), the remodeling of HDL-particles (Jauhiainen et al. 1993; Tu et al. 1993), the stimulation of hepatic very low-density lipoprotein (VLDL) secretion (Jiang et al. 2005, 2001; Lie et al. 2002; van Haperen et al. 2002), and the modification of the inflammatory

and oxidative state of lipoproteins (Jiang et al. 2005; Jiang et al. 2002; Schlitt et al. 2005; Yan et al. 2004; Yang et al. 2003).

Diet studies in different atherosclerosis-prone mouse models showed that PLTP deficiency resulted in a decreased development of atherosclerosis (Jiang et al. 2001). Overexpression of human PLTP in low-density lipoprotein receptor (LDLR) deficient mice resulted in a dose-responsive increase in susceptibility to atherosclerosis (van Haperen et al. 2002). Most functions of PLTP are potentially atherogenic: overexpression of human PLTP dose-dependently decreases HDL levels, resulting in a more atherogenic lipoprotein profile (Albers et al. 1996; van Haperen et al. 2002); overexpression of human PLTP stimulates VLDL secretion by the liver (Lie et al. 2002; van Haperen et al. 2002); lipoprotein oxidizability increases following adenoviral overexpression of PLTP in atherosclerosis-prone mice (Jiang et al. 2002; Yang et al. 2003). On the other hand, PLTP has anti-atherogenic potential as well, because it increases the formation of preβ-HDL, an HDL subfraction that is a very efficient acceptor of cellular cholesterol and therefore an important mediator in reverse cholesterol transport (Jiang et al. 1996; Lie et al. 2001; van Haperen et al. 2000). Apparently, PLTP displays both pro- and anti-atherogenic properties, of which the experimental approaches used so far demonstrate that the net effect is proatherogenic (Desrumaux et al. 2003; Jiang et al. 2001; Lie et al. 2004; van Haperen et al. 2002; Yang et al. 2003). However, it is important to notice that PLTP is a lipid transfer protein and thus very sensitive to changes in lipaemia (Rye et al. 1998). Depending on the metabolic status, the proatherogenic effects of PLTP could outweigh the antiatherogenic effects, or vice versa. A different experimental approach might shed more light on the exact physiological role of PLTP in lipoprotein metabolism and atherogenesis. Therefore, we generated a new mouse model with conditional expression of the human PLTP gene, using the tetracycline-responsive gene system that was developed by using the *Escherichia coli* tetracycline resistance Tn10 operon (Gossen and Bujard 1992). This mouse model consists of two transgenes. The first transgene is an improved version of the reverse tetracycline transactivator, 2S-M2 [rtTA2S-M2, developed by Urlinger et al. (Urlinger et al. 2000)], under the control of a ubiquitously expressed hnRNP A2 (heterogeneous nuclear ribonucleoprotein A2) coding sequence. The second transgene is a human PLTP encoding sequence under the transcriptional control of a tetracycline operator and minimal cytomegalovirus promoter (Gossen and Bujard 1992; Orth et al. 2000). The crossbreeding of these two transgenic mouse lines results in a mouse that gives us the opportunity to activate the expression of human PLTP by administration of doxycycline. In the future, using this mouse model, it will be possible to use new and refined approaches studying the physiological role of PLTP. For example, we will be able to investigate the effect of acute elevation of PLTP activity on pre-existing atherosclerosis. In this study, we describe the features of our conditional mouse model. Using this model we studied some effects of acute changes in PLTP activity on lipoprotein metabolism.

Methods

DNA constructs

Two constructs were used to generate transgenic mice that would allow externally inducible expression of PLTP: (1) hnRNP-rtTA-SV40, which consists of a recent version of the tetracycline-controlled transactivator rtTA2S-M2 (Urlinger et al. 2000) under the control of an hnRNP A2 coding sequence (Antoniou et al. 2003). This sequence consists of an 8 kB methylation-free island, containing the incomplete HP1γ (heterochromatin protein 1γ) promoter, first exon and a part of the first intron of genomic human hnRNP A2, which is expressed ubiquitously (Kamma et al. 1999) (Fig. 1a). (2) TRE-hPLTP, which consists of a human PLTP encoding sequence under the transcriptional control of a Tetracycline Response Element (TRE) containing eight tetracycline operators, and minimal cytomegalovirus promoter (P_{minCMV}) (Gossen and Bujard 1992; Orth et al. 2000) (Fig. 1b). This construct was generated using the pTRE vector (GenBank Accession #U89931) of ClonTech (ClonTech Laboratories, CA, USA). The human PLTP encoding sequence was constructed from genomic human PLTP, of which the sequences upstream from exon 1 were removed, and the genomic sequences between the *Bam*HI sites in exons 5 and 8 were replaced by the equivalent cDNA sequence (264 bp) (Day et al. 1994).

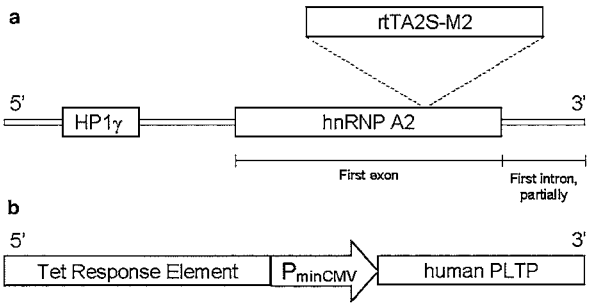

Fig. 1 Schematic diagram showing constructs used for generating transgenic mice. (**a**) hnRNP-rtTA-SV40, which consists of a tetracycline-controlled transactivator (rtTA2S-M2) under the control of an hnRNP A2 coding sequence. This sequence consists of an 8 kb methylation-free island, containing the incomplete HP1(and promoter, first exon and a part of the first intron of genomic human hnRNP A2. (**b**) TRE-hPLTP, which consists of a human PLTP encoding sequence under the transcriptional control of a Tetracycline Response Element (TRE) and minimal cytomegalovirus promoter. The human PLTP encoding sequence was constructed from genomic human PLTP, of which the sequence upstream from exon 1 was removed, and the genomic sequences between the *Bam*HI sites in exons 5 and 8 were replaced by the equivalent cDNA sequence (264 bp)

Generation and treatment of transgenic mice

For both constructs, vector sequences were removed by restriction endonuclease digestion. Linear DNA was dissolved in microinjection buffer (10 mmol/L Tris–HCl, pH 7.5, 0.1 mM EDTA) at a concentration of 1–2 μg/ml. DNA constructs were microinjected into fertilized oocytes from FVB mice. These oocytes were transferred into the oviducts of pseudopregnant foster females. Transgenic founder mice were bred with FVB mice to obtain transgenic mice. FVB transgenic mice were backcrossed with C57Bl/6 mice for at least ten generations. Genotypes were identified by polymerase chain reaction (PCR) using genomic DNA from tail clips of 10-day-old mice. Primers used for the presence of the rtTA transgene: sense 5′-GGA ACA GGA GCA TCA AGT AGC-3′, antisense 5′-GCG TCA GCA GGC AGC ATA TC-3′; 32 cycles (45 s 94°C, 60 s 62°C, 90 s. 72°C). Primers used for the presence of the human PLTP transgene: sense 5′-GCC ACA GCA GGA GCT GAT GC-3′, antisense 5′-GCG GAT GGA CAC ACC CTC AGC-3′; 32 cycles (45 s 94°C, 90 s 65°C, 90 s 72°C).

Animals were kept on regular chow. Blood samples were collected from the orbital plexus. Blood was centrifuged at 2,800 rpm for 15 min at 4°C. Plasma samples were stored at −80°C until measurement of PLTP activity and quantification of plasma lipids. All experiments were performed in accordance with institutional and national regulations.

In vivo induction of PLTP activity

Mice carrying the reverse tetracycline transactivator (referred to as rtTAtg mice) were crossed with mice carrying the human PLTP gene under the control of the tetracycline-responsive element (referred to as Tre-PLTPtg mice), resulting in Tre-PLTPtg//rtTAtg mice. As controls, transgenic mice were used having only one of the transgenes, thus either lacking the rtTA transgene, or the Tre-PLTP transgene. Induction of PLTP expression needs addition of doxycycline to the drinking water. In order to mask the bitter taste of doxycycline, 5% sucrose was added. First, the mice were kept on drinking water containing 5% sucrose for 3 days, followed by drinking water containing 2 mg/ml doxycycline hydrochloride (Sigma) + 5% sucrose for 2 days. Doxycycline-containing drinking water was protected from light. PLTP activity was determined before the sucrose run-in period, after 3 days of sucrose administration and after 2 days of doxycycline administration. In an additional experiment, the turnover of the induced PLTP activity was determined. A group of Tre-PLTPtg//rtTAtg mice was treated according the regime described above,

followed by a 5-days period of normal drinking water. Every day, a small amount of blood was taken by tail bleeding, starting after the sucrose run-in period of 3 days. In these samples PLTP activity was measured.

PLTP activity assay

PLTP activity was measured using a phospholipid vesicle-HDL system, according to Speijer et al. (Speijer et al. 1991). PLTP activity is expressed as arbitrary units (AU). One arbitrary unit is equal to the level of PLTP activity found in human reference plasma, which is 13.9 μmol/ml/h.

Separation of lipoproteins

High-density lipoprotein and non-HDL fractions were separated by density gradient ultracentrifugation of freshly isolated plasma samples using a Beckman 42.2 Ti rotor (42,000 rpm, 2 h 50 min, 12°C) at d = 1.063 g/ml.

Quantification of plasma lipids

Cholesterol concentration was determined enzymatically using a Free Cholesterol C kit (WAKO) after hydrolysis of cholesteryl esters with cholesterol esterase from *Candida cylindracaea* (Boehringer). Phospholipid concentration was measured using the PAP150 kit from BioMerieux. Triglycerides were measured using a triglyceride kit from WAKO.

Protein analysis of plasma samples

In order to analyze apoAI protein mass, equal plasma samples of individual mice were subjected to electrophoresis on precast 4–15% SDS-polyacrylamide gradient gels (Bio-Rad) which were subsequently stained with Coomassie blue (Brilliant Blue R, Sigma).

mRNA expression analysis

Mice were humanely killed and organs were quickly removed and homogenized. Total RNA was isolated using the RNeasy Mini Kit (Qiagen). Isolated RNA was used as a template for reverse transcription primed by oligo(dT). cDNA was used as a template for Real-Time PCR to quantitate RNA expression using the MyIQ (Bio-Rad). Sample cycle threshold (Ct) values were determined from plots of relative fluorescence units versus PCR cycle number during exponential amplification. Relative mRNA levels were normalized to hypoxanthine–guanine phosphoribosyl transferase expression using the ΔCt method $[2^{(-\Delta Ct)}]$ and presented as arbitrary units. All experiments were repeated three times. Details on the primer sets used and PCR conditions are available upon request via e-mail (m.decrom@erasmusmc.nl).

Statistical analysis

All values are expressed as means ± standard error of the mean. Statistical analyses were done by one-way analysis of variance with Bonferroni multiple comparison tests or Student's t-tests using Intercooled Stata 8.2/SE software (Stata Corporation, College Station, TX, USA).

Results

Induction of PLTP expression

First, we tested whether inducible expression of human PLTP could be achieved in our newly generated mouse model. To this end, three experimental groups were used: Tre-PLTPtg//rtTAtg mice, Tre-PLTPtg mice and rtTAtg mice. Mice were kept on drinking water containing 5% sucrose for 3 days prior to doxycycline administration, When the effect of sucrose on plasma PLTP activity and lipid levels was measured, no statistically significant differences in plasma PLTP activity between groups before and after sucrose administration were found (Fig. 2). PLTP activity in the Tre-PLTPtg mice or rtTAtg mice (PLTP activity 2.9 ± 0.7 AU and 2.8 ± 0.7 AU, respectively) did not differ from the activity in chow-fed wildtype mice on normal drinking water (3.3 ± 0.8 AU, n = 6 mice). After 3 days, drinking water containing 5% sucrose was replaced with drinking water containing 2 mg/ml doxycycline + 5% sucrose. After 2 days, plasma PLTP activity was measured. In the Tre-PLTPtg mice and rtTAtg mice, administration of doxycycline had no effect on PLTP

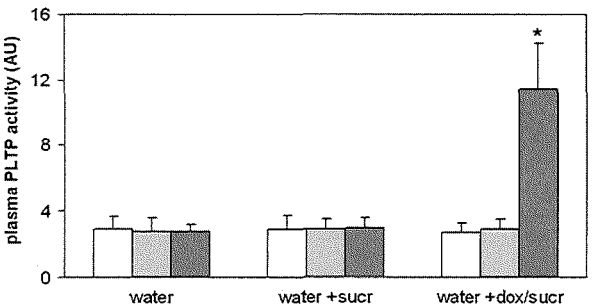

Fig. 2 Plasma PLTP activity levels. PLTP was measured in plasma samples and expressed in arbitrary units (1 AU is equal to the level of PLTP activity found in human reference plasma, 13.9 μmol/ml/h). Measurements were performed at three time points: "water" (normal drinking water), "water + sucr" (after 3 days of drinking water containing 5% sucrose), "water + dox/sucr" (after 2 days of drinking water containing 2 mg/ml doxycycline + 5% sucrose). *White bars* indicate Tre-PLTPtg mice, *gray bars* indicate rtTAtg mice, *black bars* indicate Tre-PLTPtg//rtTAtg mice. n = 15–18 mice/group. *$p < 0.001$, versus Tre-PLTPtg//rtTAtg + dox/sucr and versus Tre-PLTPtg + sucr and rtTAtg + sucr

activity. However, in the PLTPtg//rtTAtg group administration of doxycycline resulted in a strong increase in PLTP activity (from 3.0 ± 0.6 to 11.4 ± 2.8 AU, Fig. 2).

Next, the duration of the doxycycline-induced PLTP activity was determined. A group of Tre-PLTPtg//rtTAtg mice was treated according to the regime described above, followed by a 5-days period on normal drinking water. During the 2 days of treatment with doxycycline, PLTP activity increased from 2.3 ± 0.4 AU to 9.2 ± 2.2 AU (Fig. 3). PLTP activity further increased to 9.9 ± 2.3 AU after 1 day after on normal drinking water and subsequently started to decline. Five days after discontinuing the doxycycline treatment, PLTP activity had returned to the initial level.

Effects of human PLTP expression on plasma lipids and lipoproteins

Cholesterol and phospholipid levels were measured in plasma to investigate the effects of an acute increase in plasma PLTP activity on lipid metabolism. After administration of doxycycline, there was only a 10% decrease in plasma cholesterol in one of the control groups, Tre-PLTPtg mice (Fig. 4a, from

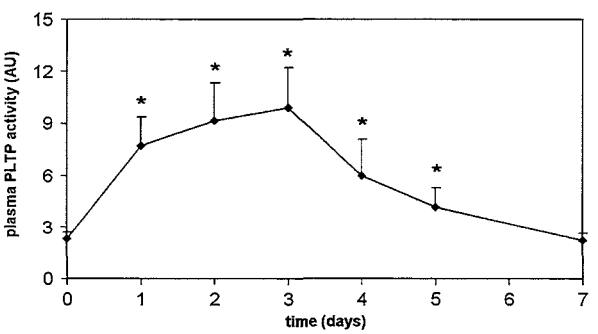

Fig. 3 Plasma PLTP activity levels in Tre-PLTPtg//rtTAtg mice. PLTP was measured in plasma samples and expressed in arbitrary units. Time points of measurement: $t = 0$ (after 3 days of drinking water containing 5% sucrose); t = 1 and t = 2 (drinking water containing 2 mg/ml doxycycline + 5% sucrose); t = 3, t = 4, t = 5, t = 7 (normal drinking water). n = 6 mice. *$p < 0.001$ versus $t = 0$

2.7 ± 0.3 mM to 2.5 ± 0.4 mM, $p < 0.02$), while plasma cholesterol levels in the other control group, rtTAtg mice, remained unchanged. In the Tre-PLTPtg//rtTAtg mice however, induction of PLTP expression by doxycycline administration resulted in a 33% decrease in plasma cholesterol (from 2.7 ± 0.4 mM to 1.8 ± 0.6 mM, $p < 0.001$). Plasma phospholipid levels were also lowered after induction of PLTP activity, by 33% (Fig. 5a, from 2.6 ± 0.4 mM to 1.7 ± 0.5 mM, $p < 0.001$). In the control groups, phospholipid levels did not change significantly following administration of doxycycline. Addition of 5% sucrose to the drinking water had no statistically significant effects on plasma cholesterol or phospholipid levels. Triglyceride levels are very low in chow-fed C57Bl/6 mice. Administration of doxycycline resulted in a decrease in triglyceride levels, both in the control groups (from 0.52 ± 0.16 to 0.35 ± 0.17 mM in Tre-PLTPtg mice, $p < 0.001$, and from 0.48 ± 0.17 mM to 0.35 ± 0.17 mM in rtTAtg mice, $p < 0.001$) as well as in the Tre-PLTPtg//rtTAtg mice (from 0.54 ± 0.19 to 0.33 ± 0.17 mM, $p < 0.001$), indicating that the decrease in plasma triglycerides is not caused by the action of PLTP.

The decreases in plasma cholesterol and phospholipids in the Tre-PLTPtg//rtTAtg mice were studied in more detail. Lipoprotein fractions were separated by ultracentrifugation. In all groups, administration of 5% sucrose significantly increased total cholesterol and phospholipids in the non-HDL fractions, which remained unchanged after the subsequent addition of doxycycline (Fig. 4c, 5c). Administration of doxycycline + 5% sucrose had no statistically significant effects on total cholesterol levels or phospholipid levels in the HDL fractions of both control groups (Fig. 4b, 5b). However, in the human PLTP-expressing Tre-PLTPtg//rtTAtg mice, HDL-cholesterol

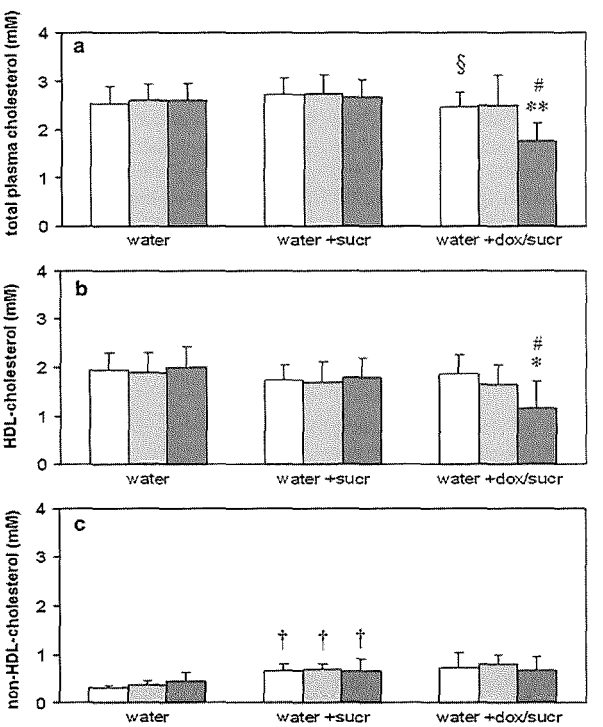

Fig. 4 Cholesterol levels. (**a**) Total plasma cholesterol, (**b**) HDL-cholesterol, (**c**) non-HDL-cholesterol. Measurements were performed at three time points: "water" (normal drinking water), "water + sucr" (after 3 days of drinking water containing 5% sucrose), "water + dox/sucr" (after 2 days of drinking water containing 2 mg/ml doxycycline + 5% sucrose). *White bars* indicate Tre-PLTPtg mice, *gray bars* indicate rtTAtg mice, *black bars* indicate Tre-PLTPtg//rtTAtg mice. n = 15–18 mice/group. $^{*}p < 0.05$, $^{**}p < 0.001$, versus Tre-PLTPtg and rtTAtg + dox/sucr. $^{\#}p < 0.001$, versus Tre-PLTPtg//rtTAtg + sucr. $^{\S}p < 0.05$, versus Tre-PLTPtg and rtTAtg + sucr. † $p < 0.01$, each group versus normal drinking water

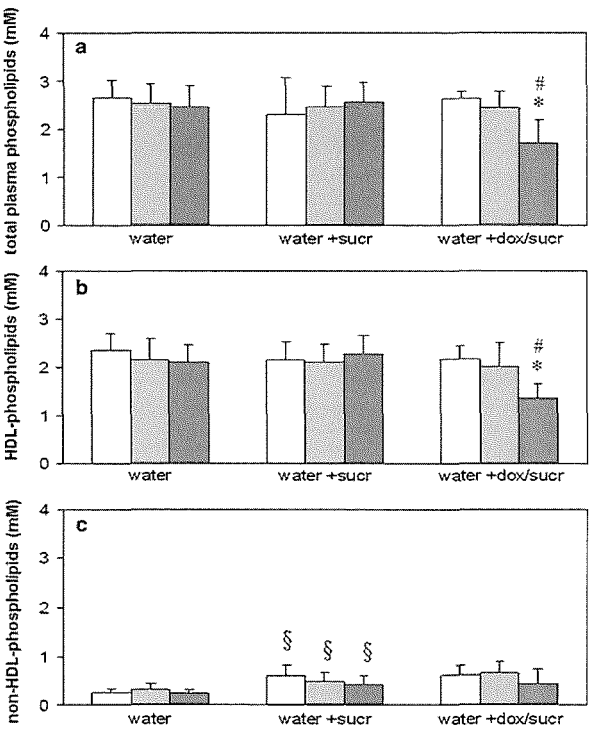

Fig. 5 Phospholipid levels. (**a**) Total plasma phospholipids, (**b**) HDL-phospholipids, (**c**) non-HDL-phospholipids. Measurements were performed at three time points: "water" (normal drinking water), "water + sucr" (after 3 days of drinking water containing 5% sucrose), "water + dox/sucr" (after 2 days of drinking water containing 2 mg/ml doxycycline + 5% sucrose). *White bars* indicate Tre-PLTPtg mice, *gray bars* indicate rtTAtg mice, *black bars* indicate Tre-PLTPtg//rtTAtg mice. n = 15–18 mice/group. $^{*}p < 0.001$, versus Tre-PLTPtg and rtTAtg + dox/sucr. $^{\#}p < 0.001$, versus Tre-PLTPtg//rtTAtg + sucr. $^{\S}p < 0.01$, each group versus normal drinking water

decreased by 35% (from 1.8 ± 0.4 mM to 1.2 ± 0.4 mM, $p < 0.001$), and HDL-phospholipid level decreased by 41% (from 2.3 ± 0.4 mM to 1.4 ± 0.3 mM, $p < 0.001$). Furthermore, plasma apoAI protein level decreased after induction of PLTP expression (Fig. 6).

mRNA expression analysis

Liver rtTA and PLTP mRNA expression levels were measured by Real-Time PCR (Table 1). RNA was isolated from livers of Tre-PLTPtg//rtTAtg mice, Tre-PLTPtg mice and rtTAtg mice that had been treated with doxycycline for 2 days, and from livers of Tre-PLTPtg//rtTAtg mice that had not received doxycycline in the drinking water. rtTAtg mice and the Tre-PLTPtg//rtTAtg mice (with and without doxycycline) showed similar levels of rtTA expression. Induction by doxycycline gave rise to a tenfold increase in PLTP mRNA (human + murine) in the inducible transgenic mouse model compared to Tre-PLTPtg//rtTAtg mice that had not been given doxycycline and therefore expressed only murine PLTP (from 1.5 ± 0.9 to 11.7 ± 2.7 AU, $p < 0.001$). In the control groups there were no differences in PLTP expression. Induction of human PLTP expression did not suppress murine PLTP mRNA levels.

In addition, the effect of an acute increase in PLTP activity on RNA expression level of various key players in lipoprotein metabolism was studied (Table 1). Compared to the control groups, induction of PLTP in the double transgenic mice did not result in a statistically significant change in RNA expression level of ATP-binding cassette A1 (ABCA1), apolipoprotein AI (apoAI), apolipoprotein B (apoB) or scavenger receptor class B type I (SRBI).

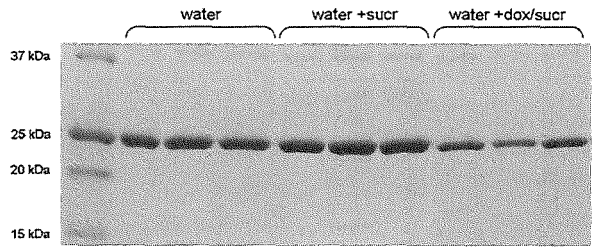

Fig. 6 ApoAI protein mass in individual plasma samples of Tre-PLTPtg//rtTAtg mice, three mice per condition (water, water with sucrose, water with doxycycline and sucrose). Plasma samples were subjected to electrophoresis on precast 4–15% SDS-polyacrylamide gradient gels and subsequently stained with Coomassie blue

Table 1 Liver mRNA analysis

		mPLTP	hPLTP	m + hPLTP	rtTA	ABCA1	apoAI	apoB	SRBI
Tre-PLTPtg	+dox	1.2 ± 0.4	0.0 ± 0.0*	1.2 ± 0.4*	0.0 ± 0.0	1.2 ± 0.2	0.9 ± 0.2	0.7 ± 0.4	1.0 ± 0.3
rtTAtg	+dox	0.9 ± 0.3	0.0 ± 0.0*	1.0 ± 0.2*	0.9 ± 0.1**	0.9 ± 0.4	0.8 ± 0.2	1.0 ± 0.9	1.1 ± 0.4
Tre-PLTPtg//rtTAtg	+dox	1.2 ± 0.5	10.5 ± 2.6	11.7 ± 2.7	1.0 ± 0.3**	1.2 ± 0.5	0.7 ± 0.2	1.0 ± 0.5	1.2 ± 0.4
Tre-PLTPtg//rtTAtg	−dox	1.5 ± 1.0	0.0 ± 0.0*	1.5 ± 0.9*	0.9 ± 0.1**	0.9 ± 0.1	0.8 ± 0.1	0.5 ± 0.1	1.0 ± 0.1

* $p < 0.001$, versus Tre-PLTPtg//rtTAtg + dox

* $p < 0.001$, versus Tre-PLTPtg + dox

In another experiment, tissue distribution of murine and human PLTP mRNA was determined in a group of four doxycycline-treated Tre-PLTPtg// rtTAtg mice by Real-Time PCR. Murine PLTP mRNA was mostly present in lung tissue, followed by liver, adipose tissue, brain and heart (Fig. 7a). Lower levels of murine PLTP mRNA were found in adrenals, spleen and kidney. Upon doxycycline-induction, human PLTP expression appeared to be strongest in liver, lung and kidney (Fig. 7b). Low levels of human PLTP mRNA were detected in spleen, heart, adrenal and adipose tissue. Only negligible amounts of human PLTP mRNA were detected in brain.

Discussion

In the present study, we describe a mouse model with conditional induction of human PLTP expression: 2 days of doxycycline administration resulted in a strong increase in plasma PLTP activity. The induction of PLTP activity not only proved to be fast, but also transient. Discontinuation of doxycycline administration via the drinking water resulted in a decline of PLTP activity to basal activity levels after 5 days. Upon induction, PLTP mRNA level in the liver increased by tenfold, while in the control groups (Tre-PLTPtg and rtTAtg) plasma PLTP activity and PLTP liver mRNA did not increase. The non-induced double transgenic mouse line (Tre-PLTPtg//rtTAtg without doxycycline) did not show any significant differences in RNA expression compared with control mice, so our inducible mouse model does not show any intrinsic background activity ("leakage") in the non-induced state. The improved version of the tetracycline-controlled transactivator we used (rtTA2S-M2) has been reported to have a low background activity (Lamartina et al. 2002, 2003; Salucci et al. 2002). Our inducible mouse model shows a broad pattern of human PLTP expression (liver, kidney > lung > spleen, heart, adrenal, adipose tissue). It is not known whether this is a reflection of the normal regulation of PLTP expression found in human tissues (Day et al. 1994), a reflection of the doxycycline distribution among tissues (Michel et al. 1984), or a combination of both. The high human PLTP expression levels in liver and kidney might be

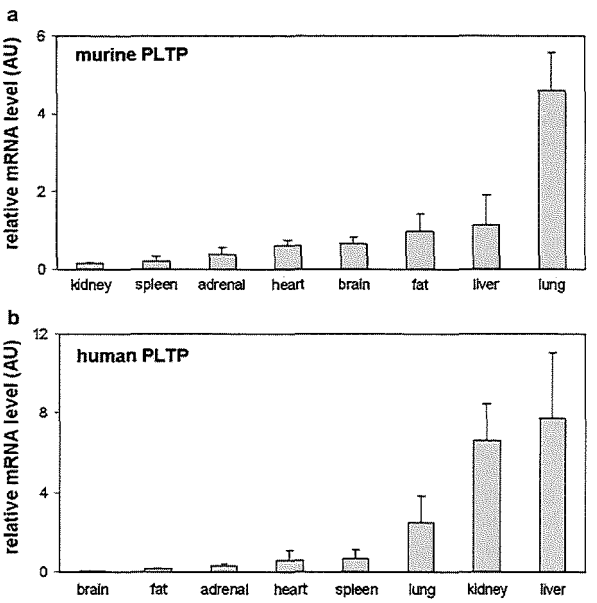

Fig. 7 mRNA expression levels. (**a**) Tissue distribution of murine PLTP mRNA expression, (**b**) Tissue distribution of human PLTP mRNA expression. Tissues were isolated from Tre-PLTPtg// rtTAtg mice supplied with drinking water containing 2 mg/ml doxycycline + 5% sucrose for 2 days and subsequently sacrificed (n = 4). mRNA levels were normalized to hypoxanthine-guanine phosphoribosyl transferase expression using the (Ct method ($2^{[-\Delta Ct]}$) and presented as arbitrary units

explained by doxycycline distribution between tissues: after oral doxycycline administration to rats, highest levels were measured in excretory organs (i.e., liver and kidney) (Michel et al. 1984). No human PLTP mRNA was detected in brain tissue, probably because doxycycline poorly crosses the blood brain barrier (Michel et al. 1984). The distribution pattern of murine PLTP in human PLTP expressing PLTPtg//rtTAtg mice is comparable with the pattern found in C57BL/6 wildtype mice (Jiang and Bruce 1995).

The increase in PLTP activity in the double transgenic mice resulted in a decrease in plasma cholesterol of 33% and a comparable decrease in plasma phospholipids. These effects are explained by a decrease in HDL-cholesterol of 35% and in HDL-phospholipids of 41%. These results are well comparable with our previous studies in human PLTP transgenic mice, in which a 2.5- to 4.5-fold increase in PLTP activity in plasma resulted in a 30–40% decrease in plasma levels of HDL-cholesterol (van Haperen et al. 2000). Concomitantly with the decrease in plasma HDL level, plasma apoAI protein level decreased. This is probably caused by a PLTP-induced increase in HDL catabolism (Föger et al. 1997). In addition, PLTP has been reported to cause proteolytic cleavage of apoAI (Jauhiainen et al. 1999). The decrease in HDL levels cannot be explained by changes in expression of SRBI, apoB or ABCA1. Compared to the control groups, induction of PLTP in the double transgenic mice did not result in statistical changes of mRNA expression levels of these genes. Therefore, the decline in HDL is probably a direct effect of PLTP.

Acute elevation of plasma PLTP activity has been studied before using adenovirus-mediated overexpression of human PLTP (Ehnholm et al. 1998; Föger et al. 1997; Yang et al. 2003). As in our inducible mouse model, also in these studies an increase in PLTP activity resulted in an acute decrease in HDL-cholesterol and HDL-phospholipids. An advantage of the inducible mouse model is that it provides a stable and controlled elevation of PLTP activity, which differs from the transient induction obtained by using an adenoviral transfection system. Another important feature of our mouse model is the efficacy to induce PLTP activity, which only requires the administration of doxycycline to the drinking water. We are well

aware of the fact that long-term administration of high doses of doxycycline might disturb intestinal flora (Riond and Riviere 1988), resulting in diarrhea and colitis. However, the doxycycline dose used in our present study was relatively low and symptoms pointing to a disturbed intestinal flora were not observed. In future experiments, it will be possible to further decrease the doxycycline dose, as maximal induction of PLTP activity was already reached at a dose of 0.25 mg/ml (data not shown). Recently, the 2S-M2-version of the tetracycline-controlled transactivator has been used in a mouse model that allowed Cre-mediated tetracycline-dependent gene expression in specific embryonic tissues (Yu et al. 2005), and in two mouse models with inducible and tissue-specific gene expression in the neuronal system of adult mice (Kerrison et al. 2005; Michalon et al. 2005). To the best of our knowledge, the present study is the first one to describe an inducible mouse model in the field of cardiovascular research.

In conclusion, the results of our study demonstrate the feasibility of our mouse model to induce an acute elevation of PLTP activity, which is easily reversible. Using this mouse model, it will be possible to study the effects of acute elevation of PLTP activity on lipoprotein metabolism and pre-existing atherosclerosis.

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References

Albers JJ, Tu AY, Paigen B, Chen H, Cheung MC, Marcovina SM (1996) Transgenic mice expressing human phospholipid transfer protein have increased HDL/non-HDL cholesterol ratio. Int J Clin Lab Res 26:262–267

Antoniou M, Harland L, Mustoe T, Williams S, Holdstock J, Yague E, Mulcahy T, Griffiths M, Edwards S, Ioannou PA, Mountain A, Crombie R (2003) Transgenes encompassing dual-promoter CpG islands from the human TBP and HNRPA2B1 loci are resistant to heterochromatin-mediated silencing. Genomics 82:269–279

Day JR, Albers JJ, Lofton-Day CE, Gilbert TL, Ching AF, Grant FJ, O'Hara PJ, Marcovina SM, Adolphson JL (1994) Complete cDNA encoding human phospholipid transfer protein from human endothelial cells. J Biol Chem 269:9388–9391

Desrumaux CM, Mak PA, Boisvert WA, Masson D, Stupack D, Jauhiainen M, Ehnholm C, Curtiss LK (2003) Phospholipid transfer protein is present in human atherosclerotic lesions and is expressed by macrophages and foam cells. J Lipid Res 44:1453–1461

Ehnholm S, van Dijk KW, van't Hof B, van der Zee A, Olkkonen VM, Jauhiainen M, Hofker M, Havekes L, Ehnholm C (1998) Adenovirus mediated overexpression of human phospholipid transfer protein alters plasma HDL levels in mice. J Lipid Res 39:1248–1253

Föger B, Santamarina-Fojo S, Shamburek RD, Parrot CL, Talley GD, Brewer HB Jr (1997) Plasma phospholipid transfer protein. Adenovirus-mediated overexpression in mice leads to decreased plasma high density lipoprotein (HDL) and enhanced hepatic uptake of phospholipids and cholesteryl esters from HDL. J Biol Chem 272:27393–27400

Gossen M, Bujard H (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 89:5547–5551

Jauhiainen M, Metso J, Pahlman R, Blomqvist S, van Tol A, Ehnholm C (1993) Human plasma phospholipid transfer protein causes high density lipoprotein conversion. J Biol Chem 268:4032–4036

Jauhiainen M, Huuskonen J, Baumann M, Metso J, Oka T, Egashira T, Hattori H, Olkkonen VM, Ehnholm C (1999) Phospholipid transfer protein (PLTP) causes proteolytic cleavage of apolipoprotein A-I. J Lipid Res 40:654–664

Jiang XC, Bruce C (1995) Regulation of murine plasma phospholipid transfer protein activity and mRNA levels by lipopolysaccharide and high cholesterol diet. J Biol Chem 270:17133–17138

Jiang X, Francone OL, Bruce C, Milne R, Mar J, Walsh A, Breslow JL, Tall AR (1996) Increased prebeta-high density lipoprotein, apolipoprotein AI, and phospholipid in mice expressing the human phospholipid transfer protein and human apolipoprotein AI transgenes. J Clin Invest 98:2373–2380

Jiang XC, Qin S, Qiao C, Kawano K, Lin M, Skold A, Xiao X, Tall AR (2001) Apolipoprotein B secretion and atherosclerosis are decreased in mice with phospholipid-transfer protein deficiency. Nat Med 7:847–852

Jiang XC, Tall AR, Qin S, Lin M, Schneider M, Lalanne F, Deckert V, Desrumaux C, Athias A, Witztum JL, Lagrost L (2002) Phospholipid transfer protein deficiency protects circulating lipoproteins from oxidation due to the enhanced accumulation of vitamin E. J Biol Chem 277:31850–31856

Jiang XC, Li Z, Liu R, Yang XP, Pan M, Lagrost L, Fisher EA, Williams KJ (2005) Phospholipid transfer protein deficiency impairs apolipoprotein-B secretion from hepatocytes by stimulating a proteolytic pathway through a relative deficiency of vitamin E and an increase in intracellular oxidants. J Biol Chem 280:18336–18340

Kamma H, Horiguchi H, Wan L, Matsui M, Fujiwara M, Fujimoto M, Yazawa T, Dreyfuss G (1999) Molecular characterization of the hnRNP A2/B1 proteins: tissue-specific expression and novel isoforms. Exp Cell Res 246:399–411

Kerrison JB, Duh EJ, Yu Y, Otteson DC, Zack DJ (2005) A system for inducible gene expression in retinal ganglion cells. Invest Ophthalmol Vis Sci 46:2932–2939

Lamartina S, Roscilli G, Rinaudo CD, Sporeno E, Silvi L, Hillen W, Bujard H, Cortese R, Ciliberto G, Toniatti C (2002) Stringent control of gene expression in vivo by using novel doxycycline-dependent trans-activators. Hum Gene Ther 13:199–210

Lamartina S, Silvi L, Roscilli G, Casimiro D, Simon AJ, Davies ME, Shiver JW, Rinaudo CD, Zampaglione I, Fattori E, Colloca S, Gonzalez Paz O, Laufer R, Bujard H, Cortese R, Ciliberto G, Toniatti C (2003) Construction of an rtTA2(s)-m2/tts(kid)-based transcription regulatory switch that displays no basal activity, good inducibility, and high responsiveness to doxycycline in mice and non-human primates. Mol Ther 7:271–280

Lie J, de Crom R, Jauhiainen M, van Gent T, van Haperen R, Scheek L, Jansen H, Ehnholm C, van Tol A (2001) Evaluation of phospholipid transfer protein and cholesteryl ester transfer protein as contributors to the generation of pre beta-high-density lipoproteins. Biochem J 360:379–385

Lie J, de Crom R, van Gent T, van Haperen R, Scheek L, Lankhuizen I, van Tol A (2002) Elevation of plasma phospholipid transfer protein in transgenic mice increases VLDL secretion. J Lipid Res 43:1875–1880

Lie J, de Crom R, van Gent T, van Haperen R, Scheek L, Sadeghi-Niaraki F, van Tol A (2004) Elevation of plasma phospholipid transfer protein increases the risk of atherosclerosis despite lower apolipoprotein B-containing lipoproteins. J Lipid Res 45:805–811

Michalon A, Koshibu K, Baumgartel K, Spirig DH, Mansuy IM (2005) Inducible and neuron-specific gene expression in the adult mouse brain with the rtTA2S-M2 system. Genesis 43:205–212

Michel G, Mosser J, Olle J (1984) Pharmacokinetics and tissue localization of doxycycline polyphosphate and doxycycline hydrochloride in the rat. Eur J Drug Metab Pharmacokinet 9:149–153

Orth P, Schnappinger D, Hillen W, Saenger W, Hinrichs W (2000) Structural basis of gene regulation by the tetracycline inducible Tet repressor-operator system. Nat Struct Biol 7:215–219

Riond JL, Riviere JE (1988) Pharmacology and toxicology of doxycycline. Vet Hum Toxicol 30:431–443

Rye KA, Jauhiainen M, Barter PJ, Ehnholm C (1998) Triglyceride-enrichment of high density lipoproteins enhances their remodelling by phospholipid transfer protein. J Lipid Res 39:613–622

Salucci V, Scarito A, Aurisicchio L, Lamartina S, Nicolaus G, Giampaoli S, Gonzalez-Paz O, Toniatti C, Bujard H, Hillen W, Ciliberto G, Palombo F (2002) Tight control of gene expression by a helper-dependent adenovirus vector carrying the rtTA2(s)-M2 tetracycline transactivator and repressor system. Gene Ther 9:1415–1421

Schlitt A, Liu J, Yan D, Mondragon-Escorpizo M, Norin AJ, Jiang XC (2005) Anti-inflammatory effects of phospholipid transfer protein (PLTP) deficiency in mice. Biochim Biophys Acta 1733:187–191

Speijer H, Groener JE, van Ramshorst E, van Tol A (1991) Different locations of cholesteryl ester transfer protein and phospholipid transfer protein activities in plasma. Atherosclerosis 90:159–168

Tall AR, Krumholz S, Olivecrona T, Deckelbaum RJ (1985) Plasma phospholipid transfer protein enhances transfer and exchange of phospholipids between very low density lipoproteins and high density lipoproteins during lipolysis. J Lipid Res 26:842–851

Tu AY, Nishida HI, Nishida T (1993) High density lipoprotein conversion mediated by human plasma phospholipid transfer protein. J Biol Chem 268:23098–23105

Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W (2000) Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. Proc Natl Acad Sci USA 97:7963–968

van Haperen R, van Tol A, Vermeulen P, Jauhiainen M, van Gent T, van den Berg P, Ehnholm S, Grosveld F, van der Kamp A, de Crom R (2000) Human plasma phospholipid transfer protein increases the antiatherogenic potential of high density lipoproteins in transgenic mice. Arterioscler Thromb Vasc Biol 20:1082–1088

van Haperen R, van Tol A, van Gent T, Scheek L, Visser P, van der Kamp A, Grosveld F, de Crom R (2002) Increased risk of atherosclerosis by elevated plasma levels of phospholipid transfer protein. J Biol Chem 277:48938–48943

Yan D, Navab M, Bruce C, Fogelman AM, Jiang XC (2004) PLTP deficiency improves the anti-inflammatory properties of HDL and reduces the ability of LDL to induce monocyte chemotactic activity. J Lipid Res 45:1852–1858

Yang XP, Yan D, Qiao C, Liu RJ, Chen JG, Li J, Schneider M, Lagrost L, Xiao X, Jiang XC (2003) Increased atherosclerotic lesions in apoE mice with plasma phospholipid transfer protein overexpression. Arterioscler Thromb Vasc Biol 23:1601–1607

Yu HM, Liu B, Chiu SY, Costantini F, Hsu W (2005) Development of a unique system for spatiotemporal and lineage-specific gene expression in mice. Proc Natl Acad Sci USA 102:8615–8620

Chapter 3

Acute elevation of plasma PLTP activity strongly increases pre-existing atherosclerosis

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Rien van Haperen, Geesje Dallinga-Thie, Frank Grosveld,
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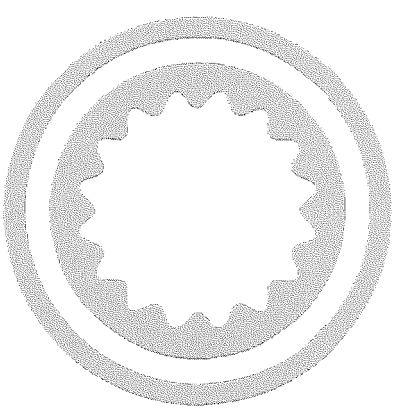

Acute Elevation of Plasma PLTP Activity Strongly Increases Pre-existing Atherosclerosis

Matthijs Moerland, Hannelore Samyn, Teus van Gent, Rien van Haperen, Geesje Dallinga-Thie, Frank Grosveld, Arie van Tol, Rini de Crom

Objective—A transgenic mouse model was generated that allows conditional expression of human PLTP, based on the tetracycline-responsive gene system, to study the effects of an acute increase in plasma PLTP activity as may occur in inflammation.

Methods and Results—The effects of an acute elevation of plasma PLTP activity on the metabolism of apolipoprotein B–containing lipoproteins and on diet-induced pre-existing atherosclerosis were determined in mice displaying a humanized lipoprotein profile (low-density lipoprotein receptor knockout background). Induced expression of PLTP strongly increases plasma VLDL levels in LDL receptor knockout mice, whereas VLDL secretion is not affected. The elevation in plasma triglyceride levels is explained by a PLTP-dependent inhibition of VLDL catabolism, which is caused, at least partly, by a decreased lipoprotein lipase activity. Together with the decreased plasma HDL levels, the acutely increased PLTP expression results in a highly atherogenic lipoprotein profile. Induction of PLTP expression leads to a further increase in size of pre-existing atherosclerotic lesions, even on a chow diet. In addition, the lesions contain more macrophages and less collagen relative to controls, suggesting a less stable lesion phenotype.

Conclusion—In conclusion, acute elevation of PLTP activity destabilizes atherosclerotic lesions and aggravates pre-existing atherosclerosis. (***Arterioscler Thromb Vasc Biol.* 2008;28:1277-1282**)

Key Words: atherosclerosis ■ lipoproteins ■ phospholipid transfer protein ■ transgenic mouse models ■ triglycerides

Studies in various atherosclerosis-prone mouse models have demonstrated that expression of human phospholipid transfer protein (PLTP) stimulates the development of atherosclerosis.[1–3] This can be explained by effects of PLTP activity on lipoprotein composition and metabolism. PLTP increases hepatic VLDL secretion[4–7] and decreases plasma HDL levels, resulting in a more atherogenic lipoprotein profile.[7–9] Furthermore, PLTP unfavorably affects the antiinflammatory and antioxidative properties of HDL particles.[6,10–12] Recently we developed a transgenic mouse model that allows conditional expression of human PLTP.[13] We hypothesize that the physiological effect of an acute increase in plasma PLTP activity differs significantly from the effects seen in mice overexpressing human PLTP innately and life-long and which may have developed compensatory mechanisms. We tested this hypothesis in transgenic mice that conditionally overexpress human PLTP and that are deficient for the LDL receptor. The first objective was to test the effects of acute elevation of plasma PLTP activity on lipoprotein metabolism. The second objective was to study the effect of the acute expression of human PLTP on diet-induced pre-existing atherosclerosis.

Materials and Methods

Animals

The generation of inducible human PLTP transgenic mice is described in detail elsewhere.[13] Briefly, 2 constructs were used to generate transgenic mice that allow inducible expression of PLTP: (1) hnRNP-rtTA-SV40, which consists of an improved version of the tetracycline-controlled transactivator rtTA2S-M2 under the control of an hnRNP A2 coding sequence, resulting in rtTAtg mice, and (2) pTet-PLTP-SV40, which consists of the human PLTP cDNA under the transcriptional control of a tetracycline operator and minimal cytomegalovirus promoter (TetO/Pcmv min), resulting in Tre-PLTPtg mice. Both Tre-PLTPtg mice and rtTAtg mice were crossed into an $LDLR^{-/-}$ background (Jackson Laboratory, Bar Harbor, ME, USA). Tre-PLTPtg/$LDLR^{-/-}$ mice and rtTAtg/$LDLR^{-/-}$ mice were crossbred, resulting in Tre-PLTPtg/rtTAtg/$LDLR^{-/-}$ mice. For convenience, these mice are referred to as "indPLTP" mice. Control mice are animals lacking either the Tre-PLTP transgene or the rtTA transgene. For determination of the genotype, genomic DNA was isolated from tail clips of 10-day-old mice and analyzed by polymerase chain reaction (PCR). Annealing temperatures and primer sequences are available on request.

After weaning, animals were fed a standard chow diet. Animals had free access to water and food. Blood samples were collected by orbital bleeding after removing food overnight. Male mice were used

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in all experiments. All procedures used in this study are in accordance with national and institutional guidelines.

Experimental Setup

Animals were subjected to 2 dietary regimes, referred to as treatment A and treatment B (supplemental Figure I, available online at http://atvb.ahajournals.org). Experiments were performed with mice of 10 to 15 weeks. Using treatment A, PLTP expression was induced immediately after switching from a high cholesterol to a normal chow diet, and its effect on lipoprotein metabolism was investigated. Two weeks after the switch from the high-fat high-cholesterol diet to the chow diet, PLTP expression was induced using treatment B and maintained for 5 weeks. This treatment was used to study the effect of PLTP expression on pre-existing atherosclerosis. In the online supplemental methods, the experimental setup is discussed in more detail.

Analysis of Plasma Activity of PLTP, Hepatic Lipase, and Lipoprotein Lipase

Plasma samples were collected by orbital puncture. Activities of PLTP, pre- and postheparin hepatic lipase (HL), and lipoprotein lipase (LPL) were analyzed as described in the supplemental methods.

Analysis of Plasma Lipids and Lipoproteins

Measurements of plasma concentration of lipids, and isolation and analysis of plasma lipoproteins were performed as described in the supplemental methods.

Determination of VLDL Secretion and VLDL Decay

VLDL secretion experiments were performed as described in the supplemental methods. VLDL decay experiments were performed with [^{3}H]cholesteryl oleyl ether labeled murine VLDL, injected intravenously in mice as a tracer. See supplemental methods for details.

Histology and Measurement of Atherosclerotic Lesions

Histological analysis of atherosclerotic lesions were performed as described in the supplemental methods.

Statistics

Data are expressed as means±SE. Differences were analyzed by 2-sample Wilcoxon rank sum tests using Intercooled Stata 8.2/SE software (Stata Corporation).

Results

PLTP-Effects on Lipoprotein Metabolism

For the first set of experiments, designed to determine the effect of acutely increased PLTP activity on lipoprotein metabolism, treatment A was followed (supplemental Figure I). At 0 weeks, no differences were observed in plasma PLTP activity, triglycerides, cholesterol, or phospholipid levels between indPLTP and control mice (Figure 1, Table). After 9 weeks of Western diet, PLTP activity, triglycerides, cholesterol, and phospholipids were increased in both groups to a similar extent (Figure 1, Table). Subsequently, Western diet was stopped and doxycycline was administered for 2 weeks. This resulted in a further 4.5-fold increase in PLTP activity in the indPLTP group, whereas PLTP activity remained unchanged in the control group (Figure 1A). Induction of PLTP in animals fed a chow diet resulted in a 3-fold increase in plasma triglycerides in the indPLTP mice, whereas in the control mice triglyceride levels returned to basal levels (Figure 1B). Similar effects were observed for cholesterol

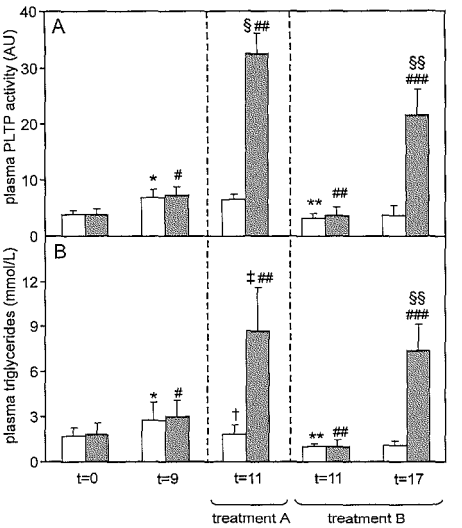


Figure 1. Plasma PLTP activity levels (A) and triglyceride levels (B). Measurements at 0 and 9 weeks. After 9 weeks, mice were subjected to either treatment A or treatment B. White bars, control mice; gray bars, indPLTP mice. n>10 mice per group. Please see supplemental materials for a detailed figure legend.

and phospholipids levels in the control group (Table). In contrast, plasma cholesterol and phospholipid levels remained elevated in the indPLTP group: the plasma cholesterol was 20.0±8.3 mmol/L and the phospholipid level 7.1±1.1 mmol/L in the indPLTP mice, versus 8.5±1.5 mmol/L and 4.4±0.6 mmol/L in the controls.

The effects of induced expression of human PLTP on plasma lipids were studied in more detail by separation of HDL and non-HDL using density ultracentrifugation. Feeding the Western diet did not influence HDL-cholesterol or HDL-phospholipid levels in either group, whereas it strongly increased non–HDL-cholesterol and non–HDL-phospholipid levels (Table, 9 weeks). The subsequent 2 weeks administration of doxycycline on a chow diet did not induce any change in HDL levels in the control mice (Table). Non–HDL-phospholipid levels returned to basal values, non–HDL-cholesterol level strongly decreased but remained slightly elevated compared to basal level (6.4±0.8 mmol/L versus 4.3±0.7 mmol/L). In contrast, HDL levels dramatically decreased in the indPLTP mice on PLTP induction by doxycycline treatment (Table, treatment A: HDL-cholesterol from 2.1±0.5 to 0.3±0.1 mmol/L, HDL-phospholipid from 1.9±0.4 to 0.4±0.0 mmol/L). Non–HDL-lipids remained increased when human PLTP expression was induced (Table).

To study the effects of expression of human PLTP on lipoprotein distribution further, pooled plasma samples obtained from 8 to 10 mice were subjected to gel filtration by fast protein liquid (FPLC) at 0, 9, and 11 weeks. At 0 weeks,

Table. Plasma Parameters

	Cholesterol					Phospholipids				
	0	9	11A	11B	17B	0	9	11A	11B	17B
control	7.9±1.0	31.4±10.1§§	8.5±1.5§§	8.0±1.9§§	9.6±2.2§	4.0±0.4	8.3±2.0§§	4.4±0.6§§	4.0±0.6§§	4.3±0.8
indPLTP	8.8±1.3	35.1±9.3##	20.0±8.3*##	8.2±1.9##	16.9±4.0*##	3.9±0.6	8.9±1.5##	7.1±1.1*#	4.1±0.6##	6.4±1.2*##
	HDL-C					HDL-PL				
	0	9	11A	11B	17B	0	9	11A	11B	17B
control	1.9±0.4	2.0±0.5	1.7±0.2	1.6±0.3§	1.7±0.4	2.0±0.4	1.7±0.4	1.8±0.2	1.6±0.3	1.8±0.4§
ndPLTP	1.9±0.6	2.1±0.5	0.3±0.1*##	1.6±0.3#	0.2±0.1*##	1.9±0.5	1.9±0.4	0.4±0.0*##	1.6±0.2#	0.4±0.0*##
	Non–HDL-C					Non–HDL-PL				
	0	9	11A	11B	17B	0	9	11A	11B	17B
control	4.3±0.7	24.7±8.0§§	6.4±0.8§§	5.8±1.4§§	5.7±1.3	1.9±0.2	6.0±1.9§§	2.1±0.4§§	1.8±0.3§§	1.9±0.3
indPLTP	4.6±0.8	27.5±5.1##	15.4±5.2*##	5.8±1.5##	12.6±2.7*##	1.9±0.3	6.0±0.9##	5.4±1.2*	1.9±0.3##	4.7±0.8*##

Cholesterol and phospholipid levels (expressed in mmol/L) in plasma and in HDL and non-HDL fractions that had been separated by density gradient ultracentrifugation of freshly isolated plasma samples. Measurements before diet ('0') and after 9 weeks of Western diet ('9'). After stopping Western diet, mice were fed either chow+doxycycline for 2 weeks (treatment A: '11A') or chow without administration of doxycycline for 2 weeks (treatment B: '11B'), followed by doxycycline administration for 6 weeks (treatment B: '17B').

n>11 mice/group.

*P<0.001 vs control.

§P<0.01, §§P<0.001: 9 vs 0, 11A vs 9, 11B vs 9, 17B vs 11B (control).

#P<0.01, ##P<0.001: 9 vs 0, 11A vs 9, 11B vs 9, 17B vs 11B (indPLTP).

cholesterol profiles of the control group and the indPLTP group did not differ (Figure 2). At 9 weeks cholesterol levels in the non-HDL size range (fractions 1 to 12) strongly increased in both groups. At 11 weeks (treatment A) the non-HDL peak strongly declined in the control mice, although the level of LDL-sized particles remained elevated (Figure 2A). In the indPLTP mice the non-HDL peak overall also declined (Figure 2B). However, in these animals a substantial lipoprotein fraction with VLDL size was clearly present after induction of PLTP expression (fractions 1 to 4).

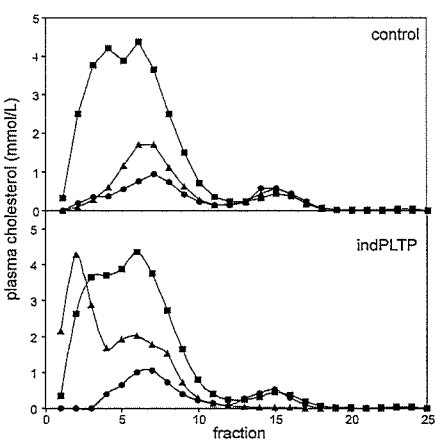


Figure 2. Cholesterol levels in FPLC fractions of plasma from control mice and indPLTP mice. Individual plasma samples were pooled (≥8 mice per pool) and subjected to FPLC at 0 (circles), 9 (squares), and 11 weeks (triangles).

To evaluate possible mechanisms explaining this observation, we investigated VLDL secretion in indPLTP and control mice at 11 weeks. All mice had been given drinking water containing both doxycycline and 5% sucrose during the last 2 weeks. Plasma triglycerides (at t=0 minutes) strongly differed between controls and indPLTP (Figure 3A), as observed before (Figure 1B). However, both groups had equal rates of VLDL triglyceride secretion. Therefore, the increase in VLDL after induction of PLTP in indPLTP mice cannot be explained by an increase in formation and secretion of these particles. Next, we evaluated possible differences in VLDL degradation. Murine VLDL was labeled with [^{3}H]-cholesteryl oleoyl ether, and tracer amounts were injected intravenously in doxycycline-treated control mice and indPLTP mice at 11 weeks of treatment A. Subsequently, the disappearance of radioactivity from the blood was monitored (Figure 3B). There is a big difference between the 2 groups in VLDL clearance during the first 15 minutes after labeled VLDL injection. After 15 minutes, already 39% of labeled VLDL had been cleared from the plasma of control mice, whereas in the indPLTP mice there had not been any clearance yet. At later time points [^{3}H]-VLDL disappeared slowly and linearly in both control and indPLTP mice. Four hours after injection, 51±5% and 84±10% of the label still remained in the plasma of the control mice and the indPLTP mice, respectively. Separation of lipoprotein particles by FPLC demonstrated that both in the indPLTP and the control mice a substantial part of the injected VLDL particles had been converted into IDL and LDL (data not shown). The tissue distribution of injected label was studied after sacrificing the animals at t=4 hours (Figure 3C). In both groups, almost all injected label that had disappeared from plasma was detected in the liver. To get more insight in the mechanism behind the initially delayed decay of VLDL-particles in the human PLTP ex-

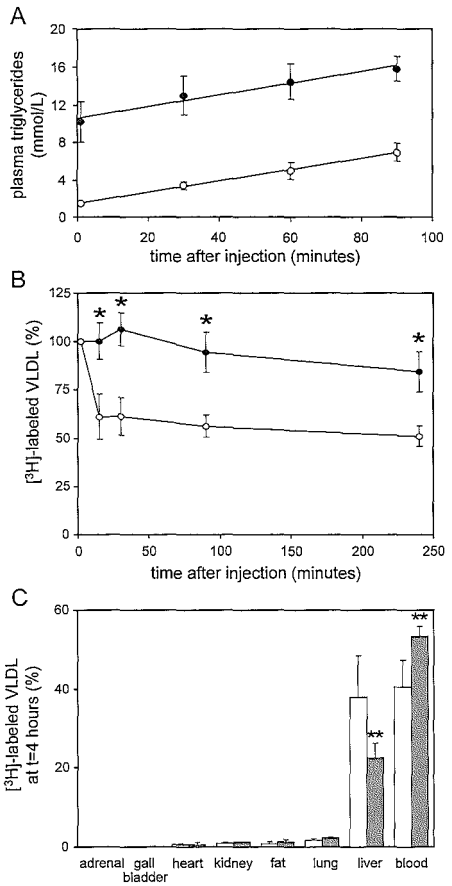


Figure 3. A, VLDL secretion: Increase in plasma triglyceride level in time. B, VLDL decay in time. Open circles, control mice; filled circles, indPLTP mice. C, Tissue distribution of [^{3}H]-label after last measurement at t=4 hours. White bars, control mice; gray bars, indPLTP mice. Please see supplemental materials for a detailed figure legend.

pressing mice, we measured plasma lipase activities in doxycycline-treated indPLTP mice and control mice. Hepatic lipase activity in preheparin plasma did not differ between the indPLTP group and the control group. However, LPL activity in postheparin plasma was significantly decreased in the indPLTP group compared to the control group (Figure 4).

The Effect of PLTP Expression on Preexisting Atherosclerosis

The second main objective was to determine the effect of acute elevation of PLTP activity on pre-existing atherosclerosis. For this purpose, treatment B was followed (supplemental Figure I). Plasma PLTP activity levels or triglyceride levels did not differ between the control mice and the indPLTP mice at 0, 9, and 11 weeks (ie, before PLTP induction; Figure 1). From 11 weeks on, doxycycline was administered for an additional 6 weeks. This resulted in a 5.5-fold increase in PLTP activity in the indPLTP group, whereas PLTP activity did not change in the control group (Figure 1A). The increased PLTP activity in the indPLTP group resulted in strongly increased plasma levels of triglycerides, cholesterol, and phospholipids (Figure 1B, Table). Separation of lipoprotein classes using density ultracentrifugation showed that on induction of PLTP activity, levels of HDL-cholesterol and HDL-phospholipids were substantially decreased whereas levels of non-HDL-cholesterol and non-HDL-phospholipids were substantially increased (Table). Atherosclerotic lesion development was determined at 9 and 17 weeks. In the control mice, discontinuing the Western diet for 8 weeks did not influence atherosclerotic lesion area significantly (Figure 5A and supplemental Figure II; $2.8\pm1.5\times10^4$ μm^2 at 9 weeks and $2.4\pm0.7\times10^4$ μm^2 at 17 weeks), but lesion composition changed. The macrophage content of the lesion decreased by 40% on switching the Western diet to a chow diet (Figure 5B and supplemental Figure II), whereas the collagen content increased 4-fold (Figure 5C and supplemental Figure II), suggesting a significant stabilization of the lesion. In the indPLTP mice however, induction of human PLTP expression resulted in a further increase in mean lesion area (Figure 5A and supplemental Figure II; from $3.1\pm1.2\times10^4$ μm^2 at 9 weeks to $5.2\pm1.3\times10^4$ μm^2 at 17 weeks), even though the mice were on a chow diet. In these mice, the relative macrophage content of the lesion remained unchanged (Figure 5B and supplemental Figure II). The collagen content increased but was significantly lower when compared to that seen in the control mice (Figure 5C and supplemental Figure II; 38.9±14.8% versus 27.2±9.5%).

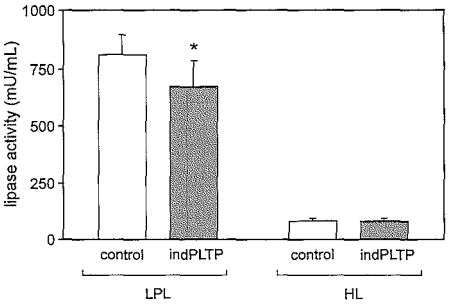


Figure 4. Lipoprotein lipase (LPL) and hepatic lipase (HL) activity in doxycycline-treated control mice (white bars) and indPLTP mice (gray bars). Lipase activity is expressed in milliunits per mL (mU/mL), 1 mU representing the release of 1 nmol fatty acid from the substrate per minute. *$P<0.01$ vs control.

Discussion

Our first objective was to investigate the effect of conditional PLTP expression on lipoprotein metabolism after changing

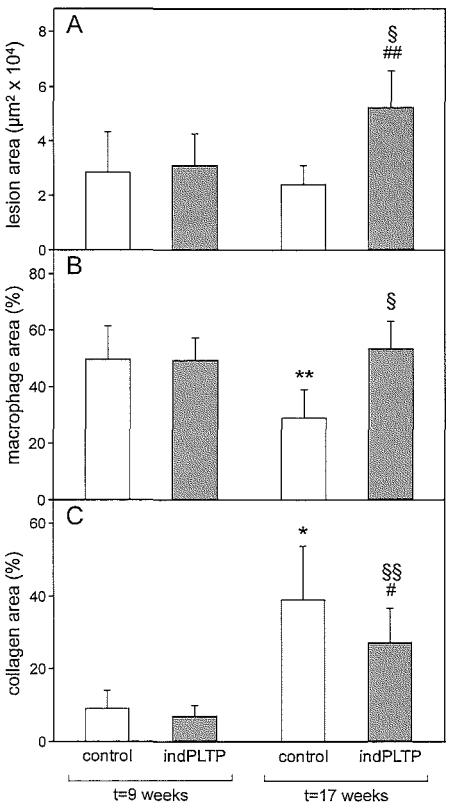


Figure 5. Atherosclerotic lesion development. A, Plaque area. B, Macrophage content. C, Collagen content. Measurements were performed in sections from the aortic root at 9 weeks and 17 weeks. White bars, control mice; gray bars, indPLTP mice. Please see supplemental materials for a detailed figure legend.

from a hyperlipidaemic state, induced by a high-fat high-cholesterol diet, to normolipidaemia induced by chow diet. HDL levels strongly decreased on induction of expression of human PLTP. In addition, we observed an important novel effect of elevated PLTP expression on VLDL catabolism, probably caused by induction of decreased LPL activity. Induction of elevated PLTP activity strongly increased plasma triglyceride, cholesterol, and phospholipid levels in the non-HDL fractions. Separation of lipoproteins by FPLC revealed that the majority of the lipids was present in VLDL-sized particles. To our knowledge this is the first demonstration that the plasma levels of apoB-containing lipoproteins or plasma triglyceride levels are actually higher after induction of PLTP expression. Apparently, the metabolic effects of an acute increase in PLTP activity differ from the effect of life-long increase in plasma PLTP activity levels in human PLTP transgenic mice. Indeed, in the acute model, the triglyceride secretion rate by the liver was not affected by overexpression of PLTP, whereas we previously showed that life-long enhanced PLTP expression results in increased hepatic triglyceride secretion.[5,7] The high lipid levels in the non-HDL fractions therefore cannot be explained by an increase in VLDL production. To examine whether these observations could be explained by a decreased catabolism of VLDL, we performed VLDL decay studies using [^{3}H]-cholesteryl oleyl ether labeled VLDL as a tracer. The absolute amount of injected VLDL-cholesterol was only 0.5% to 1% of total circulating cholesterol, an amount that was not expected to affect endogenous lipoprotein metabolism. We found that plasma clearance of [^{3}H]-labeled VLDL was delayed, and that the hepatic uptake of [^{3}H]-labeled cholesterol was strongly decreased in indPLTP mice when compared with control mice.

It is unlikely that hepatic clearance of VLDL is inhibited due to a decreased apoE content in indPLTP mice, as protein analysis of FPLC fractions showed that VLDL in doxycycline-treated indPLTP mice contained normal amounts of apoE (data not shown). Already a very low level of apoE is sufficient to normalize plasma cholesterol levels in apoE-deficient mice.[14] Next, we examined whether PLTP may affect VLDL lipolysis. Lipoprotein lipase (LPL) plays an important role in this process by hydrolyzing the triglycerides in VLDL particles.[15–17] In addition, HL and LPL may act as a ligand between low density lipoproteins and hepatic lipoprotein binding sites. Indeed, plasma LPL activity measured in postheparin plasma under optimal conditions was significantly decreased in indPLTP mice. Thus the delayed VLDL clearance could, at least partly, be explained by a decrease in the plasma level of LPL, causing a limited turnover of VLDL triglycerides in peripheral tissues.

Our second main objective was to determine the effect of increased PLTP activity on pre-existing atherosclerotic lesions, which had been induced by feeding a high-fat high-cholesterol diet for 9 weeks. Induction of high plasma PLTP activity levels in the indPLTP mice not only decreased HDL levels but also strongly increased plasma triglyceride, cholesterol, and phospholipid levels in the non-HDL fraction. In the control mice, the lesion size at 9 and 17 weeks was similar. Lesion macrophage content was strongly decreased, whereas collagen content was significantly increased. This is in accordance with previous experiments in which the regression of pre-existing atherosclerotic lesions was studied.[18–21] A decrease in lesion area on withdrawal of an atherosclerosis-inducing diet is only observed in foam cell–containing fatty streaks. For lesions containing more advanced characteristics, such as fibrous caps and necrotic cores, a decrease in lesion area may not be observed, but generally macrophage content of the lesions strongly decreases on withdrawal of the high-fat high-cholesterol diet. In addition, collagen content of the lesions increases,[22] resulting in lesion stabilization. In contrast to the situation observed in the control mice, the unfavorable lipoprotein profile observed in the indPLTP resulted in a further increase in lesion area. Although the Western diet had been stopped, the lesion area had almost doubled compared to the situation seen at 9 weeks, indicating a strong atherogenic effect of PLTP. No regression of the

macrophage percentage was observed. Also the collagen percentage was remarkably lower than that seen in control mice, indicating that high plasma PLTP activity levels stimulate an increase in lesion size and also inhibit lesion stabilization, which may be caused by the elevated plasma VLDL levels. Besides, a PLTP-induced inflammatory response might very well contribute to the formation of atherosclerotic lesion. Plasma PLTP activity is increased during acute systemic inflammation,[23] a situation that is comparable with the acute increase in plasma PLTP activity that is induced in our mouse model.

In conclusion, using a novel mouse model with inducible expression of PLTP we found that increased plasma PLTP activity strongly enhances plasma VLDL levels by a PLTP-dependent inhibition of VLDL catabolism. In combination with the PLTP-dependent decrease in plasma HDL levels, this results in a strongly atherogenic lipoprotein profile and may account for the PLTP-dependent size increase of pre-existing atherosclerotic lesions and the decreased lesion stability, even after replacement of the Western diet by chow diet. Our study supplies new evidence that high systemic PLTP activity unfavorably affects the cardiovascular system.

Acknowledgments

A.J. Zonneveld, Department of Clinical Chemistry, Erasmus MC is gratefully thanked for the determination of lipase activities.

Disclosures

None.

References

1. van Haperen R, van Tol A, Vermeulen P, Jauhiainen M, van Gent T, van den Berg P, Ehnholm S, Grosveld F, van der Kamp A, de Crom R. Human plasma phospholipid transfer protein increases the antiatherogenic potential of high density lipoproteins in transgenic mice. *Arterioscler Thromb Vasc Biol.* 2000;20:1082–1088.
2. Lie J, de Crom R, van Gent T, van Haperen R, Scheek L, Sadeghi-Niaraki F, van Tol A. Elevation of plasma phospholipid transfer protein increases the risk of atherosclerosis despite lower apolipoprotein B-containing lipoproteins. *J Lipid Res.* 2004;45:805–811.
3. Yang XP, Yan D, Qiao C, Liu RJ, Chen JG, Li J, Schneider M, Lagrost L, Xiao X, Jiang XC. Increased atherosclerotic lesions in apoE mice with plasma phospholipid transfer protein overexpression. *Arterioscler Thromb Vasc Biol.* 2003;23:1601–1607.
4. Jiang XC, Qin S, Qiao C, Kawano K, Lin M, Skold A, Xiao X, Tall AR. Apolipoprotein B secretion and atherosclerosis are decreased in mice with phospholipid-transfer protein deficiency. *Nat Med.* 2001;7:847–852.
5. Lie J, de Crom R, van Gent T, van Haperen R, Scheek L, Lankhuizen I, van Tol A. Elevation of plasma phospholipid transfer protein in transgenic mice increases VLDL secretion. *J Lipid Res.* 2002;43:1875–1880.
6. Jiang XC, Li Z, Liu R, Yang XP, Pan M, Lagrost L, Fisher EA, Williams KJ. Phospholipid transfer protein deficiency impairs apolipoprotein-B secretion from hepatocytes by stimulating a proteolytic pathway through a relative deficiency of vitamin E and an increase in intracellular oxidants. *J Biol Chem.* 2005;280:18336–18340.
7. van Haperen R, van Tol A, van Gent T, Scheek L, Visser P, van der Kamp A, Grosveld F, de Crom R. Increased risk of atherosclerosis by elevated plasma levels of phospholipid transfer protein. *J Biol Chem.* 2002;277:48938–48943.
8. Foger B, Santamarina-Fojo S, Shamburek RD, Parrot CL, Talley GD, Brewer HB Jr. Plasma phospholipid transfer protein. Adenovirus-mediated overexpression in mice leads to decreased plasma high density lipoprotein (HDL) and enhanced hepatic uptake of phospholipids and cholesteryl esters from HDL. *J Biol Chem.* 1997;272:27393–27400.
9. Ehnholm S, van Dijk KW, van 't Hof B, van der Zee A, Olkkonen VM, Jauhiainen M, Hofker M, Havekes L, Ehnholm C. Adenovirus mediated overexpression of human phospholipid transfer protein alters plasma HDL levels in mice. *J Lipid Res.* 1998;39:1248–1253.
10. Jiang XC, Tall AR, Qin S, Lin M, Schneider M, Lalanne F, Deckert V, Desrumaux C, Athias A, Witztum JL, Lagrost L. Phospholipid transfer protein deficiency protects circulating lipoproteins from oxidation due to the enhanced accumulation of vitamin E. *J Biol Chem.* 2002;277:31850–31856.
11. Yan D, Navab M, Bruce C, Fogelman AM, Jiang XC. PLTP deficiency improves the anti-inflammatory properties of HDL and reduces the ability of LDL to induce monocyte chemotactic activity. *J Lipid Res.* 2004;45:1852–1858.
12. Schlitt A, Liu J, Yan D, Mondragon-Escorpizo M, Norin AJ, Jiang XC. Anti-inflammatory effects of phospholipid transfer protein (PLTP) deficiency in mice. *Biochim Biophys Acta.* 2005;1733:187–191.
13. Moerland M, Anghelescu N, Samyn H, van Haperen R, van Gent T, Strouboulis J, van Tol A, Grosveld F, de Crom R. Inducible expression of phospholipid transfer protein (PLTP) in transgenic mice: acute effects of PLTP on lipoprotein metabolism. *Transgenic Res.* 2007;16:503–513.
14. Hasty AH, Linton MF, Swift LL, Fazio S. Determination of the lower threshold of apolipoprotein E resulting in remnant lipoprotein clearance. *J Lipid Res.* 1999;40:1529–1538.
15. Wong H, Schotz MC. The lipase gene family. *J Lipid Res.* 2002;43:993–999.
16. Jansen H, Verhoeven AJ, Sijbrands EJ. Hepatic lipase: a pro- or anti-atherogenic protein? *J Lipid Res.* 2002;43:1352–1362.
17. Goldberg IJ. Lipoprotein lipase and lipolysis: central roles in lipoprotein metabolism and atherogenesis. *J Lipid Res.* 1996;37:693–707.
18. Tangirala RK, Pratico D, FitzGerald GA, Chun S, Tsukamoto K, Maugeais C, Usher DC, Pure E, Rader DJ. Reduction of isoprostanes and regression of advanced atherosclerosis by apolipoprotein E. *J Biol Chem.* 2001;276:261–266.
19. Tangirala RK, Tsukamoto K, Chun SH, Usher D, Pure E, Rader DJ. Regression of atherosclerosis induced by liver-directed gene transfer of apolipoprotein A-I in mice. *Circulation.* 1999;100:1816–1822.
20. Gijbels MJ, van der Cammen M, van der Laan LJ, Emeis JJ, Havekes LM, Hofker MH, Kraal G. Progression and regression of atherosclerosis in APOE3-Leiden transgenic mice: an immunohistochemical study. *Atherosclerosis.* 1999;143:15–25.
21. Moghadasian MH, Godin DV, McManus BM, Frohlich JJ. Lack of regression of atherosclerotic lesions in phytosterol-treated apo E-deficient mice. *Life Sci.* 1999;64:1029–1036.
22. Aikawa M, Rabkin E, Okada Y, Voglic SJ, Clinton SK, Brinckerhoff CE, Sukhova GK, Libby P. Lipid lowering by diet reduces matrix metalloproteinase activity and increases collagen content of rabbit atheroma: a potential mechanism of lesion stabilization. *Circulation.* 1998;97:2433–2444.
23. Barlage S, Frohlich D, Bottcher A, Jauhiainen M, Muller HP, Noetzel F, Rothe G, Schutt C, Linke RP, Lackner KJ, Ehnholm C, Schmitz G. ApoE-containing high density lipoproteins and phospholipid transfer protein activity increase in patients with a systemic inflammatory response. *J Lipid Res.* 2001;42:281–290.

Online Supplemental Data

Supplementary Materials and Methods

Experimental setup

Animals were subjected to two dietary regimes, referred to as treatment A and treatment B. Experiments were started after the mice reached an age between 10 and 15 weeks. Using treatment A, the effect of PLTP expression on lipoprotein metabolism was investigated under diet-induced high lipidaemic conditions. The animals were fed a high-fat high-cholesterol diet ('Western diet") for a period of 9 weeks. After stopping the Western diet the mice were fed standard chow diet for 2 weeks, together with 1 mg/ml doxycycline + 5% sucrose added to the drinking water. After these 2 weeks, VLDL secretion and VLDL decay experiments were performed (methods described below). At 0 weeks (before Western diet), 9 weeks (after Western diet) and 11 weeks (after doxycycline treatment) plasma PLTP activity and plasma lipids were measured and lipoprotein profiles were obtained by gel filtration (methods described below).
Using treatment B, the effect of PLTP expression on pre-existing atherosclerosis was determined. The first 9 weeks the mice followed the same regime of Western diet as in treatment A. After 9 weeks of feeding Western diet, 8 mice of both the indPLTP and the control group were sacrificed to determine atherosclerotic lesion development. The remaining mice were fed standard chow diet again for 2 weeks, receiving normal drinking water, in order to obtain basal plasma lipid levels before induction of PLTP activity. After these 2 weeks, 1 mg/ml doxycycline + 5% sucrose was added to the drinking water. At 17 weeks, mice were sacrificed and atherosclerotic lesion development was determined. In addition, plasma PLTP activity and plasma lipids were measured (methods described below).

Plasma PLTP activity

PLTP activity was measured using a phospholipid vesicle-HDL system, according to Speijer et al.[1] PLTP activity is expressed as arbitrary units (AU). One AU is equal to the level of PLTP activity found in human reference plasma, which is 13.9 μmol/ml/h.

Plasma lipase activities

Plasma lipase activities were analyzed in pre-heparin plasma and post-heparin plasma using a gum acacia-stabilized glycerol ^{14}C trioleate emulsion as described by Jansen et al.[2]. Plasma pre-heparin lipase activity reflects hepatic lipase (HL) activity. Lipase activity in post-heparin plasma minus pre-heparin HL activity reflects lipoprotein lipase (LPL) activity[3]. All assays were performed in the presence of heat-inactivated plasma as a source of apoCII. Lipase activities are expressed in milliunits (mU), 1 mU representing the release of 1 nmol fatty acid from the substrate per minute.

Quantification of plasma lipids

Cholesterol concentration was determined enzymatically using a Free Cholesterol C kit (WAKO) after hydrolysis of cholesteryl esters with cholesterol esterase from *Candida cylindracea* (Boehringer). Triglycerides were measured using a triglyceride kit from WAKO. Phospholipids were measured using the PAP150 kit from BioMerieux.

Separation of lipoproteins

HDL and non-HDL fractions were separated by density gradient ultracentrifugation of freshly isolated plasma samples (Beckman 42.2 Ti rotor, 42000 rpm, 2h50min, 12 °C, d=1.063 g/ml).

Plasma lipoprotein profiles by gel filtration

Lipoprotein profiles in EDTA-plasma were obtained using HR 10/30 fast performance liquid chromatography (FPLC) columns in tandem that were filled with Superose 6 and Superdex 200 (preparation grade, Pharmacia Biotechnology) respectively. Columns were equilibrated and run in a 2 mM phosphate buffer containing 0.9% (w/v) NaCl, 0.02% (w/v) NaN_3 and 5 mM EDTA (pH 7.4). Pooled plasma samples obtained from 8-10 mice

were filtered through 0.8 μm filters (Pall Corporation). 0.5 ml of plasma was loaded onto the Superose 6 column. The separation was performed at 4°C with a flow rate of 0.1 ml/min, fractions of 0.8 ml were collected.

Determination of VLDL secretion

VLDL secretion experiments were performed as described previously[4]. VLDL secretion was measured in mice that had overnight access to drinking water containing doxycycline and 5% sucrose, but no access to chow. Mice were injected intravenously with 10% (w/v) Triton WR1339 (Sigma, St Louis, MO) (500 mg/kg body weight) dissolved in 0.9% NaCl. After injection of Triton WR1339, blood samples were drawn at appropriate time points (up to 90 min) and triglyceride levels were measured as described above. The triglyceride accumulation in plasma was linear during this period. Hepatic triglyceride secretion rate was calculated from the slope of the line and expressed as mmol/L per minute.

Determination of VLDL decay

VLDL was isolated by density ultracentrifugation of freshly isolated plasma of wildtype mice (Beckman 70.1 Ti rotor, 40400 rpm, 20 h, 12 °C, d=1.006 g/ml). Isolated VLDL was radioactively labeled using an adapted protocol for the labeling of LDL designed by Groener et al.[5] VLDL was labeled by incubation with [^{3}H]cholesteryl oleyl ether containing liposomes for 24 hours at 37 °C. After labeling, VLDL was re-isolated by a one-step gradient ultracentrifugation as described by Redgrave et al.[6] To determine the *in vivo* VLDL decay, 200 μl of [^{3}H]-labeled VLDL (cholesterol 0.55 mM, 20*10^6 dpm/ml) was injected intravenously in mice that had access to drinking water containing 5% sucrose, but no access to chow. At t=2, 15, 30 and 90 minutes after VLDL injection a small amount of blood was taken by tail bleeding. At t=4 hours after injection a larger blood sample was collected from the orbital plexus after which the mice were killed by cervical dislocation. Liver, adrenals, kidney, gall bladder, lungs, adipose tissue and heart were removed. To determine VLDL decay (expressed as percentage of plasma [^{3}H]-label measured at t=2 minutes), the decrease of [^{3}H]-label in the plasma samples over time was measured. Tissue distribution of [^{3}H]-label (expressed as percentage of the injected dose)

was determined by dissolving tissues overnight in Soluene 350 (Perkin Elmer), followed by treatment with isopropanol and peroxide, after which radioactivity was measured.

Histology and measurement of atherosclerotic lesions

The mice were anesthetized with isoflurane, the thorax was opened and the animals were subjected to perfusion fixation through the left ventricle of the heart using 4% phosphate-buffered formalin. The heart was dissected and 7 μm thick cryosections of the aortic valves were made. For quantification of atherosclerosis, sections were stained with Oil Red O and hematoxilin. Atherosclerotic lesion area was measured in five sections at intervals of 90 μm using image processing by NIH-based Scion Image and analyzing software (available at www.scioncorp.com) according to Paigen et al.[7] Macrophages were detected by immunohistochemical analysis using an indirect peroxidase antibody conjugate method, with an anti-CD68 monoclonal antibody. Sections were counterstained with Nuclear Fast Red. Collagen was stained using the Picro-Sirius Red method and examined by crossed polars microscopy[8, 9]. Macrophage and collagen percentage of atherosclerotic lesions was measured using Clemex Vision image analysis system (Clemex Technologies, Quebec, Canada).

References

1. Speijer H, Groener JE, van Ramshorst E, van Tol A. Different locations of cholesteryl ester transfer protein and phospholipid transfer protein activities in plasma. *Atherosclerosis.* 1991;90:159-168.
2. Jansen H, Lammers R, Baggen MG, Wouters NM, Birkenhager JC. Circulating and liver-bound salt-resistant hepatic lipases in the golden hamster. *Biochim Biophys Acta.* 1989;1001:44-49.
3. Dallinga-Thie GM, Zonneveld-de Boer AJ, van Vark-van der Zee LC, van Haperen R, van Gent T, Jansen H, De Crom R, van Tol A. Appraisal of hepatic lipase and lipoprotein lipase activities in mice. *J Lipid Res.* 2007;48:2788-2791.
4. Lie J, de Crom R, van Gent T, van Haperen R, Scheek L, Lankhuizen I, van Tol A. Elevation of plasma phospholipid transfer protein in transgenic mice increases VLDL secretion. *J Lipid Res.* 2002;43:1875-1880.
5. Groener JE, Pelton RW, Kostner GM. Improved estimation of cholesteryl ester transfer/exchange activity in serum or plasma. *Clin Chem.* 1986;32:283-286.
6. Redgrave TG, Roberts DC, West CE. Separation of plasma lipoproteins by density-gradient ultracentrifugation. *Anal Biochem.* 1975;65:42-49.
7. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis.* 1987;68:231-240.
8. Puchtler H, Waldrop FS, Valentine LS. Fluorescence microscopic distinction between elastin and collagen. *Histochemie.* 1973;35:17-30.
9. Junqueira LC, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem J.* 1979;11:447-455.

Detailed Figure Legends

Figure 1.

Plasma PLTP activity levels (A) and triglyceride levels (B). PLTP activity was measured in plasma samples and is expressed as arbitrary units (AU; 1 AU is equal to the level of PLTP activity found in human reference plasma, i.e. 13.9 μmol/mL/h). Measurements at 0 (t=0) and 9 weeks (t=9; after 9 weeks of Western diet). After 9 weeks, mice were subjected to either treatment A or treatment B. Treatment A: measurement at 11 weeks (t=11; after 2 weeks of chow and drinking water containing doxycycline/sucrose), treatment B: measurements at 11 weeks (t=11; after 2 weeks of chow and normal drinking water) and 17 weeks (t=17; after 6 weeks of chow and drinking water containing doxycycline/sucrose). White bars: control mice, gray bars: indPLTP mice. n>10 mice per group.

$p<0.001$ versus control at 0 weeks (*), at 9 weeks (**)
$p<0.001$ versus indPLTP at 0 weeks (#), at 9 weeks (##), at 11 weeks/B (###)
$p<0.001$ versus control at 11 weeks/A (§),at 17 weeks (§§)
$p<0.01$ versus control at 9 weeks (†)
$p<0.01$ versus control at 11 weeks/A (‡)

Figure 3.

A: VLDL secretion: Increase in plasma triglyceride level (mmol/L) in time. Open circles: control mice (n=4), filled circles: indPLTP mice (n=8). There is no statistically significant difference between the secretion rates. B: VLDL decay in time. Values are expressed as a percentage of [^{3}H]-label measured at t=2 minutes. Open circles: control mice (n=5), filled circles: indPLTP mice (n=5). C: Tissue distribution of [^{3}H]-label after last measurement at t=4 hours. Values are expressed as a percentage of the total injected dose. White bars: control mice (n=5), gray bars: indPLTP mice (n=5).

* $p<0.001$, ** $p<0.01$ versus control.

Figure 5.

Atherosclerotic lesion development. A: Plaque area (expressed in $\mu m^2 \times 10^4$), B: macrophage content of atherosclerotic lesions (expressed as percentage of total lesion area), C: collagen content of atherosclerotic lesions (expressed as percentage of total lesion area). Measurements were performed in sections from the aortic root (see Methods) at 9 weeks (after 9 weeks of Western diet) and 17 weeks (after 2 weeks of chow + 6 weeks of chow and doxycycline administration). White bars: control mice, gray bars: indPLTP mice. 8-10 mice per group.

$p<0.001$ (*), $p<0.01$ (**) versus control at 9 weeks

$p<0.001$ (#), $p<0.01$ (##) versus indPLTP at 9 weeks

$p<0.001$ (§), $p<0.05$ (§§) versus control at 17 weeks

Supplementary Figures

Figure I.

Experimental setup. Treatment A and treatment B: shared run-in period of 9 weeks on Western diet, followed by 2 weeks on chow with administration of doxycycline (treatment A), or 2 weeks on chow and subsequently 6 weeks on chow with administration of doxycycline (treatment B).

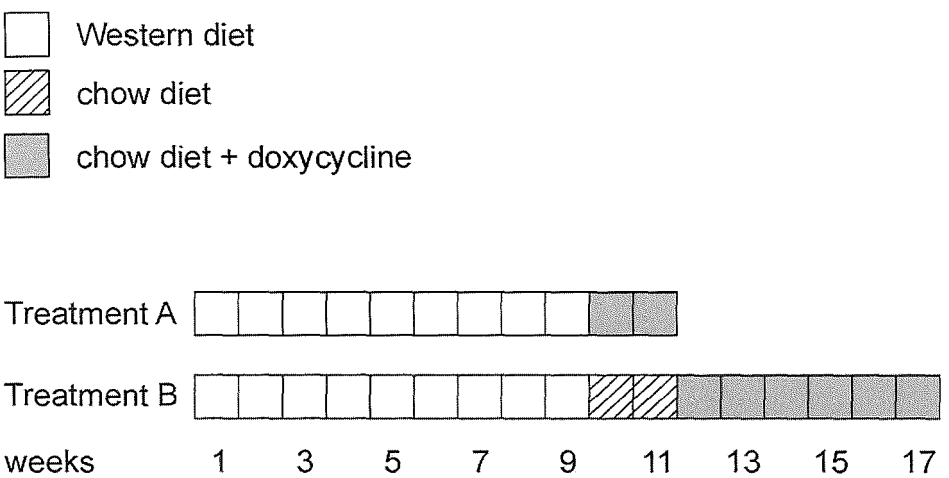
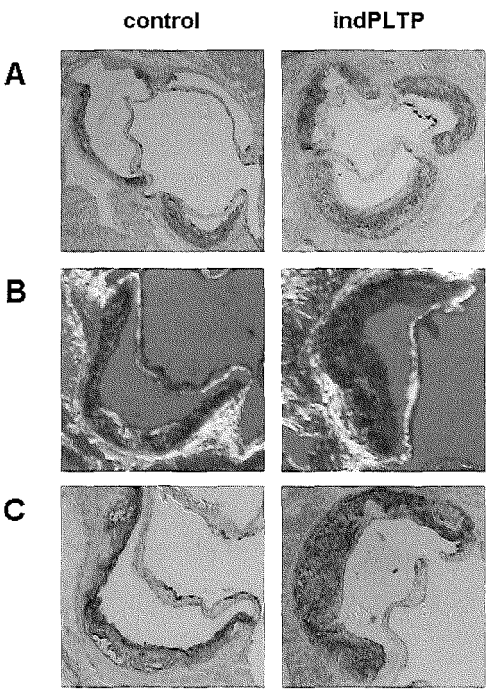


Figure II.

Histological staining of atherosclerotic lesions in control mice and indPLTP mice at t=17 weeks (9 weeks of high fat high cholesterol diet + 2 weeks of chow + 6 weeks of chow and doxycycline). A: Lipid staining (red) in representative cross-sections from the aortic root, B: Collagen staining (bright yellow and orange/red) in representative cross-sections of one aortic valve, C: Macrophage staining (black) in representative cross-sections of one aortic valve. See supplementary methods for details.



Chapter 4

Plasma phospholipid transfer activity is essential for increased atherogenesis in PLTP transgenic mice: a mutation-inactivation study

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Rien van Haperen, Jari Metso, Frank Grosveld,
Matti Jauhiainen, Arie van Tol, and Rini de Crom

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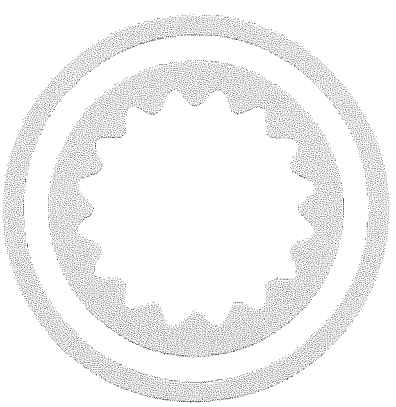

Plasma phospholipid transfer activity is essential for increased atherogenesis in PLTP transgenic mice: a mutation-inactivation study

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Abstract Plasma phospholipid transfer protein (PLTP) interacts with HDL particles and facilitates the transfer of phospholipids from triglyceride (TG)-rich lipoproteins to HDL. Overexpressing human PLTP in mice increases the susceptibility to atherosclerosis. In human plasma, high-active and low-active forms of PLTP exist. To elucidate the contribution of phospholipid transfer activity to changes in lipoprotein metabolism and atherogenesis, we developed mice expressing mutant PLTP, still able to associate with HDL but lacking phospholipid transfer activity. In mice heterozygous for the LDL receptor, effects of the mutant and normal human PLTP transgene (mutPLTP tg and PLTP tg, respectively) were compared. In PLTP tg mice, plasma PLTP activity was increased 2.9-fold, resulting in markedly reduced HDL lipid levels. In contrast, in mutPLTP tg mice, lipid levels were not different from controls. Furthermore, hepatic VLDL-TG secretion was stimulated in PLTP tg mice, but not in mutPLTP tg mice. When mice were fed a cholesterol-enriched diet, atherosclerotic lesion size in PLTP tg mice was increased more than 2-fold compared with control mice, whereas in mutPLTP tg mice, there was no change. Our findings demonstrate that PLTP transfer activity is essential for the development of atherosclerosis in PLTP transgenic mice, identifying PLTP activity as a possible target to prevent atherogenesis, independent of plasma PLTP concentration.—Samyn, H., M. Moerland, T. van Gent, R. van Haperen, J. Metso, F. Grosveld, M. Jauhiainen, A. van Tol, and R. de Crom. **Plasma phospholipid transfer activity is essential for increased atherogenesis in PLTP transgenic mice: a mutation-inactivation study.** ***J. Lipid Res.*** **2008.** 49: **2504–2512.**

Supplementary key words phospholipid transfer protein • lipoproteins • atherosclerosis

Phospholipid transfer protein (PLTP) is a multifunctional protein secreted by various cell types into the plasma, where it associates with HDL particles. Plasma PLTP has a central role in cholesterol and lipoprotein metabolism. It mediates the transfer of phospholipids between lipoprotein particles (1, 2). In addition, PLTP in vitro is able to transfer other lipophilic substances such as diacylglycerol (3), cholesterol (4), lipopolysaccharide (5, 6), and α-tocopherol (7), an important anti-oxidant. Furthermore, plasma PLTP has been identified as an HDL conversion factor. It remodels HDL to generate large particles and small lipid-poor pre-β-HDL (8–10). In vitro, HDL conversion depends on phospholipid transfer activity of PLTP (11).

Studies using genetically modified mouse models have provided more insight into the (patho-) physiological role of PLTP in lipoprotein metabolism and the development of atherosclerosis. PLTP-deficient mice have markedly reduced HDL levels (12). These mice are more resistant to atherosclerosis development; this is partly attributable to diminished secretion and lower levels of apolipoprotein B (apoB)-containing lipoproteins and to the increase in bioavailability of vitamin E in these particles (13, 14). When overexpressing human PLTP in mice, elevation of plasma PLTP activity levels results in a dose-dependent decrease in HDL levels, despite an increased production of pre-β-HDL particles (15). Hepatic VLDL secretion rates are increased in PLTP transgenic mice (16). We previously generated different mouse lines with either ubiquitous or liver-specific overexpression of human PLTP. Regardless of the site of PLTP production, a positive correlation between plasma PLTP activity and atherosclerotic plaque size was

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Abbreviations: apoA-I, apolipoprotein A-I; AU, arbitrary unit; FPLC, fast-protein liquid chromatography; FXR, farnesoid X receptor; HFHC, high-fat, high-cholesterol; IAA, iodoacetate; LDLR, LDL receptor; mutPLTP, mutant PLTP; PLTP, phospholipid transfer protein; TC, total cholesterol; tg, transgenic; TG, triglyceride.

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observed, suggesting that systemic PLTP is the cause of the increased susceptibility to developing atherosclerosis in these mouse models (16). Exactly how elevated PLTP levels lead to increased atherosclerosis is still not fully understood.

Until now, studies regarding the relationship between PLTP and atherosclerosis in mice focused on the absence of PLTP protein in plasma (PLTP knockout mice) or on the presence of functional PLTP with phospholipid transfer activity.

However, in human plasma, as well as in plasma of mice with adenoviral overexpression of human PLTP, two forms of PLTP have been described (17, 18), which may be regulated differently (19). In addition to the catalytically active form, a low-active form with unkown function also exists. To provide more insight into the role of PLTP transfer activity and PLTP mass in vivo, we generated a new mouse model with overexpression of a mutant human PLTP protein in the liver. The mutant has previously been described in vitro by Huuskonen et al. (20). The mutation in the N-terminal lipid binding pocket of the PLTP protein interfered with its phospholipid transfer activity in vitro, but did not affect binding to HDL. Our newly developed mouse model expresses human PLTP that associates with HDL in plasma, but lacks phospholipid transfer activity.

In the present study, we investigate the importance of PLTP transfer activity in lipoprotein metabolism, pre-β-HDL formation, hepatic VLDL secretion, and the development of atherosclerosis, by comparing mice expressing human PLTP with and without transfer activity with their control littermates.

MATERIALS AND METHODS

Animals

The generation of transgenic mice with liver-specific expression of human PLTP has been described previously (16). In short, the complete human PLTP sequence was isolated and the genomic DNA sequence between the *BamHI* sites in exons 5 and 8 was replaced by the equivalent cDNA sequence. The albumin promoter and enhancer sequences were cloned into the first exon of the human PLTP gene, and a 6.5 kb fragment containing the 3 α-fetoprotein enhancer elements was cloned downstream from exon 16. The PLTP transgenic (PLTP tg) mice used in the present study are derived from the mouse line referred to as A3 in our previous work (16). Mutant PLTP transgenic (mutPLTP tg) mice were generated as described for PLTP transgenic mice, but a mutation was introduced in the cDNA sequence of the construct. The replacement of TGG nucleotides by CTT through PCR-based site-directed mutagenesis changed the leucine residue 196 in the N-terminal pocket of PLTP into tryptophan (L196W) (20). Transgenic mice were crossed into a C57BL/6 background for at least eight generations. Transgenic mice hemizygous for human PLTP or mutant human PLTP were crossed with LDL receptor (LDLR)-deficient mice (obtained from Jackson Laboratory) to obtain PLTP transgenic*LDLR$^{+/-}$ and mutant PLTP transgenic*LDLR$^{+/-}$, or LDLR$^{+/-}$ control littermates. For convenience, these mice are referred to as "PLTP tg" mice, "mutPLTP tg" mice, and "control" mice, respectively. All mice used in the study were kept on regular chow diet or on an atherogenic high-fat, high-cholesterol (HFHC) diet containing 40% (w/w) sucrose, 15% (w/w) cocoa butter, 1% (w/w) cholesterol, and 0.5% (w/w) cholate (diet N; Hope Farms, Woerden, The Netherlands) for 13 weeks. Only male mice were used in the study. The mice were 10–12 weeks old at the beginning of the diet studies. All animals had free access to food and water. To perform plasma analyses, blood was collected from the orbital plexus after an overnight fasting period. All animal experiments were carried out in compliance with national and institutional guidelines.

PLTP mRNA expression analysis

After systemic perfusion of mice with PBS, organs were excised, and total RNA was isolated (RNeasy mini kit, Qiagen) and used as a template for reverse transcription (iScript cDNA synthesis kit, Biorad). Quantitative PLTP RNA expression analysis was performed using a MyIQ 5.0 detection system (Biorad). A SYBR®Green PCR reagents kit (Eurogentec), and primers specific for human or murine PLTP were used (sequences available upon request). cDNA quantities were normalized to the amount of hypoxanthine–guanine phosphoribosyl transferase cDNA, using the ΔCt method [$2^{(-\Delta Ct)}$], and presented as arbitrary units (AUs). Averages were taken from at least three individual runs, each sample in triplicate.

PLTP activity and mass

Plasma PLTP activity levels were determined by measuring the transfer of radiolabeled phospholipid from liposomes to exogenous HDL, as described previously (15), with a minor modification: liposomes were made using [^{14}C]-labeled instead of [^{3}H]-labeled phosphatidylcholine (Amersham). Plasma PLTP activity was expressed as AUs, where one AU equals 13.9 mmol/l/h, the level of PLTP activity that is found in human reference plasma. To determine the transfer of cholesterol, liposomes containing radiolabeled cholesterol (Amersham) were used. The transfer of α-tocopherol was determined using a micromethod with reverse-phase HPLC, with detection by fluorescence at 292/324 nm (21).

To determine hepatic phospholipid transfer activity levels, livers of PLTP tg, mutPLTP tg, and control mice were perfused in situ with ice-cold PBS, after which a section of approximately 0.2 g was homogenized in a buffer containing 50 mmol/l Tris-HCl (pH 7.4), 5 mmol/l EDTA, and 250 mmol/l sucrose. After centrifugation (16,000 *g*, 10 min, 4°C), supernatant was collected and phospholipid transfer activity was determined using the same protocol as for plasma PLTP. PLTP activity was calculated per milligram liver tissue, and expressed as AU. Hepatic phospholipid transfer activity of PLTP$^{-/-}$ mice (kindly donated by Dr. X-C. Jiang, Brooklyn, NY) was subtracted in order to express PLTP-specific activity.

Human PLTP mass in plasma was measured as described previously (22), using a sandwich-type ELISA using two monoclonal antibodies with specificity for human PLTP. The concentration of plasma PLTP was expressed as micrograms/milliliter.

Separation of plasma lipoproteins

Plasma HDL fractions and non-HDL fractions were separated by ultracentrifugation of freshly isolated plasma samples at a density of 1.063 g/ml as described previously (16). Lipoprotein size distribution was determined by gel filtration chromatography. Plasma samples from 10 to 15 mice were pooled and passed through a 0.45 μm filter (Millipore), and 0.5 ml was applied onto a fast-protein liquid chromatography (FPLC) system containing a Superdex 200 prepgrade column and a Superose 6 prepgrade column connected in tandem. Columns were equilibrated with buffer containing 65 mmol/l sucrose, 225 mmol/l mannitol, 10 mmol/l Tris-HCl (pH 8.1), 5 mmol/l EDTA, and 0.02%

NaN_3 to obtain optimal PLTP recovery (23). After running (at 4°C, 0.1 ml/min), 0.8 ml fractions were collected and analyzed for total cholesterol (TC) levels and PLTP mass.

Plasma liver lipid analyses

For the measurement of TC, a Free Cholesterol C kit from Wako was used, with cholesterol esterase from *Candida cylindracea* (Boehringer-Mannheim) to hydrolyze cholesteryl esters. Phospholipids were measured enzymatically with a PAP150 kit from BioMérieux. Triglycerides (TGs) were determined using a triglyceride kit from Wako.

Determination of pre-β-HDL levels and formation

Pre-β-HDL levels were determined by crossed immunoelectrophoresis. Plasma samples were incubated for 5 h at 37°C in the presence of 1 mmol/l iodoacetate (IAA) to inhibit LCAT activity, or kept at 4°C in the absence of IAA. Five microliters of plasma was loaded per well. First-dimension agarose gel (1%) electrophoresis separated lipoproteins with pre-β- and α-mobility under nonreducing, nondenaturing conditions and low ionic strength. Second-dimension electrophoresis in a 1% agarose gel containing 7.5% polyclonal rabbit anti-mouse apoA-I antiserum quantified apoA-I in the samples. Gels were stained with Coomassie Brilliant Blue R250 and dried. Areas under pre-β- and α-HDL peaks were calculated for each condition. The relative pre-β-HDL area was expressed as a percentage of the total HDL area. Pre-β-HDL formation was determined as the difference between relative pre-β-HDL levels with and without incubation with IAA.

VLDL secretion experiments

Following an overnight fasting period, mice were injected intravenously with 10% (w/v) Triton WR1339 (Tyloxapol, Sigma) dissolved in PBS, at a concentration of 500 mg/kg body weight. At different time points after injection, blood samples were taken from the orbital plexus, and plasma TG levels were determined as described above. The rate of TG secretion was calculated from the TG accumulation in time, and expressed as mmol/l/min.

Quantification of atherosclerotic plaque size

After 13 weeks of the HFHC diet, mice were euthanized. Perfusion of hearts was performed in situ, with subsequent fixation using 4% PBS-buffered formalin. The hearts with aortic arch were excised and processed for cryosectioning. Serial 7 μm-thick sections of the valves in the aortic root were stained with Oil Red O and Mayer's hematoxilin. Atherosclerotic lesion area was measured in five sections at intervals of 90 μm using image processing by National Institutes of Health-based Scion Image and analyzing software (www.scioncorp.com) according to Paigen et al. (24). Mean lesion area per section was calculated for each mouse, and expressed as μm^2.

Statistics

All values are expressed as mean ± SD. Differences were anayzed by two-sample Wilcoxon rank sum tests using Intercooled Stata 8.2/SE software (Stata Corporation, College Station, TX). Statistical significance was assumed when $P < 0.05$.

RESULTS

Expression of human PLTP in transgenic mice

To investigate the role of PLTP-mediated phospholipid transfer in vivo, the effects of overexpressing human PLTP with transfer activity and mutant human PLTP without transfer activity were compared in mice with an $LDLR^{+/-}$ background (PLTP tg and mutPLTP tg mice, respectively). First, we analyzed PLTP expression in PLTP tg, mutPLTP tg, and control ($LDLR^{+/-}$) mice.

RNA of different tissues (liver, lung, spleen, kidney, adrenals, and adipose tissue) was isolated and mRNA expression of murine and human PLTP was determined by real-time PCR. Human PLTP was expressed specifically in the liver of PLTP transgenic mice, whereas endogenous PLTP was expressed in all tissues tested (not shown). The overexpression of human PLTP in the liver did not affect endogenous PLTP mRNA levels, because these were not different between groups (**Fig. 1A**).

To check whether an increased PLTP expression in the liver was associated with changes in phospholipid transfer activity, we measured hepatic PLTP activity. PLTP activity in the livers of PLTP tg mice was approximately 2.5-fold higher than in mutPLTP tg and control mice. MutPLTP tg and control mice showed comparable activity levels (Fig. 1B).

Because PLTP is a protein secreted by the liver cells into the plasma, we quantified human PLTP protein levels as well as total PLTP activity levels in the mouse plasma. Because the assay to determine PLTP mass detects exclusively human PLTP, no signal was found in plasma from control mice. PLTP mass in mutPLTP tg mice was higher than in PLTP tg mice (Fig. 1C). Total plasma PLTP activity in PLTP tg mice was 2.9-fold higher than in control mice, whereas in mutPLTP tg mice, it did not exceed the endogenous mouse PLTP activity level (Fig. 1D). In addition to the reduced transfer activity of phospholipids, the transfer activity of cholesterol and α-tocopherol in plasma of mutPLTP tg mice was decreased compared with PLTP tg mice (cholesterol transfer in mutPLTP tg mice: 30% of transfer in PLTP tg mice; α-tocopherol transfer: 22% of transfer in PLTP tg mice; both $P < 0.05$), indicating that the mutation does not selectively affect phospholipid transfer.

Effect of PLTP transfer activity on lipid metabolism

To determine whether the mutation influenced the ability of PLTP to associate with HDL, plasma lipoproteins were separated by FPLC, and TC as well as human PLTP mass were measured in the different fractions (**Fig. 2A**). Two major cholesterol peaks were distinguished in the plasma of both transgenic mouse groups, one peaking in fraction 8 and another in fraction 15, representing LDL and HDL particles, respectively. In both mouse models, human PLTP mass peaked in fractions 11–13, where a subfraction of large HDL particles, carrying PLTP, is present. These findings indicate that the in vivo association of PLTP protein with HDL particles was not affected by the mutation.

We studied the physiological role of PLTP transfer activity in lipid metabolism. TC, phospholipid, and TG were measured in total plasma samples and in plasma subfractions obtained after ultracentrifugation. Consistent with earlier findings, PLTP tg mice showed markedly decreased TC and HDL-cholesterol (HDL-C) levels compared with control mice (**Table 1**). In mutPLTP tg mice however, lipid values did not differ from those of control littermates. Gel

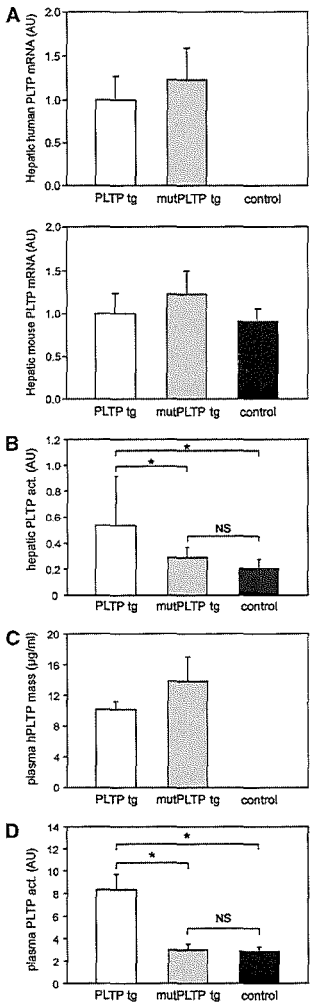

Fig. 1. Phospholipid transfer protein (PLTP) expression. A: Hepatic PLTP mRNA. The expression of human and mouse PLTP in livers of PLTP transgenic (PLTP tg), mutant PLTP transgenic (mutPLTP tg), and control mice was analyzed by real-time PCR. Values are expressed as arbitrary units (AUs) ± SD, n = 4 per group. B: Hepatic PLTP activity. PLTP activity in the liver is represented as the total phospholipid transfer activity in 1 mg liver tissue subtracted from the phospholipid transfer activity found in 1 mg liver tissue of PLTP-deficient mice. Values are expressed as AUs ± SD, n = 4–11 per group. *$P < 0.05$; NS, not significant. C: Plasma PLTP mass. Human PLTP protein was quantified using a sandwich-type ELISA. Values are expressed as µg/ml ± SD, n = 17–20 per group. D: Plasma PLTP activity. Values are expressed as AUs ± SD, n = 16–20 per group. *$P < 0.001$; NS, not significant.

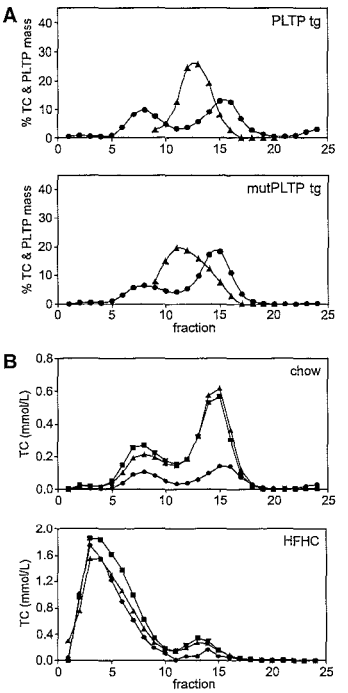

Fig. 2. Total cholesterol (TC) and PLTP distribution among plasma lipoprotein particles. Pooled plasma of PLTP tg, mutPLTP tg, and control mice (n = 10–15 per group) was subjected to fast-protein liquid chromatography (FPLC). A: TC (circles) and PLTP mass (triangles) in the FPLC fractions. Values are represented as fractional percentages of total amount of TC and PLTP mass measured in the column eluate. B: TC among lipoprotein particles in PLTP tg (circles), mutPLTP tg (triangles), and control mice (squares) on a chow or high-fat, high-cholesterol (HFHC) diet. Values are expressed as mmol/l.

filtration profiles confirmed these findings (Fig. 2B; upper graph). The same was found for phospholipid levels, whereas plasma TG levels were not different among the three groups (data not shown). To investigate whether the liver-specific overexpression of human PLTP in mice has consequences for liver lipid levels, cholesterol, phospholipid, and TG levels were determined in liver extracts. No significant differences between the mouse groups were observed (data not shown).

Effect of PLTP transfer activity on pre-β-HDL formation and VLDL secretion

Previous studies have shown that PLTP enhances the formation of pre-β-HDL particles (15). To investigate the importance of PLTP transfer activity in this process, total plasma was incubated for 5 h with iodoacetic acid, an LCAT inhibitor, after which crossed immunoelectrophoresis ex-

TABLE 1. Plasma total cholesterol

	HFHC diet		
	0 Weeks	5 Weeks	13 Weeks
	mmol/l		
Plasma TC			
PLTP tg	1.54 ± 0.24[a,b]	9.90 ± 1.92	11.55 ± 3.74
MutPLTP tg	3.54 ± 0.36	11.61 ± 1.65	9.68 ± 2.38
Control	3.64 ± 0.49	11.10 ± 2.33	10.36 ± 2.86
HDL-C			
PLTP tg	0.65 ± 0.18[a,b]	0.31 ± 0.05[a,b]	0.30 ± 0.11[a,b]
MutPLTP tg	2.10 ± 0.31	1.02 ± 0.29	1.18 ± 0.57
Control	2.18 ± 0.41	1.27 ± 0.31	1.38 ± 0.57
non-HDL-C			
PLTP tg	0.73 ± 0.24	9.01 ± 1.72	11.24 ± 3.81
MutPLTP tg	1.06 ± 0.07	9.66 ± 2.17	8.34 ± 2.18
Control	0.96 ± 0.12	9.24 ± 2.01	8.79 ± 2.87

HFHC, high-fat, high-cholesterol; TC, total cholesterol; PLTP, phospholipid transfer protein; PLTP tg, PLTP transgenic; mutPLTP tg, mutant PLTP transgenic; HDL-C, HDL cholesterol. Values are expressed as mean ± SD.

[a] $P < 0.001$ compared with control mice.

[b] $P < 0.001$ compared with mutPLTP tg mice.

periments were performed and mouse apoA-I was visualized (**Fig. 3**). In the absence of IAA, no pre-β-HDL could be detected in plasma (not shown). After incubation, 3.1% of total apoA-I was found in the pre-β-HDL subfraction in control mice (Fig. 3C) and 3.9% in mutPLTP tg mice (Fig. 3B), whereas in the PLTP tg mice, pre-β-HDL represented 28.1% of total plasma apoA-I (Fig. 3A), demonstrating the importance of active plasma PLTP for the production of pre-β-HDL.

To investigate the contribution of PLTP activity to the increased secretion of apoB-containing lipoproteins in mice with overexpression of PLTP, we performed VLDL-TG secretion experiments in vivo. As expected, TG secretion rates in PLTP tg mice were increased (1.4-fold) compared with those in control mice (**Fig. 4**). In contrast, mutPLTP tg mice without enhanced PLTP transfer activity did not stimulate VLDL secretion compared with control littermates. These findings show the role of active PLTP in hepatic VLDL secretion, whereas inactive PLTP has no effect.

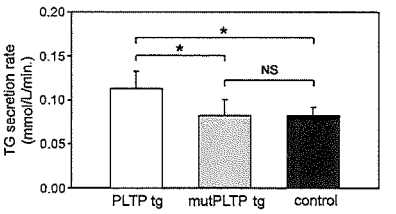

Fig. 4. Hepatic triglyceride (TG) secretion. Plasma TG levels were measured in plasma of PLTP tg (n = 6), mutPLTP tg (n = 23), and control mice (n = 12), at different time points after injection of mice with Triton WR1339. TG secretion rate was calculated using the slope of plasma TG secretion over time. Values are expressed as mmol/l/min ± SD. *$P < 0.005$; NS, not significant.

Effect of PLTP transfer activity on plasma lipids and the development of atherosclerosis in mice on a HFHC diet

To study whether PLTP activity influences the development of atherosclerosis, mice were fed a HFHC, cholate-containing diet for 13 weeks. At regular time points after starting the diet, a blood sample was taken and lipid analyses were performed. After 13 weeks, mice were euthanized and the extent of atherosclerosis was quantified. The cholesterol-enriched diet increased TC and non-HDL-C, whereas it decreased HDL-C in all mice. PLTP tg mice showed a more profound decrease in HDL-C than did mutPLTP tg mice or control littermates (Table 1; Fig. 2B). PLTP activity was increased 1.5- to 2.5-fold in hyperlipidemic mice compared with mice on a chow diet ($P < 0.05$). The diet also induced PLTP mass levels, 1.5- and 1.7-fold in PLTP tg and mutPLTP tg mice, respectively ($P < 0.05$). After incubating plasma of hyperlipidemic mice for 5 h, pre-β-HDL levels (relative to total plasma HDL) increased by 13% in control mice (**Fig. 5F** versus 5C) and 15% in mutPLTP tg mice (Fig. 5E versus 5B). In PLTP tg mice, the formation of pre-β-HDL increased by 50%, and pre-β-HDL levels, which were already high before incubation, were close to 100% of total plasma HDL after incubation (Fig. 5D versus 5A). The

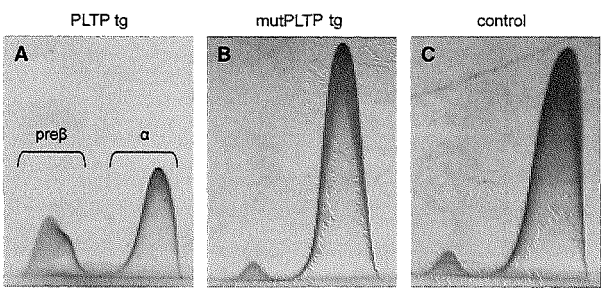

Fig. 3. Pre-β-HDL formation in mice on chow diet. Plasma of PLTP tg (A), mutPLTP tg (B), and control mice (C) was incubated with 1 mM iodoacetate (IAA) for 5 h at 37°C and subjected to crossed immunoelectrophoresis. Three to five mice per group were analyzed; one representative gel per group is shown. α- and pre-β-mobility of the HDL particles are indicated in A.

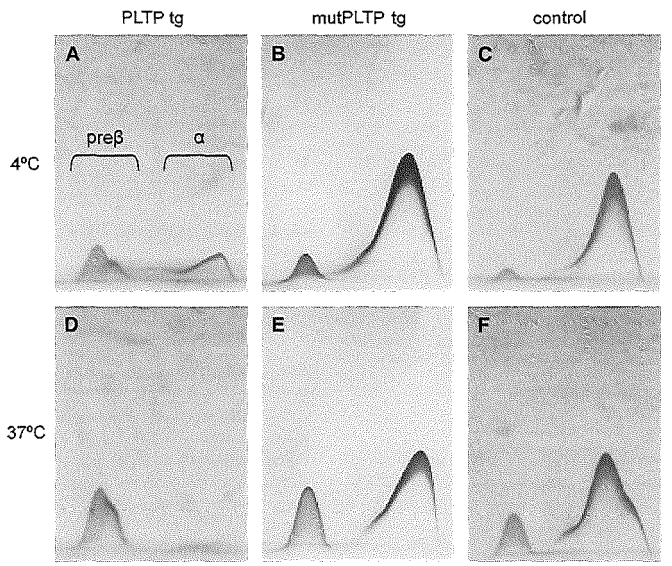

Fig. 5. Pre-β-HDL levels and formation in mice fed a HFHC diet. Plasma of PLTP tg (A, D), mutPLTP tg (B, E), and control mice (C, F) fed a HFHC diet for 13 weeks, was either kept at 4°C (upper panels), or incubated with 1 mM IAA for 5 h at 37°C (lower panels), after which it was subjected to crossed immunoelectrophoresis. Three to five mice per group were analyzed; one representative gel per group is shown. α- and pre-β-mobility of the HDL particles are indicated in A.

capacity of total plasma of hyperlipidemic mice to accept cholesterol from loaded macrophages in vitro, as investigated in cholesterol efflux experiments, was not different among the three groups (data not shown).

Finally, atherosclerotic lesion development was analyzed at the aortic valves. The overexpression of PLTP in PLTP tg mice increased the development of atherosclerotic lesions more than 2-fold compared with control mice (**Fig. 6**). In mice with overexpression of the mutant PLTP, atherosclerotic plaque sizes were not significantly different from those observed in control mice. These findings demonstrate that overexpression of PLTP with transfer activity accelerates the development of atherosclerosis, whereas mutant PLTP lacking transfer activity does not.

DISCUSSION

In human plasma, a low-active form of PLTP has been described in addition to the high-active form (18). The role of this low-active PLTP, however, is not well understood. In a Japanese cohort study, a possible protective role for serum PLTP mass in coronary heart disease was indicated, independent of PLTP activity (25). Recently, it was published that distribution of PLTP between high-activity and low-activity forms may be disturbed in peripheral arterial disease (26). The few existing studies regarding the relationship between plasma PLTP activity and cardiovascular disease in humans show contradictory findings. Whereas Schlitt et al. (27) identified PLTP activity as an independent predictive value for coronary artery disease, others found variable relationships between PLTP activity and peripheral artery disease (26, 28).

In the present study, we aimed to elucidate the role of plasma PLTP transfer activity in *1*) the modulation of HDL levels, *2*) pre-β-HDL formation, *3*) hepatic VLDL secretion, and *4*) the development of atherosclerosis in PLTP transgenic mice. Hence, we generated a mouse model with liver-specific expression of a mutant human PLTP protein, associating with HDL particles in plasma and lacking phospholipid transfer activity, and cross-bred the animals in an $LDL^{+/-}$ background.

The liver-specific expression is the consequence of a PLTP transgene driven by the albumin promoter with enhancers. A similar construct was used in previous studies for the generation of PLTP tg mice (16). These PLTP tg mice developed similar levels of atherosclerosis compared with mice with equal plasma PLTP activity levels as a result of ubiquitous expression of human PLTP, showing that increased atherosclerosis development was caused by plasma PLTP activity. Therefore, the PLTP effects we evaluated in

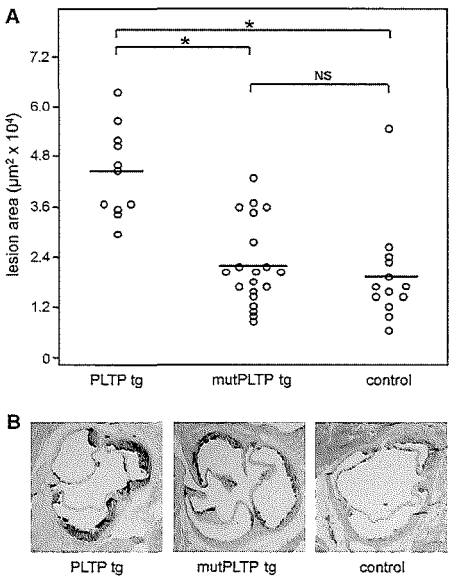

Fig. 6. Atherosclerotic lesion development Atherosclerotic lesion development was induced in PLTP tg (n = 11), mutPLTP tg (n = 20), and control (n = 13) mice by feeding a HFHC diet for 13 weeks. Cross sections of the aortic root were stained with Oil Red O to visualize the accumulation of lipids. A: Atherosclerotic lesion area of aortic valves, expressed as $\mu m^2 \times 10^4$ per section. Circles indicate individual lesion areas; horizontal lines indicate the mean lesion area per group. $^*P < 0.001$; NS, not significant. B: Representative aortic root cross sections.

the present study represent systemic effects, caused by PLTP secreted by hepatocytes into the plasma.

We focused on the role of PLTP transfer activity in vivo by introducing the L196W mutation in the PLTP protein, based on a study from Huuskonen et al. (20). This mutation at the N-terminal lipid binding pocket of PLTP resulted in a decreased phospholipid transfer activity, without affecting the HDL binding characteristics of the protein. In plasma, PLTP is found associated with plasma HDL particles (29, 30). Therefore, when studying the role of PLTP activity in lipoprotein metabolism and atherogenesis, it is important to use a mouse model in which the mutation specifically affects the phospholipid transfer activity but not the PLTP-HDL association.

PLTP is a well-known HDL conversion factor. It remodels HDL with the generation of large α-HDL particles and small lipid-poor pre-β-HDL, by a mechanism that involves fusion of unstable HDL particles and displacement of lipid-poor apoA-I molecules in vitro (8, 31). HDL-associated PLTP has been shown to be an important regulator of plasma HDL levels in vivo. In PLTP transgenic mice, catabolism of HDL particles is enhanced (32), thereby decreasing plasma HDL levels. In the present study, we showed that this PLTP-dependent HDL-lowering effect requires increased PLTP transfer activity, and should therefore be distinguished from increased HDL catabolism leading to reduced HDL levels in PLTP-deficient mice. As revealed by crossed immunoelectrophoresis, the reduction in HDL levels in PLTP tg mice is mainly due to a decrease in α-migrating HDL particles. In addition to its effect on the catabolism of mature HDL particles, PLTP plays a role in the formation of pre-β-HDL particles, which also requires PLTP transfer activity. The PLTP effects on the distribution and levels of α- and pre-β-HDL were more pronounced when mice were fed a HFHC diet. This could be explained by the increased PLTP levels found in mice fed a high-fat diet. As a reaction to cholesterol loading, the production of pre-β-HDL might be stimulated, inasmuch as pre-β-HDL has been shown to be an excellent acceptor of cholesterol (33). We found that the capacity of total plasma of hyperlipidemic mice to accept cholesterol from loaded macrophages in vitro was not different among the three groups. The decreased α-HDL levels in plasma of PLTP tg mice may compensate for the increased pre-β-HDL levels regarding its role in cholesterol efflux in vitro. The role of systemic PLTP in cholesterol efflux in vivo is yet to be determined. The big decrease in α-HDL may override the potentially athero-protective effect of increased pre-β-HDL levels in PLTP tg mice in vivo.

When measuring hepatic phospholipid transfer activity, we found an increase in PLTP tg mice, possibly explaining the increase in VLDL secretion in these mice. In contrast, in mice expressing the mutated form of PLTP, neither hepatic phospholipid transfer activity nor VLDL secretion was altered compared with controls. Elevation of PLTP activity in the liver was not associated with differences in liver lipids, suggesting that hepatic PLTP is of minor importance for lipid homeostasis in the liver of PLTP transgenic mice. Moreover, in PLTP-deficient mice, hepatic phospholipid transfer activity is about 75% of the activity measured in wild-type mice (data not shown). This is not surprising, inasmuch as several other hepatic proteins able to transfer various types of phospholipids have been described (34).

Finally, we determined the importance of PLTP activity for the development of atherosclerosis in mice fed a cholesterol-enriched, cholate-containing diet. In the mutPLTP tg mice, the extent of atherosclerosis was comparable to that in the control littermates. In PLTP tg mice, however, increased atherosclerotic lesion sizes were found, consistent with earlier findings in mice overexpressing human PLTP (10, 16). The presence of cholate in the diet has been shown to regulate PLTP gene expression in vivo by the nuclear farnesoid X receptor (FXR), because the PLTP promoter contains an FXR response element (35). However, because we used an albumin promoter to generate our transgenic mouse models, it is not likely that plasma levels of human PLTP are affected by cholate feeding. Endogenous PLTP is possibly regulated by the cholate, but we expect that mouse PLTP levels were affected to the same extent in all mice. A limitation in our study is the presence of mouse PLTP, with activity, in all mice.

Whether PLTP mass, in the absence of any transfer activity, contributes to atherosclerosis development should be investigated in additional studies in mice by crossing the mice into a PLTP knockout background. However, we do not expect endogenous PLTP to influence our findings, inasmuch as plasma lipoprotein levels in mutPLTP tg*PLTP$^{-/-}$ mice are not different when compared with those in PLTP$^{-/-}$ mice (data not shown).

An important issue is how increased PLTP expression stimulates atherosclerosis. Elevation of PLTP activity (2.9-fold) results in HDL hypercatabolism and enhanced hepatic VLDL secretion. Both aspects may be important in the process of atherogenesis, and our present results suggest that PLTP transfer activity is required for these effects. In addition to the PLTP-mediated phopholipid transfer, the transfer of cholesterol and α-tocopherol was also inhibited. These transfer activities could account, at least in part, for the pro-atherogenic potential of PLTP in vivo. In addition, O'Brien et al. (36) suggested that PLTP may be pro-atherogenic by acting as a bridging protein between lipoproteins and biglycan, one of the major extracellular proteoglycans found in human atherosclerotic lesions. It is well known that retention of lipoproteins by extracellular matrix molecules is critical in the pathogenesis of atherosclerosis (36, 37). This bridging effect of PLTP has been shown to be independent of its phospholipid transfer activity in vitro (36). Furthermore, over the last 2 years, much attention has been paid to the functionality of PLTP in macrophages (38–40). However, results between different studies were contradictory, illustrating the complexity of the in vivo situation. Whereas Vikstedt et al. (40) demonstrated that PLTP deficiency in macrophages resulted in reduced plasma PLTP activity levels and a decreased atherosclerotic lesion development, two other research groups found an increased atherosclerosis development despite lower plasma PLTP activity levels, and suggested an atheroprotective role for macrophage-derived PLTP (38, 39). The relative contribution of systemic PLTP effects and local PLTP effects to atherosclerosis development remains unclear, and a balance between pro- and anti-atherogenic properties of PLTP might determine the impact of PLTP on atherosclerosis. In the present study, we showed the importance of plasma PLTP activity in the process of atherosclerosis development. Whether PLTP acts as a ligand binding to vascular proteoglycans, playing a role in the retention of HDL, and whether PLTP activity is required for local PLTP functions within the artery wall, needs further investigation. The use of an adapted version of our mutant PLTP mouse model, allowing expression of inactive PLTP in different tissues, might be very informative for future studies.

In conclusion, this study defines the contribution of PLTP transfer activity in plasma to atherogenesis in PLTP transgenic mice. Inhibitors of plasma PLTP activity could prevent atherosclerosis development in humans with elevated PLTP activity levels.

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REFERENCES

1. Tall, A. R., and P. H. Green. 1981. Incorporation of phosphatidylcholine into spherical and discoidal lipoproteins during incubation of egg phosphatidylcholine vesicles with isolated high density lipoproteins or with plasma. *J. Biol. Chem.* **256:** 2035–2044.
2. Tall, A. R., S. Krumholz, T. Olivecrona, and R. J. Deckelbaum. 1985. Plasma phospholipid transfer protein enhances transfer and exchange of phospholipids between very low density lipoproteins and high density lipoproteins during lipolysis. *J. Lipid Res.* **26:** 842–851.
3. Rao, R., J. J. Albers, G. Wolfbauer, and H. J. Pownall. 1997. Molecular and macromolecular specificity of human plasma phospholipid transfer protein. *Biochemistry.* **36:** 3645–3653.
4. Nishida, H. I., and T. Nishida. 1997. Phospholipid transfer protein mediates transfer of not only phosphatidylcholine but also cholesterol from phosphatidylcholine-cholesterol vesicles to high density lipoproteins. *J. Biol. Chem.* **272:** 6959–6964.
5. Hailman, E., J. J. Albers, G. Wolfbauer, A. Y. Tu, and S. D. Wright. 1996. Neutralization and transfer of lipopolysaccharide by phospholipid transfer protein. *J. Biol. Chem.* **271:** 12172–12178.
6. Levels, J. H., J. A. Marquart, P. R. Abraham, A. E. van den Ende, H. O. Molhuizen, S. J. van Deventer, and J. C. Meijers. 2005. Lipopolysaccharide is transferred from high-density to low-density lipoproteins by lipopolysaccharide-binding protein and phospholipid transfer protein. *Infect. Immun.* **73:** 2321–2326.
7. Kostner, G. M., K. Oettl, M. Jauhiainen, C. Ehnholm, H. Esterbauer, and H. Dieplinger. 1995. Human plasma phospholipid transfer protein accelerates exchange/transfer of alpha-tocopherol between lipoproteins and cells. *Biochem. J.* **305:** 659–667.
8. Jauhiainen, M., J. Metso, R. Pahlman, S. Blomqvist, A. van Tol, and C. Ehnholm. 1993. Human plasma phospholipid transfer protein causes high density lipoprotein conversion. *J. Biol. Chem.* **268:** 4032–4036.
9. Tu, A. Y., H. I. Nishida, and T. Nishida. 1993. High density lipoprotein conversion mediated by human plasma phospholipid transfer protein. *J. Biol. Chem.* **268:** 23098–23105.
10. Moerland, M., H. Samyn, T. van Gent, M. Jauhiainen, J. Metso, R. van Haperen, F. Grosveld, A. van Tol, and R. de Crom. 2007. Atherogenic, enlarged, and dysfunctional HDL in human PLTP/apoA-I double transgenic mice. *J. Lipid Res.* **48:** 2622–2631.
11. Huuskonen, J., V. M. Olkkonen, C. Ehnholm, J. Metso, I. Julkunen, and M. Jauhiainen. 2000. Phospholipid transfer is a prerequisite for PLTP-mediated HDL conversion. *Biochemistry.* **39:** 16092–16098.
12. Jiang, X. C., C. Bruce, J. Mar, M. Lin, Y. Ji, O. L. Francone, and A. R. Tall. 1999. Targeted mutation of plasma phospholipid transfer protein gene markedly reduces high-density lipoprotein levels. *J. Clin. Invest.* **103:** 907–914.
13. Jiang, X. C., S. Qin, C. Qiao, K. Kawano, M. Lin, A. Skold, X. Xiao, and A. R. Tall. 2001. Apolipoprotein B secretion and atherosclerosis are decreased in mice with phospholipid-transfer protein deficiency. *Nat. Med.* **7:** 847–852.
14. Jiang, X. C., A. R. Tall, S. Qin, M. Lin, M. Schneider, F. Lalanne, V. Deckert, C. Desrumaux, A. Athias, J. L. Witztum, et al. 2002. Phospholipid transfer protein deficiency protects circulating lipoproteins from oxidation due to the enhanced accumulation of vitamin E. *J. Biol. Chem.* **277:** 31850–31856.
15. van Haperen, R., A. van Tol, P. Vermeulen, M. Jauhiainen, T. van Gent, P. van den Berg, S. Ehnholm, F. Grosveld, A. van der Kamp, and R. de Crom. 2000. Human plasma phospholipid transfer protein increases the antiatherogenic potential of high density lipoproteins in transgenic mice. *Arterioscler. Thromb. Vasc. Biol.* **20:** 1082–1088.
16. van Haperen, R., A. van Tol, T. van Gent, L. Scheek, P. Visser, A. van der Kamp, F. Grosveld, and R. de Crom. 2002. Increased risk of atherosclerosis by elevated plasma levels of phospholipid transfer protein. *J. Biol. Chem.* **277:** 48938–48943.
17. Jaari, S., K. W. van Dijk, V. M. Olkkonen, A. van der Zee, J. Metso, L. Havekes, M. Jauhiainen, and C. Ehnholm. 2001. Dynamic changes

in mouse lipoproteins induced by transiently expressed human phospholipid transfer protein (PLTP): importance of PLTP in prebeta-HDL generation. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **128:** 781–792.
18. Oka, T., T. Kujiraoka, M. Ito, T. Egashira, S. Takahashi, M. N. Nanjee, N. E. Miller, J. Metso, V. M. Olkkonen, C. Ehnholm, et al. 2000. Distribution of phospholipid transfer protein in human plasma: presence of two forms of phospholipid transfer protein, one catalytically active and the other inactive. *J. Lipid Res.* **41:** 1651–1657.
19. Jonkers, I. J., A. H. Smelt, H. Hattori, L. M. Scheek, T. van Gent, F. H. de Man, A. van der Laarse, and A. van Tol. 2003. Decreased PLTP mass but elevated PLTP activity linked to insulin resistance in HTG: effects of bezafibrate therapy. *J. Lipid Res.* **44:** 1462–1469.
20. Huuskonen, J., G. Wohlfahrt, M. Jauhiainen, C. Ehnholm, O. Teleman, and V. M. Olkkonen. 1999. Structure and phospholipid transfer activity of human PLTP: analysis by molecular modeling and site-directed mutagenesis. *J. Lipid Res.* **40:** 1123–1130.
21. Anttolainen, M., L. M. Valsta, G. Alfthan, P. Kleemola, I. Salminen, and M. Tamminen. 1996. Effect of extreme fish consumption on dietary and plasma antioxidant levels and fatty acid composition. *Eur. J. Clin. Nutr.* **50:** 741–746.
22. Oka, T., T. Kujiraoka, M. Ito, M. Nagano, M. Ishihara, T. Iwasaki, T. Egashira, N. E. Miller, and H. Hattori. 2000. Measurement of human plasma phospholipid transfer protein by sandwich ELISA. *Clin. Chem.* **46:** 1357–1364.
23. Clark, R. W., J. B. Moberly, and M. J. Bamberger. 1995. Low level quantification of cholesteryl ester transfer protein in plasma subfractions and cell culture media by monoclonal antibody-based immunoassay. *J. Lipid Res.* **36:** 876–889.
24. Paigen, B., A. Morrow, P. A. Holmes, D. Mitchell, and R. A. Williams. 1987. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis.* **68:** 231–240.
25. Yatsuya, H., K. Tamakoshi, H. Hattori, R. Otsuka, K. Wada, H. Zhang, T. Mabuchi, M. Ishikawa, C. Murata, T. Yoshida, et al. 2004. Serum phospholipid transfer protein mass as a possible protective factor for coronary heart diseases. *Circ. J.* **68:** 11–16.
26. Schgoer, W., T. Mueller, M. Jauhiainen, A. Wehinger, R. Gander, I. Tancevski, K. Salzmann, P. Eller, A. Ritsch, M. Haltmayer, et al. 2008. Low phospholipid transfer protein (PLTP) is a risk factor for peripheral atherosclerosis. *Atherosclerosis.* **196:** 219–226.
27. Schlitt, A., C. Bickel, P. Thumma, S. Blankenberg, H. J. Rupprecht, J. Meyer, and X. C. Jiang. 2003. High plasma phospholipid transfer protein levels as a risk factor for coronary artery disease. *Arterioscler. Thromb. Vasc. Biol.* **23:** 1857–1862.
28. Ruhling, K., A. Lang, F. Richard, A. van Tol, B. Eisele, V. Herzberg, and U. Till. 1999. Net mass transfer of plasma cholesteryl esters and lipid transfer proteins in normolipidemic patients with peripheral vascular disease. *Metabolism.* **48:** 1361–1366.
29. Tall, A. R. 1986. Plasma lipid transfer proteins. *J. Lipid Res.* **27:** 361–367.
30. Speijer, H., J. E. Groener, E. van Ramshorst, and A. van Tol. 1991. Different locations of cholesteryl ester transfer protein and phospholipid transfer protein activities in plasma. *Atherosclerosis.* **90:** 159–168.
31. Lusa, S., M. Jauhiainen, J. Metso, P. Somerharju, and C. Ehnholm. 1996. The mechanism of human plasma phospholipid transfer protein-induced enlargement of high-density lipoprotein particles: evidence for particle fusion. *Biochem. J.* **313:** 275–282.
32. Foger, B., S. Santamarina-Fojo, R. D. Shamburek, C. L. Parrot, G. D. Talley, and H. B. Brewer, Jr. 1997. Plasma phospholipid transfer protein. Adenovirus-mediated overexpression in mice leads to decreased plasma high density lipoprotein (HDL) and enhanced hepatic uptake of phospholipids and cholesteryl esters from HDL. *J. Biol. Chem.* **272:** 27393–27400.
33. Fielding, C. J., and P. E. Fielding. 2001. Cellular cholesterol efflux. *Biochim. Biophys. Acta.* **1533:** 175–189.
34. Wirtz, K. W. 2006. Phospholipid transfer proteins in perspective. *FEBS Lett.* **580:** 5436–5441.
35. Urizar, N. L., D. H. Dowhan, and D. D. Moore. 2000. The farnesoid X-activated receptor mediates bile acid activation of phospholipid transfer protein gene expression. *J. Biol. Chem.* **275:** 39313–39317.
36. O'Brien, K. D., S. Vuletic, T. O. McDonald, G. Wolfbauer, K. Lewis, A. Y. Tu, S. Marcovina, T. N. Wight, A. Chait, and J. J. Albers. 2003. Cell-associated and extracellular phospholipid transfer protein in human coronary atherosclerosis. *Circulation.* **108:** 270–274.
37. Williams, K. J., and I. Tabas. 1995. The response-to-retention hypothesis of early atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* **15:** 551–561.
38. Liu, R., M. R. Hojjati, C. M. Devlin, I. H. Hansen, and X. C. Jiang. 2007. Macrophage phospholipid transfer protein deficiency and ApoE secretion: impact on mouse plasma cholesterol levels and atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **27:** 190–196.
39. Valenta, D. T., N. Ogier, G. Bradshaw, A. S. Black, D. J. Bonnet, L. Lagrost, L. K. Curtiss, and C. M. Desrumaux. 2006. Atheroprotective potential of macrophage-derived phospholipid transfer protein in low-density lipoprotein receptor-deficient mice is overcome by apolipoprotein AI overexpression. *Arterioscler. Thromb. Vasc. Biol.* **26:** 1572–1578.
40. Vikstedt, R., D. Ye, J. Metso, R. B. Hildebrand, T. J. Van Berkel, C. Ehnholm, M. Jauhiainen, and M. Van Eck. 2007. 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.* **27:** 578–586.

Chapter 5

Atherogenic, enlarged, and dysfunctional HDL in human PLTP/apoA-I double transgenic mice

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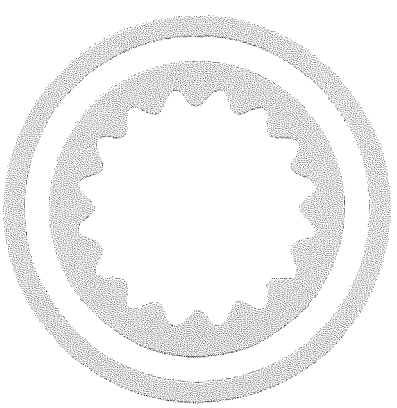

Atherogenic, enlarged, and dysfunctional HDL in human PLTP/apoA-I double transgenic mice

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Abstract In low density lipoprotein receptor (LDLR)-deficient mice, overexpression of human plasma phospholipid transfer protein (PLTP) results in increased atherosclerosis. PLTP strongly decreases HDL levels and might alter the antiatherogenic properties of HDL particles. To study the potential interaction between human PLTP and apolipoprotein A-I (apoA-I), double transgenic animals (hPLTPtg/hApoAItg) were compared with hApoAItg mice. PLTP activity was increased 4.5-fold. Plasma total cholesterol and phospholipid were decreased. Average HDL size (analyzed by gel filtration) increased strongly, hPLTPtg/hApoAItg mice having very large, LDL-sized, HDL particles. Also, after density gradient ultracentrifugation, a substantial part of the apoA-I-containing lipoproteins in hPLTPtg/hApoAItg mice was found in the LDL density range. In cholesterol efflux studies from macrophages, HDL isolated from hPLTPtg/hApoAItg mice was less efficient than HDL isolated from hApoAItg mice. Furthermore, it was found that the largest subfraction of the HDL particles present in hPLTPtg/hApoAItg mice was markedly inferior as a cholesterol acceptor, as no labeled cholesterol was transferred to this fraction. In an LDLR-deficient background, the human PLTP-expressing mouse line showed a 2.2-fold increased atherosclerotic lesion area. These data demonstrate that the action of human PLTP in the presence of human apoA-I results in the formation of a dysfunctional HDL subfraction, which is less efficient in the uptake of cholesterol from cholesterol-laden macrophages.—Moerland, M., H. Samyn, T. van Gent, M. Jauhiainen, J. Metso, R. van Haperen, F. Grosveld, A. van Tol, and R. de Crom. **Atherogenic, enlarged, and dysfunctional HDL in human PLTP/apoA-I double transgenic mice.** *J. Lipid Res.* **2007.** 48: **2622–2631.**

Supplementary key words phospholipid transfer protein • apolipoprotein A-I • cholesterol • high density lipoprotein • low density lipoprotein • atherosclerosis • lipoprotein metabolism

The incidence of coronary heart disease shows a strong inverse relationship with the concentration of plasma HDL (1). Therefore, HDL is believed to have antiatherogenic properties, which are attributed to its effects on endothelial cells, its antioxidant activity, and its role in reverse cholesterol transport, a pathway in which peripheral cellular cholesterol is transported to the liver (2–4). One important determinant of the plasma HDL concentration is phospholipid transfer protein (PLTP) activity. During lipolysis, PLTP transfers phospholipids from apolipoprotein B-containing lipoproteins to HDL (5). Furthermore, PLTP acts as an HDL conversion factor by remodeling HDL to produce large particles and small lipid-poor particles, known as preβ-HDL (6–9). The latter HDL subfraction is a very efficient acceptor of cellular cholesterol. In this way, PLTP plays an important role in the early steps of reverse cholesterol transport (10–12).

Although the involvement of PLTP in the cellular efflux of cholesterol and phospholipids is potentially antiatherogenic, different in vivo studies showed that an increase in plasma PLTP activity is associated with decreased HDL cholesterol levels (9, 13, 14) and increased atherosclerotic lesion development (15–17). The PLTP-dependent decrease in HDL levels was explained by an accelerated HDL catabolism (13). In addition to its effect on HDL cholesterol levels, PLTP may alter the antiatherogenic properties of HDL particles. For example, anti-inflammatory properties of HDL were improved in PLTP-deficient mice (18). To study the action of increased PLTP levels on HDL particles in more detail, we cross-bred human PLTP transgenic mice with human apolipoprotein A-I (apoA-I) transgenic mice, resulting in mice expressing both human PLTP and human apoA-I. Previous studies in transgenic mice showed that introduction of the human apoA-I transgene affected HDL subfraction distribution (19, 20). Expression of human apoA-I resulted in conversion of the

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Abbreviations: AcLDL, acetylated low density lipoprotein; apoA-I, apolipoprotein A-I; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; FPLC, fast-protein liquid chromatography; LDLR, low density lipoprotein receptor; PLTP, phospholipid transfer protein.

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normal relatively homogeneous mouse HDL population to a more heterogeneous population consisting of different HDL subclasses.

This difference in plasma HDL distribution may be caused by differences in physical properties between mouse and human apoA-I (i.e., molecular properties in solution, incorporation into discoidal complexes, structural stability in HDL particles) (21). PLTP binds directly to apoA-I found on the surface of HDL particles (22). The specificity of the interaction between protein (such as transfer protein or enzyme) and HDL might strongly influence the action of the protein itself. For example, cholesteryl ester transfer protein (CETP) has a much more profound effect on HDL cholesterol levels in transgenic mice when human apoA-I is expressed as well, as a result of an enhanced interaction of CETP with human apoA-I compared with mouse apoA-I (23). In transgenic mice expressing both human LCAT and human apoA-I, human LCAT has a significant preference for HDL containing human apoA-I (24).

In the present study, we investigated whether the action of human PLTP in the presence of HDL containing human apoA-I is altered compared with the situation when only mouse apoA-I is present. In a human apoA-I transgenic mouse model, we studied the effect of the introduction of the human PLTP transgene on different HDL properties: particle size and density and the ability to induce cholesterol efflux from peritoneal macrophages. In addition, the effect of the introduction of the human PLTP gene on atherosclerotic lesion development was determined in low density lipoprotein receptor (LDLR)-deficient mice expressing human apoA-I.

MATERIALS AND METHODS

Animals

Human PLTP transgenic mice were described previously (9). In this study, we used mice with a human genomic PLTP construct in which PLTP expression is controlled by its native promoter, resulting in a moderately increased PLTP activity (line P4) (16). Human apoA-I transgenic mice (19) and LDLR-deficient mice were purchased from Jackson Laboratory. All mice were crossed for >15 generations to a C57Bl/6J background. Human PLTP transgenic mice were cross-bred with human apoA-I transgenic mice to obtain hPLTPtg/hApoAItg mice, hemizygous for both transgenes. As a control, hemizygous hApoAItg mice were used. For the atherosclerosis experiments, hApoAItg mice and hPLTPtg/hApoAItg mice were cross-bred with LDLR knockout mice to obtain hApoAItg/LDLR$^{-/-}$ and hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice. For determination of the genotype, genomic DNA was isolated from tail clips of 10 day old mice and analyzed by PCR. Annealing temperatures and primer sequences are available upon request.

Male mice were used in all experiments. After weaning, animals were fed a standard chow diet. Experiments in the hApoAItg and hPLTPtg/hApoAItg mice were performed at an age between 10 and 15 weeks. The development of atherosclerosis was studied in hApoAItg/LDLR$^{-/-}$ and hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice. Animals between 10 and 15 weeks of age were put on a high-fat/high-cholesterol diet [40% (w/w) sucrose, 15% (w/w) fat, and 1% (w/w) cholesterol; Hope Farms] for 9 weeks. Animals had free access to water and food. Blood samples were collected by orbital bleeding after fasting the animals overnight. All procedures in this study were in accordance with national and institutional guidelines.

Plasma PLTP activity

PLTP activity was measured using a phospholipid vesicle-HDL system according to Speijer et al. (25). PLTP activity is expressed as arbitrary units. One arbitrary unit is equal to the level of PLTP activity found in human reference plasma, which was 13.9 μmol/ml/h.

Quantification of plasma lipids and apoA-I

Total cholesterol concentration was determined enzymatically using a free cholesterol C kit (Wako) after hydrolysis of cholesteryl esters (CEs) with cholesterol esterase from *Candida cylindracea* (Boehringer). Triglycerides were measured using a triglyceride kit from Wako. Phospholipids were measured using the PAP150 kit from BioMerieux.

Quantification of preβ-HDL by crossed immunoelectrophoresis

In pooled plasma samples obtained from 8–10 mice, preβ- and α-migrating HDL were separated by agarose gel electrophoresis under nonreducing, nondenaturing conditions as described previously (9). Proteins were transferred to nitrocellulose membranes by capillary blotting, and human apoA-I was detected using an anti-human apoA-I antibody.

Plasma lipoprotein profiles by gel filtration

Lipoprotein profiles in EDTA-plasma were obtained using HR 10/30 fast-protein liquid chromatography (FPLC) columns in tandem that were filled with Superose 6 and Superose 12 (preparation grade; Pharmacia Biotechnology). Columns were equilibrated and run in a 2 mM phosphate buffer containing 0.9% (w/v) NaCl, 0.02% (w/v) NaN_3, and 5 mM EDTA (pH 7.4). Pooled plasma samples obtained from 8–10 mice were filtered through 0.45 μm filters (Millipore). A total of 0.5 ml of plasma was loaded onto the Superose 6 column. The separation was performed at 4°C with a flow rate of 0.1 ml/min. Fractions of 0.8 ml were collected for measurements of cholesterol and phospholipids.

Plasma lipoprotein profiles by density gradient centrifugation

Individual plasma samples freshly isolated from hApoAItg mice and hPLTPtg/hApoAItg mice were pooled (at least 10 mice per pool) and subjected to density gradient ultracentrifugation in a Beckman SW60 Ti rotor, adapted from the method described previously by Redgrave, Roberts, and West (26), at the following densities: $d < 1.006$ (normally corresponding to VLDL), $1.006 < d < 1.019$ (intermediate density lipoprotein), $1.019 < d < 1.063$ (LDL), $1.063 < d < 1.21$ (HDL), and $d > 1.21$ (proteins).

Protein analysis of FPLC fractions

To determine the protein components of lipoprotein fractions, FPLC fractions were subjected to electrophoresis on 4–15% SDS-polyacrylamide gels (Bio-Rad) (27), which were subsequently stained with Coomassie Brilliant Blue R (Sigma) or with the Silver Stain Plus Kit (Bio-Rad).

Cholesterol efflux experiments with mouse peritoneal macrophages

We determined the ability of isolated mouse HDL to induce the efflux of cholesterol from acetylated low density lipoprotein

(AcLDL)-loaded peritoneal macrophages. AcLDL was prepared by isolation of LDL from human plasma by differential centrifugation (density range, 1.019–1.063), followed by acetylation by means of repeated addition of acetic anhydride. C57Bl/6J mice were elicited by intraperitoneal injection of 2 ml of 40.5 g/l Bacto® Brewer Thioglycollate Medium (Sigma) according to the manufacturer's instructions. After 3 days, peritoneal macrophages were obtained as described (28). Cultured macrophage monolayers were incubated with 500 μl of DMEM (Gibco, Invitrogen) containing 50 μg/ml AcLDL, 2% (w/v) BSA, and 1 μCi/ml [^{3}H]cholesterol for 24 h. After lipid loading, cells were washed twice, followed by an overnight equilibration incubation to remove AcLDL from cellular membranes. Subsequently, cells were washed twice again, and cholesterol efflux was initiated by incubating the macrophages for 5 h with efflux medium consisting of DMEM, 2% (w/v) BSA, and mouse HDL (4, 10, 20, or 40 μg total cholesterol/ml).

HDL of individual hApoAItg and hPLTPtg/hApoAItg mice was isolated by density gradient ultracentrifugation at a density range of 1.019–1.21, and HDL of hApoAItg/LDLR$^{-/-}$ and hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice was isolated at a density range of 1.063–1.21. In every experiment, incubations without HDL were included to measure HDL-independent cholesterol efflux. Efflux medium was collected and radioactivity was determined. Remaining cholesterol was extracted from the intact monolayers with isopropanol. Protein was measured in the cells after the addition of 0.1 M NaOH by the method of Lowry et al. (29) using BSA as a reference. Cholesterol efflux was corrected for HDL-independent efflux and subsequently calculated as cholesterol in the medium/(cellular cholesterol content + cholesterol in the medium). The resulting percentage of the total label, adjusted for HDL-independent efflux, was normalized for small differences in cell protein content between the different wells. In one experiment, individual efflux media were pooled for each group and subjected to FPLC. In the resulting lipoprotein fractions, total cholesterol and radioactivity were measured to determine the efficiency of lipoprotein particles to incorporate cellular cholesterol.

Quantification of atherosclerosis

After 9 weeks of a high-fat/high-cholesterol diet, the animals were anesthetized with isoflurane, the thorax was opened, and the animals were subjected to perfusion fixation through the left ventricle of the heart using 4% phosphate-buffered formalin. The heart was dissected and processed for cryosectioning. Cryosections of the aortic valves (7 μm thick) were stained with Oil Red O and hematoxylin. The sections were photographed with a Sony digital camera. The atherosclerotic area was measured in five sections at intervals of 90 μm according to Paigen et al. (30) using Scion Image image-analysis software (available at www.scioncorp.com).

Statistics

Data are expressed as means ± SEM. Differences were analyzed by two-sample Wilcoxon rank sum tests using Intercooled Stata 8.2/SE software (Stata Corp., College Station, TX).

RESULTS

Effects of human PLTP overexpression on plasma lipids and apoA-I levels

First, we investigated the effect of introduction of the human PLTP gene on PLTP activity and plasma lipids. hPLTPtg/hApoAItg mice had a 4.5-fold increased PLTP activity compared with hApoAItg mice (**Table 1**). Overexpression of human PLTP resulted in a decrease in total cholesterol, caused mainly by a decrease in free cholesterol level. CE levels were similar. Phospholipid levels decreased strongly. Plasma triglyceride levels did not change. Overexpression of human PLTP resulted in a 30% decrease in human apoA-I levels and a 40% decrease in mouse apoA-I levels. Mouse apoA-I levels were very low compared with human apoA-I levels, accounting for only 1–2% of total apoA-I levels (Table 1). Crossed immunoelectrophoresis showed that expression of human PLTP resulted in a decrease of apoA-I in α-HDL, whereas apoA-I in the preβ-HDL range did not decrease (**Fig. 1**). Human apoA-I plasma concentration with preβ mobility was 1.27 mg/ml (28% of total plasma human apoA-I) in hApoAItg mice versus 1.17 mg/ml (37%) in hPLTPtg/hApoAItg mice. Human apoA-I plasma concentration with α mobility was 3.26 mg/ml (72%) in hApoAItg mice versus 1.98 mg/ml (63%) in hPLTPtg/hApoAItg mice.

Effects of human PLTP overexpression on plasma lipoprotein profiles (gel filtration and density gradient centrifugation)

Next, we investigated the effect of an increased PLTP activity on the distribution of lipoprotein subclasses. Analysis of plasma lipoproteins by gel filtration showed that introduction of the human PLTP transgene in hApoAItg mice resulted in the conversion of normal HDL (top at fraction 17) into a much larger particle (top at fraction 11) with a highly increased molar ratio of cholesterol to phospholipid (**Fig. 2**, compare A with B). Next, plasma lipoproteins were separated by density gradient centrifugation to determine the density of the lipoprotein par-

TABLE 1. Plasma parameters in hApoAItg versus hPLTPtg/hApoAItg mice

Mouse	Phospholipid Transfer Protein Activity	Total Cholesterol	Phospholipid	Triglyceride	Free Cholesterol	Cholesteryl Ester	Human ApoA-I	Mouse ApoA-I
	arbitrary units			*mmol/l*			*mg/ml*	
hApoAItg	4.12 ± 0.10	5.68 ± 0.13	5.38 ± 0.12	1.20 ± 0.10	2.17 ± 0.04	3.51 ± 0.09	4.53 ± 0.09	0.10 ± 0.01
hPLTPtg/hApoAItg	18.41 ± 0.67[a]	5.07 ± 0.13[b]	3.53 ± 0.07[a]	1.16 ± 0.12	1.74 ± 0.06[a]	3.34 ± 0.10	3.15 ± 0.15[c]	0.06 ± 0.00[c]

ApoA-I, apolipoprotein A-I. Values shown are means ± SEM.
[a] $P < 0.0001$ versus hApoAItg (n = 16–17).
[b] $P < 0.01$ versus hApoAItg (n = 16–17).
[c] $P < 0.001$ versus hApoAItg (n = 16–17).

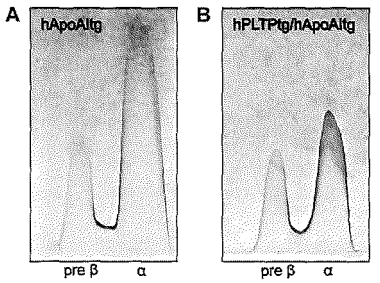

Fig. 1. Quantification of preβ-HDL by crossed immunoelectrophoresis. A: Plasma pool from hApoAItg mice. B: Plasma pool from hPLTPtg/hApoAItg mice (>10 mice per pool). The positions of preβ- and α-migrating human apolipoprotein A-I (apoA-I) are indicated.

ticles (**Table 2**). In hApoAItg mice, almost all lipoproteins were found in the HDL density range from 1.063 to 1.21 g/ml, whereas hPLTPtg/hApoAItg mice displayed lipoproteins in the HDL density range and in addition a very substantial lipoprotein fraction in the density range from 1.006 to 1.063 g/ml, which corresponds with the density of LDL.

Protein analysis of FPLC fractions

hApoAItg mice fed a standard chow diet lack substantial levels of plasma LDL (Fig. 2A, fractions 6–10, Table 2,

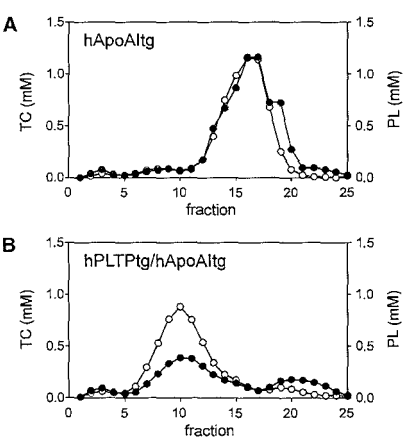

Fig. 2. Total cholesterol (TC) and phospholipid (PL) in fast-protein liquid chromatography (FPLC) fractions of plasma from hApoAItg mice (A) and hPLTPtg/hApoAItg mice (B). Individual plasma samples were pooled (>10 mice per pool) and subjected to FPLC. Total cholesterol (white circles) and phospholipid (black circles) were determined in each fraction (see Materials and Methods). Elution volumes for wild-type mice are as follows: LDL in fractions 5–10, HDL in fractions 11–20.

1.019 < d < 1.063). Introduction of the human PLTP transgene resulted in the formation of large lipoprotein particles with LDL density, as demonstrated by gel filtration and density gradient centrifugation (Fig. 2B, Table 2). To investigate whether these particles contained apoA-I, FPLC fractions were subjected to electrophoresis on a gradient SDS-polyacrylamide gel, which was subsequently stained with Coomassie blue. In hApoAItg mice, apoA-I was found mainly in fractions 12–18, which is the normal HDL range (**Fig. 3A**). In the FPLC fractions of the hPLTPtg/hApoAItg mice (Fig. 3B), apoA-I was detected not only in fractions in the HDL range but also in fractions with sizes usually corresponding with LDL particles (fractions 7–12). Apolipoprotein B patterns in fractions 7–12 did not differ between hPLTPtg/hApoAItg mice and hApoAItg mice (data not shown). These results show that concomitant expression of human PLTP and human apoA-I results in the formation of a very large, cholesterol-rich HDL particle with LDL density.

To check for the presence of apoE in the plasma lipoproteins from hPLTPtg/hApoAItg mice, lipoproteins were isolated by density gradient centrifugation and subsequently subjected to FPLC. The apolipoproteins present in the FLPC fractions were analyzed by silver staining of 4–15% SDS-polyacrylamide gels. As shown in Fig. 3C, apoA-I was present in all FPLC subfractions from the d = 1.019–1.063 g/ml range (corresponding to the normal size of LDL) and from the d = 1.063–1.21 g/ml range (corresponding to the normal size of HDL), whereas apoE could not be detected.

The ability of isolated HDL fractions to induce cholesterol efflux from mouse peritoneal macrophages

To test the functionality of the large HDL particles that are formed in hPLTPtg/hApoAItg mice, we studied the ability of HDL to induce cholesterol efflux from mouse peritoneal macrophages. Therefore, HDL fractions of both mouse lines were isolated from individual mice of both mouse lines by density gradient ultracentrifugation and used as acceptors for ^{3}H-labeled cellular cholesterol. Cholesterol efflux from macrophages induced by the large HDL isolated from hPLTPtg/hApoAItg mice was lower than that induced by HDL isolated from hApoAItg mice (**Fig. 4**), with the largest effect at an HDL cholesterol concentration of 40 μg/ml. We calculated the relative phospholipid and free cholesterol contents of the HDL particles that were used to induce cellular cholesterol efflux and found that this was similar for hApoAItg mice and hPLTPtg/hApoAItg mice. Therefore, it is unlikely that the exchange of labeled cellular cholesterol for unlabeled free HDL cholesterol differed between genotypes.

Subsequently, efflux media were collected and subjected to FPLC. In the FPLC fractions, both total cholesterol and ^{3}H counts were measured. When "normal-sized" HDL isolated from hApoAItg mice was used as an acceptor for cellular cholesterol, we found an equal distribution of cholesterol and ^{3}H counts in the FPLC fractions (**Fig. 5A**). In contrast, the profiles of labeled and unlabeled cholesterol did not completely overlap when HDL from hPLTPtg/

TABLE 2. Components of lipoprotein subclasses of pooled plasma samples, separated by density gradient ultracentrifugation

Component	d < 1.006	1.006 < d < 1.019	1.019 < d < 1.063	1.063 < d < 1.21	d > 1.21
hApoAItg					
Protein	0.05	0.02	0.12	2.72	NA
Phospholipid	0.12	0.03	0.17	2.34	0.02
Free cholesterol	0.03	0.01	0.05	0.42	0.02
Esterified cholesterol	0.04	0.02	0.14	1.53	0.05
Triglycerides	0.58	0.04	0.03	0.03	0.01
hPLTPtg/hApoAItg					
Protein	0.07	0.08	0.44	0.72	NA
Phospholipid	0.15	0.06	0.87	0.76	0.03
Free cholesterol	0.05	0.03	0.28	0.15	0.04
Esterified cholesterol	0.19	0.08	1.24	0.54	0.07
Triglycerides	0.79	0.06	0.08	0.02	0.02

Values shown are mg/ml.

hApoAItg mice was used (Fig. 5B). In this case, the largest HDL particles did not accept labeled cholesterol from the macrophages, whereas the smaller particles did (Fig. 5B, fractions 5–11 vs. fractions 11–15). To confirm this observation, another cholesterol efflux experiment was performed with HDL of hPLTPtg/hApoAItg mice that was separated by FPLC into three different subfractions (**Fig. 6A**): fractions 6–9 (very large HDL), fractions 10–14 (large HDL), and fractions 6–14 (total HDL). The ability of these subfractions to induce cholesterol efflux from ^{3}H-labeled AcLDL-loaded peritoneal macrophages was studied (Fig. 6B). Indeed, large HDL induced cholesterol efflux in a more efficient way than very large HDL. It is clear that the HDL subfraction with a very large size is an inferior acceptor in the cholesterol efflux experiments.

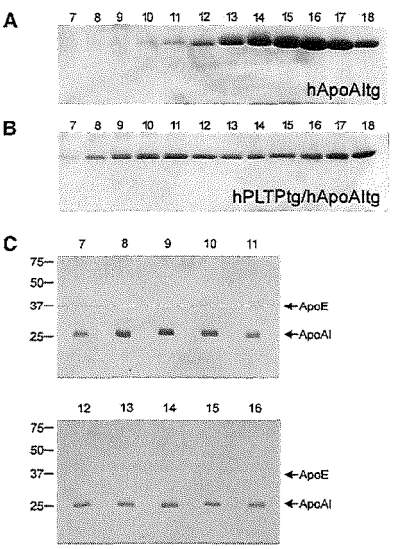

Fig. 3. ApoA-I and apoE protein levels. A, B: FPLC fractions 7–18 of hApoAItg mice (A) and hPLTPtg/hApoAItg mice (B) were subjected to electrophoresis on 10–20% SDS-polyacrylamide gels and subsequently stained with Coomassie blue. C: Plasma lipoproteins from hPLTPtg/hApoAItg mice were isolated by density gradient ultracentrifugation (see Materials and Methods). The isolated fractions with densities of 1.019–1.063 g/ml (upper gel) and 1.063–1.21 g/ml (lower gel) were subfractionated by FPLC, followed by silver staining of 4–15% SDS-polyacrylamide gels. The positions of molecular weight markers are indicated at left, and those of apoA-I and apoE are indicated at right.

Atherosclerosis experiments

To determine the effect of the expression of human PLTP on the development of atherosclerosis, hApoAItg mice and hPLTPtg/hApoAItg mice were crossed into an LDLR-deficient background. Animals were fed a high-fat/high-cholesterol diet for 9 weeks to induce the development of atherosclerosis. hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice had a 3-fold increased PLTP activity compared with hApoAItg/LDLR$^{-/-}$ mice (**Table 3**). No differences in plasma cholesterol or phospholipids between groups

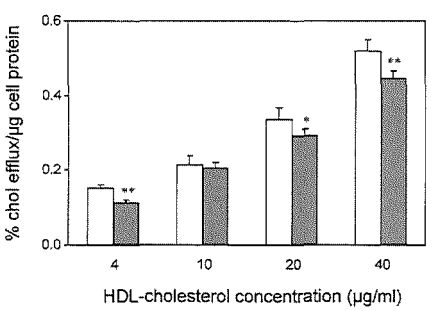

Fig. 4. Percentage cholesterol efflux per microgram of cell protein. The ability of HDL isolated from hApoAItg mice (white bars) and hPLTPtg/hApoAItg mice (gray bars) to induce cholesterol efflux from labeled acetylated low density lipoprotein (AcLDL)-loaded peritoneal macrophages was determined (see Materials and Methods). Values shown are means ± SEM obtained from >10 mice per group. * $P < 0.05$, ** $P < 0.01$ versus hApoAItg.

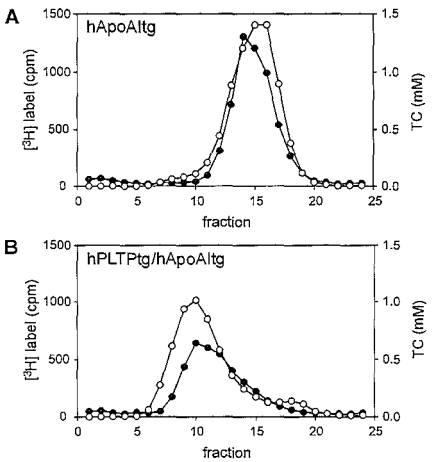

Fig. 5. Total cholesterol (TC) and ^{3}H label (counts per minute) in FPLC fractions of pooled efflux media obtained with HDL from hApoAItg mice (A) and hPLTPtg/hApoAItg mice (B). After cholesterol efflux, efflux media of individual mice were collected. Pools of efflux media (at least 10 mice per group) were subjected to FPLC. Total cholesterol (white circles) and ^{3}H label (black circles) were determined in each fraction. Elution volumes for wild-type mice are as follows: LDL in fractions 5–10, HDL in fractions 11–20.

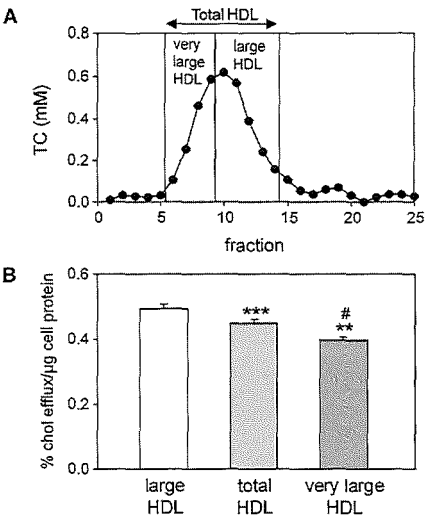

Fig. 6. Percentage cholesterol efflux per microgram of cell protein for very large HDL versus large HDL. A: A plasma pool isolated from a group of 12 hPLTPtg/hApoAItg mice was subjected to FPLC and divided into three pools: very large HDL (fractions 6–9), large HDL (fractions 10–14), and total HDL (fractions 6–14). B: The ability of the three HDL pools to induce cholesterol efflux from labeled AcLDL-loaded peritoneal macrophages was determined (see Materials and Methods). Values shown are means ± SEM of four determinations. ** $P < 0.05$, *** $P < 0.01$ versus large HDL; # $P < 0.05$ versus total HDL. TC, total cholesterol.

were observed. Overexpression of human PLTP resulted in a 60% decrease in human apoA-I levels and a 20% decrease in mouse apoA-I levels. Mouse apoA-I levels were very low compared with human apoA-I levels, accounting for only 1–3% of total apoA-I levels (Table 3). Crossed immunoelectrophoresis showed that expression of human PLTP resulted in a decrease in both α-HDL and preβ-HDL. Human apoA-I plasma concentration with preβ mobility was 0.63 mg/ml (19% of total plasma human apoA-I) in hApoAItg/LDLR$^{-/-}$ mice versus 0.29 mg/ml (26%) in hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice. Human apoA-I plasma concentration with α mobility was 2.69 mg/ml (81%) in hApoAItg/LDLR$^{-/-}$ mice versus 0.82 mg/ml (74%) in hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice.

We again studied the ability of isolated HDL to induce cholesterol efflux from mouse peritoneal macrophages. After 9 weeks of a high-fat/high-cholesterol diet, HDL from hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice and hApoAItg/LDLR$^{-/-}$ mice was isolated individually by density gradient ultracentrifugation and used as an acceptor for ^{3}H-labeled cellular cholesterol. In agreement with the results in hPLTPtg/hApoAItg mice and hApoAItg mice, HDL isolated from hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice proved to induce cholesterol efflux in a less efficient way than did HDL isolated from hApoAItg/LDLR$^{-/-}$ mice (**Fig. 7**). After 9 weeks of the high-fat/high-cholesterol diet, animals were euthanized and atherosclerotic lesion area was determined in sections of the aortic valves. Mice overexpressing human PLTP showed a 2.2-fold increased atherosclerotic lesion area (**Fig. 8**).

DISCUSSION

In the present study, overexpression of the human PLTP gene in a human apoA-I transgenic mouse line resulted in decreased total plasma cholesterol and phospholipid levels, which were caused by decreased HDL levels. In human apoA-I transgenic mice, the relatively high percentage of apoA-I in the preβ range can be attributed to the presence of the human apoA-I transgene (20, 31, 32). Overexpression of human PLTP did result in an increase in the percentage of human apoA-I in the preβ-HDL range, in accordance with our previous data in mice without human apoA-I (8, 9). However, the absolute plasma level of human apoA-I with preβ mobility did not increase in human PLTP-expressing mice, whereas the plasma level of human apoA-I with α mobility was strongly decreased. This could be explained by the fact that overexpression of PLTP increases HDL catabolism (13). In addition, PLTP has been reported to cause proteolytic cleavage of apoA-I (33). We

TABLE 3. Plasma parameters in hApoAItg/LDLR$^{-/-}$ mice versus hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice measured chow-fed (0 weeks) and after 9 weeks of a Western diet (9 weeks)

	Phospholipid Transfer Protein Activity		Total Cholesterol		Phospholipid		Human ApoA-I	Mouse ApoA-I
Mouse	0 Weeks	9 Weeks	0 Weeks	9 Weeks	0 Weeks	9 Weeks	9 Weeks	9 Weeks
	arbitrary units		*mmol/l*		*mmol/l*		*mg/ml*	
hApoAItg/LDLR$^{-/-}$	3.63 ± 0.26	7.27 ± 1.19	4.09 ± 0.35	15.95 ± 0.87	3.60 ± 0.13	9.19 ± 0.35	3.32 ± 0.24	0.06 ± 0.01
hPLTPtg/hApoAItg/LDLR$^{-/-}$	11.86 ± 0.36[a]	19.32 ± 2.00[b]	3.96 ± 0.85	16.56 ± 0.70	2.97 ± 0.40	8.70 ± 0.33	1.11 ± 0.10[a]	0.04 ± 0.00[a]

Values shown are means ± SEM.
[a] $P < 0.001$ versus hApoAItg/LDLR$^{-/-}$ (n = 7–11).
[b] $P < 0.01$ versus hApoAItg/LDLR$^{-/-}$ (n = 7–11).

found that the action of human PLTP together with human apoA-I resulted in the formation of a large CE-rich lipoprotein particle with LDL density.

We determined protein components in this lipoprotein particle and found that the main apolipoprotein was apoA-I. Therefore, we conclude that the large lipoprotein particle found in hPLTPtg/hApoAItg mice is a very large, cholesterol-enriched subspecies of HDL displaying LDL density. In vitro HDL conversion studies demonstrated that human PLTP is able to promote the conversion of a homogenous population of human HDL_3 particles into a new population with an increased average size via particle fusion (34–36). Earlier, a small increase in HDL size was observed in a human PLTP/human apoA-I double transgenic mouse model displaying a slightly increased PLTP activity (37). Ehnholm et al. (14) also showed that adenovirus-mediated overexpression of human PLTP in human apoA-I transgenic mice resulted in an increase in HDL size and a decrease in HDL density. Furthermore, human apoA-I transgenic mice treated with fenofibrate showed an increase in the expression of murine PLTP in the liver through stimulation of the transcription factor peroxisome proliferator-activated receptor α. The resulting increase in PLTP activity accounted for the HDL size enlargement in plasma from treated animals (38, 39).

Enlarged HDL species have been described in a variety of other mouse models, such as scavenger receptor class B type I knockout mice, hepatic lipase knockout mice, human LCAT transgenic mice, and hepatocyte nuclear factor-1α knockout mice (40–43). In these mouse models, HDL levels were increased and lipoprotein particles were enriched with apoE and CEs. The only mouse model that displays, like our hPLTPtg/hApoAItg mouse model, an increase in HDL size concomitant with a decrease in HDL levels is a model in which apoM is selectively silenced by small interfering RNA (44). It is unknown whether PLTP plays a role in the molecular mechanisms by which apoM affects HDL particle size.

Not only HDL concentration but also HDL composition may be important for its antiatherogenic properties. Scavenger receptor class B type I-deficient mice and mice overexpressing human LCAT, displaying enlarged HDL subspecies, showed an increase in atherosclerotic lesion development despite highly increased levels of HDL cholesterol (40, 42). Human studies confirm the hypothesis

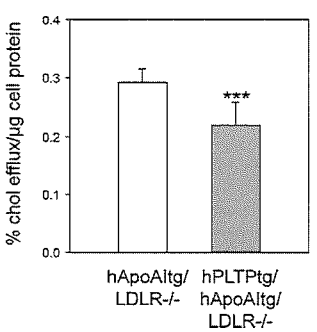

Fig. 7. Percentage cholesterol efflux per microgram of cell protein. The ability of HDL isolated from hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice versus hApoAItg/LDLR$^{-/-}$ mice (fed a Western diet) to induce cholesterol efflux from labeled AcLDL-loaded peritoneal macrophages was determined (see Materials and Methods). Values shown are means ± SEM obtained from >10 mice per group. *** $P < 0.001$ versus hApoAItg/LDLR$^{-/-}$ mice.

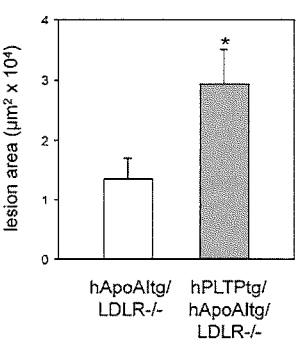

Fig. 8. Atherosclerotic lesion development in hApoAItg/LDLR$^{-/-}$ mice versus hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice. Plaque area was measured in sections from the aortic root (see Materials and Methods). Values shown are means ± SEM obtained from at least 12 mice per group. * $P < 0.05$ versus hApoAItg/LDLR$^{-/-}$ mice.

that HDL size and composition are the main determinants of susceptibility to atherosclerosis development. The inhibitory effect of small dense HDL subfractions isolated from healthy human subjects on the expression of cell adhesion proteins is superior to that of larger and less dense HDL particles (45). This might be of physiopathological relevance in the initial steps of atherogenesis, in which monocytes are arrested on the surface of endothelial cells.

Recently, it was shown that hyperalphalipoproteinemia in cardiac transplant recipients is associated with the formation of partially dysfunctional HDL (46). Other studies showed that average HDL size and CE content increase as a result of low CETP activity (47–50). Also, the large HDL particles that are formed in our double transgenic mice are rich in CEs. In the absence of CETP, CEs cannot be transferred to apoB-containing lipoproteins. Possibly, the expression of CETP in our hPLTPtg/hApoAItg mice would prevent the accumulation of esterified cholesterol in the core of the HDL particle, thereby reducing HDL particle size. Interestingly, expression of CETP corrected dysfunctional HDL in LCAT transgenic mice, thereby reducing the development of atherosclerosis (51). In CETP-deficient subjects, it was reported that these large CE-rich HDL particles have reduced acceptor capacity for cholesterol from lipid-laden macrophages (52, 53). These findings indicate that measuring HDL functionality might be as important as measuring HDL concentration for assessing cardiovascular risk.

In our study, cholesterol efflux capacity of the enlarged HDL isolated from hPLTPtg/hApoAItg mice was decreased compared with that of HDL from hApoAItg mice. The cholesterol efflux experiment in which the efflux of cellular lipids to lipoprotein particles in fractions 6–9 was compared directly with the efflux of lipids to lipoprotein particles in fractions 10–14 confirmed the hypothesis that the very large HDL particles are inefficient at inducing cellular cholesterol efflux. These results show that PLTP may act proatherogenically not only by decreasing plasma HDL levels but also by changing HDL particle composition. Interestingly, Tall and colleagues (54) recently discovered that the enlarged HDL (HDL_2) that is found in patients with CETP deficiency has an increased capacity to promote cellular cholesterol efflux, but only when apoE content of the HDL particle was high. When apoE was removed from the particle, HDL lost this ability. We checked the apoE content of the enlarged HDL particles in hPLTPtg/hApoAItg mice by silver staining of apolipoproteins on SDS-polyacrylamide gels, but no apoE was detected.

Lipoprotein enrichment with apoE is not essential for the formation of large HDL, as there are reports of enlarged HDL in apoE-deficient mice (41). In our mouse model, the absence of an increased apoE content of the large HDL might be caused by the overexpression of human apoA-I. Probably, the large excess of human apoA-I in the HDL particles, including the large HDL in the double transgenic mice, leads to a repression of apoE incorporation. Also, the expression of apoA-II is strongly repressed in our human apoA-I transgenic mouse line, as has been reported previously (19). We analyzed the distribution of apoA-II in FPLC fractions of both genotypes (Coomassie blue staining of SDS-polyacrylamide gels) and found that apoA-II levels were negligible and that there were no differences between genotypes (data not shown). Furthermore, the substantial reduction in the ratio of surface to core lipoprotein components, which may occur in hPLTPtg/hApoAItg mice, might contribute to the dissociation of apolipoproteins from the HDL particles (55).

In hPLTPtg/hApoAItg mice, the size increase renders the apoE-poor HDL particle less efficient at accepting cellular cholesterol. Also, when both mouse lines were crossed into an LDLR-deficient background, HDL isolated from the human PLTP-expressing mice was less efficient at promoting cellular cholesterol efflux. Unfortunately, it is not feasible to perform the same set of analyses for the HDL of animals with an LDLR-deficient background as we did for the HDL of hApoAItg and hPLTPtg/hApoAItg mice. The reason is that LDLR-deficient animals have predominant levels of LDL, which precludes the isolation of large HDL, which is in the same density range. However, the reduced capacity of the HDL to induce cellular cholesterol efflux may explain, at least in part, the observation that human apoA-I transgenic mice overexpressing human PLTP have an increased susceptibility to the development of atherosclerotic lesions.

In conclusion, our data show that in the presence of human apoA-I, human PLTP not only decreases plasma HDL levels but also unfavorably affects the functionality of the remaining HDL particles. When crossed into an atherosclerosis-prone background, mice expressing human PLTP show a strong increase in susceptibility to the development of atherosclerosis. Our findings strengthen the opinion that high plasma PLTP activity levels might be harmful for cardiovascular health.

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REFERENCES

1. Miller, G. J., and N. E. Miller. 1975. Plasma-high-density-lipoprotein concentration and development of ischaemic heart disease. *Lancet.* **1:** 16–19.
2. Barter, P. J., S. Nicholls, K. A. Rye, G. M. Anantharamaiah, M. Navab, and A. M. Fogelman. 2004. Antiinflammatory properties of HDL. *Circ. Res.* **95:** 764–772.
3. Assmann, G., and A. M. Gotto, Jr. 2004. HDL cholesterol and protective factors in atherosclerosis. *Circulation.* **109:** III8–III14.
4. Ohashi, R., H. Mu, X. Wang, Q. Yao, and C. Chen. 2005. Reverse cholesterol transport and cholesterol efflux in atherosclerosis. *QJM.* **98:** 845–856.
5. Tall, A. R., S. Krumholz, T. Olivecrona, and R. J. Deckelbaum. 1985. Plasma phospholipid transfer protein enhances transfer and exchange of phospholipids between very low density lipoproteins and high density lipoproteins during lipolysis. *J. Lipid Res.* **26:** 842–851.
6. Jauhiainen, M., J. Metso, R. Pahlman, S. Blomqvist, A. van Tol, and C. Ehnholm. 1993. Human plasma phospholipid transfer protein causes high density lipoprotein conversion. *J. Biol. Chem.* **268:** 4032–4036.

7. Tu, A. Y., H. I. Nishida, and T. Nishida. 1993. High density lipoprotein conversion mediated by human plasma phospholipid transfer protein. *J. Biol. Chem.* **268:** 23098–23105.
8. Lie, J., R. de Crom, M. Jauhiainen, T. van Gent, R. van Haperen, L. Scheek, H. Jansen, C. Ehnholm, and A. van Tol. 2001. Evaluation of phospholipid transfer protein and cholesteryl ester transfer protein as contributors to the generation of pre beta-high-density lipoproteins. *Biochem. J.* **360:** 379–385.
9. van Haperen, R., A. van Tol, P. Vermeulen, M. Jauhiainen, T. van Gent, P. van den Berg, S. Ehnholm, F. Grosveld, A. van der Kamp, and R. de Crom. 2000. Human plasma phospholipid transfer protein increases the antiatherogenic potential of high density lipoproteins in transgenic mice. *Arterioscler. Thromb. Vasc. Biol.* **20:** 1082–1088.
10. Oram, J. F., G. Wolfbauer, A. M. Vaughan, C. Tang, and J. J. Albers. 2003. Phospholipid transfer protein interacts with and stabilizes ATP-binding cassette transporter A1 and enhances cholesterol efflux from cells. *J. Biol. Chem.* **278:** 52379–52385.
11. Wolfbauer, G., J. J. Albers, and J. F. Oram. 1999. Phospholipid transfer protein enhances removal of cellular cholesterol and phospholipids by high-density lipoprotein apolipoproteins. *Biochim. Biophys. Acta.* **1439:** 65–76.
12. Lee-Rueckert, M., R. Vikstedt, J. Metso, C. Ehnholm, P. T. Kovanen, and M. Jauhiainen. 2006. Absence of endogenous phospholipid transfer protein impairs ABCA1-dependent efflux of cholesterol from macrophage foam cells. *J. Lipid Res.* **47:** 1725–1732.
13. Foger, B., S. Santamarina-Fojo, R. D. Shamburek, C. L. Parrot, G. D. Talley, and H. B. Brewer, Jr. 1997. Plasma phospholipid transfer protein. Adenovirus-mediated overexpression in mice leads to decreased plasma high density lipoprotein (HDL) and enhanced hepatic uptake of phospholipids and cholesteryl esters from HDL. *J. Biol. Chem.* **272:** 27393–27400.
14. Ehnholm, S., K. W. van Dijk, B. van't Hof, A. van der Zee, V. M. Olkkonen, M. Jauhiainen, M. Hofker, L. Havekes, and C. Ehnholm. 1998. Adenovirus mediated overexpression of human phospholipid transfer protein alters plasma HDL levels in mice. *J. Lipid Res.* **39:** 1248–1253.
15. Lie, J., R. de Crom, T. van Gent, R. van Haperen, L. Scheek, F. Sadeghi-Niaraki, and A. van Tol. 2004. Elevation of plasma phospholipid transfer protein increases the risk of atherosclerosis despite lower apolipoprotein B-containing lipoproteins. *J. Lipid Res.* **45:** 805–811.
16. van Haperen, R., A. van Tol, T. van Gent, L. Scheek, P. Visser, A. van der Kamp, F. Grosveld, and R. de Crom. 2002. Increased risk of atherosclerosis by elevated plasma levels of phospholipid transfer protein. *J. Biol. Chem.* **277:** 48938–48943.
17. Yang, X. P., D. Yan, C. Qiao, R. J. Liu, J. G. Chen, J. Li, M. Schneider, L. Lagrost, X. Xiao, and X. C. Jiang. 2003. Increased atherosclerotic lesions in apoE mice with plasma phospholipid transfer protein overexpression. *Arterioscler. Thromb. Vasc. Biol.* **23:** 1601–1607.
18. Yan, D., M. Navab, C. Bruce, A. M. Fogelman, and X. C. Jiang. 2004. PLTP deficiency improves the anti-inflammatory properties of HDL and reduces the ability of LDL to induce monocyte chemotactic activity. *J. Lipid Res.* **45:** 1852–1858.
19. Rubin, E. M., B. Y. Ishida, S. M. Clift, and R. M. Krauss. 1991. Expression of human apolipoprotein A-I in transgenic mice results in reduced plasma levels of murine apolipoprotein A-I and the appearance of two new high density lipoprotein size subclasses. *Proc. Natl. Acad. Sci. USA.* **88:** 434–438.
20. Walsh, A., Y. Ito, and J. L. Breslow. 1989. High levels of human apolipoprotein A-I in transgenic mice result in increased plasma levels of small high density lipoprotein (HDL) particles comparable to human HDL3. *J. Biol. Chem.* **264:** 6488–6494.
21. Gong, E. L., C. S. Tan, M. I. Shoukry, E. M. Rubin, and A. V. Nichols. 1994. Structural and functional properties of human and mouse apolipoprotein A-I. *Biochim. Biophys. Acta.* **1213:** 335–342.
22. Pussinen, P. J., M. Jauhiainen, J. Metso, L. E. Pyle, Y. L. Marcel, N. H. Fidge, and C. Ehnholm. 1998. Binding of phospholipid transfer protein (PLTP) to apolipoproteins A-I and A-II: location of a PLTP binding domain in the amino terminal region of apoA-I. *J. Lipid Res.* **39:** 152–161.
23. Hayek, T., T. Chajek-Shaul, A. Walsh, L. B. Agellon, P. Moulin, A. R. Tall, and J. L. Breslow. 1992. An interaction between the human cholesteryl ester transfer protein (CETP) and apolipoprotein A-I genes in transgenic mice results in a profound CETP-mediated depression of high density lipoprotein cholesterol levels. *J. Clin. Invest.* **90:** 505–510.
24. Francone, O. L., E. L. Gong, D. S. Ng, C. J. Fielding, and E. M. Rubin. 1995. Expression of human lecithin-cholesterol acyltransferase in transgenic mice. Effect of human apolipoprotein AI and human apolipoprotein AII on plasma lipoprotein cholesterol metabolism. *J. Clin. Invest.* **96:** 1440–1448.
25. Speijer, H., J. E. Groener, E. van Ramshorst, and A. van Tol. 1991. Different locations of cholesteryl ester transfer protein and phospholipid transfer protein activities in plasma. *Atherosclerosis.* **90:** 159–168.
26. Redgrave, T. G., D. C. Roberts, and C. E. West. 1975. Separation of plasma lipoproteins by density-gradient ultracentrifugation. *Anal. Biochem.* **65:** 42–49.
27. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
28. McCarron, R. M., D. K. Goroff, J. E. Luhr, M. A. Murphy, and H. B. Herscowitz. 1984. Methods for the collection of peritoneal and alveolar macrophages. *Methods Enzymol.* **108:** 274–284.
29. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
30. Paigen, B., A. Morrow, P. A. Holmes, D. Mitchell, and R. A. Williams. 1987. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis.* **68:** 231–240.
31. Castro, G., L. P. Nihoul, C. Dengremont, C. de Geitere, B. Delfly, A. Tailleux, C. Fievet, N. Duverger, P. Denefle, J. C. Fruchart, et al. 1997. Cholesterol efflux, lecithin-cholesterol acyltransferase activity, and pre-beta particle formation by serum from human apolipoprotein A-I and apolipoprotein A-I/apolipoprotein A-II transgenic mice consistent with the latter being less effective for reverse cholesterol transport. *Biochemistry.* **36:** 2243–2249.
32. Lee, J. Y., L. Lanningham-Foster, E. Y. Boudyguina, T. L. Smith, E. R. Young, P. L. Colvin, M. J. Thomas, and J. S. Parks. 2004. Prebeta high density lipoprotein has two metabolic fates in human apolipoprotein A-I transgenic mice. *J. Lipid Res.* **45:** 716–728.
33. Jauhiainen, M., J. Huuskonen, M. Baumann, J. Metso, T. Oka, T. Egashira, H. Hattori, V. M. Olkkonen, and C. Ehnholm. 1999. Phospholipid transfer protein (PLTP) causes proteolytic cleavage of apolipoprotein A-I. *J. Lipid Res.* **40:** 654–664.
34. Lusa, S., M. Jauhiainen, J. Metso, P. Somerharju, and C. Ehnholm. 1996. The mechanism of human plasma phospholipid transfer protein-induced enlargement of high-density lipoprotein particles: evidence for particle fusion. *Biochem. J.* **313:** 275–282.
35. Korhonen, A., M. Jauhiainen, C. Ehnholm, P. T. Kovanen, and M. Ala-Korpela. 1998. Remodeling of HDL by phospholipid transfer protein: demonstration of particle fusion by 1H NMR spectroscopy. *Biochem. Biophys. Res. Commun.* **249:** 910–916.
36. Lagrost, L., A. Athias, B. Herbeth, V. Guyard-Dangremont, Y. Artur, F. Paille, P. Gambert, and C. Lallemant. 1996. Opposite effects of cholesteryl ester transfer protein and phospholipid transfer protein on the size distribution of plasma high density lipoproteins. Physiological relevance in alcoholic patients. *J. Biol. Chem.* **271:** 19058–19065.
37. Jiang, X., O. L. Francone, C. Bruce, R. Milne, J. Mar, A. Walsh, J. L. Breslow, and A. R. Tall. 1996. Increased prebeta-high density lipoprotein, apolipoprotein AI, and phospholipid in mice expressing the human phospholipid transfer protein and human apolipoprotein AI transgenes. *J. Clin. Invest.* **98:** 2373–2380.
38. Berthou, L., N. Duverger, F. Emmanuel, S. Langouet, J. Auwerx, A. Guillouzo, J. C. Fruchart, E. Rubin, P. Denefle, B. Staels, et al. 1996. Opposite regulation of human versus mouse apolipoprotein A-I by fibrates in human apolipoprotein A-I transgenic mice. *J. Clin. Invest.* **97:** 2408–2416.
39. Bouly, M., D. Masson, B. Gross, X. C. Jiang, C. Fievet, G. Castro, A. R. Tall, J. C. Fruchart, B. Staels, L. Lagrost, et al. 2001. Induction of the phospholipid transfer protein gene accounts for the high density lipoprotein enlargement in mice treated with fenofibrate. *J. Biol. Chem.* **276:** 25841–25847.
40. Berard, A. M., B. Foger, A. Remaley, R. Shamburek, B. L. Vaisman, G. Talley, B. Paigen, R. F. Hoyt, Jr., S. Marcovina, H. B. Brewer, Jr., et al. 1997. High plasma HDL concentrations associated with enhanced atherosclerosis in transgenic mice overexpressing lecithin-cholesteryl acyltransferase. *Nat. Med.* **3:** 744–749.
41. Mezdour, H., R. Jones, C. Dengremont, G. Castro, and N. Maeda. 1997. Hepatic lipase deficiency increases plasma cholesterol but reduces susceptibility to atherosclerosis in apolipoprotein E-deficient mice. *J. Biol. Chem.* **272:** 13570–13575.
42. Van Eck, M., J. Twisk, M. Hoekstra, B. T. Van Rij, C. A. Van der Lans, I. S. Bos, J. K. Kruijt, F. Kuipers, and T. J. Van Berkel. 2003.

Differential effects of scavenger receptor BI deficiency on lipid metabolism in cells of the arterial wall and in the liver. *J. Biol. Chem.* **278:** 23699–23705.
43. Shih, D. Q., M. Bussen, E. Sehayek, M. Ananthanarayanan, B. L. Shneider, F. J. Suchy, S. Shefer, J. S. Bollileni, F. J. Gonzalez, J. L. Breslow, et al. 2001. Hepatocyte nuclear factor-1alpha is an essential regulator of bile acid and plasma cholesterol metabolism. *Nat. Genet.* **27:** 375–382.
44. Wolfrum, C., M. N. Poy, and M. Stoffel. 2005. Apolipoprotein M is required for prebeta-HDL formation and cholesterol efflux to HDL and protects against atherosclerosis. *Nat. Med.* **11:** 418–422.
45. Ashby, D. T., K. A. Rye, M. A. Clay, M. A. Vadas, J. R. Gamble, and P. J. Barter. 1998. Factors influencing the ability of HDL to inhibit expression of vascular cell adhesion molecule-1 in endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **18:** 1450–1455.
46. Sviridov, D., J. Chin-Dusting, P. Nestel, B. Kingwell, A. Hoang, B. Olchawa, J. Starr, and A. Dart. 2006. Elevated HDL cholesterol is functionally ineffective in cardiac transplant recipients: evidence for impaired reverse cholesterol transport. *Transplantation.* **81:** 361–366.
47. Asztalos, B. F., K. V. Horvath, K. Kajinami, C. Nartsupha, C. E. Cox, M. Batista, E. J. Schaefer, A. Inazu, and H. Mabuchi. 2004. Apolipoprotein composition of HDL in cholesteryl ester transfer protein deficiency. *J. Lipid Res.* **45:** 448–455.
48. de Grooth, G. J., J. A. Kuivenhoven, A. F. Stalenhoef, J. de Graaf, A. H. Zwinderman, J. L. Posma, A. van Tol, and J. J. Kastelein. 2002. Efficacy and safety of a novel cholesteryl ester transfer protein inhibitor, JTT-705, in humans: a randomized phase II dose-response study. *Circulation.* **105:** 2159–2165.
49. Brousseau, M. E., M. R. Diffenderfer, J. S. Millar, C. Nartsupha, B. F. Asztalos, F. K. Welty, M. L. Wolfe, M. Rudling, I. Bjorkhem, B. Angelin, et al. 2005. Effects of cholesteryl ester transfer protein inhibition on high-density lipoprotein subspecies, apolipoprotein A-I metabolism, and fecal sterol excretion. *Arterioscler. Thromb. Vasc. Biol.* **25:** 1057–1064.
50. Clark, R. W., T. A. Sutfin, R. B. Ruggeri, A. T. Willauer, E. D. Sugarman, G. Magnus-Aryitey, P. G. Cosgrove, T. M. Sand, R. T. Wester, J. A. Williams, et al. 2004. Raising high-density lipoprotein in humans through inhibition of cholesteryl ester transfer protein: an initial multidose study of torcetrapib. *Arterioscler. Thromb. Vasc. Biol.* **24:** 490–497.
51. Foger, B., M. Chase, M. J. Amar, B. L. Vaisman, R. D. Shamburek, B. Paigen, J. Fruchart-Najib, J. A. Paiz, C. A. Koch, R. F. Hoyt, et al. 1999. Cholesteryl ester transfer protein corrects dysfunctional high density lipoproteins and reduces aortic atherosclerosis in lecithin cholesterol acyltransferase transgenic mice. *J. Biol. Chem.* **274:** 36912–36920.
52. Ishigami, M., S. Yamashita, N. Sakai, T. Arai, K. Hirano, H. Hiraoka, K. Kameda-Takemura, and Y. Matsuzawa. 1994. Large and cholesteryl ester-rich high-density lipoproteins in cholesteryl ester transfer protein (CETP) deficiency can not protect macrophages from cholesterol accumulation induced by acetylated low-density lipoproteins. *J. Biochem. (Tokyo).* **116:** 257–262.
53. Ohta, T., R. Nakamura, K. Takata, Y. Saito, S. Yamashita, S. Horiuchi, and I. Matsuda. 1995. Structural and functional differences of subspecies of apoA-I-containing lipoprotein in patients with plasma cholesteryl ester transfer protein deficiency. *J. Lipid Res.* **36:** 696–704.
54. Matsuura, F., N. Wang, W. Chen, X. C. Jiang, and A. R. Tall. 2006. HDL from CETP-deficient subjects shows enhanced ability to promote cholesterol efflux from macrophages in an apoE- and ABCG1-dependent pathway. *J. Clin. Invest.* **116:** 1435–1442.
55. Ryan, R. O., S. Yokoyama, H. Liu, H. Czarnecka, K. Oikawa, and C. M. Kay. 1992. Human apolipoprotein A-I liberated from high-density lipoprotein without denaturation. *Biochemistry.* **31:** 4509–4514.

Chapter 6

Sex differences in atherosclerosis in mice with elevated phospholipid transfer protein activity are related to decreased plasma high density lipoproteins and not to increased production of triglycerides

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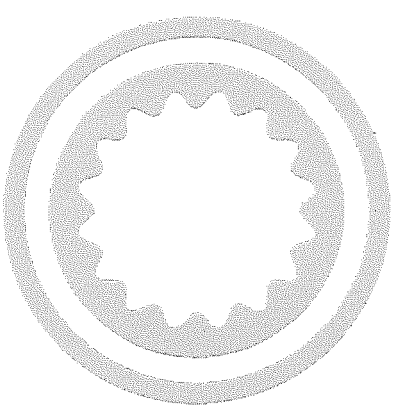

Sex differences in atherosclerosis in mice with elevated phospholipid transfer protein activity are related to decreased plasma high density lipoproteins and not to increased production of triglycerides

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Abstract

Plasma phospholipid transfer protein (PLTP) has atherogenic properties in genetically modified mice. PLTP stimulates hepatic triglyceride secretion and reduces plasma levels of high density lipoproteins (HDL). The present study was performed to relate the increased atherosclerosis in PLTP transgenic mice to one of these atherogenic effects. A humanized mouse model was used which had decreased LDL receptor expression and was transgenic for human cholesterylester transfer protein (CETP) in order to obtain a better resemblance to the plasma lipoprotein profile present in humans. It is well known that female mice are more susceptible to atherosclerosis than male mice. Therefore, we compared male and female mice expressing human PLTP. The animals were fed an atherogenic diet and the effects on plasma lipids and lipoproteins, triglyceride secretion and the development of atherosclerosis were measured. The development of atherosclerosis was sex-dependent. This effect was stronger in PLTP transgenic mice, while PLTP activity levels were virtually identical. Also, the rates of hepatic secretion of triglycerides were similar. In contrast, plasma levels of HDL were about 2-fold lower in female mice than in male mice after feeding an atherogenic diet. We conclude that increased atherosclerosis caused by overexpression of PLTP is related to a decrease in HDL, rather than to elevated hepatic secretion of triglycerides.

Keywords: Atherosclerosis; Lipid metabolism; Lipoprotein; Transgenic animal model

Atherosclerosis is the leading cause of death and morbidity in industrialized countries [1–3]. It is generally accepted that an elevated level of plasma cholesterol is an important risk factor for atherosclerosis. While the level of cholesterol in low density lipoproteins (LDL) correlates positively with the incidence of atherosclerotic disease, cholesterol in high density lipoproteins (HDL) appears to protect against the development of atherosclerosis [1,2,4]. HDL is thought to have several anti-atherogenic properties. First, HDL is involved in the reverse cholesterol transport pathway, by which excess cholesterol is transported back to the liver for excretion [5–7]. In addition, HDL has both anti-inflammatory and anti-oxidant properties [4,8–10]. One of the proteins involved in HDL metabolism is plasma phospholipid transfer protein (PLTP) [11]. We previously demonstrated that transgenic mice overexpressing human PLTP to various activity levels are more prone to the development of diet-induced atherosclerosis in a PLTP-dose dependent way [12]. Concomitantly, the plasma level of HDL was decreased. We tentatively concluded that the increase in atherosclerosis is probably caused by this decrease in plasma HDL. However,

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in addition to an effect on HDL, PLTP has an effect on the production of very low density lipoproteins (VLDL), which was first described in PLTP deficient mice [13]. The decreased atherosclerosis found in these mice was explained by decreased synthesis of apoB containing lipoproteins by the liver. We examined whether elevated PLTP activity levels would affect hepatic VLDL secretion. Indeed, PLTP over-expressing mice were found to have an increase in the synthesis of apoB containing lipoproteins [12,14], which could be an alternative explanation for the increased susceptibility to atherosclerosis development found in these mice. Thus, elevated PLTP has two atherogenic effects on lipoprotein metabolism, i.e., increasing hepatic VLDL secretion and decreasing plasma levels of HDL. In order to find out which of these mechanisms is the most important one for the development of atherosclerosis, we first determined whether male and female mice with similar levels of overexpression of PLTP differ in their susceptibility to atherosclerosis. Sex differences have been found in mouse models, including the commonly used low density lipoprotein receptor (LDLR) deficient mice and apolipoprotein (apo) E deficient mice [15–17]. In female mice, the surface area of atherosclerotic lesions is larger than in male mice. The reason for this difference is unclear. The present data show that the susceptibility to diet-induced atherosclerosis is also sex-dependent in PLTP overexpressing mice, enabling us to relate these differences with the effects on apoB containing lipoproteins versus the effects on HDL. In the present studies, we used humanized transgenic mouse models which have decreased levels of LDL receptors and express human cholesteryl ester transfer protein (CETP) [14,18]. CETP is normally not expressed in mice, but is involved in the same pathways of lipoprotein metabolism as PLTP and may be crucially involved in the process of atherosclerosis in man [19,20]. Moreover, the plasma lipoprotein profile of these mice have a closer resemblance to the profile found in humans than that of normal mice.

1. Methods

1.1. Animals

The human PLTP transgenic mice (huPLTPtg) were described before (line P1; [12,21]). LDL receptor knockout mice were purchased from Jackson Laboratory. Human CETP transgenic mice (huCETPtg) were kindly provided by Dr. A.R. Tall (Columbia University, New York). All mice were in C57BL/6J background for at least 8 generations. LDLR$^{+/-}$/huCETPtg and LDLR$^{+/-}$/huCETPtg/huPLTPtg mice were created by crossbreeding. After weening, animals were kept on a chow diet (Hope Farms, The Netherlands). For the induction of atherosclerosis, mice were fed a high fat high cholesterol (HFHC) diet for 14 weeks, which contained 40% w/w sucrose 15% w/w fat, 1% w/w cholesterol and 0.5% w/w sodium cholate (Hope Farms, The Netherlands). Mice were 12 weeks old at the beginning of the diet studies. Animals were housed under standard conditions and had free access to water and food. After fasting overnight, blood samples were collected from the orbital plexus by using Vitrex™ sodium-heparinized micropipettes (80 IU) (Modulohm A/S, Copenhagen, Denmark) and immediately stored on ice. Blood was centrifuged at 2700 rpm for 15 min at 4 °C. Plasma was either used directly or stored in small aliquots at −80 °C before analysis. All experiments were performed according to national and institutional guidelines. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

1.2. Separation of plasma lipoproteins by density gradient centrifugation

Lipoprotein fractions (HDL and non-HDL) used in Table 1 were collected following ultracentrifugation at density 1.063 g/mL in a Beckman 42.2 Ti rotor (42,000 rpm, 3 h, 12 °C). The non-HDL fraction includes VLDL, IDL and LDL.

1.3. Quantification of cholesterol

Total plasma cholesterol (TC) was determined enzymatically with the Free Cholesterol C kit no. 274-47109 (WAKO, Neuss, Germany) after hydrolysis of cholesteryl esters with cholesterol esterase from *Candida cylindracea* (Boehringer, Mannheim, Germany).

1.4. Plasma activity assays

CETP and PLTP activity assays were performed as described before [18]. The activities are expressed as arbitrary units (AU); 1 AU is the activity found in human reference pool plasma. The activities are: CETP 216 nmol/mL/h; PLTP 14 μmol/mL/h.

1.5. In vivo hepatic VLDL secretion

VLDL secretion experiments were performed using the Triton WR1339 method, as described [14].

1.6. Histological assessment of atherosclerosis

After 14 weeks of HFHC diet feeding, mice were sacrificed after blood collection as described in the animal section. The hearts were dissected, stored in phosphate buffered 4% formaldehyde until processed. Atherosclerotic areas in the aortic valves were quantified by computer assisted measurements as described [12,22]. To evaluate whether PLTP has an effect on accumulation of free cholesterol in atherosclerotic lesions, the presence of cholesterol clefts in the sections was evaluated as described [23].

1.7. Statistical analysis

Data are expressed as means±SEM. Differences between two groups of mice were analyzed by two sample Wilcoxon rank-sum tests by using Intercooled Stata 6.0 software (Stata corporation, College Station, TX, USA).

Table 1
Plasma levels of lipoprotein cholesterol on chow and HFHC diet

Genotype	LDLR$^{+/-}$/huCETPtg		LDLR$^{+/-}$/huCETPtg/huPLTPtg	
Sex	Male	Female	Male	Female
Non-HDL-C, chow	0.8±0.1	1.1±0.1[a]	0.6±0.1[b]	0.8±0.1[a,b]
HDL-C, chow	1.7±0.1	1.8±0.1	1.1±0.1[b]	1.0±0.1[b]
Non-HDL-C, HFHC	9.9±1.3	8.2±0.9	7.3±0.8[c]	6.9±0.7
HDL-C, HFHC	1.4±0.1	0.6±0.1[a]	0.6±0.1[b]	0.3±0.03[a,b]

Cholesterol (C) concentrations are in mmol/L.
n=14–20 per group.
[a] P<0.001, male versus female of same genotype.
[b] P<0.001, LDLR$^{+/-}$/huCETPtg versus LDLR$^{+/-}$/huCETPtg/huPLTPtg of same sex.
[c] P=0.002, LDLR$^{+/-}$/huCETPtg versus LDLR$^{+/-}$/huCETPtg/huPLTPtg of same sex.

2. Results

2.1. Levels of plasma cholesterol

Throughout the present study, comparisons are made between LDLR$^{+/-}$/huCETPtg and LDLR$^{+/-}$/huCETPtg/huPLTPtg mice of both sexes. As shown in Fig. 1A, there is no difference in the plasma level of cholesterol (total cholesterol) between male and female LDLR$^{+/-}$/huCETPtg mice. In contrast, plasma cholesterol is somewhat higher in females than in males in LDLR$^{+/-}$/huCETPtg/huPLTPtg mice . In either sex, plasma cholesterol is much lower in the LDLR$^{+/-}$/huCETPtg/huPLTPtg mice compared with LDLR$^{+/-}$/huCETPtg mice. HDL was separated from VLDL+LDL via ultracentrifugation in order to study the distribution of cholesterol between the lipoproteins (Table 1). Plasma levels of HDL-cholesterol are decreased in both chow-fed male and female LDLR$^{+/-}$/huCETPtg/huPLTPtg mice when compared to LDLR$^{+/-}$/huCETPtg mice. The difference is −35% for the male mice and −44% for the female mice. Non-HDL-cholesterol is lower as well, albeit by a smaller percentage. The concentrations of HDL-cholesterol are not different between males and females of either genotype. In these mice, fed a regular chow diet, a minor fraction of plasma cholesterol is in non-HDL. As expected, the plasma levels of non-HDL cholesterol are greatly increased after feeding the mice a HFHC diet (Table 1). However, there are no statistically significant differences in non-HDL cholesterol between males and females of either genotype. The non-HDL cholesterol levels are somewhat lower in plasma from LDLR$^{+/-}$/huCETPtg/huPLTPtg mice when compared to LDLR$^{+/-}$/huCETPtg mice, but this difference was only statistically significant in males. In contrast, striking differences were found in HDL cholesterol levels. These were about 2-fold higher in male mice than in female mice in either genotype. In both sexes, LDLR$^{+/-}$/huCETPtg/huPLTPtg mice have lower levels of plasma HDL cholesterol than LDLR$^{+/-}$/huCETPtg mice. The lowest concentration of plasma HDL cholesterol was found in female LDLR$^{+/-}$/huCETPtg/huPLTPtg mice. The lower HDL levels in females cannot be attributed to higher CETP activity, which was actually 26% lower (LDLR$^{+/-}$/huCETPtg) and 40% lower (LDLR$^{+/-}$/huCETPtg/huPLTPtg) in females than in males (Fig. 3B).

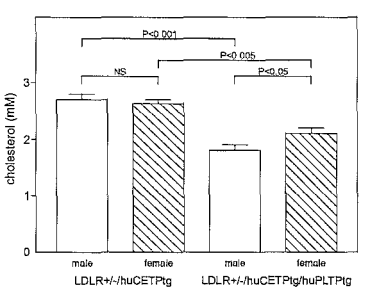

Fig. 1. Total plasma cholesterol concentration in transgenic mice on chow diet. Measurements represent means±SEM of 14–20 mice.

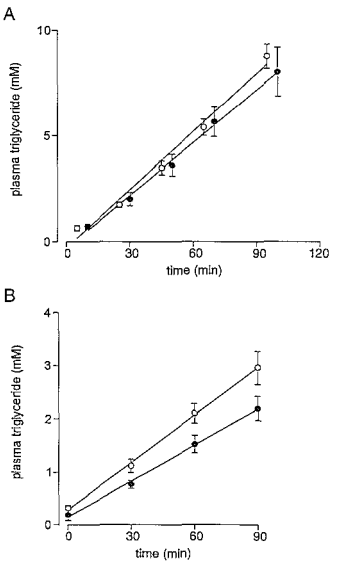

Fig. 2. Hepatic secretion of triglycerides in transgenic mice on chow diet (A) or HFHC diet (B). Plasma levels of triglycerides were measured at the indicated times in male (open circles) and female (closed circles) LDLR$^{+/-}$/huCETPtg/huPLTPtg mice after administration of Triton WR 1339. Measurements represent means±SEM of 7 (A) or 10 (B) mice per group.

2.2. VLDL triglyceride secretion

PLTP is known to stimulate hepatic secretion of VLDL [13,14]. Therefore, a comparison was made between male and female LDLR$^{+/-}$/huCETPtg/huPLTPtg mice in a set of experiments investigating the hepatic secretion of VLDL triglycerides by the Triton WR1339 method (Fig. 2A). No significant differences between males and females are observed. After correction for body weight, the rates of VLDL secretion between these two groups are identical (males: 195±40 μmol/kg/h; females: 195±48 μmol/kg/h). In order to check whether the atherogenic diet could possibly induce a sex-specific difference, VLDL secretion was also measured in animals fed the HFHC diet. As shown in Fig. 2B, the rate of VLDL triglyceride secretion is considerably lower in these animals than in animals fed a chow diet. However, there is no statistically significant difference in the VLDL secretion rates after correction for body weight

between males and females (males 69±9 μmol/kg/h; females 54±6 μmol/kg/h; $P=0.18$).

2.3. Plasma activities of CETP and PLTP

Fig. 3 shows plasma CETP and PLTP activities in male and female mice fed a HFHC diet for 14 weeks. While the plasma activities of CETP and PLTP in mice expressing either only PLTP or the combination of the two lipid transfer proteins have been described in previous studies from our laboratory [14,18], we did not investigate possible sex-related differences. As expected, PLTP activity is much higher in LDLR$^{+/-}$/huCETPtg/huPLTPtg mice than in LDLR$^{+/-}$/huCETPtg mice. PLTP activity is not different between males and females from either genotype (Fig. 3A). CETP activity is identical in male LDLR$^{+/-}$/huCETPtg mice and male LDLR$^{+/-}$/huCETPtg/huPLTPtg mice. In female mice, CETP activity is slightly increased in LDLR$^{+/-}$/huCETPtg/huPLTPtg mice versus LDLR$^{+/-}$/huCETPtg mice. CETP activity is lower in females than in males in both genotypes (Fig. 3B). The HFHC diet causes a small increase in PLTP activity, but a strong induction of CETP activity in mice of both genotypes and of both sexes (PLTP activity: 124±6%, $P<0.05$; CETP activity: 344±13%, $P<0.001$; results not shown).

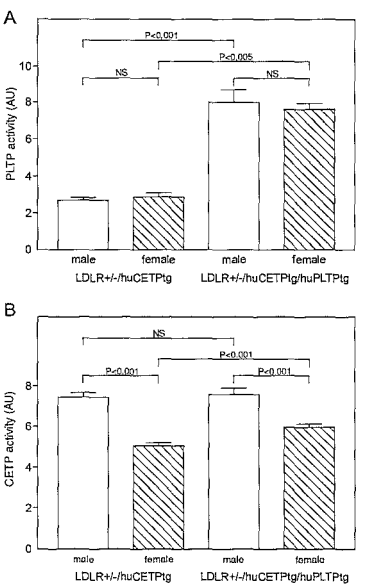

Fig. 3. Activities of CETP and PLTP in plasma from transgenic mice fed a HFHC diet. (A) PLTP activities. 1 AU is equal to 14 μmol/mL/h. (B) CETP activities. 1 AU is equal to 216 nmol/mL/h. Data represent means±SEM of 17–19 mice.

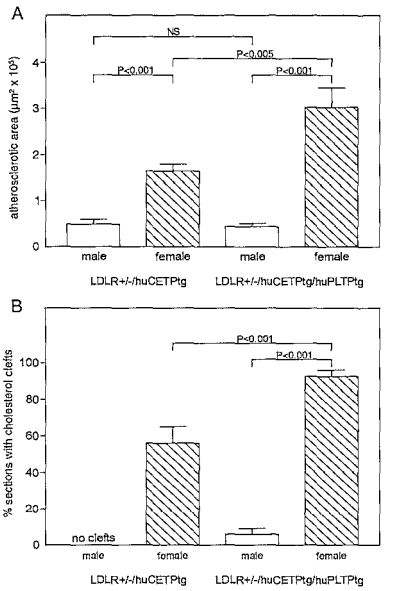

Fig. 4. Atherosclerosis in transgenic mice fed a HFHC diet. (A) Atherosclerotic lesion areas were measured in 5 sections per animal from the aortic root. (B) Percentage of sections containing free cholesterol clefts in the aortic lesion. Data represent means±SEM of 11–18 mice.

2.4. Atherosclerosis

Diet-induced atherosclerosis was evaluated by measuring the atherosclerotic area in the aortic root (Fig. 4A). Male mice show small lesion areas that are similar in size between the two genotypes. In contrast, female mice develop substantial lesion areas in both genotypes. When comparing sexes, female mice show 3.4-fold (LDLR$^{+/-}$/huCETPtg mice) and 7.0-fold (LDLR$^{+/-}$/huCETPtg/huPLTPtg mice) larger lesion areas than male mice (both $P< 0.001$) (Fig. 4A). PLTP overexpression in female mice results in a 1.8-fold further increase ($P<0.005$) in aortic lesion area, while no effect was detected in male mice. In addition, female mice overexpressing human PLTP show more severe plaques with larger numbers of cholesterol clefts (Fig. 4B). In male mice, the severity of the plaque appears higher too, as no cholesterol clefts are observed in LDLR$^{+/-}$/huCETPtg mice, while a modest percentage of the plaques in the LDLR$^{+/-}$/huCETPtg/huPLTPtg mice do contain cholesterol clefts. Representative images are shown in Fig. 5. Finally, we investigated whether there was a relation between the plasma concentration of HDL and the size of the atherosclerotic lesions in the LDLR$^{+/-}$/huCETPtg/huPLTPtg mice. As shown in Fig. 6, a strong correlation was observed for the

female mice ($P=0.006$). In the male mice, in which the differences in lesion size are much smaller, the same relation seems to be present, but just failed to reach statistical significance ($P=0.076$).

3. Discussion

We previously described potentially anti-atherogenic effects of elevated expression of PLTP [21]. However, when we investigated PLTP transgenic mice with different levels of PLTP expression, and consequently of plasma PLTP activity, a clear PLTP-dose dependent increase in atherosclerosis susceptibility was observed [12]. This was found both in mouse models in which PLTP expression was driven by its autologous promoter, as well as in mice with hepatocyte-specific PLTP expression [12]. All these studies were performed in male mice only which were backcrossed to C57BL/6 background for at least 5 generations.

In the present study male and female mice were used in which PLTP expression was driven by its autologous promoter and which were backcrossed to C57BL/6 background for at least 8 generations. In these animals, we investigated whether a sex difference in susceptibility to diet-induced atherosclerosis exists in PLTP transgenic mice, and whether such a difference would be indicative for the mechanism involved in the increased development of atherosclerosis in PLTP overexpressing mice. In addition, we performed our study in more human-like mice with decreased LDL receptors and expressing CETP, which we described previously [24].

Higher susceptibility to diet-induced atherosclerosis in female mice has been noticed already by pioneer researchers in the 1980's and has been confirmed since then by many others [15–

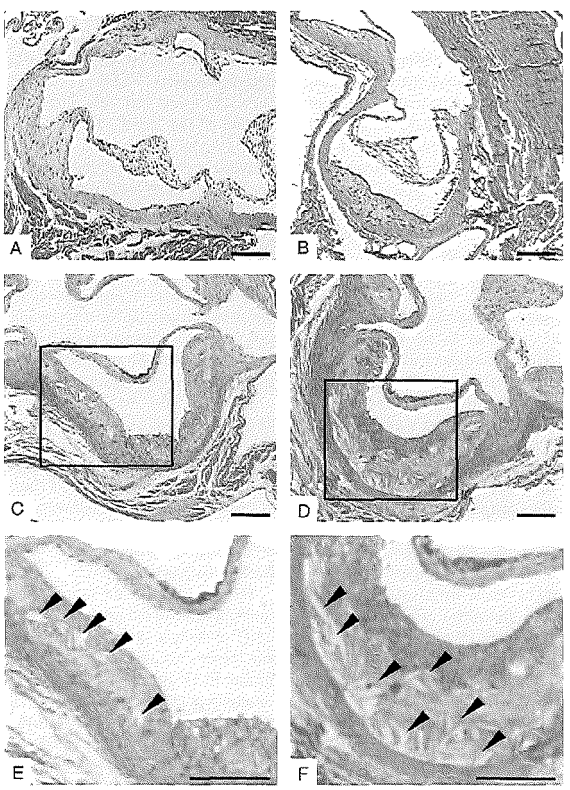

Fig. 5. Atherosclerotic lesions in the aortic valves. Photomicrographs showing representative atherosclerotic lesions in aortic valves. (A) male LDLR$^{+/-}$/huCETPtg mice; (B) male LDLR$^{+/-}$/huCETPtg/huPLTPtg mice; (C and E) female LDLR$^{+/-}$/huCETPtg mice; (D and F) female LDLR$^{+/-}$/huCETPtg/huPLTPtg mice. (E and F) are magnifications of the boxed parts of panels C and D, respectively. Arrowheads point to cholesterol clefts. Scale bars: 200 μm.

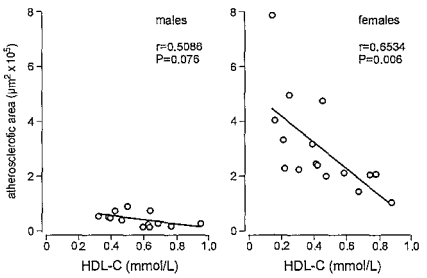

Fig. 6. Correlation between plasma levels of HDL and atherosclerotic lesion size. Plasma concentration of HDL-cholesterol (HDL-C) was measured and plotted against the size of the atherosclerotic lesions in individual male (left panel) and female (right panel) LDLR$^{+/-}$/huCETPtg/huPLTPtg mice. *r*- and *P*-values are indicated.

17,25–29]. However, the reason for this difference is still unknown, and so is the reason why this sex-difference appears to be opposite to the situation in humans (where males have more atherosclerosis at younger age than females). We did not intend to solve this paradox in the present study. Instead, we used this difference as a starting point and found that the PLTP transgenic female mice in our study have a 7-fold higher level of atherosclerosis than the PLTP transgenic male mice. More atherosclerosis was also found in female than in male mice in the control group (LDLR$^{+/-}$/huCETPtg mice). These results expand those from Arai et al. [16], who compared atherosclerosis between males and females in LDLR$^{+/-}$ mice. A similar sex difference in atherosclerotic lesion area was found in both studies.

The genetic background is known to be an important parameter for the extent of atherosclerosis in mice [30]. The mice used in the present study are all in C57Bl6 background, which is the most susceptible strain for diet-induced atherosclerosis. When LDLR$^{+/-}$/huCETPtg mice are compared with LDLR$^{+/-}$/huCETPtg/huPLTPtg mice, the atherosclerotic lesion areas are not different in male mice. In a previous report studying male mice without the CETP gene with various levels of PLTP overexpression, we demonstrated that the development of atherosclerosis was PLTP-dose dependent [12]. However, in the transgenic mouse line with the lowest level of PLTP overexpression (line P1), the difference in atherosclerotic lesion formation with the controls did not reach statistical significance in male mice [12]. This is in agreement with our current findings, in which the same line of PLTP transgenic mice has been used for the generation of the LDLR$^{+/-}$/huCETPtg/huPLTPtg mice. Both the stimulation of VLDL secretion and the decrease of plasma HDL-cholesterol levels were statistically significant in line P1 mice compared to non-transgenic controls [12]. In the present study, the effect on plasma HDL is confirmed in the male mice. Although there is no obvious increase of the atherosclerotic area, the number of lesions with cholesterol clefts is increased. Taken together, these results suggest that a moderate overexpression of PLTP is atherogenic in male mice, although the effect is too small to be detected unambiguously in the mouse models used. In contrast, the atherogenic effect of elevation of PLTP activity is very obvious in female mice (Fig. 4A: $P<0.005$ in LDLR$^{+/-}$/huCETPtg mice versus LDLR$^{+/-}$/huCETPtg/huPLTPtg mice). Thus, under circumstances where the susceptibility to atherosclerosis is increased, in this case by female sex, the atherogenicity of elevated PLTP becomes much more pronounced and therefore easier to detect.

This situation offered the opportunity to evaluate the contribution of two known atherogenic effects of PLTP on lipoprotein metabolism, i.e., the stimulatory effect on hepatic VLDL secretion and the lowering effect on plasma HDL. The rate of VLDL secretion in LDLR$^{+/}$/huCETPtg/huPLTPtg mice was similar in male and female mice, either on chow or on HFHC diet. On HFHC diet the female mice even tended to have lower VLDL secretion rates than male mice, but this difference was not statistically significant. VLDL triglyceride secretion is considerably less on HFHC diet when compared to the situation on chow diet, due to the high hepatic cholesterol levels which are caused by the HFHC diet, resulting in cholesterol-rich and triglyceride-poor VLDL [31]. Female LDLR$^{+/}$/huCETPtg/huPLTPtg mice had very low levels of plasma HDL after feeding of the HFHC diet, much lower than male LDLR$^{+/}$/huCETPtg/huPLTPtg mice. Therefore, we conclude that the decrease in plasma HDL has a more substantial contribution to the atherogenicity of elevated PLTP than the stimulation of VLDL secretion.

Apart from the level of HDL, other factors associated with HDL or involved in HDL metabolism could be involved. However, various measurements in these and previous studies, including the activities of paraoxonase, platelet-activating factor acetyl hydrolase, hepatic triglyceride lipase, lipoprotein lipase, and lecithin:cholesterol acyl transferase did not reveal any obvious differences between males and females that could explain differences in atherosclerosis susceptibility.

Recent studies have shown that PLTP is expressed by macrophages and is present in human atherosclerotic lesions [32,33]. In the transgenic mouse models we used, PLTP is expressed by a wide variety of cells and tissues [12]. Therefore, the possible contribution of PLTP secreted by the macrophages in the lesions to the atherosclerotic process cannot be determined from the present study. In order to dissect the role of macrophage derived PLTP, bone marrow transplantation studies could be used [34].

By nature, mice do not express CETP. Therefore, mice could be considered poor models to study atherosclerosis, as CETP is thought to be a key player in atherosclerosis [19]. For this reason, we studied in the present as well as in previous studies PLTP function in the presence of CETP [14,24]. In a comparison of the atherosclerotic lesion areas with published data [12,16], it appears that in the present study the contribution of the CETP transgene to the development of atherosclerosis is relatively small. This is in agreement with previous findings from our group [24], and also with earlier reports from others in which the same line of CETP transgenic mice was used to study atherosclerosis in a background of either LDLR deficiency or apoE deficiency [35], showing that

a moderate increase in atherosclerosis was found only when several time points were taken into account, but not for any individual time point. The HFHC diet resulted in a marked increase in CETP activity, as reported before [35,36]. It is likely that CETP activity levels have to be increased even much more in order to find a clear atherogenic effect, as was the case in transgenic mice overexpressing simian CETP [37] and in transgenic rats overexpressing human CETP [38]. Still, from all these studies CETP emerges as an atherogenic protein, which is further corroborated by the finding that CETP inhibition reduces atherosclerosis in rabbits [39]. Therefore, the lower CETP activity in female mice compared to male mice on the HFHC diet found in the present study is probably not related to the sex differences observed in the susceptibility to atherosclerosis.

In conclusion, we report here that female mice with moderate overexpression of PLTP are more prone to the development of diet-induced atherosclerosis than male mice, probably due to extremely low levels of plasma HDL. HDL has several anti-atherogenic properties, including a key role in reverse cholesterol transport as well as anti-oxidant and anti-inflammatory properties. The relative importance of these different mechanisms warrants further investigation.

Acknowledgements

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References

[1] A.J. Lusis, Atherosclerosis, Nature 407 (2000) 233–241.
[2] C.K. Glass, J.L. Witztum, Atherosclerosis. the road ahead, Cell 104 (2001) 503–516.
[3] D. Steinberg, Atherogenesis in perspective: hypercholesterolemia and inflammation as partners in crime, Nat. Med. 8 (2002) 1211–1217.
[4] P. Libby, Managing the risk of atherosclerosis: the role of high-density lipoprotein, Am. J. Cardiol. 88 (2001) 3N–8N.
[5] D. Sviridov, P. Nestel, Dynamics of reverse cholesterol transport: protection against atherosclerosis, Atherosclerosis 161 (2002) 245–254.
[6] G. Assmann, J.R. Nofer, Atheroprotective effects of high-density lipoproteins, Annu. Rev. Med. 54 (2003) 321–341.
[7] H.B. Brewer Jr., S. Santamarina-Fojo, New insights into the role of the adenosine triphosphate-binding cassette transporters in high-density lipoprotein metabolism and reverse cholesterol transport, Am. J. Cardiol. 91 (2003) 3E–11E.
[8] B.J. Van Lenten, M. Navab, D. Shih, A.M. Fogelman, A.J. Lusis, The role of high-density lipoproteins in oxidation and inflammation, Trends Cardiovasc. Med. 11 (2001) 155–161.
[9] J.R. Nofer, B. Kehrel, M. Fobker, B. Levkau, G. Assmann, A. von Eckardstein, HDL and arteriosclerosis: beyond reverse cholesterol transport, Atherosclerosis 161 (2002) 1–16.
[10] P. Barter, J. Kastelein, A. Nunn, R. Hobbs, High density lipoproteins (HDLs) and atherosclerosis; the unanswered questions, Atherosclerosis 168 (2003) 195–211.
[11] A. van Tol, Phospholipid transfer protein, Curr. Opin. Lipidol. 13 (2002) 135–139.
[12] R. van Haperen, A. van Tol, T. van Gent, L. Scheek, P. Visser, A. van der Kamp, F. Grosveld, R. de Crom, Increased risk of atherosclerosis by elevated plasma levels of phospholipid transfer protein, J. Biol. Chem. 277 (2002) 48938–48943.
[13] X.C. Jiang, S. Qin, C. Qiao, K. Kawano, M. Lin, A. Skold, X. Xiao, A.R. Tall, Apolipoprotein B secretion and atherosclerosis are decreased in mice with phospholipid-transfer protein deficiency, Nat. Med. 7 (2001) 847–852.
[14] J. Lie, R. De Crom, T. Van Gent, R. Van Haperen, L. Scheek, I. Lankhuizen, A. Van Tol, Elevation of plasma phospholipid transfer protein in transgenic mice increases VLDL secretion, J. Lipid Res. 43 (2002) 1875–1880.
[15] J.H. van Ree, W.J. van den Broek, V.E. Dahlmans, P.H. Groot, M. Vidgeon-Hart, R.R. Frants, B. Wieringa, L.M. Havekes, M.H. Hofker, Diet-induced hypercholesterolemia and atherosclerosis in heterozygous apolipoprotein E-deficient mice, Atherosclerosis 111 (1994) 25–37.
[16] T. Arai, N. Wang, M. Bezouevski, C. Welch, A.R. Tall, Decreased atherosclerosis in heterozygous low density lipoprotein receptor-deficient mice expressing the scavenger receptor BI transgene, J. Biol. Chem. 274 (1999) 2366–2371.
[17] A. Daugherty, Mouse models of atherosclerosis, Am. J. Med. Sci. 323 (2002) 3–10.
[18] J. Lie, R. de Crom, M. Jauhiainen, T. van Gent, R. van Haperen, L. Scheek, H. Jansen, C. Ehnholm, A. van Tol, Evaluation of phospholipid transfer protein and cholesteryl ester transfer protein as contributors to the generation of prebeta-high-density lipoproteins, Biochem. J. 360 (2001) 379–385.
[19] P.J. Barter, H.B. Brewer Jr., M.J. Chapman, C.H. Hennekens, D.J. Rader, A.R. Tall, Cholesteryl ester transfer protein: a novel target for raising HDL and inhibiting atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 160–167.
[20] P. Parini, L.L. Rudel, Is there a need for cholesteryl ester transfer protein inhibition? Arterioscler. Thromb. Vasc. Biol. 23 (2003) 374–375.
[21] R. van Haperen, A. van Tol, P. Vermeulen, M. Jauhiainen, T. van Gent, P. van den Berg, S. Ehnholm, F. Grosveld, A. van der Kamp, R. de Crom, Human plasma phospholipid transfer protein increases the antiatherogenic potential of high density lipoproteins in transgenic mice, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 1082–1088.
[22] R. Van Haperen, M. De Waard, E. Van Deel, B. Mees, M. Kutryk, T. Van Aken, J. Hamming, F. Grosveld, D.J. Duncker, R. De Crom, Reduction of blood pressure, plasma cholesterol and atherosclerosis by elevated endothelial nitric oxide, J. Biol. Chem. 277 (2002) 48803–48807.
[23] D.J. Delsing, E.H. Offerman, W. van Duyvenvoorde, H. van Der Boom, E. C. de Wit, M.J. Gijbels, A. van Der Laarse, J.W. Jukema, L.M. Havekes, H. M. Princen, Acyl-CoA:cholesterol acyltransferase inhibitor avasimibe reduces atherosclerosis in addition to its cholesterol-lowering effect in ApoE*3-Leiden mice, Circulation 103 (2001) 1778–1786.
[24] J. Lie, R. de Crom, T. van Gent, R. van Haperen, L. Scheek, F. Sadeghi-Niaraki, A. van Tol, Elevation of plasma phospholipid transfer protein increases the risk of atherosclerosis despite lower apolipoprotein B-containing lipoproteins, J. Lipid Res. 45 (2004) 805–811.
[25] C.H. Warden, C.C. Hedrick, J.H. Qiao, L.W. Castellani, A.J. Lusis, Atherosclerosis in transgenic mice overexpressing apolipoprotein A-II, Science 261 (1993) 469–472.
[26] M.J. Callow, J. Verstuyft, R. Tangirala, W. Palinski, E.M. Rubin, Atherogenesis in transgenic mice with human apolipoprotein B and lipoprotein (a), J. Clin. Invest. 96 (1995) 1639–1646.
[27] D.A. Purcell-Huynh, R.V. Farese Jr., D.F. Johnson, L.M. Flynn, V. Pierotti, D.L. Newland, M.F. Linton, D.A. Sanan, S.G. Young, Transgenic mice expressing high levels of human apolipoprotein B develop severe atherosclerotic lesions in response to a high-fat diet, J. Clin. Invest. 95 (1995) 2246–2257.
[28] S.D. Hughes, J. Verstuyft, E.M. Rubin, HDL deficiency in genetically engineered mice requires elevated LDL to accelerate atherogenesis, Arterioscler. Thromb. Vasc. Biol. 17 (1997) 1725–1729.
[29] M.P. de Winther, M.J. Gijbels, K.W. van Dijk, P.J. van Gorp, H. suzuki, T. Kodama, R.R. Frants, L.M. Havekes, M.H. Hofker, Scavenger receptor deficiency leads to more complex atherosclerotic lesions in APOE3Leiden transgenic mice, Atherosclerosis 144 (1999) 315–321.
[30] H.M. Dansky, S.A. Charlton, J.L. Sikes, S.C. Heath, R. Simantov, L.F.

Levin, P. Shu, K.J. Moore, J.L. Breslow, J.D. Smith, Genetic background determines the extent of atherosclerosis in ApoE-deficient mice, Arterioscler. Thromb. Vasc. Biol. 19 (1999) 1960–1968.
[31] S.H. Zhang, R.L. Reddick, B. Burkey, N. Maeda, Diet-induced atherosclerosis in mice heterozygous and homozygous for apolipoprotein E gene disruption, J. Clin. Invest. 94 (1994) 937–945.
[32] K.D. O'Brien, S. Vuletic, T.O. McDonald, G. Wolfbauer, K. Lewis, A.Y. Tu, S. Marcovina, T.N. Wight, A. Chait, J.J. Albers, Cell-associated and extracellular phospholipid transfer protein in human coronary atherosclerosis, Circulation (2003) 270–274.
[33] B.A. Laffitte, S.B. Joseph, M. Chen, A. Castrillo, J. Repa, D. Wilpitz, D. Mangelsdorf, P. Tontonoz, The phospholipid transfer protein gene is a liver X receptor target expressed by macrophages in atherosclerotic lesions, Mol. Cell. Biol. 23 (2003) 2182–2191.
[34] S. Fazio, M.F. Linton, Mouse models of hyperlipidemia and atherosclerosis, Front. Biosci. 6 (2001) D515–D525.
[35] A.S. Plump, L. Masucci-Magoulas, C. Bruce, C.L. Bisgaier, J.L. Breslow, A.R. Tall, Increased atherosclerosis in ApoE and LDL receptor gene knock-out mice as a result of human cholesteryl ester transfer protein transgene expression, Arterioscler. Thromb. Vasc. Biol. 19 (1999) 1105–1110.
[36] L. Masucci-Magoulas, A. Plump, X.C. Jiang, A. Walsh, J.L. Breslow, A.R. Tall, Profound induction of hepatic cholesteryl ester transfer protein transgene expression in apolipoprotein E and low density lipoprotein receptor gene knockout mice. A novel mechanism signals changes in plasma cholesterol levels, J. Clin. Invest. 97 (1996) 154–161.
[37] K.R. Marotti, C.K. Castle, T.P. Boyle, A.H. Lin, R.W. Murray, G.W. Melchior, Severe atherosclerosis in transgenic mice expressing simian cholesteryl ester transfer protein, Nature 364 (1993) 73–75.
[38] V.L. Herrera, S.C. Makrides, H.X. Xie, H. Adari, R.M. Krauss, U.S. Ryan, N. Ruiz-Opazo, Spontaneous combined hyperlipidemia, coronary heart disease and decreased survival in Dahl salt-sensitive hypertensive rats transgenic for human cholesteryl ester transfer protein, Nat. Med. 5 (1999) 1383–1389.
[39] H. Okamoto, F. Yonemori, K. Wakitani, T. Minowa, K. Maeda, H. Shinkai, A cholesteryl ester transfer protein inhibitor attenuates atherosclerosis in rabbits, Nature 406 (2000) 203–207.

Chapter 7

Novel roles of hepatic lipase and phospholipid transfer protein in VLDL as well as HDL metabolism

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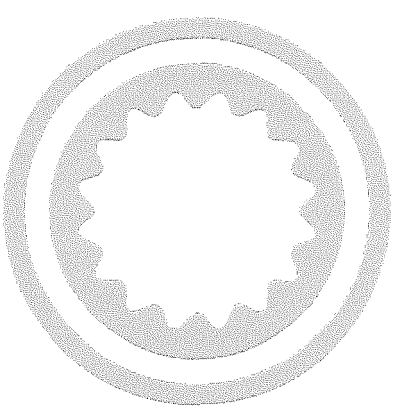

Novel Roles of Hepatic Lipase and Phospholipid Transfer Protein in VLDL as well as HDL Metabolism

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Abstract

***Objective*: Elevated plasma phospholipid transfer protein (PLTP) expression may increase atherosclerosis in mice by reducing plasma HDL and increasing hepatic VLDL secretion. Hepatic lipase (HL) is a lipolytic enzyme involved in several aspects of the same pathways of lipoprotein metabolism. We investigated whether the effects of elevated PLTP activity are compromised by HL deficiency.**

***Methods and Results*: HL deficient mice were crossbred with PLTP transgenic (PLTPtg) mice and studied in the fasted state. Plasma triglycerides were decreased in HL deficiency, explained by reduced hepatic triglyceride secretion. In PLTPtg mice, HL activity and plasma triglycerides were also decreased. HL deficiency mitigated or even abolished the stimulatory effect of elevated PLTP activity on hepatic triglyceride secretion. HL deficiency had a modest incremental effect on plasma HDL, which remained present in PLTP transgenic/HL-/- mice, thereby partially compensating the decrease in HDL caused by elevation of PLTP activity. HDL decay experiments showed that the fractional turnover rate of HDL cholesteryl esters was delayed in HL deficient mice, increased in PLTPtg mice and intermediate in PLTPtg mice in a HL-/- background.**

***Conclusions*: HL affects VLDL secretion by the liver, while elevated PLTP activity lowers plasma HDL cholesterol by stimulating the plasma turnover and hepatic uptake of HDL cholesteryl esters. HL is not required for the increase in hepatic triglyceride secretion or for the lowering of HDL cholesterol induced by PLTP overexpression.**

Introduction

Phospholipid transfer protein (PLTP) is a key regulator in lipid and lipoprotein metabolism. It is expressed in a variety of tissues and cells, and secreted into the plasma, where it is found associated with HDL particles. PLTP has an essential role in HDL metabolism by transferring phospholipids between lipoproteins and modulating the size and composition of HDL particles.[1,2] We previously generated a series of transgenic mouse models in which the human PLTP protein is expressed.[3] In these mice, we found a PLTP-dose dependent decrease in plasma HDL levels, associated with an increased susceptibility to diet-induced atherosclerosis. In PLTP deficient mice, the development of atherosclerosis is reduced, even though, in this condition too, lower HDL levels were found.[4] Hepatocytes from PLTP deficient mice show an impaired secretion of apolipoprotein (apo) B containing lipoproteins, possibly explaining the reduction of atherosclerosis development in PLTP deficient mice. The role of PLTP in apoB lipoprotein metabolism has also been investigated in our transgenic mice.[3,5] Overexpression of PLTP resulted in a moderate increase in VLDL secretion. Taken together, the exact molecular mechanism by which PLTP influences lipid metabolism and thereby affects atherogenesis is still unclear.

In addition to PLTP, a number of other proteins are critically involved in the regulation of lipoprotein metabolism, that might lead to interactions by affecting each other's functions. From previous work from our laboratory and others, it could be concluded that PLTP and cholesterol ester transfer protein (CETP), the other lipid transfer protein that is active in human plasma, act independently from each other.[6,7]

In addition to CETP, hepatic lipase (HL) is another candidate protein that might affect the function of PLTP in plasma. HL is a lipolytic enzyme which catalyzes hydrolysis of triglycerides and phospholipids, and in this way is involved in conversion of VLDL into LDL particles, as well as in HDL remodeling.[8-10] Thus, HL is involved in the same two pathways in lipoprotein biochemistry as PLTP, i.e. VLDL and HDL metabolism. In HL knockout mouse models, elevated plasma HDL cholesterol levels are found, but effects on plasma triglyceride levels appear to be absent, at least in the fasted state.[11] This is surprising since hypertriglyceridemia could be expected, because HL has both a lipolytic role in hydrolyzing triglycerides of the apoB-containing lipoproteins IDL and LDL, and a non-lipolytic function in facilitating the uptake of apoB-containing lipoprotein particles by

the liver.[10] Therefore we investigated the absence of hypertriglyceridemia in fasted mice with complete HL deficiency. To our surprise we found decreased instead of increased triglyceride levels and hypothesized that HL is necessary for optimal hepatic production and secretion of VLDL, a possibility never studied before.
In the present study, we investigated the possibility of functional relations between PLTP and HL. To evaluate HL function, we compared wild type mice with HL deficient mice. To study PLTP function, we used two PLTP transgenic mouse models that we generated previously: one with 4-5 fold and another with 8 fold elevation of plasma PLTP activity. We argued that if PLTP effects would be dependent on HL activity, these would be compromised in HL deficiency. PLTP transgenic mice were crossed with HL deficient mice and effects on hepatic triglyceride secretion and HDL cholesteryl ester turnover were measured.

Methods

Mice

PLTP transgenic mice were generated as described before.[3] As in our previous studies, we used PLTP transgenic mice with two different expression levels resulting in a 4-5 fold elevation of plasma PLTP activity (P4 mice) and in an 8-fold increase in PLTP activity (A2 mice).[3,12] $HL^{-/-}$ mice were obtained from Jackson Laboratory and were originally generated and described by Homanics *et al.*[11] Mice transgenic for PLTP and deficient for HL were obtained by cross-breeding. All animals were in C57BL/6J background (> 99.5%). Only male animals were used in the present study. All animal experiments were performed in compliance with institutional (Erasmus MC, Rotterdam, the Netherlands) and national guidelines.

PLTP activity, lipase activities, plasma and hepatic lipids, plasma apolipoproteins

Plasma samples were collected after an overnight fast. PLTP activity levels were measured using an exogenous substrate assay as described previously[3] and expressed as arbitrary units, in which 1 arbitrary unit is the PLTP activity found in human reference plasma, which is equivalent to 14 μmol/ml/hour. HL activity was measured in pre-heparin plasma, as well as in post-heparin plasma (collected at 5 min after injection of 200 units of heparin/ kg body weight into a tail vein) after inhibition of LPL activity with a specific anti-LPL antibody and LPL activity was measured in post-heparin plasma as described.[13] Plasma levels of total cholesterol or triglycerides were measured with commercially available kits as described.[3] HDL and non-HDL fractions were obtained by tube slicing after density gradient ultracentrifugation using a Beckman 42.2 rotor (42,000 rpm, 2 h 50 min, 12°C) at d = 1.063. Lipid levels in liver was measured after extracting the lipids from hepatic tissue

using a mixture of chloroform/methanol as described by Bligh and Dyer[14] and expressed as mg lipid per whole organ.
Lipoprotein fractions were obtained by density gradient ultra centrifugation of plasma samples in a Beckman SW60 Ti rotor (36000 rpm, 21 hr, 12°C).

Triglyceride secretion experiments

The hepatic secretion rate of triglycerides was measured after an overnight fast as described before[5] by injecting animals with Triton WR1339 and collecting plasma samples at various time points until 90 minutes after injection.

HDL decay measurements

HDL was isolated from wild type mice and radiolabeled with the non-degradable label[^{3}H]-cholesterol oleyl ether using the methods described by Groener *et al.*[15] Animals were injected with 700,000 dpm of radiolabeled HDL. Blood samples were collected from the orbital plexus under isoflurane anaesthesia at the indicated time points and radioactivity was determined in isolated plasma by liquid scintillation counting. In separate experiments, in order to correct for variation in the initial concentrations of plasma HDL-cholesterol, animals were injected with radiolabeled HDL supplemented with unlabeled HDL. The PLTP transgenic mice in an HL proficient background were adjusted to the level of plasma HDL found in wild type mice (1.9 mMol/L); the PLTP transgenic mice in an HL deficient background were adjusted to the level of plasma HDL found in HL deficient mice (2.1 mMol/L). See legend of figure 6 for details. Tissue radioactivities in tissues were determined at the end of the decay experiments by liquid scintillation counting.

Statistics

Data are expressed as means ± SD. Differences between the various mouse lines were analyzed by ANOVA, followed by Bonferroni correction. Differences between two groups of mice were analyzed by two sample Wilcoxon rank-sum tests. The tests were performed using Intercooled Stata 8.2 SE software (Stata Corporation, College Station, TX, USA).

Results

Plasma PLTP activity

First we checked whether HL deficiency would interfere with plasma PLTP activity. As shown in Fig.1, wild type and HL$^{-/-}$ mice have similar plasma PLTP activity levels. As reported before, PLTP activity in plasma is elevated in P4 mice and even more so in A2 mice. In these animals, HL deficiency did not affect plasma PLTP activity (figure1).

Plasma HL and LPL activity

Subsequently, we analyzed whether overexpression of PLTP affects HL or LPL activity. Both enzymes are partly bound to proteoglycans on the cell surface and can be released in plasma after injection of heparin.[16] In wild type mice, HL activity is almost quantitatively present in pre-heparin plasma.[13] However, in PLTP transgenic mice this has not been measured before. As shown in figure 2A, pre-heparin HL activities in P4 and A2 mice are 37% en 26% respectively of the activity found in wild type mice, while the post-heparin HL activity is unchanged. These results indicate that although PLTP overexpression does not affect the total post-heparin HL activity, a substantial part of HL is bound to cellular proteoglycans in mice overexpressing PLTP and can be released by heparin. Total post-heparin LPL activity is shown in figure 2B. In the PLTP transgenic lines there was no significant change in LPL activity compared to the wild type animals.

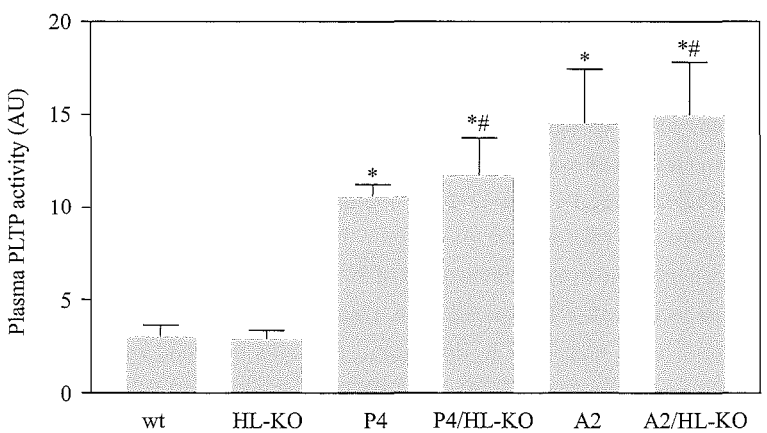

Figure 1. PLTP activity levels in plasma. Plasma was collected from mice with the indicated genotypes by orbital bleedings. PLTP activity was measured as described in Methods and expressed in arbitrary units (AU). 1 AU is equivalent to the PLTP activity found in human reference plasma, i.e. 14 μmol/ml/hour. Values are expressed as mean ± S.D. n > 12 animals/ group.

*P<0.001 vs wt (tested for all genotypes)

#P<0.001 vs $HL^{-/-}$ (tested only for P4/ $HL^{-/-}$ mice and A2/$HL^{-/-}$ mice)

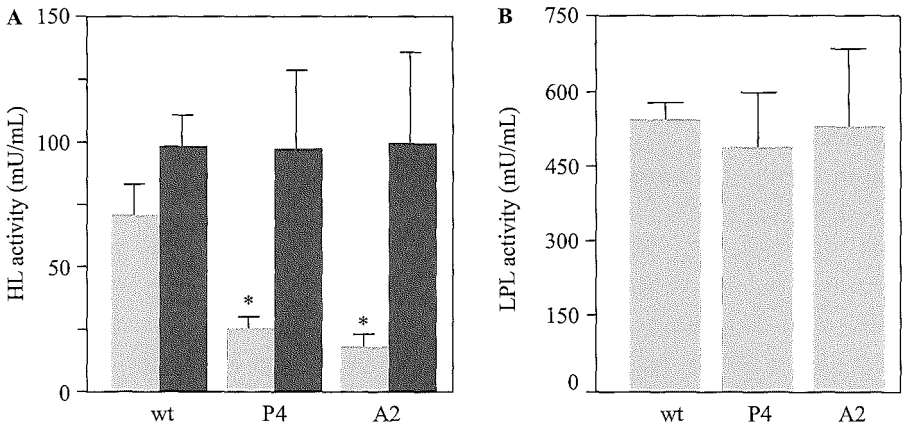

Figure 2. Plasma lipase activities in PLTP transgenic mice and wild type mice. HL activities were measured in plasma before (grey bars) and 5 min after (black bars) injection of heparin (A), while LPL activities were measured in plasma samples collected 5 min after injection of heparin (B), as described in Methods. Values are expressed as mean ± S.D. n = 6 animals/group.
*P<0.001 vs wt (tested for all genotypes)

Plasma lipids and lipoproteins

Measurements of total cholesterol (TC) in plasma are provided in figure 3A. Total plasma cholesterol was increased with 21% in $HL^{-/-}$ mice in comparison with wild type animals, confirming previous reports. As we reported before, elevated expression of PLTP resulted in a strong decrease in total plasma cholesterol. This effect could be partly reversed by HL deficiency as an increase in total cholesterol of 28% and 108% was found in $P4/HL^{-/-}$ and in $A2/HL^{-/-}$ mice compared to P4 and A2 mice, respectively. When non-HDL cholesterol and HDL-cholesterol were measured in lipoprotein fractions obtained by ultracentrifugation, similar effects were observed (Table 1). Effects on plasma phospholipids (PL) largely reflect those on plasma TC (figure 3B and Table 1).

Plasma triglyceride concentrations in P4 and A2 mice were decreased to 46% and 14% of the triglyceride concentration found in wild type mice (figure 3C). $HL^{-/-}$, $P4/HL^{-/-}$ and $A2/HL^{-/-}$ mice have 46%, 23% and 18% of the triglyceride concentration found in wild type mice.

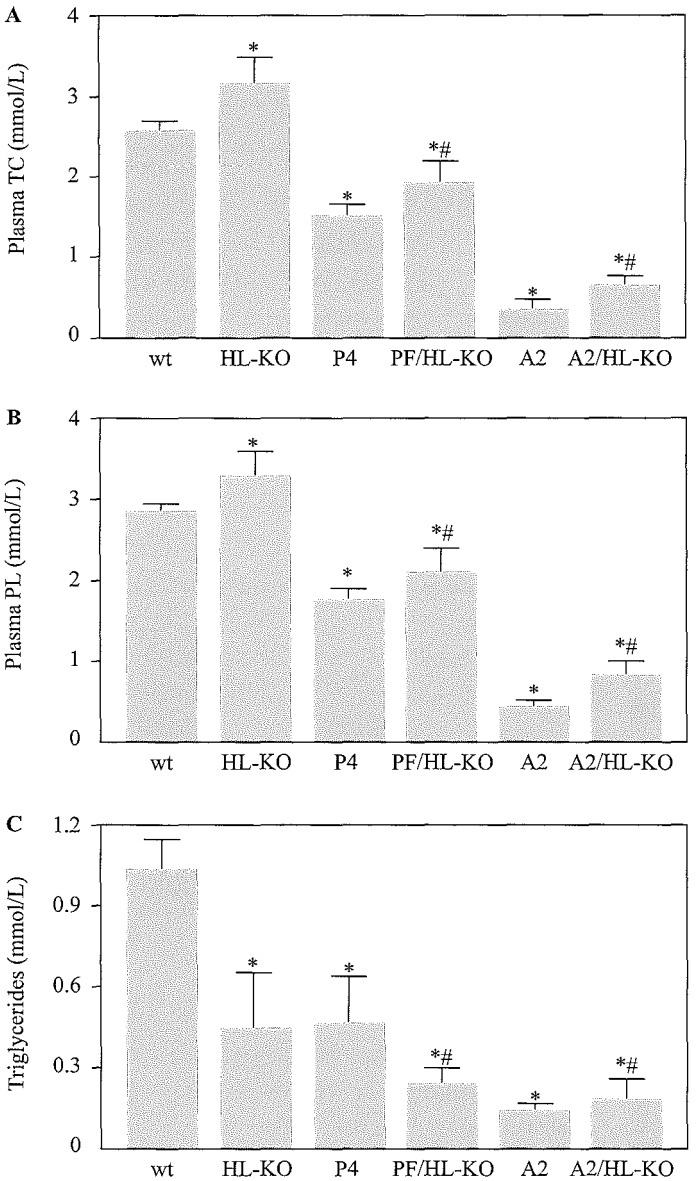

Figure 3. Plasma lipid concentrations. Plasma was collected from mice with the indicated genotypes by orbital bleedings. Total cholesterol (A), phopholipids (B) and triglycerides (C) were measured as described in Methods and expressed in mmol/L. Values are expressed as mean ± S.D. n > 12 animals/group.

*P<0.001 vs wt (tested for all genotypes)

#P<0.001 vs $HL^{-/-}$ (tested only for P4/ $HL^{-/-}$ mice and A2/$HL^{-/-}$ mice)

Table 1. Plasma total cholesterol (TC) and phosholipids (PL) levels in non-HDL and HDL lipoprotein fractions

	non-HDL TC	HDL TC	non-HDL PL	HDL PL
wt	0.73 ± 0.06	1.86 ± 0.16	0.67 ± 0.07	2.19 ± 0.1
$HL^{-/-}$	1.01 ± 0.18*	2.10 ± 0.22*	0.85 ± 0.12*	2.42 ± 0.23*
P4	0.74 ± 0.11	0.76 ± 0.07*	0.70 ± 0.10	1.08 ± 0.07*
P4/ $HL^{-/-}$	0.90 ± 0.18* #	1.03 ± 0.19* ##	0.68 ± 0.17##	1.42 ± 0.21* ##
A2	0.26 ± 0.10*	0.11 ± 0.03*	0.13 ± 0.06*	0.26 ± 0.04*
A2/ $HL^{-/-}$	0.31 ± 0.09* ##	0.36 ± 0.08* ##	0.19 ± 0.06* ##	0.62 ± 0.13* ##

Values are in mmol/L. n >10 animals.
*P<0.001 vs wt
#P<0.05 vs $HL^{-/-}$
##P<0.001 vs $HL^{-/-}$

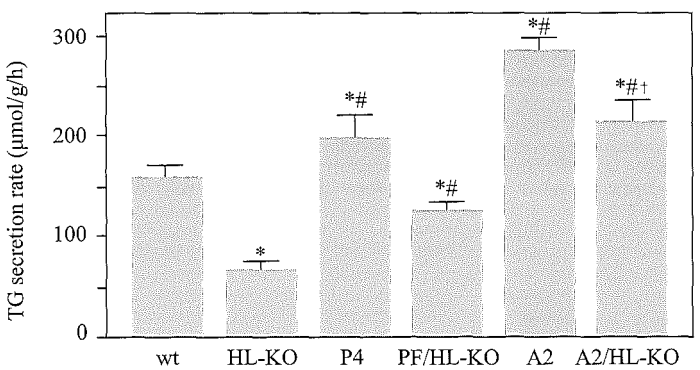

Figure 4. Secretion rates of hepatic triglycerides. Animals were injected with Triton WR1339. Plasma was collected from mice with the indicated genotypes by orbital bleedings at 2, 30, 60, and 90 minutes after injection. Triglyceride concentrations were measured and the triglycerides secretion rates were calculated using these data. Values are expressed as mean ± S.D. n > 5 animals/group.
*P<0.05 vs wt; #P<0.05 vs $HL^{-/-}$; $P<0.05 vs P4; †P<0.05 vs A2

Triglyceride secretion

We have previously shown that elevated plasma PLTP activity levels in P4 and A2 mice result in increased hepatic triglyceride secretion in fasted mice.[3] To investigate the possible involvement of HL in this PLTP effect, we analyzed triglyceride secretion in mice with

various genotypes (figure 4). Surprisingly, HL deficiency by itself decreased the secretion rate of triglycerides by 57% as compared to wt mice. As a result of HL deficiency, the increased TG secretion due to PLTP overexpression (P4) is eliminated. So the lowering effect of HL deficiency on triglyceride secretion is sufficiently large to counterbalance the stimulatory effect of elevated PLTP expression on triglyceride secretion normally seen in P4 mice. Overall this results in a relatively small decrease in triglyceride secretion, by only 20%, in P4/HL-/- mice compared to wild type mice. VLDL secretion in mice with the highest PLTP expression (A2) is strongly increased in comparison with wild type mice and remains higher than in wild type mice in the absence of HL. The dramatic effect of HL deficiency on triglyceride secretion did not result in differences in the lipid content of the liver (not shown).

HDL turnover experiments

To find an explanation for the differences in plasma HDL-cholesterol concentration between the various mouse lines, we measured the turnover rates of HDL cholesteryl esters. Animals were injected with [^{3}H]-cholesterol oleyl ether labeled HDL, as a tracer for HDL cholesteryl esters, and plasma samples were analyzed for radioactivity at the indicated time points (figure 5). $HL^{-/-}$ mice showed a delayed fractional turnover of HDL cholesteryl esters compared with wild type mice, with half-lives of the labeled HDL cholesteryl esters of 6.77 ± 0.31 hours versus 5.29 ± 0.29 hours, respectively. In contrast, the fractional turnover of HDL cholesteryl esters was faster in mice with elevated PLTP expression, as compared with wild type mice. When the half-life of the injected dose was calculated, these differences appeared to be highly statistically significant (Table 2, all differences $P<0.001$). In P4/ $HL^{-/-}$ mice and A2/$HL^{-/-}$ mice, the decay of labeled HDL is delayed in comparison with P4 and A2 mice, respectively, but still faster than in wild type mice (figure 5, Table 2). Obviously, the initial plasma concentrations of HDL were highly different between the various mouse lines. Therefore, we performed a similar set of experiments, in which we corrected for the initial plasma concentrations of HDL by injecting unlabeled HDL into the PLTP transgenic mice, thereby achieving similar concentrations of plasma HDL at the start of the experiment (for details see the legend to figure 6). As expected, the differences in fractional HDL cholesteryl ester turnover rate are reduced, but the half-life in the wild type mice remains clearly longer than in the PLTP transgenic mice. Moreover, the half-life of HDL cholesterylesters in the $HL^{-/-}$ mice remains longer than in the PLTP transgenic mice in an $HL^{-/-}$ background (figure 6, Table 2). Tissue uptake of radioactive HDL cholesteryl ether was measured in several organs. In both sets of experiments, uptake by the liver (Table 2) was quantitatively most important.

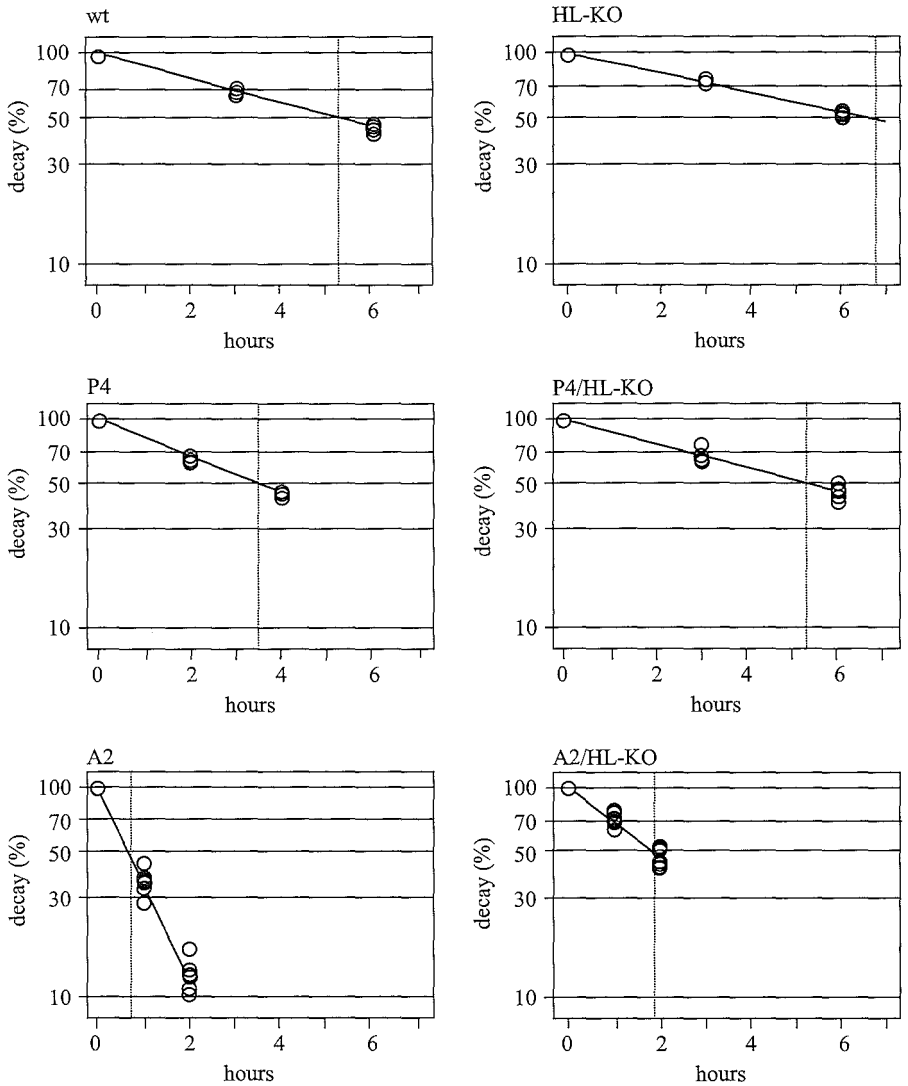

Figure 5. HDL decay in plasma. Isolated mouse HDL was radiolabeled with non-degradable [^{3}H] cholesteryl oleyl ether, which served as a tracer for HDL cholesteryl esters, and injected intraveneously in mice (700,000 dpm/animal). Plasma samples were collected at the indicated time points and radioactivity was measured by scintillation counting. n = 6-8 animals/group. The half-lifes of the tracer were calculated from these data for individual mice. These are indicated by the vertical dashed lines (means) and provided in Table 2 (means ± S.D).

Concomitantly, mouse HDL was injected to correct for initial differences in the concentration of plasma HDL-cholesterol. P4 mice (mean of 0.8 mMol/L HDL cholesterol) and A2 mice (mean of 0.1 mMol/L HDL cholesterol) received a bolus-injection of mouse HDL to restore the plasma level to the level found in wt mice (on average 1.9 mMol/L HDL cholesterol), while P4/ $HL^{-/-}$ mice (mean of 1.0 mMol/L HDL cholesterol) and A2/$HL^{-/-}$ mice (mean of 0.4 mMol/L HDL cholesterol) received a bolus-injection of mouse HDL to restore the plasma level to the level found in $HL^{-/-}$ mice (on average 2.1 mMol/L HDL cholesterol). The total plasma volume of mice was taken as 4.37% of body weight for calculations. n = 6–8 animals/group. The half-lifes of the tracer were calculated from these data for individual mice. These are indicated by the vertical dashed lines (means) and provided in Table 2 (means ± S.D)

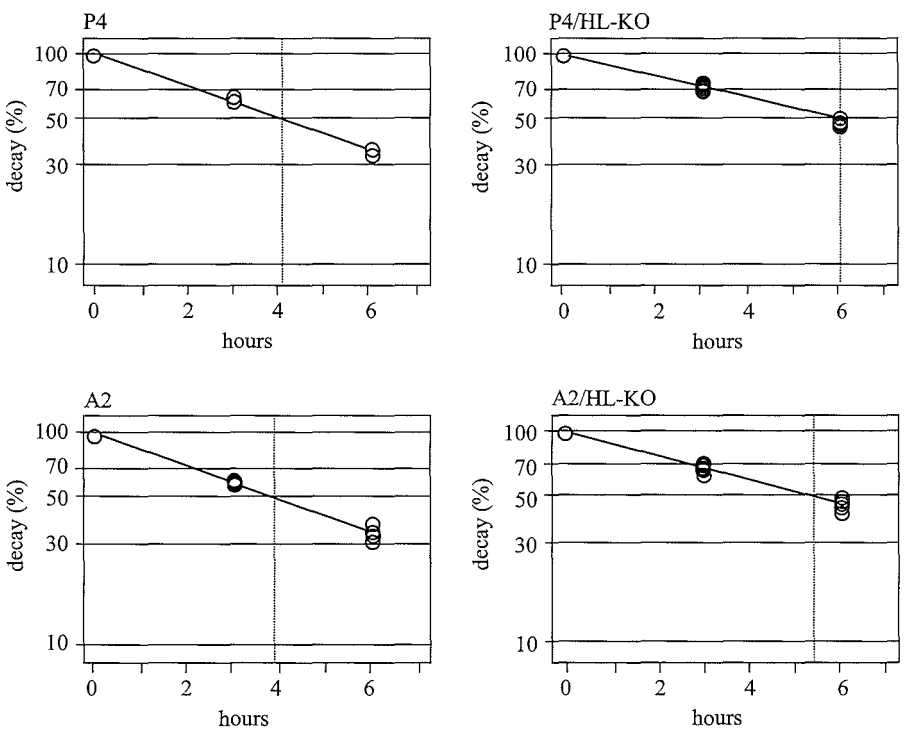

Figure 6. HDL decay in plasma following supplementation with exogenous HDL. Isolated mouse HDL was radiolabeled with non-degradable [^{3}H] cholesteryl oleyl ether, which served as a tracer for HDL cholesteryl esters, and injected intravenously in mice (700,000 dpm/animal).

Table 2. Plasma decay and hepatic uptake of radioactively labeled HDL cholesteryl oleyl ether

genotype	wt	$HL^{-/-}$	P4	P4/ $HL^{-/-}$	A2	A2/ $HL^{-/-}$
$t_{1/2}$ (1)	5.29 ± 0.29	6.77 ± 0.31*	3.48 ± 0.11*	5.32 ± 0.47#	0.68 ± 0.08*	1.92 ± 0.29*#
% ID in plasma (1)	45.1 ± 1.81	53.7 ± 1.5	33.3 ± 3.23	45.9 ± 2.14	13.0 ± 2.3	47.4 ± 5.05
% ID in liver (1)	26.5 ± 2.32	17.4 ± 1.25	38.0 ± 4.11	23.6 ± 0.95	71.6 ± 3.5	39.5 ± 5.38
$t_{1/2}$ (2)	5.29 ± 0.29	6.77 ± 0.31*	4.10 ± 0.15*	6.04 ± 0.23#	3.90 ± 0.23*	5.40 ± 0.37#
% ID in plasma (2)	45.1 ± 1.81	53.7 ± 1.5	33.1 ± 1.96	49.7 ± 1.06	32.3 ± 1.3	46.4 ± 2.45
% ID in liver (2)	26.5 ± 2.32	17.4 ± 1.25	32.5 ± 2.37	22.8 ± 1.59	27.9 ± 2.9	25.3 ± 3.24

Values are $t_{1/2}$ in hours or percentage of the injected dose of radioactivity at the end of the experiment (% ID) without adjustment of initial plasma HDL levels [$t_{1/2}$ (1)] and $t_{1/2}$ in hours or % ID after adjustment of initial plasma HDL levels [$t_{1/2}$ (2)]. For more details see legends to figures 5 and 6. n = 6-8 animals.

*P<0.001 vs wt

#P<0.005 vs $HL^{-/-}$

Discussion

Earlier studies in mouse models showed that PLTP affects hepatic triglyceride and apoB secretion and HDL metabolism.[4,17,18] In the present study we investigated whether HL, a lipolytic enzyme which is thought to play an important role in lipoprotein metabolism,[8,10,19] interferes with the effects of PLTP. To this end, we crossbred HL deficient mice with PLTP transgenic mice and studied the effects of HL and PLTP on VLDL and HDL metabolism. As published earlier, elevated expression of PLTP normally results in increased secretion of triglyceride-rich lipoproteins by the liver.[3,5] We now report that elevated expression of PLTP also stimulates hepatic triglyceride secretion in the absence of HL (figure 4). Of note, plasma PLTP activity in either wild type or human PLTP transgenic mice was not affected by deficiency of HL (figure 1). Both HL and LPL are partly bound to extracellular heparin sulfate proteoglycan groups and can be released to the plasma compartment by injection of heparin.[16] Post-heparin LPL activity and HL activity were unchanged in PLTP transgenic mice. However, in contrast to the situation in wild type animals,[13] a substantial part of the HL activity was bound to cellular proteoglycans and is released by heparin injection, leading to striking differences in HL activity in pre-heparin plasma of wild type versus PLTP overexpressing mice. This might be induced by the decreased HDL levels in PLTP overexpressing mice, as HL is normally bound to HDL. Compared with wild type mice, HL deficient animals have a moderate increase in total plasma cholesterol and HDL-cholesterol, which is in agreement with earlier findings.[11,20] This effect is corroborated by studies in HL transgenic mice, showing a decrease in HDL.[21,22] Elevated expression of PLTP results in a decrease of plasma HDL, as shown by us before in transgenic mice[3,12] and by others using adenoviral methodology.[17,23,24] Only high-level overexpression of PLTP (A2 line) decreased both HDL and non-HDL cholesterol and phospholipid concentrations (see Table 1). As expected, plasma phospholipids show changes similar to plasma cholesterol and HDL cholesterol, since the bulk of plasma phospholipids is present in HDL. Overexpression of PLTP in HL deficient mice results in intermediate levels of plasma HDL, indicating that PLTP overexpression in an $HL^{-/-}$ background lowers HDL-cholesterol concentrations but not to the same extent as found in a wild type background. These results suggest that the effects of PLTP on plasma HDL-cholesterol concentration do not involve HL, and vice versa that effects of HL on plasma HDL-cholesterol concentration are still present in conditions of elevated plasma PLTP activity.

We now report that plasma triglyceride levels are reduced in PLTP transgenic mice (figure 3C). This appears to be in contrast with the previously reported stimulation of hepatic triglyceride secretion.[3,5] A stimulation of VLDL degradation is therefore suggested. LPL activity was virtually unchanged, so the decreased plasma triglyceride levels in PLTP

transgenic mice are not easily explained, but may be related to the role of PLTP in the transfer of surface fragments of VLDL during lipolysis by lipoprotein lipase.
In contrast, the striking reduction of plasma triglycerides in fasted HL deficient mice can be easily explained by our finding of decreased hepatic VLDL secretion. The decrease in triglyceride secretion found in HL deficient mice could be due to a rate-limiting effect on substrate availability for VLDL synthesis,[8,25] a phenomenon which has also been suggested in humans.[26] It must be noted that there is no evidence for decreased VLDL production rates in human subjects with partial HL deficiency.[27,28] Decreased plasma triglycerides were not reported in two seminal papers on HL deficient mice.[11] However, there are important differences between the earlier studies and our current experiments. The animals used by Homanics *et al.*,[11] were much older (>6 months), while the animals used by Qiu *et al.*,[11] were 10-14 weeks and were in a non-fasted state. The animals we used were fasted overnight, so their plasma lipid levels, in particular triglycerides, cannot be compared with those reported by Qiu *et al.* Moreover, the animals described in the first papers were in a 129 background or in a mixed genetic background (C57BL6 / 129), while we used mice in C57BL/6 background. Therefore, different effects on plasma triglycerides might be explained by differences in nutritional state, genetic background and age (animals used in the present study were 12-15 weeks).

The atherogenic potential of elevated PLTP activity may be attributed to its HDL-lowering effect, because many of the anti-atherogenic mechanisms are dependent on HDL levels.[29] In humans, HL activity and HDL-cholesterol are inversely correlated.[8] Animal studies confirmed this relation.[8,10,30] In general, plasma HDL levels are mostly determined by HDL clearance rather than production.[31] Therefore we studied plasma HDL cholesteryl ester turnover in our mouse models in more detail and found evidence for a delay in plasma decay of labeled HDL cholesteryl esters in HL knockout mice, when compared to wild type animals, which can be explained by the hydrolysis of HDL phospholipids by HL in wild type animals. In contrast, PLTP transgenic mice showed a PLTP-dose dependent accelerated decay of radiolabeled HDL. This effect was partly prevented by deficiency of HL. There may be a particular caveat in previous work showing that elevated PLTP results in accelerated decay of labeled HDL components.[17] Because the plasma levels of HDL in wild-type mice and PLTP transgenic mice are widely different, this will strongly affect the rate of clearance of injected tracer, the radiolabeled HDL. Therefore, we now also performed decay experiments in which we corrected for the initial HDL-cholesterol concentration by injection of unlabeled mouse HDL. In these experiments, the observed differences were smaller, but still clearly present. Therefore we can conclude that HL deficiency retards and PLTP expression accelerates the fractional turnover of HDL cholesteryl esters.
Finally, an important question is whether our findings provide further insight in the role of

both HL and PLTP in atherosclerosis. In the present study we did not use dietary conditions and mouse models that are suitable for measuring the development of atherosclerosis in coronary vessels or aorta. For HL, both atherogenic and anti-atherogenic properties have been reported and therefore the exact role of HL in atherogenesis is still unclear.[8,10] HL activity was reported to be a risk factor for atherosclerosis in humans,[32] and indeed HL activity is clearly elevated in high-risk conditions, e.g. type 2 diabetes with low insulin sensitivity.[33,34] Also in mouse models, different results with respect to atherogenesis have been reported depending on the mouse model used.[18,19,31,32] We now report that total HL deficiency results in a marked reduction in hepatic triglyceride secretion, explaining the decreased triglyceride levels in overnight fasted mice. However, low HL activity in man does not result in low plasma triglycerides or decreased VLDL production rates. In fact there is evidence for opposite effects.[27] In the case of PLTP, there are reports showing that PLTP activity is elevated in human subjects at risk for atherosclerosis development, including patients with obesitas and type 1 or type 2 diabetes mellitus.[2,35] PLTP has been proposed as an independent risk factor[36] and as a potential target for anti-atherosclerotic drug therapy.[37] In mice, overexpression of PLTP is clearly atherogenic, as shown by us and others.[3,12,24] Now we report that elevation of plasma PLTP activity leads to high rates of plasma HDL cholesteryl ester turnover. A substantial part of these HDL lipids is taken up by the liver (Table 2), probably via SRBI, indicating that this step of reverse cholesterol transport is stimulated by PLTP. However, rapid hepatic uptake of HDL cholesteryl esters, induced by PLTP-mediated HDL conversion, may ultimately enhance degradation of small HDL particles in the kidneys, resulting in very low plasma concentrations of apo A-I and HDL, as can be observed in PLTP transgenic mice. In the end such a low HDL profile may turn out to be pro-atherogenic. Further studies, including atherosclerosis-susceptible mouse models, are needed to address these issues.

In conclusion, we provide evidence for novel roles of both HL and PLTP in VLDL and HDL metabolism. In addition to its role in HDL metabolism, HL appears to be very important in hepatic VLDL secretion, while elevated PLTP activity lowers plasma HDL cholesterol by stimulating the plasma turnover and uptake of HDL cholesteryl esters by the liver. HL is not required for the increase in hepatic triglyceride secretion by PLTP overexpression or for the lowering of HDL cholesterol by PLTP overexpression.

References

1. Huuskonen J, Olkkonen VM, Jauhiainen M, Ehnholm C. The impact of phospholipid transfer protein (PLTP) on HDL metabolism. *Atherosclerosis.* 2001;155:269-281.
2. van Tol A. Phospholipid transfer protein. *Curr Opin Lipidol.* 2002;13:135-139.
3. Van Haperen R, Van Tol A, Van Gent T, Scheek L, Visser P, Van Der Kamp A, Grosveld F, De Crom R. Increased risk of atherosclerosis by elevated plasma levels of phospholipid transfer protein. *J Biol Chem.* 2002;277:48938-48943.
4. Jiang XC, Qin S, Qiao C, Kawano K, Lin M, Skold A, Xiao X, Tall AR. Apolipoprotein B secretion and atherosclerosis are decreased in mice with phospholipid-transfer protein deficiency. *Nat Med.* 2001;7:847-852.
5. Lie J, De Crom R, Van Gent T, Van Haperen R, Scheek L, Lankhuizen I, Van Tol A. Elevation of plasma phospholipid transfer protein in transgenic mice increases VLDL secretion. *J Lipid Res.* 2002;43:1875-1880.
6. Kawano K, Qin SC, Lin M, Tall AR, Jiang X. Cholesteryl Ester Transfer Protein and Phospholipid Transfer Protein Have Nonoverlapping Functions in Vivo. *J Biol Chem.* 2000;275:29477-29481.
7. Lie J, de Crom R, Jauhiainen M, van Gent T, van Haperen R, Scheek L, Jansen H, Ehnholm C, van Tol A. Evaluation of phospholipid transfer protein and cholesteryl ester transfer protein as contributors to the generation of prebeta-high- density lipoproteins. *Biochem J.* 2001;360:379-385.
8. Jansen H, Verhoeven AJ, Sijbrands EJ. Hepatic lipase: a pro- or anti-atherogenic protein? *J Lipid Res.* 2002;43:1352-1362.
9. Jansen H. Hepatic lipase: friend or foe and under what circumstances? *Curr Atheroscler Rep.* 2004;6:343-347.
10. Santamarina-Fojo S, Gonzalez-Navarro H, Freeman L, Wagner E, Nong Z. Hepatic lipase, lipoprotein metabolism, and atherogenesis. *Arterioscler Thromb Vasc Biol.* 2004;24:1750-1754.
11. Homanics GE, de Silva HV, Osada J, Zhang SH, Wong H, Borensztajn J, Maeda N. Mild dyslipidemia in mice following targeted inactivation of the hepatic lipase gene. *J Biol Chem.* 1995;270:2974-2980.
12. Lie J, De Crom R, Van Gent T, Van Haperen R, Scheek L, Sadeghi-Niaraki F, Van Tol A. Elevation of plasma phospholipid transfer protein increases the risk of atherosclerosis in spite of lowering apolipoprotein B containing lipoproteins. *J Lipid Res.* 2004;45:805-811.
13. Dallinga-Thie GM, Zonneveld-de Boer AJ, van Vark-van der Zee LC, van Haperen R, van Gent T, Jansen H, De Crom R, van Tol A. Appraisal of hepatic lipase and lipoprotein lipase activities in mice. *J Lipid Res.* 2007;48:2788-2791.
14. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol.* 1959;37:911-917.
15. Groener JE, da Col PG, Kostner GM. A hyperalphalipoproteinaemic family with normal cholesteryl ester transfer/exchange activity. *Biochem J.* 1987;242:27-31.
16. Hasham SN, Pillarisetti S. Vascular lipases, inflammation and atherosclerosis. *Clin Chim Acta.* 2006;372:179-183.
17. Foger B, Santamarina-Fojo S, Shamburek RD, Parrot CL, Talley GD, Brewer HB, Jr. Plasma phospholipid transfer protein. Adenovirus-mediated overexpression in mice leads to decreased plasma high density lipoprotein (HDL) and enhanced hepatic uptake of phospholipids and cholesteryl esters from HDL. *J Biol Chem.* 1997;272:27393-27400.
18. Van Tol A, Jauhiainen M, De Crom R, Ehnholm C. Role of phospholipid transfer protein in high-density lipoprotein metabolism: insights from studies in transgenic mice. *Int J Tissue React.* 2000;22:79-84.

19. Qiu S, Bergeron N, Kotite L, Krauss RM, Bensadoun A, Havel RJ. Metabolism of lipoproteins containing apolipoprotein B in hepatic lipase-deficient mice. *J Lipid Res.* 1998;39:1661-1668.

20. Mezdour H, Jones R, Dengremont C, Castro G, Maeda N. Hepatic lipase deficiency increases plasma cholesterol but reduces susceptibility to atherosclerosis in apolipoprotein E-deficient mice. *J Biol Chem.* 1997;272:13570-13575.

21. Busch SJ, Barnhart RL, Martin GA, Fitzgerald MC, Yates MT, Mao SJ, Thomas CE, Jackson RL. Human hepatic triglyceride lipase expression reduces high density lipoprotein and aortic cholesterol in cholesterol-fed transgenic mice. *J Biol Chem.* 1994;269:16376-16382.

22. Braschi S, Couture N, Gambarotta A, Gauthier BR, Coffill CR, Sparks DL, Maeda N, Schultz JR. Hepatic lipase affects both HDL and ApoB-containing lipoprotein levels in the mouse. *Biochim Biophys Acta.* 1998;1392:276-290.

23. Ehnholm S, van Dijk KW, van 't Hof B, van der Zee A, Olkkonen VM, Jauhiainen M, Hofker M, Havekes L, Ehnholm C. Adenovirus mediated overexpression of human phospholipid transfer protein alters plasma HDL levels in mice. *J Lipid Res.* 1998;39:1248-1253.

24. Yang XP, Yan D, Qiao C, Liu RJ, Chen JG, Li J, Schneider M, Lagrost L, Xiao X, Jiang XC. Increased atherosclerotic lesions in apoE mice with plasma phospholipid transfer protein overexpression. *Arterioscler Thromb Vasc Biol.* 2003;23:1601-1607.

25. Jacobs RL, Devlin C, Tabas I, Vance DE. Targeted deletion of hepatic CTP:phosphocholine cytidylyltransferase alpha in mice decreases plasma high density and very low density lipoproteins. *J Biol Chem.* 2004;279:47402-47410.

26. Watts GF, Riches FM, Humphries SE, Talmud PJ, van Bockxmeer FM. Genotypic associations of the hepatic secretion of VLDL apolipoprotein B-100 in obesity. *J Lipid Res.* 2000;41:481-488.

27. Tilly-Kiesi M, Schaefer EJ, Knudsen P, Welty FK, Dolnikowski GG, Taskinen MR, Lichtenstein AH. Lipoprotein metabolism in subjects with hepatic lipase deficiency. *Metabolism.* 2004;53:520-525.

28. Ruel IL, Lamarche B, Mauger JF, Badellino KO, Cohn JS, Marcil M, Couture P. Effect of fenofibrate on plasma lipoprotein composition and kinetics in patients with complete hepatic lipase deficiency. *Arterioscler Thromb Vasc Biol.* 2005;25:2600-2607.

29. Barter PJ, Rye KA. Relationship between the concentration and antiatherogenic activity of high-density lipoproteins. *Curr Opin Lipidol.* 2006;17:399-403.

30. Jansen H, van Tol A, Hulsmann WC. On the metabolic function of heparin-releasable liver lipase. *Biochem Biophys Res Commun.* 1980;92:53-59.

31. Lewis GF. Determinants of plasma HDL concentrations and reverse cholesterol transport. *Curr Opin Cardiol.* 2006;21:345-352.

32. Dugi KA, Brandauer K, Schmidt N, Nau B, Schneider JG, Mentz S, Keiper T, Schaefer JR, Meissner C, Kather H, Bahner ML, Fiehn W, Kreuzer J. Low hepatic lipase activity is a novel risk factor for coronary artery disease. *Circulation.* 2001;104:3057-3062.

33. Riemens SC, van Tol A, Scheek LM, Dullaart RP. Plasma cholesteryl ester transfer and hepatic lipase activity are related to high-density lipoprotein cholesterol in association with insulin resistance in type 2 diabetic and non-diabetic subjects. *Scand J Clin Lab Invest.* 2001;61:1-9.

34. Al Majali K, Cooper MB, Staels B, Luc G, Taskinen MR, Betteridge DJ. The effect of sensitisation to insulin with pioglitazone on fasting and postprandial lipid metabolism, lipoprotein modification by lipases, and lipid transfer activities in type 2 diabetic patients. *Diabetologia.* 2006;49:527-537.

35. Colhoun HM, Taskinen MR, Otvos JD, Van Den Berg P, O'Connor J, Van Tol A. Relationship of phospholipid transfer protein activity to HDL and apolipoprotein B-containing lipoproteins in subjects with and without type 1 diabetes. *Diabetes.* 2002;51:3300-3305.

36. Schlitt A, Bickel C, Thumma P, Blankenberg S, Rupprecht HJ, Meyer J, Jiang XC. High plasma phospholipid transfer protein levels as a risk factor for coronary artery disease. *Arterioscler Thromb Vasc Biol.* 2003;23:1857-1862.

37. Tall AR, Lalanne F. Phospholipid transfer protein and atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2003;23:1484-1485.

Chapter 8

Reduction of HDL levels lowers plasma PLTP expression and affects its distribution among lipoproteins in mice

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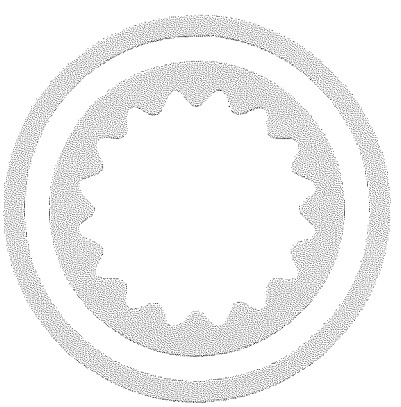
Submitted for publication



Reduction of HDL levels lowers plasma PLTP expression and affects its distribution among lipoproteins in mice

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Abstract

Phospholipid transfer protein (PLTP) is associated with HDL particles in plasma, where it transfers phospholipids between lipoproteins and remodels HDL particles. Tangier disease patients, with a mutated ABCA1 transporter, have extremely low plasma HDL concentration and reduced PLTP activity levels, a phenotype that is also observed in mice lacking ABCA1. We investigated whether low HDL levels and low PLTP activity are mechanistically related. Firstly, we studied PLTP expression and distribution among lipoproteins in mice lacking ABCA1 ($ABCA1^{-/-}$). Parallel to the strong reduction in PLTP activity in plasma of $ABCA1^{-/-}$ mice, decreased PLTP protein levels were observed. Neither PLTP synthesis in liver or macrophages nor the ability of the macrophages to secrete PLTP were impaired in $ABCA1^{-/-}$ mice. However, the PLTP activity level in the medium of cultured macrophages was determined by HDL levels in the medium. PLTP was associated with HDL particles in wild type mice, whereas in $ABCA1^{-/-}$ mice, PLTP was associated with VLDL and LDL particles. Secondly, we treated different mouse models with varying plasma HDL and PLTP levels (wild type, $ABCA1^{-/-}$, $apoE^{-/-}$ and PLTPtg mice, overexpressing human PLTP) with a synthetic LXR ligand, and investigated the relationship between LXR-mediated PLTP induction and HDL levels in plasma. Plasma PLTP activity in wild type mice was induced 5.6-fold after LXR activation, whereas in $ABCA1^{-/-}$, $apoE^{-/-}$ and PLTPtg mice, all having reduced HDL levels, induction of PLTP activity was 2.4- , 3.2- and 2.0-fold, respectively. The less pronounced PLTP induction in these mice compared to wild type mice was not caused by a decreased PLTP gene expression in liver or macrophages. Our findings

indicate that the extent of LXR-mediated PLTP induction depends on plasma HDL levels. In conclusion, we demonstrate that ABCA1 deficiency in mice affects plasma PLTP level and distribution through an indirect effect on HDL metabolism. In addition, we show that the extent of LXR-mediated PLTP induction is HDL-dependent. These findings indicate that plasma HDL level is an important regulator of plasma PLTP and might play a role in the stabilisation of PLTP in plasma.

Introduction

Tangier disease is a genetic disorder of which one of the main characteristics is the nearly complete absence of plasma high density lipoproteins (HDL).[1] The defective gene responsible for this disorder was identified as the ATP-binding cassette (ABC) transporter A1.[2-5] By mediating efflux of cellular phospholipids and cholesterol to lipid-free or lipid-poor apoAI, ABCA1 plays a pivotal role in the maturation of HDL particles. Moreover, macrophage-ABCA1 facilitates the removal of excess cholesterol from foam cells, thereby preventing the development of atherosclerosis.[6] Since the discovery of the role of ABCA1 in Tangier disease, many studies have been performed in animal models lacking ABCA1. Deficiency of ABCA1 in mice produces a phenotype comparable to that of human Tangier disease.[7,8] Besides the extremely low plasma HDL levels, a defective phospholipid transfer between lipoproteins has been found in plasma of Tangier disease patients and ABCA1 deficient mice, resulting from reduced phospholipid transfer protein (PLTP) activity.[9,10]
Plasma PLTP plays a key role in lipoprotein and HDL metabolism. Being an important HDL conversion factor, PLTP remodels HDL particles resulting in the formation of large fused α-HDL and small lipid-poor preβ-HDL particles.[11,12] Preβ-HDL is an efficient acceptor of cholesterol from lipid-loaded peripheral cells *in vitro*. Furthermore, it has been reported that PLTP is involved in ABCA1-dependent cholesterol efflux *in vitro*.[13,14] PLTP may also have a role in the formation of nascent HDL particles by the liver, as studied using PLTP deficient hepatocytes.[15]
However, the role of PLTP in HDL metabolism *in vivo* is more complex and not fully understood. PLTP deficient mice have reduced HDL levels, as a result of a defective maturation of HDL particles.[16] It has also been demonstrated that PLTP deficient HDL particles have enhanced anti-inflammatory properties.[17] Elevation of plasma PLTP activity in mice leads to an increased formation of preβ-HDL particles, but also to the hypercatabolism of mature α-HDL, the latter effect being postulated as the most likely cause of the increased susceptibility to atherosclerosis in mice overexpressing PLTP.[18-20]
ABCA1 and PLTP are both proteins with an essential role in HDL metabolism in liver and macrophages. We tried to clarify the mechanism by which PLTP activity levels are affected

by the absence of ABCA1, and demonstrated that PLTP levels and distribution in plasma of mice lacking ABCA1 are altered due to the strong reduction of HDL particles.
To further investigate the relationship between plasma HDL levels and PLTP expression, we activated the nuclear liver X receptor (LXR) *in vivo* in different mouse models. LXR is a nuclear receptor controlling the transcription of genes involved in the regulation of lipid metabolism and reverse cholesterol transport, the pathway in which cholesterol is transported from peripheral tissues to the liver for excretion.[21] PLTP and ABCA1 both have been identified as LXR target genes.[22-24] LXR activation in wild type mice is known to increase both plasma HDL levels and PLTP activity levels.[23] We treated different mouse models with varying plasma HDL and PLTP levels (wild type, ABCA1 deficient, apoE deficient and PLTP transgenic mice) with a synthetic LXR ligand, and investigated the relationship between LXR-mediated PLTP induction and HDL levels in plasma. Using this strategy, we demonstrated that LXR-induced elevation of plasma PLTP levels is influenced by plasma HDL levels in these mice.

Materials and methods

Animals

The generation of transgenic mice with expression of human PLTP (PLTPtg), controlled by its native promoter, has been described previously.[19] ApoE deficient mice (apoE$^{-/-}$) were purchased from Jackson Laboratory. ABCA1 deficient mice (ABCA1$^{-/-}$) were kindly donated by Dr. Francone.[7] All mice were crossed >15 generations to a C57Bl/6J background. Only male mice between 10 and 18 weeks old were used in the study. The animals were kept on regular chow diet, and had free access to food and water.
To perform plasma analyses, blood was taken from the orbital plexus after an overnight fasting period. To study the effects of LXR activation *in vivo*, mice were treated once daily with either synthetic LXR ligand TO-901317 ('T1317'; Cayman Chemical, ITK Diagnostics BV) at a dose of 50 mg/kg/day resuspended in sunflower seed oil (Sigma) with 5% ethanol, or vehicle alone given by oral gavage for 10 days. At day 11, mice were euthanized after overnight fasting. Blood was collected, peritoneal cells were harvested and livers were excised and stored at -80°C for later processing.
All animal experiments were carried out in compliance with national and institutional guidelines.

PLTP activity and mass

PLTP activity in plasma or culture medium was determined by measuring the transfer of radiolabelled phospholipids from liposomes to exogenous HDL, as described before.[25,26] PLTP activity is expressed as arbitrary units (AU) in plasma or AU in cell culture medium per milligram cell protein. One AU equals 13.9 mmol/L/h, the level of PLTP activity found in human reference plasma. Hepatic phospholipid transfer activity, calculated per mg liver tissue, was determined as described,[26] and expressed as AU. Hepatic phospholipid transfer activity of PLTP-/- mice (kindly donated by Dr. X-C. Jiang, Brooklyn, NY) was subtracted in order to express PLTP-specific activity. Human PLTP mass in plasma from PLTPtg mice was measured as described previously,[26,27] using a sandwich-type ELISA using 2 monoclonal antibodies with specificity for human PLTP. The concentration of plasma PLTP is expressed as μg/mL.

Isolation of unstimulated peritoneal cells, flow cytometric analysis and cell sorting

Resident peritoneal cells were washed from the unstimulated cavities of mice by peritoneal lavage with 5 ml of ice-cold sterile PBS. The lavage fluid from 2 to 4 mice was pooled and used for cell sorting after FACS analysis. Cells were washed with PBS (GibcoBRL), passed through a 0.2 μ-filter (Falcon), and washed subsequently with PBS containing 10% heat-inactivated filtered FCS (GibcoBRL) and 1% penicillin/streptomycin (GibcoBRL) ("PBS-FCS"). Cells were counted, and 3-6x 10^6 cells were transferred to FACS tubes. After centrifugation at 1200 rpm for 10 min. at 4°C, supernatant was removed and cells were incubated on ice with 100 μl of the primary rat mAb F4/80, a marker specific for mouse macrophages[28] for 30 min. Cells were washed 2x with PBS-FCS and incubated for another 30 min. with the secondary FITC-conjugated goat anti-rat IgG antibody (diluted 1:500), which was pre-incubated for 10 minutes at room temperature with 2% normal mouse serum. After washing 2 more times, cells were resuspended in 0.5 ml PBS-FCS. Flow cytometric analysis was performed using a FACScan (Becton Dickinson). Dead cells were eliminated by negatively selecting for Hoechst 33258 (1 μg/ml) (Molecular Probes) labelled cells. Cells were sorted based on their forward/side scatter and on their $F4/80^+$ expression. Sorted cells were dissolved in RLT buffer (Qiagen), after which they were immediately processed for quantitative RT-PCR.

Hepatic and macrophage mRNA expression analysis

Total RNA of liver and macrophages was isolated using an RNeasy® mini and micro kit (Qiagen), respectively. Quantitative RNA expression analyses were performed as described.[26] cDNA quantities were normalized to the amount of hypoxanthine-guanine phosphoribosyl transferase cDNA, using the ΔCt method $[2^{(-\Delta Ct)}]$, and presented as arbitrary units (AUs). Averages were taken from at least three individual runs, each sample in triplicate.

Macrophage cell culture and in vitro PLTP secretion experiments

For *in vitro* PLTP secretion experiments, bone marrow-derived or thioglycollate-elicited peritoneal macrophages from PLTPtg mice either expressing or lacking ABCA1 were harvested as described before.[29,30] Bone marrow-derived macrophages were plated onto 6-well dishes and cultured in the presence of Macrophage Colony Stimulating Factor (Biosource International) as described.[30] Thioglycollate-elicited peritoneal macrophages were plated onto 24-well dishes and loaded as described before.[31] Cells were incubated with medium consisting of DMEM, 0.2% BSA, either in the absence or the presence of 4% serum of wild type or ABCA1 deficient mice, or in the presence of different amounts of human HDL (1 to 20 μg/mL cholesterol). At different time points after addition of the medium, culture medium was collected and PLTP activity in the medium was quantified. In the experiments in which serum was added to the medium, control incubations with serum alone were included. PLTP activity measured in the wells with serum alone was subtracted in order to express macrophage-specific PLTP secretion. Cells were lysed in 0.1 mol/L NaOH with 1% SDS, and cell proteins were quantified.[31] PLTP activity is expressed as arbitrary units per milligram cell protein (AU/mg).

Separation of plasma lipoproteins and plasma lipid analyses

Plasma HDL and non-HDL fractions were separated by ultracentrifugation of freshly isolated plasma samples at a density of 1.063 g/ml.[18] Lipoprotein size distribution in pooled mouse plasma was determined by gel filtration chromatography as described before.[26] FPLC fractions were analysed for total cholesterol (TC) and PLTP activity levels. TC in plasma and fractions was measured enzymatically with a commercially available kit (Wako).

Statistics

All values are expressed as mean ± SD. Differences were analyzed by two-sample Wilcoxon ranksum tests using Intercooled Stata 8.2/SE software (Stata Corporation, College Station, Texas USA). Statistical significance was assumed when $p<0.05$.

Results

PLTP expression in ABCA1 deficient mice

We studied the effect of ABCA1 deficiency on PLTP expression and distribution in mice. In accordance with previous findings,[10] plasma PLTP activity level in mice lacking ABCA1 ($ABCA1^{-/-}$) was reduced by 85% compared to wild type (wt) mice. Deficiency of ABCA1 in mice overexpressing human PLTP (PLTPtg) also reduced total PLTP activity (figure1A). This reduction in PLTP activity was not caused by inactivation of plasma PLTP, as it

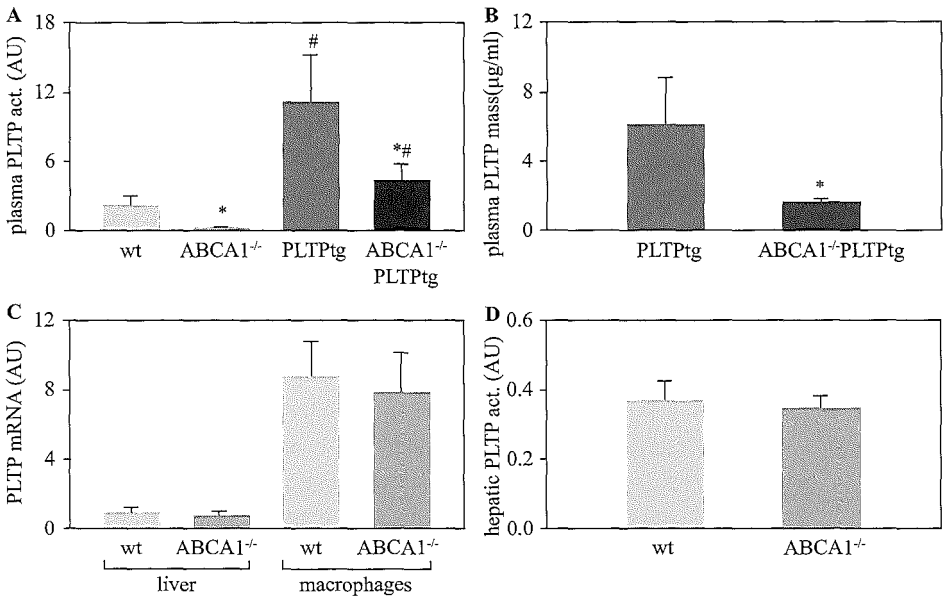

Figure 1. PLTP expression in mice with or without ABCA1. A) Plasma PLTP activity. Values are expressed as arbitrary units (AU) ± S.D. n=4-6 mice per group. *$p<0.001$ versus wt or PLTPtg; #$p<0.001$ versus wt or ABCA1$^{-/-}$. B) Plasma PLTP mass. Human PLTP protein was quantified using a Sandwich-type ELISA. Values are expressed as µg/mL ± S.D. n=4 mice per group. *$p<0.001$ versus PLTPtg. C) Hepatic and macrophage PLTP mRNA. The expression of PLTP in livers and FACS-sorted peritoneal macrophages was analyzed by real-time PCR. Values are expressed as arbitrary units (AU) ± S.D. n=3-4 mice per group. D) Hepatic PLTP activity. PLTP-mediated transfer in the liver is represented as the total phospholipid transfer activity in 1 mg liver tissue subtracted with the phospholipid transfer activity found in 1 mg liver tissue of PLTP deficient mice. Values are expressed as arbitrary units (AU) ± S.D. n=6 mice per group.

was associated with a similar decrease in PLTP mass in plasma of PLTPtg mice lacking ABCA1 (figure 1B). To investigate whether PLTP gene expression was affected by ABCA1 deficiency in mice, we isolated RNA from liver and peritoneal macrophages, two important sources of systemic PLTP,[18,30] and we quantified PLTP mRNA. Because thioglycollate-injection affects plasma PLTP activity levels,[30] we isolated non-elicited peritoneal cells and pooled the cells of 2 to 4 mice. We subjected the cells onto a FACS to sort the mature macrophages, and performed gene expression analyses. Hepatic and macrophage mRNA expression levels of murine PLTP were not different between wt and ABCA1$^{-/-}$ mice (figure 1C). Unlike plasma PLTP activity, hepatic PLTP activity levels were not different between the two groups (figure 1D). These results indicate that PLTP expression is not affected in ABCA1$^{-/-}$ mice, suggesting that the decrease in plasma PLTP levels in ABCA1$^{-/-}$ mice is

caused by a decreased secretion of PLTP into the circulation or by an increased catabolism of systemic PLTP.

PLTP secretion by macrophages

To study whether the reduced PLTP levels in ABCA1$^{-/-}$ mice could be explained by a decreased secretion of PLTP from cells lacking ABCA1, we cultured macrophages of mice overexpressing human PLTP either expressing or lacking ABCA1 (PLTPtg and ABCA1$^{-/-}$PLTPtg mice, respectively), as this is the cell type with the relatively highest PLTP expression (figure 1C). PLTP activity was measured in the culture medium of the macrophages at different time points. Surprisingly, we found slightly higher PLTP activity levels in medium from thioglycollate-elicited peritoneal macrophages of ABCA1$^{-/-}$PLTPtg mice after 8 and 24 hours compared to cells of PLTPtg mice (figure 2A). Similar results were found for PLTP activity from cultured bone marrow-derived macrophages (figure 2B). These findings demonstrate that the ability of ABCA1 deficient cells to secrete PLTP is not impaired and thus do not explain the decreased plasma PLTP levels in these mice. This also indicates that a systemic rather than a cellular effect accounts for the decrease in PLTP. We investigated whether PLTP activity levels are determined by HDL levels. Therefore, we cultured PLTPtg macrophages in the presence of serum from ABCA1$^{-/-}$ or wt mice, or in the presence of increasing HDL concentrations. Phospholipid transfer activity levels of secreted PLTP in the medium at all time points was approximately 25% lower in the presence of serum from ABCA1$^{-/-}$ mice compared to serum from wt mice. In the absence of serum, PLTP activity was significantly lower compared to serum from wt and ABCA1$^{-/-}$ mice (approximately 40 and 25%, respectively) (figure 2C). When adding increasing amounts of HDL to the culture medium, we found a dose-dependent increase in PLTP activity levels, which reached a maximum level at 10 μg/mL HDL-C (figure 2D). These findings indicate that PLTP activity levels are determined by HDL levels *in vitro* and that reduced PLTP levels in plasma of ABCA1 deficient mice might be explained by an indirect effect of ABCA1 via the reduction of plasma HDL levels.

PLTP distribution in ABCA1 deficient mice

To study whether the distribution of active PLTP among lipoproteins was affected by the reduced HDL levels in ABCA1$^{-/-}$ mice, we performed FPLC analyses on mouse plasma. As expected, in wt mice PLTP activity peaked in fractions 11-13, at the position of large HDL particles, indicating that PLTP is associated with HDL particles (figure 3A). However, in ABCA1 deficient mice PLTP was found mainly in fractions 5-8, at the position of LDL particles (figure 3B), indicating that PLTP can be associated with other lipoprotein particles when HDL particles are scarce or absent.

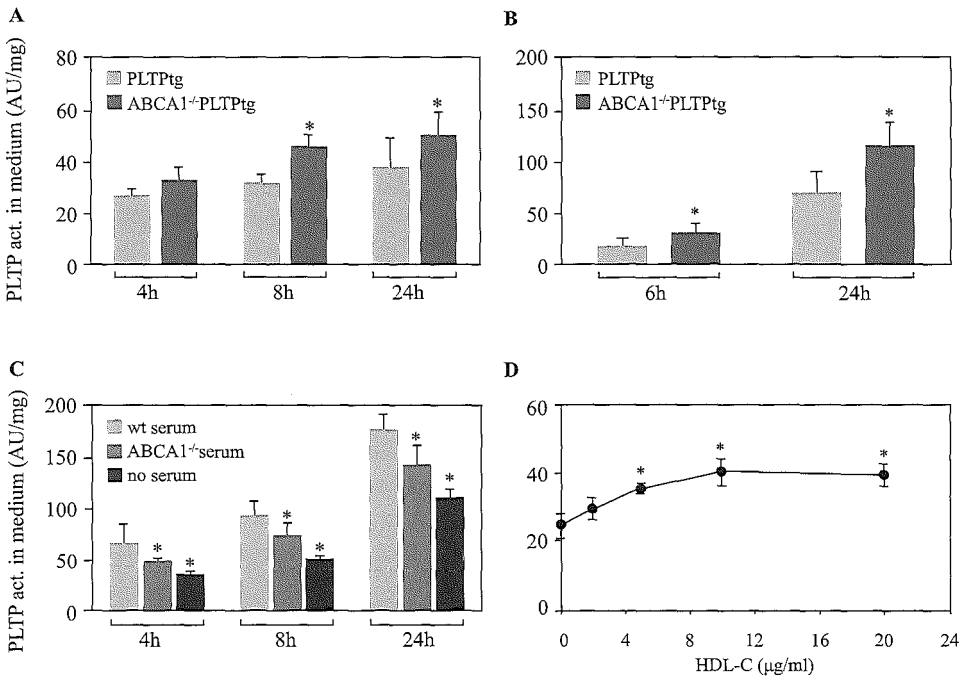

Figure 2. Secretion of active PLTP from macrophages *in vitro*.

(A-B) PLTP activity in culture medium of thioglycollate-elicited peritoneal macrophages (A) or bone marrow-derived macrophages (B) isolated from PLTPtg and ABCA1$^{-/-}$PLTPtg mice. *p<0.05 versus PLTPtg. (C-D) PLTP activity in culture medium of thioglycollate-elicited peritoneal macrophages of PLTPtg mice either in the absence or in the presence of serum from wt or ABCA1$^{-/-}$ mice (C), or in the presence of HDL (D). *p<0.05 versus serum from wt mice (C) or versus 0 µg/mL HDL cholesterol (D). Values are expressed as arbitrary units (AU) per milligram cell protein ± S.D. n= cells from 4-6 mice per group.

Effect of LXR activation on plasma PLTP

To investigate the relationship between plasma HDL levels and PLTP expression more into detail, we treated mouse models with varying plasma HDL and PLTP levels with a synthetic LXR ligand ('T1317'): wild type (wt), ABCA1 deficient (ABCA1$^{-/-}$), apoE deficient (apoE$^{-/-}$) and PLTPtg mice. After 10 days of treatment, plasma lipid and lipoprotein analyses were performed and the effect of lipoprotein levels on LXR-mediated PLTP induction was studied.

In accordance with previous findings,[23] activation of LXR in wt mice was associated with elevation of TC and the formation of enlarged HDL particles (Table 1+ figure 4A). T1317-treatment increased PLTP activity by 5.6-fold, and PLTP activity was found at the position of the large HDL particles (figure 4A).

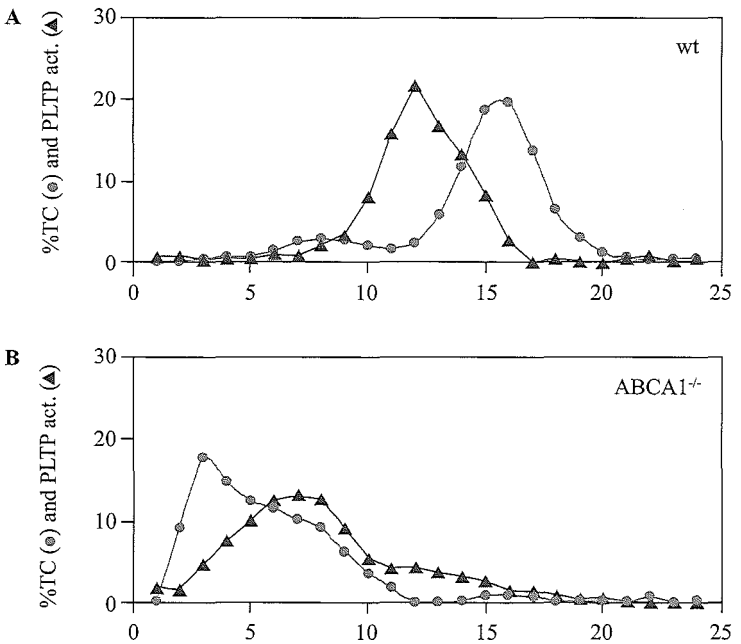

Figure 3. Distribution of active PLTP among plasma lipoprotein particles
Pooled plasma of wild type (A) and ABCA1$^{-/-}$ (B) mice was subjected to FPLC. TC (circles) and PLTP activity (triangles) were measured in the fractions. Values are represented as fractional percentages of total amount of TC and PLTP activity measured in the column eluate. n=4 mice per group.

ABCA1$^{-/-}$ mice have strongly reduced plasma cholesterol and HDL-C levels compared to wt mice (10% and 4% of wt levels, respectively), and plasma PLTP activity level is decreased (18% of wt). Treatment of ABCA1$^{-/-}$ mice with the synthetic LXR ligand resulted in increased TC levels compared to non-treated animals, but only in the VLDL-sized fractions (Fig.4B). T1317-treatment increased PLTP activity only 2.4-fold in ABCA1$^{-/-}$ mice. Importantly, in T1317-treated ABCA1$^{-/-}$ mice, PLTP was associated with VLDL and LDL particles (figure 4B).
ApoE$^{-/-}$ mice have increased TC levels (300% of wt) but reduced HDL-C levels (18% of wt), and plasma PLTP activity levels is 64% of the level in wild type mice. Treatment of these mice with the T1317 compound led to increased plasma TC levels, due to the induction of TC in the non-HDL sized fractions. Plasma PLTP activity level was increased 3.2-fold by LXR stimulation. Despite the high non-HDL/HDL ratio in apoE$^{-/-}$ mice, PLTP was found at the position of HDL particles (figure 4C).

PLTPtg mice have elevated PLTP activity levels (350% compared to wt) and reduced HDL levels (50% compared to wt). After treatment of PLTPtg mice with an LXR agonist, TC levels were increased. HDL-cholesterol levels were slightly increased, and presence of enlarged HDL was seen on gel filtration profiles (figure 4C). Total PLTP activity in T1317-treated PLTPtg mice was 2.0-fold higher than in non-treated PLTPtg mice. Also PLTP mass, representing the level of human PLTP protein in plasma,[27] was increased in treated mice (Table 1). Despite the high PLTP levels and reduced HDL levels compared to wt mice, PLTP was found associated with HDL particles (figure 4C). These results demonstrate that the strongest induction of PLTP after LXR stimulation is found when HDL levels are high. FPLC analyses demonstrated that PLTP is associated with HDL particles except in ABCA1$^{-/-}$ mice, suggesting a main role of HDL in determining PLTP position among lipoprotein particles.

Although PLTP is induced in all mice after LXR stimulation, the increase in plasma PLTP activity levels is less pronounced in mice with lower HDL levels (3.2-, 2.4- and 2.0-fold in apoE$^{-/-}$, ABCA1$^{-/-}$ and PLTPtg mice, respectively) than in wt mice (5.6-fold). We investigated whether these differences in induction of plasma PLTP after T1317-treatment could be explained by a reduced transcription of PLTP in mice with reduced HDL levels. The increase of mouse PLTP mRNA levels upon treatment was not significantly different in liver or macrophages between the three groups (data not shown).

Table 1. Plasma PLTP and lipids

genotype	treatment	total plasma TC (mmol/L)	d>1.063 TC (mmol/L)	plasma PLTP activity (AU)	plasma PLTP mass (µg/mL)
wt	- T1317	3.8 ± 0.6	2.8 ± 0.2	233 ± 54	N/A
	+ T1317	5.8 ± 0.8 [b]	3.6 ± 0.5 [b]	1313 ± 244 [b]	N/A
ABCA1$^{-/-}$	- T1317	0.4 ± 0.1 [a]	0.1 ± 0.1 [a]	43 ± 9 [a]	N/A
	+ T1317	1.1 ± 0.2 [a,b]	0.2 ± 0.1 [a]	103 ± 34 [a,b]	N/A
ApoE$^{-/-}$	- T1317	11.4 ± 1.0 [a]	0.5 ± 0.1 [a]	149 ± 26 [a]	N/A
	+ T1317	30.0 ± 3.4 [a,b]	0.7 ± 0.1 [a]	482 ± 115 [a,b]	N/A
PLTPtg	- T1317	2.0 ± 0.3 [a]	1.4 ± 0.2 [a]	809 ± 351 [a]	30 ± 4
	+ T1317	2.9 ± 0.8 [a,b]	1.7 ± 0.3 [a,b]	1620 ± 873 [a,b]	78 ± 11 [b]

Values represent mean ± SD

[a] $p < 0.05$ compared to wild type mice

[b] $p < 0.05$ compared to - T1317

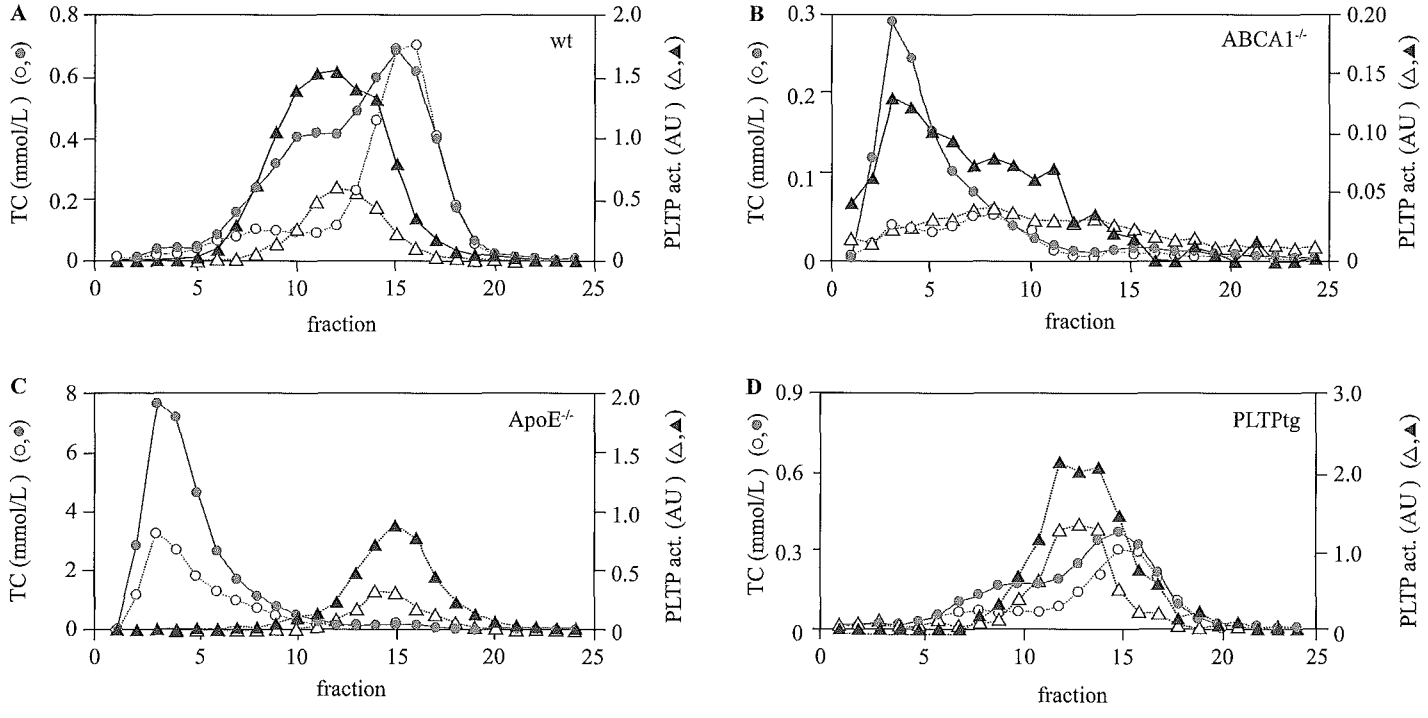

Figure 4. Effect of LXR stimulation on PLTP distribution among plasma lipoprotein particles

Pooled plasma of wild type (A), ABCA1$^{-/-}$ (B), apoE$^{-/-}$ (C), and PLTPtg (D) mice treated with synthetic LXR agonist TO-901317 ('T1317'; 50 mg/kg/day) or placebo (sunflower seed oil +5% EtOH) was subjected to FPLC. TC and PLTP activity were measured in the fractions.

Open circles: TC-T1317; open triangles: PLTP activity-T1317; closed circles: TC+T1317; closed triangles: PLTP activity+T1317. TC values are expressed as mmol/L and values of PLTP activity as arbitrary units (AU). n=3-4 mice per group.

Discussion

In the first part of our study, we report that 1) PLTP activity and protein levels are reduced in ABCA1 deficient mice; 2) this is not caused by a decreased gene expression of PLTP in liver or macrophages, nor by an impaired ability of macrophages to secrete PLTP *in vitro*; 3) PLTP activity levels in the medium of macrophage cultures depend on HDL levels in the medium; 4) PLTP is associated with HDL particles in wild type mice, whereas in ABCA1 deficient mice, PLTP is associated with VLDL and LDL particles.

Reduced PLTP activity levels in patients with Tangier disease and in mice lacking ABCA1 have been reported previously,[9,10] but it was unknown whether this is associated with decreased PLTP protein levels or with the inactivation of PLTP. Because our assay to quantify PLTP protein levels is dependent on monoclonal antibodies specific for human PLTP, we used mice expressing human PLTP, and found that plasma PLTP protein levels are reduced in mice lacking ABCA1 compared to mice with functional ABCA1. We subsequently studied PLTP gene expression in liver and macrophages, two important sources of PLTP synthesis, from both mouse models. We previously generated mice with the overexpression of human PLTP specifically in the liver[18] or in macrophages,[30] and showed that human PLTP contributed significantly to systemic PLTP activity levels in both models. In the present study, we did not find differences in PLTP mRNA expression in liver or macrophages between ABCA1 deficient mice and wild type mice, neither in the hepatic PLTP activity, indicating that PLTP production is not affected in the absence of ABCA1. Hence, we investigated the possibility that PLTP secretion by ABCA1 deficient macrophages is decreased, but found that the ability of macrophages to secrete PLTP *in vitro* is not impaired by ABCA1 deficiency. We cannot exclude the possibility that PLTP interacts with ABCA1 at the cell surface, as demonstrated previously by *in vitro* experiments,[14] which could explain the lower PLTP activity levels in the medium of ABCA1 expressing cells compared to medium of ABCA1 deficient cells. However, we also demonstrated that the levels of active PLTP in the medium of cultured macrophages are increased by HDL in the medium. HDL may either directly stimulate PLTP secretion, or stabilize or activate secreted PLTP. HDL-dependent activation of PLTP is probably not the explanation, because plasma PLTP mass *in vivo* is also reduced in the absence of ABCA1, as shown in our mouse models. PLTP is associated with large, mature HDL particles in wild type mice. In ABCA1$^{-/-}$ mice however, in which HDL levels are reduced to less than 5% of wild type levels, we found that PLTP was associated mainly with LDL particles. Therefore we decided to study the relationship between PLTP and HDL levels more into detail.

In the second part of our study, we activated LXR in mice, thereby inducing changes in lipoprotein metabolism and PLTP expression. We investigated whether induced PLTP levels and PLTP distribution in plasma were influenced by the plasma lipoprotein levels

and distribution. We report that 1) LXR activation induces changes both in lipoprotein metabolism and in PLTP expression in mice; 2) the increase in plasma PLTP activity levels is less pronounced in mice with reduced HDL levels; 3) murine PLTP gene expression in liver and macrophages upon LXR activation is induced to the same extent in all mice; 4) Plasma HDL is an important determinating factor of the distribution of PLTP among the different lipoprotein classes.

PLTP has been identified as a target gene of LXR in liver and macrophages,[22,23] together with a whole network of other proteins with an important role in cholesterol metabolism and reverse cholesterol transport. Administration of a synthetic LXR ligand in wild type mice results in an increase in HDL level, HDL-enlargement and enhancement of macrophage reverse cholesterol transport.[23,32] An important role for PLTP in LXR-mediated HDL enlargement in wild type mice has been suggested.[23] We found that PLTP was induced in all mice, even in the almost complete absence of HDL. However, plasma PLTP activity after LXR treatment was less induced in ABCA1$^{-/-}$, apoE$^{-/-}$ and PLTPtg mice compared to wt mice. Increase in liver and macrophage PLTP mRNA expression after treatment was similar in all mice, demonstrating that PLTP is a direct LXR target gene and the LXR ligand probably induces PLTP synthesis to the same extent in all mice. Therefore, the difference in extent to which plasma PLTP is increased after LXR activation is most likely a systemic effect, and is correlated with plasma HDL levels. It is remarkable that in PLTPtg mice with HDL levels of about 50% of wild type mice, plasma PLTP activity was increased only 2-fold after LXR activation, whereas in ABCA1$^{-/-}$ mice with extremely low HDL levels, PLTP was increased 2.4-fold. The fact that absolute plasma PLTP activity levels in non-treated PLTPtg mice are already much higher than in other mouse models (3.5- and 18.8-fold higher than in wt and ABCA1$^{-/-}$ mice, respectively), could explain this finding.

Furthermore, we demonstrated that plasma HDL is an important determinating factor of the distribution of PLTP among lipoprotein particles. In wt mice, PLTP activity peaked at the position of large HDL particles, which is in line with findings in human plasma.[25,27] After T1317-treatment, more enlarged HDL particles were found and PLTP activity peak was broader, but no clear leftward shift was observed. In apoE$^{-/-}$ and PLTPtg mice, PLTP was also found associated with HDL particles. Elevation of plasma non-HDL levels (in apoE$^{-/-}$ mice) or elevation of PLTP activity levels (in PLTPtg mice) by LXR activation did not affect the position of PLTP on HDL. In ABCA1$^{-/-}$ mice, LXR activation led to an increase in VLDL levels, in accordance to previous findings,[33] and PLTP was found associated mainly with these VLDL particles. The exact reason of the leftward shift of the PLTP activity peak to larger lipoprotein classes after treatment is not clear, but might be due to the change in the composition of VLDL particles after treatment with an LXR agonist.[34] It has been reported previously that HDL in ABCA1$^{-/-}$ mice is present as small lipid-poor preβ-HDL, with no detectable HDL within the larger α-HDL size interval.[10] The absence of

large, mature α-HDL particles most likely accounts for the unusual position of active PLTP in plasma of ABCA1 deficient mice.

PLTP has multiple effects, cellular and systemic, which all include "association" of PLTP with HDL particles. The importance of PLTP in HDL metabolism is intensively studied *in vitro*, and PLTP might interfere in several steps of the reverse cholesterol transport pathway. A role for macrophage-PLTP is suggested in the ABCA1-dependent cholesterol efflux, where PLTP may function to shuttle lipids between cells and existing HDL particles.[35] Furthermore, PLTP remodels HDL particles with the generation of cholesterol acceptor particles.[11,12] Also a role of PLTP in the retention of HDL on atherosclerotic plaque extracellular matrix is suggested, as PLTP may mediate HDL binding to cellular proteoglycans *in vitro*.[36] In addition, PLTP may also play an important role in the maturation of HDL particles, by mediating early lipidation of apoAI.[15] HDL particles may stabilize PLTP but when present at high concentrations, PLTP on its turn may destabilize HDL particles, resulting in an increased catabolism.[20] In our study, we found that LXR activation in PLTPtg mice was associated with increased HDL levels as well as high plasma PLTP activity levels. Activation of LXR induces several anti-inflammatory and anti-atherogenic mechanisms in mice.[37] Whether either these LXR-mediated anti-atherogenic effects or the pro-atherogenic effects of high systemic PLTP levels predominate in the process of atherogenesis, needs to be further investigated.

In the present study, we demonstrated the importance of HDL levels for PLTP levels, PLTP distribution among lipoprotein particles, and LXR-mediated PLTP induction in plasma. These findings indicate that plasma HDL level is an important regulator of PLTP and that HDL particles might play a role in the stabilisation of PLTP in plasma.

References

1. Hoffman HN, Fredrickson DS. Tangier disease (familial high density lipoprotein deficiency). Clinical and genetic features in two adults. *Am J Med.* 1965;39:582-593.
2. Marcil M, Brooks-Wilson A, Clee SM, Roomp K, Zhang LH, Yu L, Collins JA, van Dam M, Molhuizen HO, Loubster O, Ouellette BF, Sensen CW, Fichter K, Mott S, Denis M, Boucher B, Pimstone S, Genest J, Jr., Kastelein JJ, Hayden MR. Mutations in the ABC1 gene in familial HDL deficiency with defective cholesterol efflux. *Lancet.* 1999;354:1341-1346.
3. Brooks-Wilson A, Marcil M, Clee SM, Zhang LH, Roomp K, van Dam M, Yu L, Brewer C, Collins JA, Molhuizen HO, Loubser O, Ouelette BF, Fichter K, Ashbourne-Excoffon KJ, Sensen CW, Scherer S, Mott S, Denis M, Martindale D, Frohlich J, Morgan K, Koop B, Pimstone S, Kastelein JJ, Genest J, Jr., Hayden MR. Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat Genet.* 1999;22:336-345.
4. Bodzioch M, Orso E, Klucken J, Langmann T, Bottcher A, Diederich W, Drobnik W, Barlage S, Buchler C, Porsch-Ozcurumez M, Kaminski WE, Hahmann HW, Oette K, Rothe G, Aslanidis C, Lackner KJ, Schmitz G. The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat Genet.* 1999;22:347-351.
5. Rust S, Rosier M, Funke H, Real J, Amoura Z, Piette JC, Deleuze JF, Brewer HB, Duverger N, Denefle P, Assmann G. Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. *Nat Genet.* 1999;22:352-355.
6. Cavelier C, Lorenzi I, Rohrer L, von Eckardstein A. Lipid efflux by the ATP-binding cassette transporters ABCA1 and ABCG1. *Biochim Biophys Acta.* 2006;1761:655-666.
7. McNeish J, Aiello RJ, Guyot D, Turi T, Gabel C, Aldinger C, Hoppe KL, Roach ML, Royer LJ, de Wet J, Broccardo C, Chimini G, Francone OL. High density lipoprotein deficiency and foam cell accumulation in mice with targeted disruption of ATP-binding cassette transporter-1. *Proc Natl Acad Sci U S A.* 2000;97:4245-4250.
8. Orso E, Broccardo C, Kaminski WE, Bottcher A, Liebisch G, Drobnik W, Gotz A, Chambenoit O, Diederich W, Langmann T, Spruss T, Luciani MF, Rothe G, Lackner KJ, Chimini G, Schmitz G. Transport of lipids from golgi to plasma membrane is defective in tangier disease patients and Abc1-deficient mice. *Nat Genet.* 2000;24:192-196.
9. von Eckardstein A, Chirazi A, Schuler-Luttmann S, Walter M, Kastelein JJ, Geisel J, Real JT, Miccoli R, Noseda G, Hobbel G, Assmann G. Plasma and fibroblasts of Tangier disease patients are disturbed in transferring phospholipids onto apolipoprotein A-I. *J Lipid Res.* 1998;39:987-998.
10. Francone OL, Subbaiah PV, van Tol A, Royer L, Haghpassand M. Abnormal phospholipid composition impairs HDL biogenesis and maturation in mice lacking Abca1. *Biochemistry.* 2003;42:8569-8578.
11. Jauhiainen M, Metso J, Pahlman R, Blomqvist S, van Tol A, Ehnholm C. Human plasma phospholipid transfer protein causes high density lipoprotein conversion. *J Biol Chem.* 1993;268:4032-4036.
12. Tu AY, Nishida HI, Nishida T. High density lipoprotein conversion mediated by human plasma phospholipid transfer protein. *J Biol Chem.* 1993;268:23098-23105.
13. 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.
14. Oram JF, Wolfbauer G, Vaughan AM, Tang C, Albers JJ. Phospholipid transfer protein interacts with and stabilizes ATP-binding cassette transporter A1 and enhances cholesterol efflux from cells. *J Biol Chem.* 2003;278:52379-52385.

15. Siggins S, Bykov I, Hermansson M, Somerharju P, Lindros K, Miettinen TA, Jauhiainen M, Olkkonen VM, Ehnholm C. Altered hepatic lipid status and apolipoprotein A-I metabolism in mice lacking phospholipid transfer protein. *Atherosclerosis.* 2007;190:114-123.

16. Jiang XC, Qin S, Qiao C, Kawano K, Lin M, Skold A, Xiao X, Tall AR. Apolipoprotein B secretion and atherosclerosis are decreased in mice with phospholipid-transfer protein deficiency. *Nat Med.* 2001;7:847-852.

17. Yan D, Navab M, Bruce C, Fogelman AM, Jiang XC. PLTP deficiency improves the anti-inflammatory properties of HDL and reduces the ability of LDL to induce monocyte chemotactic activity. *J Lipid Res.* 2004;45:1852-1858.

18. van Haperen R, van Tol A, van Gent T, Scheek L, Visser P, van der Kamp A, Grosveld F, de Crom R. Increased risk of atherosclerosis by elevated plasma levels of phospholipid transfer protein. *J Biol Chem.* 2002;277:48938-48943.

19. van Haperen R, van Tol A, Vermeulen P, Jauhiainen M, van Gent T, van den Berg P, Ehnholm S, Grosveld F, van der Kamp A, de Crom R. Human plasma phospholipid transfer protein increases the antiatherogenic potential of high density lipoproteins in transgenic mice. *Arterioscler Thromb Vasc Biol.* 2000;20:1082-1088.

20. Foger B, Santamarina-Fojo S, Shamburek RD, Parrot CL, Talley GD, Brewer HB, Jr. Plasma phospholipid transfer protein. Adenovirus-mediated overexpression in mice leads to decreased plasma high density lipoprotein (HDL) and enhanced hepatic uptake of phospholipids and cholesteryl esters from HDL. *J Biol Chem.* 1997;272:27393-27400.

21. Wang X, Rader DJ. Molecular regulation of macrophage reverse cholesterol transport. *Curr Opin Cardiol.* 2007;22:368-372.

22. Laffitte BA, Joseph SB, Chen M, Castrillo A, Repa J, Wilpitz D, Mangelsdorf D, Tontonoz P. The phospholipid transfer protein gene is a liver X receptor target expressed by macrophages in atherosclerotic lesions. *Mol Cell Biol.* 2003;23:2182-2191.

23. Cao G, Beyer TP, Yang XP, Schmidt RJ, Zhang Y, Bensch WR, Kauffman RF, Gao H, Ryan TP, Liang Y, Eacho PI, Jiang XC. Phospholipid transfer protein is regulated by liver X receptors in vivo. *J Biol Chem.* 2002;277:39561-39565.

24. Costet P, Luo Y, Wang N, Tall AR. Sterol-dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor. *J Biol Chem.* 2000;275:28240-28245.

25. Speijer H, Groener JE, van Ramshorst E, van Tol A. Different locations of cholesteryl ester transfer protein and phospholipid transfer protein activities in plasma. *Atherosclerosis.* 1991;90:159-168.

26. Samyn H, Moerland M, van Gent T, van Haperen R, Metso J, Grosveld F, Jauhiainen M, van Tol A, de Crom R. Plasma phospholipid transfer activity is essential for increased atherogenesis in PLTP transgenic mice: a mutation-inactivation study. *J Lipid Res.* 2008;49:2504-2512.

27. Oka T, Kujiraoka T, Ito M, Egashira T, Takahashi S, Nanjee MN, Miller NE, Metso J, Olkkonen VM, Ehnholm C, Jauhiainen M, Hattori H. Distribution of phospholipid transfer protein in human plasma: presence of two forms of phospholipid transfer protein, one catalytically active and the other inactive. *J Lipid Res.* 2000;41:1651-1657.

28. Austyn JM, Gordon S. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur J Immunol.* 1981;11:805-815.

29. McCarron RM, Goroff DK, Luhr JE, Murphy MA, Herscowitz HB. Methods for the collection of peritoneal and alveolar macrophages. *Methods Enzymol.* 1984;108:274-284.

30. van Haperen R, Samyn H, Moerland M, van Gent T, Peeters M, Grosveld F, van Tol A, de Crom R. Elevated expression of phospholipid transfer protein in bone marrow derived cells causes atherosclerosis. *PLoS ONE.* 2008;3:e2255.

31. Moerland M, Samyn H, van Gent T, Jauhiainen M, Metso J, van Haperen R, Grosveld F, van Tol A, de Crom R. Atherogenic, enlarged, and dysfunctional HDL in human PLTP/apoA-I double transgenic mice. *J Lipid Res.* 2007;48:2622-2631.

32. Naik SU, Wang X, Da Silva JS, Jaye M, Macphee CH, Reilly MP, Billheimer JT, Rothblat GH, Rader DJ. Pharmacological activation of liver X receptors promotes reverse cholesterol transport in vivo. *Circulation.* 2006;113:90-97.

33. Plosch T, Kok T, Bloks VW, Smit MJ, Havinga R, Chimini G, Groen AK, Kuipers F. Increased hepatobiliary and fecal cholesterol excretion upon activation of the liver X receptor is independent of ABCA1. *J Biol Chem.* 2002;277:33870-33877.

34. Grefhorst A, Elzinga BM, Voshol PJ, Plosch T, Kok T, Bloks VW, van der Sluijs FH, Havekes LM, Romijn JA, Verkade HJ, Kuipers F. Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. *J Biol Chem.* 2002;277:34182-34190.

35. Oram JF, Wolfbauer G, Tang C, Davidson WS, Albers JJ. An amphipathic helical region of the N-terminal barrel of phospholipid transfer protein is critical for ABCA1-dependent cholesterol efflux. *J Biol Chem.* 2008;283:11541-11549.

36. O'Brien KD, Vuletic S, McDonald TO, Wolfbauer G, Lewis K, Tu AY, Marcovina S, Wight TN, Chait A, Albers JJ. Cell-associated and extracellular phospholipid transfer protein in human coronary atherosclerosis. *Circulation.* 2003;108:270-274.

37. Tontonoz P, Mangelsdorf DJ. Liver X receptor signaling pathways in cardiovascular disease. *Mol Endocrinol.* 2003;17:985-993.

Chapter 9

Elevated expression of phospholipid transfer protein in bone marrow derived cells causes atherosclerosis

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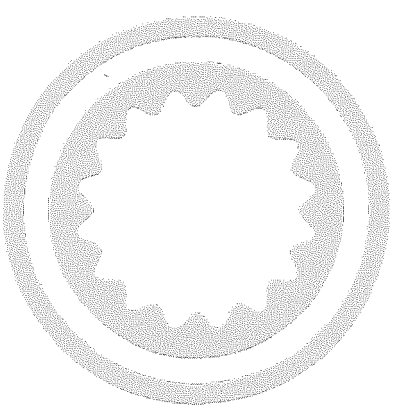

Elevated Expression of Phospholipid Transfer Protein in Bone Marrow Derived Cells Causes Atherosclerosis

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Abstract

Background: Phospholipid transfer protein (PLTP) is expressed by various cell types. In plasma, it is associated with high density lipoproteins (HDL). Elevated levels of PLTP in transgenic mice result in decreased HDL and increased atherosclerosis. PLTP is present in human atherosclerotic lesions, where it seems to be macrophage derived. The aim of the present study is to evaluate the atherogenic potential of macrophage derived PLTP.

Methods and Findings: Here we show that macrophages from human PLTP transgenic mice secrete active PLTP. Subsequently, we performed bone marrow transplantations using either wild type mice (PLTPwt/wt), hemizygous PLTP transgenic mice (huPLTPtg/wt) or homozygous PLTP transgenic mice (huPLTPtg/tg) as donors and low density lipoprotein receptor deficient mice (LDLR−/−) as acceptors, in order to establish the role of PLTP expressed by bone marrow derived cells in diet-induced atherogenesis. Atherosclerosis was increased in the huPLTPtg/wt→LDLR−/− mice (2.3-fold) and even further in the huPLTPtg/tg→LDLR−/− mice (4.5-fold) compared with the control PLTPwt/wt→LDLR−/− mice (both $P<0.001$). Plasma PLTP activity levels and non-HDL cholesterol were increased and HDL cholesterol decreased compared with controls (all $P<0.01$). PLTP was present in atherosclerotic plaques in the mice as demonstrated by immunohistochemistry and appears to co-localize with macrophages. Isolated macrophages from PLTP transgenic mice do not show differences in cholesterol efflux or in cytokine production. Lipopolysaccharide activation of macrophages results in increased production of PLTP. This effect was strongly amplified in PLTP transgenic macrophages.

Conclusions: We conclude that PLTP expression by bone marrow derived cells results in atherogenic effects on plasma lipids, increased PLTP activity, high local PLTP protein levels in the atherosclerotic lesions and increased atherosclerotic lesion size.

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Introduction

Phospholipid transfer protein (PLTP) is a plasma protein with the capacity to transfer phospholipids between liposomes and lipoproteins in vitro [1–4]. In addition, PLTP can also transfer vitamin E [5] and is active as a conversion factor of high density lipoproteins (HDL) [6,7]. HDL are generally considered anti-atherogenic lipoproteins by virtue of their role in cholesterol excretion (reverse cholesterol transport) [8], although other anti-atherosclerotic properties of HDL have been described as well [9,10]. Therefore, it was suggested that PLTP has a role in the development of atherosclerosis, based on its relation with HDL function [11,12]. In vivo, phospholipids become available for PLTP-mediated transfer during lipolysis of triglyceride-rich lipoproteins, mostly chylomicrons and very low density lipoproteins (VLDL), by the enzyme lipoprotein lipase [12]. The phospholipids are transferred from triglyceride-rich lipoproteins to HDL. PLTP is able to bind to and facilitate the transfer of several types of phospholipids, including phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol, as well as sphingomyeline [13]. In plasma, PLTP is mostly bound to HDL [11].

In genetically modified mouse models the relation between the activity levels of PLTP in plasma, HDL levels, HDL subclass distribution and the development of atherosclerosis was studied in more detail. We found that a 2.5-fold increased plasma PLTP activity in transgenic mice resulted in a 30% decrease in plasma HDL-cholesterol levels compared with wild type animals [14]. This represents total HDL, however. The formation of a specific subfraction of HDL, termed preβ-HDL, appeared to be increased in plasma from transgenic mice. Although preβ-HDL is a quantitatively minor HDL subfraction, it is believed to be a very efficient acceptor of cellular cholesterol and therefore an elevated production of preβ-HDL might result in a strong induction of reverse cholesterol transport [15]. Therefore, we suggested that PLTP increased the anti-atherogenic potential of HDL [14]. Subsequently Jiang and co-workers showed that PLTP deficiency was anti-atherogenic, however [16], in spite of decreased levels of

plasma HDL. The effect was attributed to a reduced production of apolipoprotein (apo)B containing lipoproteins by the liver. Indeed, we provided further evidence of an atherogenic role of PLTP in studies in a series of PLTP transgenic mouse lines with increasing levels of plasma PLTP activity, showing a PLTP-dose dependent enhancement of atherosclerosis [17]. We also provided in vivo evidence that PLTP is involved in the secretion of very low density lipoproteins (VLDL) [17,18], but there was no PLTP-dose dependent increase of this effect in the series of PLTP transgenic mice tested [17]. In contrast, we observed a clear PLTP-dose dependent reduction of plasma HDL levels in parallel to the induction of atherosclerosis and therefore we concluded that elevated plasma PLTP activity in transgenic mice is atherogenic because it decreases plasma HDL. PLTP could also influence the atherogenicity of plasma lipoproteins by decreasing the vitamin E content of apoB-containing lipoproteins, resulting in increased susceptibility for oxidation [19].

PLTP is expressed in a wide variety of cells and tissues in humans [20], mice [21] and human PLTP transgenic mice [14]. In addition, the presence of PLTP in human atherosclerotic lesions has been demonstrated [22,23], which probably originates from macrophages. In order to elucidate the role of PLTP derived from bone marrow derived cells, including macrophages, to atherosclerosis, we performed in vitro studies with macrophages from PLTP transgenic mice and performed bone marrow transplantations from PLTP transgenic mice to low density lipoprotein (LDL) receptor deficient mice and subsequently studied the process of diet-induced atherosclerosis as influenced by these bone marrow transplantations.

Methods

Mice

$LDLR^{-/-}$ mice were obtained from the Jackson Laboratory (Bar Arbor, ME) and were in C57BL6/J background. Mice expressing enhanced green fluorescent protein (EGFP) under the control of the chicken beta-actin promotor and cytomegalovirus enhancer were originally generated by Okabe et al. and were in C57BL6/J background [24,25]. Human PLTP transgenic mice were generated in our laboratory as described before and are derived from the P4 line [17]. The transgene in these animals contains the complete human PLTP gene, including approximately 15 kb 5′ natural flanking region and 3 kb 3′ natural flanking region. The transgene is driven by its autologous human promoter. PLTP transgenic mice were crossbred to C57BL6/J background for >15 generations. Subsequently, homozygous PLTP transgenic mice were obtained by crossbreeding hemizygous PLTP transgenic mice. Animals were provided with food and water ad libitum. Food was either regular chow, or a high fat, high cholesterol diet containing 40% (w/w) sucrose, 15% (w/w) cocoa butter, and 0.25% (w/w) cholesterol (diet W; Hope Farms, Woerden, The Netherlands). The numbers of animals used for measurements are indicated in the Figure legends. Only male mice were used. All of the procedures in this study were in accordance with national and institutional guidelines.

Bone marrow transplantation

On the day of donor cell injection, $LDLR^{-/-}$ mice of 12 weeks old were conditioned by 900 rads of γ-irradiation from a ^{137}Cs source, which was administered in a split dose, with a 3 hours interval. Cells were injected i.v. into the tail veins. Each recipient received 5×10^6 bone marrow cells isolated from the femurs and tibias of the primary donors. Injected animals were provided with 0.16% Neomycin (Sigma-Aldrich)-supplemented water. Animals were kept on a regular chow diet until 9 weeks after transplantation. During the next 9 weeks, animals were fed a high fat, high cholesterol diet.

Analysis of plasma lipids, lipoproteins, PLTP concentration and PLTP activity

Blood samples were obtained via orbital bleedings after an overnight fast at 1 week before the bone marrow transplantation, just before the start of the diet and at the end of the 9 weeks high fat, high cholesterol diet feeding. Plasma lipids (total cholesterol, phospholipids and triglycerides) were measured using commercially available kits (Wako Chemicals). Lipoprotein fractions were obtained by ultracentrifugation of plasma samples in a Beckman 42.2 Ti rotor (42000 rpm, 3 h, 12°C) at d = 1.063 g/ml. Tubes were sliced, and two fractions were collected: non-HDL (VLDL+LDL), d<1.063 g/ml; and HDL, d>1.063 g/ml. PLTP activity in plasma was measured as described previously [14] and expressed in arbitrary units (AU). 1 AU is equivalent to the activity found in human reference plasma, which is 14 μmol/ml/h. PLTP concentration was measured by ELISA as described [18], using PLTP antibodies which were kindly donated by Dr. H. Hattori (BML Incorporated, Saitama, Japan). PLTP specific activity was calculated as the ratio (PLTP activity in AU)/(human PLTP concentration in mg/L).

Histological analyses and quantification of atherosclerotic lesions

Animals were anesthetized using isoflurane, and in situ fixation was performed via the left ventricle of the heart using phosphate-buffered formaldehyde (4%, v/v). Sectioning of the aortic root, hematoxilin/eosin staining, the collection of digital images and the quantification of atherosclerotic lesions were performed as described before [26].

Immunohistochemistry was performed using antibodies directed against CD68 to detect macrophages (AbD Serotec, Kidlington, UK), against CD31 to detect endothelial cells (Hycult Biotechnology BV, Uden, The Netherlands), or against vascular smooth muscle cells alpha-actin (Sigma Chemical Co., Zwijndrecht, The Netherlands) or against PLTP (kind gift of Dr. Matti Jauhiainen, Helsinki, Finland).

Cell culture studies

For the isolation of primary macrophages mice were i.p. injected with 3 mL of 40.5 g/L Bacto® Brewer Thioglycollate Medium (Difco International B.V., Leeuwarden, The Netherlands). After three days the animals are anesthetized using isoflurane and macrophages are isolated from the peritoneal cavity in 0.34 M sucrose. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Invitrogen, Breda, The Netherlands) supplemented with 10% (v/v) fetal bovine serum and 100 U/mL penicillin/streptomycin. Cells were seeded on 6 wells plates (3×10^6 cells/well) and kept in culture for 24 hours. Alternatively, macrophages derived from bone marrow in vitro were obtained by isolating bone marrow cells from tibias and femurs, seeding the cells on 6-wells plates (3×10^5 cells/well) and culturing them in the presence of 10 ng/ml Macrophage Colony Stimulating Factor (Biosource International, Camarillo CA, USA) [27]. The concentrations of TNF-α, IFN-γ, IL-4, or IL-10 in the supernatants were measured using commercially available ELISAs (BD Biosciences Pharmingen, Alphen aan den Rijn, The Netherlands). PLTP activity was measured after 24 hours of culture in the supernatant with the same assay as used for the PLTP activity measurements in plasma. In some cases, cells were challenged by

adding 100 ng/mL bacterial lipopolysaccharide (from E. coli serotype 0111:B4, Sigma, Chemical Co., Zwijndrecht, The Netherlands). In order to measure the cholesterol loading and efflux capacity [28], macrophages were seeded on 3 cm dishes (3×10^6 cells/dish). After 2 hours, the medium was replaced with DMEM supplemented with 1% (v/v) fetal bovine serum, 100 U/mL penicillin/streptomycin, 30 μg/mL acetylated LDL (acLDL) [29], 1 μg/mL Acyl CoA:cholesterol acyltransferase inhibitor (gift from Sandoz AG, Basel, Switzerland) and 0.33 μCi [^{3}H]-cholesterol/mL for 22 hours. Efflux was monitored by incubating these loaded cells in DMEM containing 0.2% (w/v) fat-free BSA (Sigma Chemical Co., #6003, Zwijndrecht, The Netherlands) and 100 μg/mL human HDL and sampling the medium at 1, 2, 4, and 6 hours. At the end of the efflux study, cell associated radioactive cholesterol was measured and the percentage of cholesterol efflux was calculated. Cells were lysed in 0.1 M NaOH/ 0.5% (w/v) SDS and cellular protein was determined using the Lowry method [30].

Statistics

All of values are expressed as mean±S.E. Statistical analyses are by one-way analysis of variance with Bonferroni multiple comparison tests performed with Intercooled Stata 6.0 software (Stata Corp., College Station, TX, USA).

Results

PLTP production by mouse macrophages

In a first set of experiments, peritoneal macrophages were collected from three groups of mice: wild type control animals ($PLTP^{wt/wt}$), hemizygous human PLTP transgenic mice ($huPLTP^{tg/wt}$) and homozygous human PLTP transgenic mice ($huPLTP^{tg/tg}$). Macrophages were kept in culture for 24 hours before PLTP activity was measured in the culture medium. As shown in Fig. 1, PLTP activity could be measured in the medium of cells from $PLTP^{wt/wt}$ mice, demonstrating that PLTP is produced and excreted by macrophages. When macrophages from $huPLTP^{tg/wt}$ and $huPLTP^{tg/tg}$ mice were tested, increased PLTP activity was found (+125% and +233%, respectively, both $p<0.01$). From these results we conclude that macrophages from transgenic PLTP mice have an increased production and secretion of the transgenic protein.

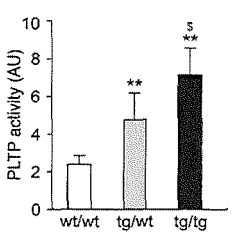

Figure 1. PLTP production by mouse macrophages. Peritoneal macrophages were collected from either C57BL/6J wild type (wt/wt), hemizygous huPLTP transgenic (tg/wt) and homozygous huPLTP transgenic (tg/tg) mice and kept in culture for 24 hours. Subsequently, PLTP activity was measured in a sample of the culture medium. PLTP activity is expressed as arbitrary units (AU; 1 AU is equivalent to 14 μmol/mL/h). N = cells from 6 mice per group. ** $p<0.005$ versus wt/wt; $ $p<0.01$ versus tg/wt.
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Effects of bone marrow transplantation on plasma PLTP activity

In order to study the contribution of PLTP expression by bone marrow derived cells on plasma PLTP activity, we performed bone marrow transplantation experiments from $PLTP^{wt/wt}$, $huPLTP^{tg/wt}$ and $huPLTP^{tg/tg}$ mice to lethally irradiated LDL-receptor deficient ($LDLR^{-/-}$) mice. Following the transplantation, animals were allowed to recover for 9 weeks. Subsequently, animals were fed a high fat, high cholesterol diet for another 9 weeks period. Blood samples were collected one week before irradiation, just before starting the diet and at the end of the 9 weeks diet period (time points -10 weeks, 0 weeks and 9 weeks, relative to the start of the diet, respectively). In addition to the transplanted animals, groups of $LDLR^{-/-}$ mice and $LDLR^{-/-}$/$huPLTP^{tg/wt}$ mice were subjected to the same series of experiments, except the irradiation and bone marrow transplantation. Blood samples were collected at 0 weeks and 9 weeks. Animals were age matched to the mice in the transplantation groups.

As shown in Fig. 2A (left panel), plasma PLTP activity in $LDLR^{-/-}$/$huPLTP^{tg/wt}$ mice were approximately 3-fold higher than in $LDLR^{-/-}$ mice when fed a chow diet (0 weeks). After feeding the animals a high fat, high cholesterol diet for 9 weeks, plasma PLTP activity in both $LDLR^{-/-}$ and $LDLR^{-/-}$/$huPLTP^{tg/wt}$ mice was increased by about 2-fold when compared to chow fed animals of the same genotype.

Fig. 2A (right panel) also shows the plasma PLTP activity in transplanted animals. The transplantation procedure itself resulted in a significant increase in PLTP activity, ranging from 29% in the $PLTP^{wt/wt}\rightarrow LDLR^{-/-}$ mice to 88% in the $huPLTP^{tg/tg}\rightarrow LDLR^{-/-}$ mice (all $p<0.001$). After feeding the animals a high fat, high cholesterol diet, a further increase in plasma PLTP activity was observed ranging from 82% in the $PLTP^{wt/wt}\rightarrow LDLR^{-/-}$ mice to 113% in the $huPLTP^{tg/tg}\rightarrow LDLR^{-/-}$ mice (all $p<0.001$). Under both dietary conditions, plasma PLTP activity did not differ between $huPLTP^{tg/wt}\rightarrow LDLR^{-/-}$ and $huPLTP^{tg/tg}\rightarrow LDLR^{-/-}$ mice.

Fig. 2B shows the concentration of human PLTP in plasma from transplanted animals. As there is no ELISA available for mouse PLTP, mass of plasma PLTP could only be measured in the $huPLTP^{tg/wt}\rightarrow LDLR^{-/-}$ and $huPLTP^{tg/tg}\rightarrow LDLR^{-/-}$ mice. The PLTP concentration appeared to be approximately twice as much in the latter animals. The high fat, high cholesterol diet resulted in a two-fold increase in PLTP mass. The specific activity of plasma PLTP was not affected by the diet (Fig. 2C). Interestingly however, the specific activity in the $huPLTP^{tg/tg}\rightarrow LDLR^{-/-}$ mice was significantly lower than in the $huPLTP^{tg/wt}\rightarrow LDLR^{-/-}$ mice, implying that part of the huPLTP in the $huPLTP^{tg/tg}\rightarrow LDLR^{-/-}$ mice is in the inactive form.

Effects of bone marrow transplantation on plasma lipids and lipoproteins

When fed a regular chow diet, plasma cholesterol levels were slightly decreased in $LDLR^{-/-}$/$huPLTP^{tg/wt}$ mice when compared to $LDLR^{-/-}$ mice (Fig. 3A, left panel). However, this difference was absent after feeding the animals a high fat, high cholesterol diet for 9 weeks. In the transplanted animals, no major differences in plasma cholesterol concentration were observed between the various groups on chow diet (Fig. 3A, right panel). However, after feeding the animals a high fat, high cholesterol diet for 9 weeks an increase in cholesterol concentration was observed in $PLTP^{wt/wt}\rightarrow LDLR^{-/-}$, $huPLTP^{tg/wt}\rightarrow LDLR^{-/-}$, and $huPLTP^{tg/tg}\rightarrow LDLR^{-/-}$ mice. Effects on plasma phospholipids and triglycerides largely mirrored those on plasma cholesterol (Fig. 3B,C). In order to further investigate these differences, HDL

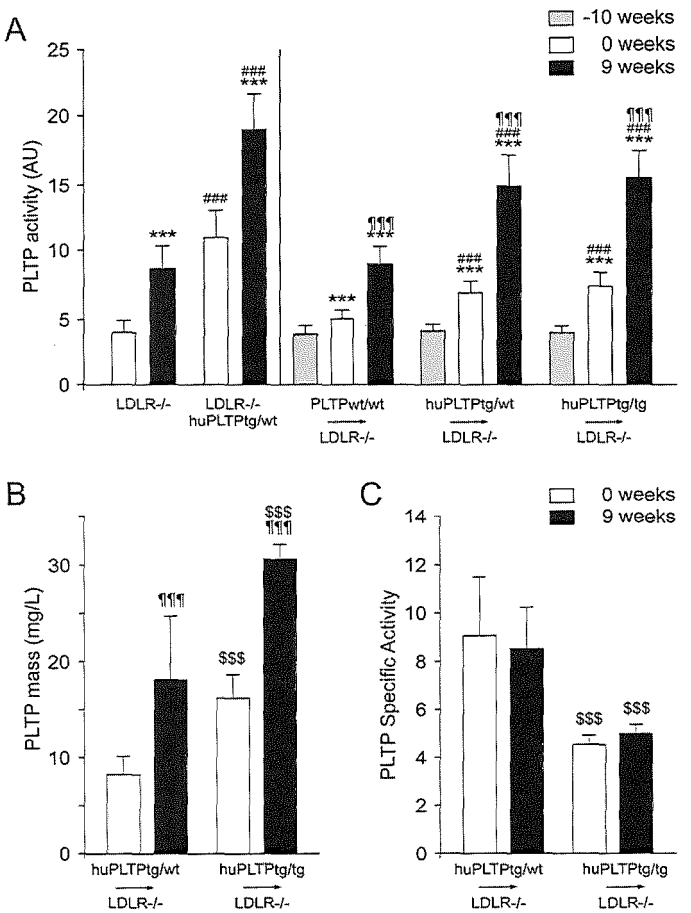

Figure 2. Plasma PLTP levels. A. PLTP activity was measured in plasma samples and expressed in arbitrary units as described in the legend of Fig. 1. Left panel: plasma samples were collected from non-transplanted LDLR$^{-/-}$ and LDLR$^{-/-}$/huPLTP$^{tg/wt}$ mice just before the start of the high fat, high cholesterol diet (0 weeks, white bars) and after 9 weeks of the diet (9 weeks, black bars). *** $p<0.001$ versus 0 weeks (same genotype), ### $p<0.001$ versus LDLR$^{-/-}$ mice (on the same diet). Right panel: plasma samples were collected from PLTP$^{wt/wt}$→LDLR$^{-/-}$, huPLTP$^{tg/wt}$→LDLR$^{-/-}$, and huPLTP$^{tg/tg}$→LDLR$^{-/-}$ recipient mice at 1 week before the start of the transplantation procedure (i.e. at 10 weeks before the start of the diet: −10 weeks, grey bars), just before the start of the diet (0 weeks, white bars) and after 9 weeks of diet (9 weeks, black bars). *** $p<0.001$ versus −10 weeks (same genotype); ### $p<0.001$ versus PLTP$^{wt/wt}$→LDLR$^{-/-}$ mice (on the same diet); ¶¶¶ $p<0.001$ versus 0 weeks (same genotype). B. Mass of human PLTP in plasma from mice was measured by ELISA as described in Methods in the Supplemental Data. C. Specific Activities of PLTP were calculated using the ratio between the activity in AU and the mass in mg/L. ¶¶¶ $p<0.001$ versus 0 weeks (same genotype); $$$ $p<0.001$ versus PLTP$^{tg/wt}$→LDLR$^{-/-}$ mice (on the same diet). N = 11–15 mice per group.
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and non-HDL was separated in plasma samples of the animals after 9 weeks on a high fat, high cholesterol diet. The mice transplanted with huPLTP transgenic bone marrow cells showed an increase in non-HDL cholesterol (Fig. 4A) and a decrease in HDL-cholesterol (Fig. 4B) when compared with the mice transplanted with wild type bone marrow cells.

Effects of bone marrow transplantation on atherosclerosis

After feeding the animals a high fat, high cholesterol diet for 9 weeks, atherosclerosis was quantified by measuring the area of atherosclerotic lesions in the aortic root. In agreement with previous studies from our laboratory [17,31], we found that

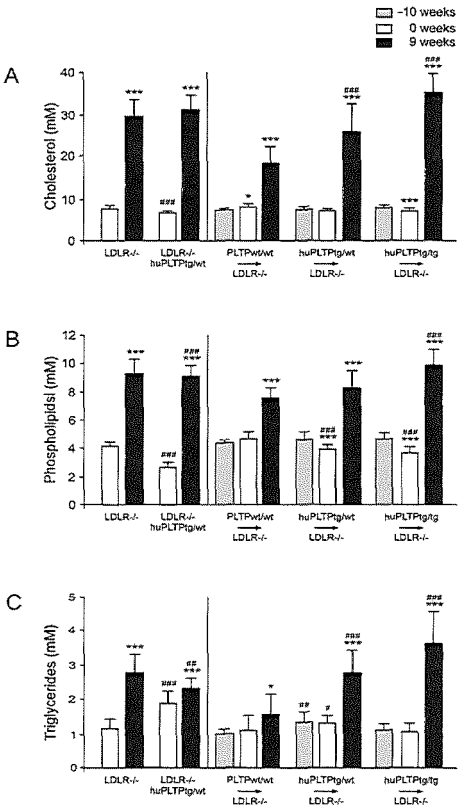

Figure 3. Plasma lipid levels. Plasma levels of cholesterol (A), phospholipids (B) and triglycerides (C) were measured as described in Methods. Left panels: Plasma samples were collected from non-transplanted LDLR$^{-/-}$ and LDLR$^{-/-}$/huPLTP$^{tg/wt}$ mice just before the start of the high fat, high cholesterol diet (0 weeks, white bars) and after 9 weeks of the diet (9 weeks, black bars). *** $p<0.001$ versus 0 weeks (same genotype), ### $p<0.001$ versus LDLR$^{-/-}$ mice (on the same diet). Right panels: plasma samples were collected from PLTP$^{wt/wt}$ →LDLR$^{-/-}$, huPLTP$^{tg/wt}$→LDLR$^{-/-}$, and huPLTP$^{tg/tg}$→LDLR$^{-/-}$ recipient mice at 1 week before the start of the transplantation procedure (i.e. at 10 weeks before the start of the diet: −10 weeks, grey bars), just before the start of the diet (0 weeks, white bars) and after 9 weeks of diet (9 weeks, black bars). N=11–15 mice per group. * $p<0.05$, *** $p<0.001$ versus −10 weeks (same genotype); ### $p<0.001$ versus PLTP$^{wt/wt}$→LDLR$^{-/-}$ mice (on the same diet).
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elevated levels of PLTP result in a strong increase (312%) in diet-induced atherosclerosis in non-transplanted mice (Fig. 5A, left panel; $p<0.001$). In transplanted mice, a clear increase in atherosclerotic lesion area was observed (Fig. 5A, right panel; all differences $p<0.001$). Compared to PLTP$^{wt/wt}$→LDLR$^{-/-}$ mice, huPLTP$^{tg/wt}$→LDLR$^{-/-}$ mice have an increase of 128% in atherosclerotic lesion area, while huPLTP$^{tg/tg}$→LDLR$^{-/-}$ mice have an increase of 348% in atherosclerotic lesion area.

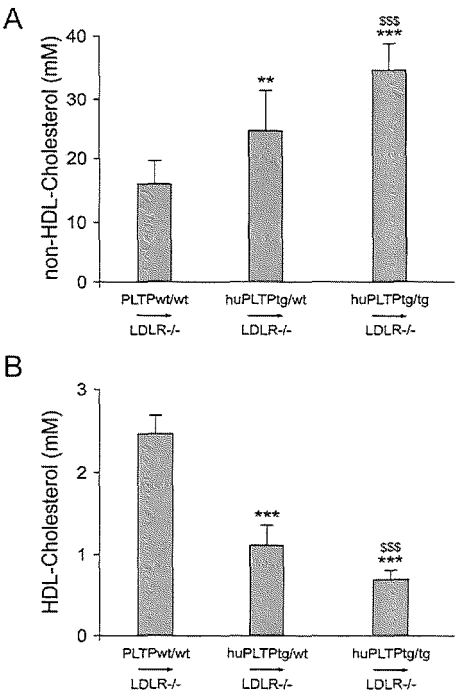

Figure 4. Plasma levels of non-HDL cholesterol and HDL cholesterol. Plasma was collected from PLTP$^{wt/wt}$→LDLR$^{-/-}$, huPLTP$^{tg/wt}$→LDLR$^{-/-}$, and huPLTP$^{tg/tg}$→LDLR$^{-/-}$ recipient mice at the end of the high fat, high cholesterol diet period (i.e., at time of sacrifice) and separated into two fractions: d<1.063 g/L (non-HDL) and d>1.063 g/L (HDL) by ultracentrifugation. In both fractions, the cholesterol concentration was measured. A: non-HDL cholesterol; B: HDL-cholesterol. N=11–15 mice per group. ** $p<0.01$, *** $p<0.001$ versus PLTP$^{wt/wt}$→LDLR$^{-/-}$ mice; $$$ $p<0.001$ versus huPLTP$^{tg/wt}$ →LDLR$^{-/-}$ mice.
doi:10.1371/journal.pone.0002255.g004

The presence of PLTP expressing macrophages in the aortic lesions was confirmed by immunohistochemistry. As illustrated in Fig. 5, the plaques from both non-transplanted LDLR$^{-/-}$/huPLTP$^{tg/wt}$ mice (Fig. 5B) as those from recipient huPLTP$^{tg/tg}$ →LDLR$^{-/-}$ mice (Fig. 5C) contain considerable amounts of macrophages while PLTP is co-localized in similar areas of the lesions.

In order to be more conclusive on the nature of the bone marrow derived cells, we performed an additional set of experiments using actin-EGFP mice as donors and LDLR$^{-/-}$ mice as acceptors, using exactly the same experimental procedure to obtain diet-induced atherosclerosis. Sections from the aortic root were inspected using confocal microscopy after immunohistochemistry with antibodies directed against CD68 as a marker for macrophages or VSMC α-actin as a marker for VSMCs. DAPI staining was used to visualize nuclei. Bone marrow derived donor cells, identified by the GFP signal, are clearly present in the

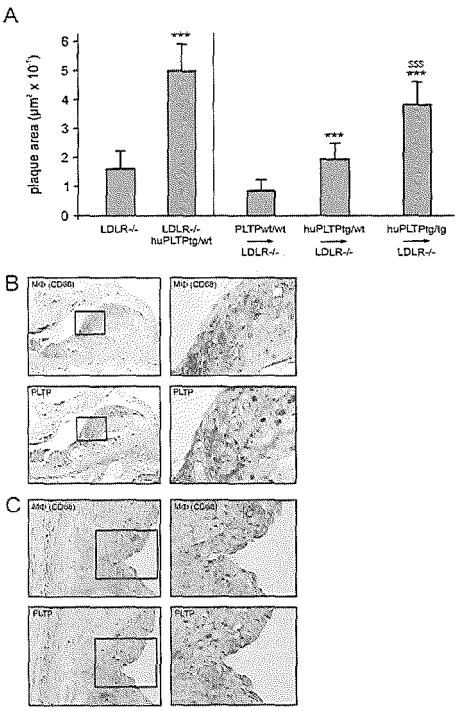

Figure 5. Atherosclerosis in non-transplanted and transplanted mice. A. Plaque area was measured in sections from the aortic root (see Methods). Left panel: Plaque area in non-transplanted $LDLR^{-/-}$ and $LDLR^{-/-}$/$huPLTP^{tg/wt}$ mice. *** $p<0.001$ versus $LDLR^{-/-}$ mice. Right panel: Plaque area in $PLTPwt/wt \rightarrow LDLR^{-/-}$, $huPLTP^{tg/wt} \rightarrow LDLR^{-/-}$, and $huPLTP^{tg/tg} \rightarrow LDLR^{-/-}$ recipient mice. N = 11–15 mice per group. *** $p<0.001$ versus $PLTP^{wt/wt} \rightarrow LDLR^{-/-}$ mice; $$$ *** $p<0.001$ versus $huPLTP^{tg/wt} \rightarrow LDLR^{-/-}$ mice. B, C. Immunohistochemistry of atherosclerotic lesions from non-transplanted $LDLR^{-/-}$/$huPLTP^{tg/wt}$ mice (B) and from transplanted $huPLTP^{tg/tg} \rightarrow LDLR^{-/-}$ mice (C), stained for macrophages (MΦ) with anti-CD68 and for PLTP and counterstained with Nuclear-fast red (serial sections). The right panels are magnifications of the boxed parts in the left panels. Original magnifications: 100x (B) and 250x (C).
doi:10.1371/journal.pone.0002255.g005

subendothelial space of the early lesions and co-localize to a great extent with CD68 (Fig. 6A,B). In the advanced lesions, the cell-rich regions show significant co-localization of the GFP and CD68 signals, while the necrotic core stills allows detection of CD68, but GFP is no longer expressed (Fig. 6C,D). In early lesions, VSMCs are almost exclusively located in the media (Fig. 6B). In advanced lesions, VSMCs can be detected in the fibrous cap (Fig. 6D), but they do not co-localize with GFP, demonstrating that also the VSMCs in the lesions are not donor-derived. As shown in Fig. 6A–D, isolated nuclei can be detected of endothelial cells covering the lesions without GFP signal, demonstrating that these cells are not donor-derived. Positive staining for endothelial cells was performed by using an antibody directed against CD31 (Fig. 6E,F). The endothelial lining in early (Fig. 6E) and in advanced (Fig. 6F) lesions is continuous and does not express GFP, implying that the endothelial cells are not donor derived.

Characterization of PLTP expressing macrophages

In order to investigate the mechanism by which mice transplanted with bone marrow cells expressing elevated levels of PLTP develop more atherosclerosis, we performed a series of in vitro studies using primary macrophages from $PLTP^{wt/wt}$, $huPLTP^{tg/wt}$, and $huPLTP^{tg/tg}$ mice, respectively.

First we examined whether PLTP expression in macrophages affects their capacity to perform cellular cholesterol efflux. To this end, macrophages were loaded with radiolabeled cholesterol using acLDL and efflux to the medium was studied during six hours using human HDL as an acceptor. There was no difference in cholesterol loading between macrophages from $PLTP^{wt/wt}$ and $huPLTP^{tg/tg}$ mice (42.87+/−8.25 versus 44.26+/−8.67% [^{3}H]cholesterol/mg cell protein). No difference in efflux to HDL was observed (Fig. 7A). Consequently, the amount of radioactive cholesterol that remained cell-associated at the end of the efflux period was also similar (not shown).

Possible differences between the inflammatory status of the macrophages were studied by comparing the level of the cytokines, TNF-α, IFN-γ, IL-4, and IL-10, by ELISA. No differences were found in medium from macrophages obtained from $PLTP^{wt/wt}$, $huPLTP^{tg/wt}$, and $huPLTP^{tg/tg}$ mice before or after stimulation with LPS (results not shown). These results indicate that there is no difference in cytokine production by macrophages from these mice.

Subsequently, we studied whether activated macrophages have different PLTP activity. Therefore, we challenged the macrophages with bacterial lipopolysaccharide (LPS), and measured PLTP activity in the medium from the stimulated macrophages. As shown in Fig. 7B, stimulation of macrophages from $PLTP^{wt/wt}$ mice with LPS resulted in approximately 70% increase in PLTP activity. Unstimulated macrophages from $huPLTP^{tg/wt}$ or $huPLTP^{tg/tg}$ mice secreted approximately 2.5 to 4 fold more PLTP in the culture medium than macrophages from $PLTP^{wt/wt}$ mice. LPS stimulation resulted in a further 7.5 fold increase of PLTP activity in the culture medium from macrophages from $huPLTP^{tg/wt}$ or $huPLTP^{tg/tg}$ mice. In order to investigate whether this in vitro effect would be reflected by similar effects in vivo, we measured plasma PLTP activity levels in both $PLTP^{wt/wt}$ and $huPLTP^{tg/tg}$ mice that were treated with thioglycolate. In both cases, this treatment resulted in a statistically significant ($P<0.002$) increase in plasma PLTP activity (Fig. 7C).

Finally, we cultured macrophages from bone marrow in order to consolidate these findings. Comparing macrophages from $PLTP^{wt/wt}$ and $huPLTP^{tg/tg}$ mice, we found much more PLTP activity in the medium of the $huPLTP^{tg/tg}$ cells. PLTP activity could be significantly increased in cells from both $PLTP^{wt/wt}$ and $huPLTP^{tg/tg}$ mice after treatment with LPS (not shown).

Discussion

The main findings of this study are: (1) Mouse macrophages express and secrete PLTP while mouse macrophages from human PLTP transgenic mice express and secrete increased levels of PLTP; (2) Bone marrow transplantations using PLTP transgenic mice as donors induce elevated plasma PLTP activity levels in the acceptor mice; (3) PLTP overexpression results in decreased plasma levels of HDL cholesterol and in increased plasma levels of non-HDL cholesterol; (4) Transplantation with PLTP expressing bone marrow cells results in increased (2–4.5 fold) atherosclerosis with abundant intra-plaque presence of PLTP.

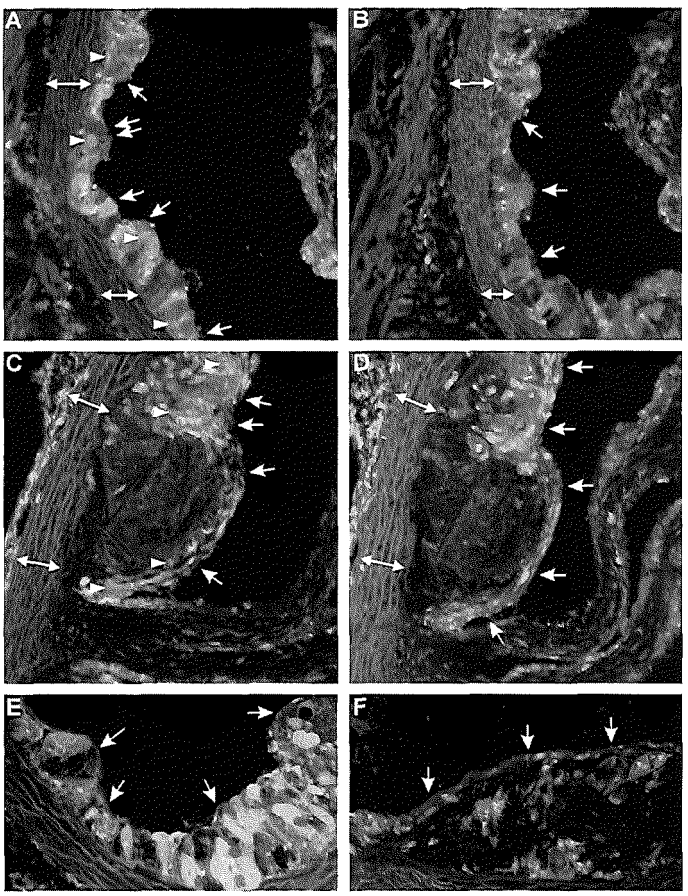

Figure 6. Transplantations with β-actin GFP mice→LDLR$^{-/-}$ mice. A–D: Early lesions (A,B) and advanced lesions (C,D) with donor cells expressing GFP (green), CD 68 (marker for macrophages) in red (A,C) and α-actin (marker for VSMCs) in red (B,D) and nuclei stained with DAPI (blue). Endothelial cells covering the lesions do not express GFP (nuclei indicated with arrows). Co-localization of GFP and CD68 results in an orange color (arrowheads). The necrotic core in the advanced lesion (located centrally in C and D) is diffusely positive for CD68 but does not show any GFP signal. The media is marked with a double arrow (↔). E,F: Early lesion (E) and advanced lesion (F) with donor cells expressing GFP (green), and CD 31 (marker for endothelial cells) in red (arrows). Representative pictures from N = 6 animals are shown. Original magnifications: 200X.
doi:10.1371/journal.pone.0002255.g006

The potential relevance of PLTP production by macrophages is emphasized by three independent studies, showing that PLTP protein is present in human atherosclerotic lesions [22,23,32]. Although intriguing, the presence of PLTP in atherosclerotic lesions per se does not prove a direct involvement in the process of atherosclerosis and therefore the significance of these findings is unclear. In humans, the causal relation between PLTP and atherosclerosis is still uncertain [33]. Plasma PLTP activity is elevated in patients suffering from diabetes mellitus type 1 [34] and type 2 [35,36]. In addition, increased PLTP is found in obese individuals [37–40] while it is decreased with weight loss [41]. The elevated plasma PLTP activity in patients with type 2 diabetes mellitus is positively correlated with the carotid intima-media thickness [42].

In order to find out what the contribution of PLTP expressing macrophages to diet induced atherosclerosis would be, we performed bone marrow transplantation experiments.

9 weeks after bone marrow transplantation, a moderate increase in plasma PLTP activity was found in the animals transplanted with PLTP$^{wt/wt}$ cells. In the animals transplanted with PLTP transgenic cells, a further increase was observed. Feeding the animals a high fat, high cholesterol diet for 9 weeks, resulted in a

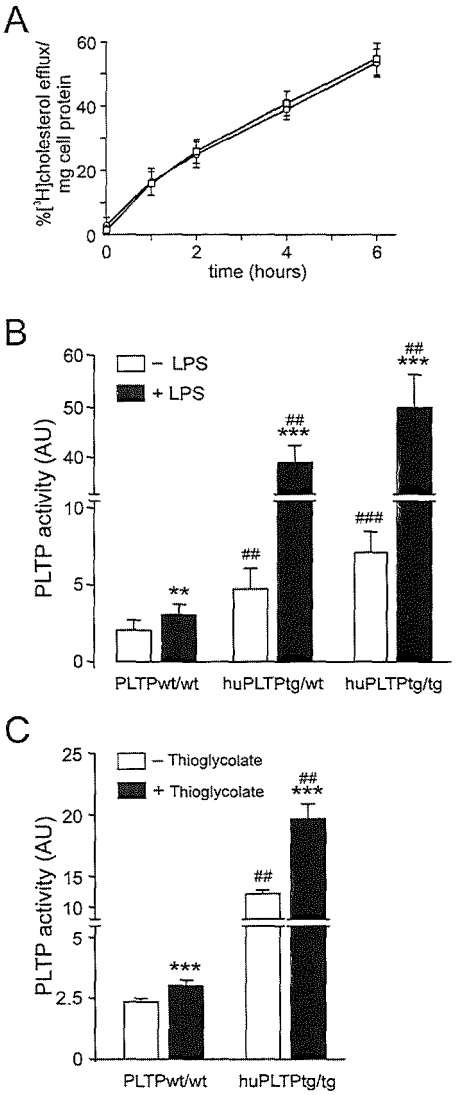

Figure 7. Characteristics of mouse macrophages. A. Cholesterol efflux from cultured peritoneal macrophages. Cells were loaded with radioactive cholesterol as described in Methods. Subsequently, culture medium with human HDL as an acceptor was added. Left panel: Radioactivity was measured in aliquots from the cultured medium taken at 0, 1, 2, 4, and 6 hours. Circles: macrophages from C57BL/6J mice, squares: macrophages from huPLTP$^{tg/tg}$ mice. Differences were not statistically significant. B. PLTP activity measured in the medium of macrophages cultured for 24 hours in the absence (white bars) or presence (black bars) of 100 ng/mL LPS. Cells from 6 mice were used per group. C. PLTP activity measured in plasma samples from mice treated with thioglycolate. N=6 mice per group. ** $p<0.005$, *** $p<0.001$ versus cells without LPS or thioglycolate (same genotype), ## $p<0.005$, ### $p<0.001$ versus PLPT$^{wt/wt}$ (same culture medium, with or without LPS or thioglycolate).
doi:10.1371/journal.pone.0002255.g007

striking increase in plasma PLTP activity. It has been demonstrated that cholesterol loading of macrophages induces a strong (2–3 fold) induction of PLTP mRNA, PLTP protein secretion and an increased PLTP activity in the medium conditioned by these cells [23,32]. Our results show that in animals fed a high fat, high cholesterol diet, plasma from huPLTP$^{tg/wt}$→LDLR$^{-/-}$ and huPLTP$^{tg/tg}$→LDLR$^{-/-}$ mice have a 66–75% increase in PLTP activity when compared to plasma from PLTP$^{wt/wt}$→LDLR$^{-/-}$ mice. Thus we conclude that a considerable amount of macrophage PLTP is excreted into the blood.

An increase in plasma PLTP activity affects plasma lipids. Plasma cholesterol is not affected by the moderate increase in PLTP activity caused by the bone marrow transplantation procedure itself. After 9 weeks of high fat, high cholesterol diet plasma cholesterol is elevated, as expected, but a further increase is observed in the huPLTP$^{tg/wt}$→LDLR$^{-/-}$ and huPLTP$^{tg/tg}$→LDLR$^{-/-}$ mice. So, PLTP expression by bone marrow derived cells affects not only plasma PLTP activity levels, but also plasma lipids.

Because PLTP activity can be directly measured in plasma, our experiments clearly show that PLTP is excreted by bone marrow derived cells into the plasma compartment. Probably this is the cause of the observed decrease in plasma HDL cholesterol, which is in agreement with previous results from our laboratory [14,17,31] and others [43,44], and which is probably caused by an enhanced catabolism of HDL [43]. Most likely, this effect will contribute to the observed enhanced atherosclerosis in mice transplanted with huPLTP transgenic bone marrow. In addition however, there might be a local effect of PLTP produced by macrophages in the vascular wall. Although PLTP overexpressing macrophages do not show any difference in cholesterol efflux capacity or cytokine production, a very strong induction of PLTP production is observed in macrophages treated with LPS. So, activated macrophages present in the inflammatory environment of atherosclerotic plaques probably excrete large amounts of PLTP. This is confirmed by immunohistochemistry data in the present study. The high local PLTP production might result in a further increase of the inflammatory status of the lesions. Additional support for this idea is provided by studies showing that PLTP deficiency is associated with anti-inflammatory effects [45,46], and by a study in which an association was found between PLTP activity and inflammatory markers in patients with cardiovascular disease [47]. Alternatively, extracellular PLTP might act as a trap for HDL. PLTP is able to bind to the extracellular matrix proteoglycan biglycan [23] and thus act as a bridging molecule to bind HDL. In vitro studies showed that this action is independent of PLTP's phospholipid transfer activity. By trapping HDL in the atherosclerotic lesion, PLTP could interfere with the reverse cholesterol transport activity of HDL and thus enhance lipid accumulation, or actually, inhibit lipid removal, and hence stimulate the development of the plaque. It is uncertain whether this HDL retention may contribute to the decreased plasma levels of HDL observed in mice transplanted with huPLTP transgenic bone marrow.

While macrophage expression of PLTP has both systemic and local effects, both of which might be atherogenic, the steep increase in atherosclerotic lesion size of almost 2-fold between

huPLTP$^{tg/wt}$→LDLR$^{-/-}$ and huPLTP$^{tg/tg}$→LDLR$^{-/-}$ mice is not completely reflected by the differences in the systemic effects, as these mice have similar plasma PLTP activities. This suggests that local effects of PLTP expression might indeed play an important role. In addition, the huPLTP$^{tg/tg}$→LDLR$^{-/-}$ mice show a reduced specific activity of PLTP. This could indicate that the huPLTP$^{tg/tg}$ →LDLR$^{-/-}$ mice have relatively more inactive PLTP [48], which may not have phospholipids transfer activity, but which might still have some of the other effects described above. Also, PLTP secreted by the huPLTP$^{tg/tg}$ bone marrow derived cells in the transplanted animals finds itself in a plasma environment with very little HDL. As active PLTP is normally bound to HDL, this situation could destabilize PLTP resulting in partial PLTP inactivation and in a decrease in PLTP specific activity.

It should be noted that bone marrow derived cells include cells other than macrophages, like endothelial cells and VSMCs. Therefore, we performed a set of control experiments with β-actin GFP donor mice. It became clear that using our experimental conditions, the contribution from bone marrow derived endothelial cells or VSMCs to the atherosclerotic lesion is undetectable, while there is a significant number of bone marrow derived macrophages in the lesions.

Recently, the susceptibility to diet-induced atherosclerosis was studied in mice after bone marrow transplantations with bone marrow cells from wild type and PLTP deficient mice. Strikingly different results were reported by two independent research groups, leading to opposite conclusions [49,50]. While Valenta et al [49] propose an athero-protective role for macrophage derived PLTP, Vikstedt et al [50] show evidence for an atherogenic function of macrophage derived PLTP. Both groups used LDLR$^{-/-}$ mice as acceptors, and either wild type or PLTP$^{-/-}$ mice as donors. All mice had the same genetic background (C57BL/6J), and the same strain of PLTP deficient mice were used. When comparing mice transplanted with PLTP$^{-/-}$ bone marrow with mice transplanted with wild type bone marrow, Valenta et al found an increase in atherosclerotic lesion area in of 30% (measured in the aortic valves) or 28% (measured in the entire aorta), while Vikstedt et al found a decrease of 29% (measured in the aortic valves). In both studies, plasma PLTP activity is lower in mice transplanted with PLTP$^{-/-}$ bone marrow than in mice transplanted with wild type bone marrow when fed a chow diet, and the difference becomes larger in mice fed an atherogenic diet. In the study by Vikstedt et al this leads to a significant decrease in plasma cholesterol levels and an increase in HDL, which may explain the decreased atherosclerosis found in mice transplanted with PLTP$^{-/-}$ bone marrow. In the study by Valenta et al there is increased atherosclerosis while plasma cholesterol levels are hardly changed. Thus, in the latter study postulated that plasma PLTP is atherogenic, while PLTP which is locally active in the vascular wall may have anti-atherogenic potential, possibly by local stimulation of macrophage cholesterol efflux.

Vikstedt et al have summarized the major differences in experimental set-up: 1) Vikstedt et al used female mice as acceptors while Valenta et al used male mice; 2) Vikstedt et al fed the mice an atherogenic diet with 0.25% cholesterol for 9 weeks while the diet used by Valenta et al contained 1.25% cholesterol and was fed for 16 weeks; 3) Vikstedt et al allowed the mice to recover from the bone marrow transplantation procedure for 8 weeks before the atherogenic diet was supplied while Valenta et al applied a recovery time of only 4 weeks. Vikstedt et al do not indicate if any of these differences could be the deciding factor to explain the different outcomes of their studies.

Very recently, Valenta et al confirmed their previous findings in a study in which they used LDLR$^{-/-}$/PLTP$^{-/-}$ mice as acceptors [51]. Here, they applied the same experimental set-up as in their earlier work. In the discussion of this recent paper, the differences with the experimental set-up followed by Vikstedt et al are listed, but there are no conclusions about what might really cause the different outcomes.

We believe that the difference in gender between the recipients in the studies using PLTP deficient mice is not a likely explanation for the conflicting results. Although it is well-known that the susceptibility of female and male mice to atherosclerosis is different, the reason for this phenomenon is unknown, and, to the best of our knowledge, there are no reports where opposite outcomes of an intervention with respect to atherosclerosis susceptibility between males and females have been described. In fact, we have shown in a previous study that in animals with elevated PLTP activity, both males and females have more atherosclerosis [52].

The difference in diet-regime may be an essential factor. The diet used by Valenta et al contains five times as much cholesterol as the diet used by Vikstedt et al and was applied much longer. Therefore, this is a significantly more aggressive way of induction of atherosclerosis. It is conceivable that this has affected the outcome of the study.

The difference in recovery time may be an essential factor as well. Vikstedt et al allowed the animals to recover for eight weeks, compared to four weeks in the study of Valenta et al. Four weeks is really a very short time, in which the hematopoietic system has not yet been stably reconstituted. It is conceivable, that PLTP deficiency affects the repopulation of monocytes/macrophages. Therefore, the development of atherosclerotic lesions may be affected by differences in recovery time. Also, the reconstitution of resident macrophages in lung and liver, both important sites of PLTP expression, may not yet be stable after only four weeks of recovery [53].

We studied the effects of elevated PLTP expression using transgenic mouse models that have been developed in our own laboratory. The interpretation of our results is independent from the conflicting results that have been reported using PLTP$^{-/-}$ mice. Our experimental set-up is comparable to the one used by Vikstedt et al, as we also fed the mice a diet containing 0.25% cholesterol for 9 weeks, and we used an even slightly longer recovery period (9 weeks). Their results are in line with our findings, as we report an increase of atherosclerosis in mice transplanted with PLTP overexpressing bone marrow, while they found decreased atherosclerosis in mice transplanted with PLTP$^{-/-}$ bone marrow cells.

In conclusion, we showed that elevated macrophage expression of PLTP results in increased atherosclerosis. This is probably directly related to the effects on plasma lipoproteins caused by PLTP expression by bone marrow derived cells. In addition, local effects in the atherosclerotic lesion could contribute to this process. These results indicate that the presence of PLTP in human atherosclerotic lesions probably contributes to the pathologic process and that inhibition of PLTP accumulation in atherosclerotic lesions may be a valuable target for therapy.

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Author Contributions

Conceived and designed the experiments: FG Rd HS MM Tv MP Av Rv. Performed the experiments: Tv MP Rv. Analyzed the data: Tv Rv. Wrote the paper: FG Rd HS MM Tv MP Av Rv.

References

1. Tall AR (1980) Studies on the transfer of phosphatidylcholine from unilamellar vesicles into plasma high density lipoproteins in the rat. J Lipid Res 21: 354–363.
2. Damen J, Regts J, Scherphof G (1982) Transfer of [14C]phosphatidylcholine between liposomes and human plasma high density lipoprotein. Partial purification of a transfer-stimulating plasma factor using a rapid transfer assay. Biochim Biophys Acta 712: 444–452.
3. Tall AR, Abreu E, Shuman J (1983) Separation of a plasma phospholipid transfer protein from cholesterol ester/phospholipid exchange protein. J Biol Chem 258: 2174–2180.
4. Albers JJ, Cheung MC (2004) Emerging roles for phospholipid transfer protein in lipid and lipoprotein metabolism. Curr Opin Lipidol 15: 255–260.
5. Kostner GM, Oettl K, Jauhiainen M, Ehnholm C, Esterbauer H, et al. (1995) Human plasma phospholipid transfer protein accelerates exchange/transfer of alpha-tocopherol between lipoproteins and cells. Biochem J 305: 659–667.
6. Tu AY, Nishida HI, Nishida T (1993) High density lipoprotein conversion mediated by human plasma phospholipid transfer protein. J Biol Chem 268: 23098–23105.
7. Jauhiainen M, Metso J, Pahlman R, Blomqvist S, van Tol A, et al. (1993) Human plasma phospholipid transfer protein causes high density lipoprotein conversion. J Biol Chem 268: 4032–4036.
8. Assmann G, Gotto AM Jr (2004) HDL cholesterol and protective factors in atherosclerosis. Circulation 109: III8–14.
9. Ansell BJ, Navab M, Watson KE, Fonarow GC, Fogelman AM (2004) Anti-inflammatory properties of HDL. Rev Endocr Metab Disord 5: 351–358.
10. Barter PJ, Nicholls S, Rye KA, Anantharamaiah GM, Navab M, et al. (2004) Antiinflammatory properties of HDL. Circ Res 95: 764–772.
11. Huuskonen J, Olkkonen VM, Jauhiainen M, Ehnholm C (2001) The impact of phospholipid transfer protein (PLTP) on HDL metabolism. Atherosclerosis 155: 269–281.
12. van Tol A (2002) Phospholipid transfer protein. Curr Opin Lipidol 13: 135–139.
13. Jiang XC, Bruce C, Mar J, Lin M, Ji Y, et al. (1999) Targeted mutation of plasma phospholipid transfer protein gene markedly reduces high-density lipoprotein levels. J Clin Invest 103: 907–914.
14. van Haperen R, van Tol A, Vermeulen P, Jauhiainen M, van Gent T, et al. (2000) Human plasma phospholipid transfer protein increases the antiatherogenic potential of high density lipoproteins in transgenic mice. Arterioscler Thromb Vasc Biol 20: 1082–1088.
15. Fielding CJ, Fielding PE (2001) Cellular cholesterol efflux. Biochim Biophys Acta 1533: 175–189.
16. Jiang XC, Qin S, Qiao C, Kawano K, Lin M, et al. (2001) Apolipoprotein B secretion and atherosclerosis are decreased in mice with phospholipid-transfer protein deficiency. Nat Med 7: 847–852.
17. Van Haperen R, Van Tol A, Van Gent T, Scheek L, Visser P, et al. (2002) Increased risk of atherosclerosis by elevated plasma levels of phospholipid transfer protein. J Biol Chem 277: 48938–48943.
18. Lie J, De Crom R, Van Gent T, Van Haperen R, Scheek L, et al. (2002) Elevation of plasma phospholipid transfer protein in transgenic mice increases VLDL secretion. J Lipid Res 43: 1875–1880.
19. Jiang XC, Tall AR, Qin S, Lin M, Schneider M, et al. (2002) Phospholipid transfer protein deficiency protects circulating lipoproteins from oxidation due to the enhanced accumulation of vitamin E. J Biol Chem 277: 31850–31856.
20. Day JR, Albers JJ, Lofton-Day CE, Gilbert TL, Ching AF, et al. (1994) Complete cDNA encoding human phospholipid transfer protein from human endothelial cells. J Biol Chem 269: 9388–9391.
21. Albers JJ, Wolfbauer G, Cheung MC, Day JR, Ching AF, et al. (1995) Functional expression of human and mouse plasma phospholipid transfer protein: effect of recombinant and plasma PLTP on HDL subspecies. Biochim Biophys Acta 1258: 27–34.
22. Laffitte BA, Joseph SB, Chen M, Castrillo A, Repa J, et al. (2003) The phospholipid transfer protein gene is a liver X receptor target expressed by macrophages in atherosclerotic lesions. Mol Cell Biol 23: 2182–2191.
23. O'Brien KD, Vuletic S, McDonald TO, Wolfbauer G, Lewis K, et al. (2003) Cell-Associated and Extracellular Phospholipid Transfer Protein in Human Coronary Atherosclerosis. Circulation.
24. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y (1997) 'Green mice' as a source of ubiquitous green cells. FEBS Lett 407: 313–319.
25. Peeters M, Ling KW, Oziemlak A, Robin C, Dzierzak E (2005) Multipotential hematopoietic progenitor cells from embryos developed in vitro engraft unconditioned W41/W41 neonatal miceB. Haematologica 90: 734–739.
26. van Haperen R, de Waard M, van Deel E, Mees B, Kutryk M, et al. (2002) Reduction of blood pressure, plasma cholesterol, and atherosclerosis by elevated endothelial nitric oxide. J Biol Chem 277: 48803–48807.
27. Bouma G, Nikolic T, Coppens JM, van Helden-Meeuwsen CG, Leenen PJ, et al. (2005) NOD mice have a severely impaired ability to recruit leukocytes into sites of inflammation. Eur J Immunol 35: 225–235.
28. Rothblat GH, Bamberger M, Phillips MC (1986) Reverse cholesterol transport. Methods Enzymol 129: 628–644.
29. Basu SK, Goldstein JL, Anderson GW, Brown MS (1976) Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. Proc Natl Acad Sci U S A 73: 3178–3182.
30. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275.
31. Lie J, De Crom R, Van Gent T, Van Haperen R, Scheek L, et al. (2004) Elevation of plasma phospholipid transfer protein increases the risk of atherosclerosis in spite of lowering apolipoprotein B containing lipoproteins. J Lipid Res 45: 805–811.
32. Desrumaux CM, Mak PA, Boisvert WA, Masson D, Stupack D, et al. (2003) Phospholipid transfer protein is present in human atherosclerotic lesions and is expressed by macrophages and foam cells. J Lipid Res 44: 1453–1461.
33. Stein O, Stein Y (2005) Lipid transfer proteins (LTP) and atherosclerosis. Atherosclerosis 178: 217–230.
34. Colhoun HM, Taskinen MR, Otvos JD, Van Den Berg P, O'Connor J, et al. (2002) Relationship of Phospholipid Transfer Protein Activity to HDL and Apolipoprotein B-Containing Lipoproteins in Subjects With and Without Type 1 Diabetes. Diabetes 51: 3300–3305.
35. Riemens SC, van Tol A, Sluiter WJ, Dullaart RP (1998) Plasma phospholipid transfer protein activity is related to insulin resistance: impaired acute lowering by insulin in obese Type II diabetic patients. Diabetologia 41: 929–934.
36. Desrumaux C, Athias A, Bessede G, Verges B, Farnier M, et al. (1999) Mass concentration of plasma phospholipid transfer protein in normolipidemic, type IIa hyperlipidemic, type IIb hyperlipidemic, and non-insulin-dependent diabetic subjects as measured by a specific ELISA. Arterioscler Thromb Vasc Biol 19: 266–275.
37. Dullaart RP, Sluiter WJ, Dikkeschei LD, Hoogenberg K, Van Tol A (1994) Effect of adiposity on plasma lipid transfer protein activities: a possible link between insulin resistance and high density lipoprotein metabolism. Eur J Clin Invest 24: 188–194.
38. Cheung MC, Knopp RH, Retzlaff B, Kennedy H, Wolfbauer G, et al. (2002) Association of plasma phospholipid transfer protein activity with IDL and buoyant LDL: impact of gender and adiposity. Biochim Biophys Acta 1587: 53–59.
39. Kaser S, Sandhofer A, Foger B, Ebenbichler CF, Igelseder B, et al. (2001) Influence of obesity and insulin sensitivity on phospholipid transfer protein activity. Diabetologia 44: 1111–1117.
40. Murdoch SJ, Carr MC, Hokanson JE, Brunzell JD, Albers JJ (2000) PLTP activity in premenopausal women. Relationship with lipoprotein lipase, HDL, LDL, body fat, and insulin resistance. J Lipid Res 41: 237–244.
41. Murdoch SJ, Kahn SE, Albers JJ, Brunzell JD, Purnell JQ (2003) PLTP activity decreases with weight loss: changes in PLTP are associated with changes in subcutaneous fat and FFA but not IAF or insulin sensitivity. J Lipid Res 44: 1705–1712.
42. de Vries R, Dallinga-Thie GM, Smit AJ, Wolffenbuttel BH, van Tol A, et al. (2006) Elevated plasma phospholipid transfer protein activity is a determinant of carotid intima-media thickness in type 2 diabetes mellitus. Diabetologia 49: 398–404.
43. Foger B, Santamarina-Fojo S, Shamburek RD, Parrot CL, Talley GD, et al. (1997) Plasma phospholipid transfer protein. Adenovirus-mediated overexpression in mice leads to decreased plasma high density lipoprotein (HDL) and enhanced hepatic uptake of phospholipids and cholesteryl esters from HDL. J Biol Chem 272: 27393–27400.
44. Ehnholm S, van Dijk KW, van 't Hof B, van der Zee A, Olkkonen VM, et al. (1998) Adenovirus mediated overexpression of human phospholipid transfer protein alters plasma HDL levels in mice. J Lipid Res 39: 1248–1253.
45. Yan D, Navab M, Bruce C, Fogelman AM, Jiang XC (2004) PLTP deficiency improves the anti-inflammatory properties of HDL and reduces the ability of LDL to induce monocyte chemotactic activity. J Lipid Res 45: 1852–1858.
46. Schlitt A, Liu J, Yan D, Mondragon-Escorpizo M, Norin AJ, et al. (2005) Anti-inflammatory effects of phospholipid transfer protein (PLTP) deficiency in mice. Biochim Biophys Acta 1733: 187–191.
47. Cheung MC, Brown BG, Marino Larsen EK, Frutkin AD, O'Brien KD, et al. (2005) Phospholipid transfer protein activity is associated with inflammatory markers in patients with cardiovascular disease. Biochim Biophys Acta 1762: 131–137.
48. Karkkainen M, Oka T, Olkkonen VM, Metso J, Hattori H, et al. (2002) Isolation and partial characterization of the inactive and active forms of human plasma phospholipid transfer protein (PLTP). J Biol Chem 277: 15413–15418.
49. Valenta DT, Ogier N, Bradshaw G, Black AS, Bonnet DJ, et al. (2006) Atheroprotective Potential of Macrophage-Derived Phospholipid Transfer Protein in Low-Density Lipoprotein Receptor-Deficient Mice Is Overcome by Apolipoprotein AI Overexpression. Arterioscler Thromb Vasc Biol.
50. Vikstedt R, Ye D, Metso J, Hildebrand RB, Van Berkel TJ, et al. (2007) 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 27: 578–586.
51. Valenta DT, Bulgrien JJ, Bonnet DJ, Curtiss LK (2008) Macrophage PLTP is atheroprotective in LDLr-deficient mice with systemic PLTP deficiency. J Lipid Res 49: 24–32.
52. Lie J, Moerland M, van Gent T, van Haperen R, Scheek L, et al. (2006) Sex differences in atherosclerosis in mice with elevated phospholipid transfer protein activity are related to decreased plasma high density lipoproteins and not to increased production of triglycerides. Biochim Biophys Acta 1761: 1070–1077.
53. Jordan CT, Lemischka IR (1990) Clonal and systemic analysis of long-term hematopoiesis in the mouse. Genes Dev 4: 220–232.

Chapter 10

Elevation of systemic PLTP, but not macrophage-PLTP, impairs macrophage reverse cholesterol transport in transgenic mice

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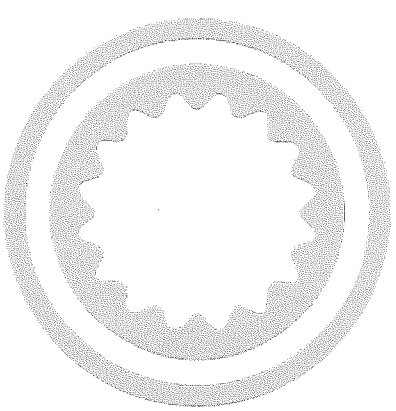

Elevation of systemic PLTP, but not macrophage-PLTP, impairs macrophage reverse cholesterol transport in transgenic mice

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ABSTRACT

Phospholipid transfer protein (PLTP) is a multifunctional protein synthesized by various cell types and secreted into the plasma. Plasma PLTP is able to transfer phospholipids between lipoproteins and modulate HDL particles. Mice with overexpression of human PLTP have an increased ability to generate preβ-HDL, reduced total HDL levels and an increased susceptibility to atherosclerosis. As the macrophage is a key component of the atherosclerotic lesion and an important site of PLTP expression, we investigated the role of systemic and peripheral PLTP in macrophage cholesterol efflux and reverse cholesterol transport (RCT) *in vivo*. We used an assay in which ^{3}H-labelled cholesterol-loaded macrophages were injected intraperitoneally into recipient mice, and radioactivity was quantified in plasma, liver and faeces.

Firstly, wild type macrophages were injected into wild type, PLTP transgenic (PLTPtg) and apoAI transgenic (apoAItg) mice. While plasma ^{3}H-tracer levels in apoAItg mice were increased compared with wild type mice, they were reduced in PLTPtg mice. Moreover, overexpression of PLTP significantly decreased faecal ^{3}H-tracer levels compared with wild type and apoAItg mice. Secondly, wild type mice were injected with peritoneal macrophages derived from PLTPtg or wild type mice. No significant difference in the amount of ^{3}H-tracer in plasma, liver or faeces was found between the two groups of mice.

Our findings demonstrate that macrophage cholesterol efflux and RCT to faeces is impaired in PLTP transgenic mice, and that elevation of macrophage-PLTP does not affect RCT, indicating that higher systemic PLTP levels may promote atherosclerosis development by decreasing the rate of macrophage RCT.

1. Introduction

Macrophage cholesterol efflux and reverse cholesterol transport (RCT) are believed to inhibit atherogenesis by transporting excess cholesterol from macrophages to the liver for biliary excretion. An inverse association between high density lipoprotein (HDL) levels and atherosclerosis is shown in many studies, and is explained by the ability of HDL to promote RCT [1,2]. One protein with a key role in HDL metabolism and possibly also in RCT is phospholipid transfer protein (PLTP).

PLTP is expressed by various cell types and secreted into the plasma, where it transfers phospholipids between lipoproteins [3] and modulates the size and composition of HDL particles, generating lipid-poor preβ-HDL [4,5], an efficient acceptor of cellular cholesterol *in vitro*. Mice with elevated PLTP activity levels have an increased ability to generate preβ-HDL [6,7]. Nevertheless, systemic PLTP has been identified as pro-atherogenic *in vivo*. Mice overexpressing human PLTP have elevated plasma PLTP levels, resulting in a decrease in total HDL levels and an increased development of atherosclerosis [8].

As PLTP is highly expressed in macrophages and colocalised with foam cells in atherosclerotic lesions [9–11], recent studies have focused on the role of macrophage-derived PLTP in the development of atherosclerosis, thereby revealing conflicting results. Bone marrow transplantation studies using PLTP deficient macrophages resulted in either decreased [12] or increased atherosclerosis [13–15]. Mice transplanted with bone marrow of PLTP overexpressing mice had an increased susceptibility to diet-induced atherosclerosis [16]. The exact mechanism of atherogenicity and the contribution of local PLTP effects in the vessel wall to atherosclerosis development are not fully understood. Peripheral PLTP could be involved in macrophage cholesterol efflux, as suggested from

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in vitro studies. Purified PLTP, added to cultured macrophages, enhanced ABCA1-mediated cholesterol efflux, suggesting an anti-atherogenic role for PLTP [17]. Active PLTP, preincubated with HDL particles, enhanced macrophage cholesterol efflux through the generation of efficient cholesterol acceptor particles [18]. The importance of intracellular PLTP for macrophage cholesterol efflux however, remains unclear. In cultures of macrophages lacking PLTP, a potential athero-protective role of PLTP in cholesterol efflux has been reported [19], but no effect on cholesterol efflux was found by others using different experimental conditions [15,20,21].

In the present study, we investigated the role of systemic and peripheral PLTP in macrophage RCT *in vivo*. We used a validated assay in which labelled cholesterol originating from mouse peritoneal macrophages was traced in plasma, liver and faeces [22,23]. We studied the effect of systemic PLTP on macrophage cholesterol efflux to plasma and subsequent transport to faeces, as well as the role of macrophage-PLTP, using macrophages from mice overexpressing human PLTP, in cholesterol efflux and RCT *in vivo*.

2. Materials and methods

2.1. Animals

The generation of transgenic mice with expression of human PLTP ("PLTPtg"), controlled by its native promoter, has been described previously [6]. Human apoAI transgenic mice ("apoAItg") were purchased from Jackson Laboratory. All mice had a C57Bl/6J background. Male mice between 10 and 18 weeks old were used and kept on regular chow diet. Blood was collected from the orbital plexus. All animal experiments were carried out in compliance with national and institutional guidelines.

2.2. PLTP activity

PLTP activity in plasma or culture medium was determined as described before [6]. PLTP activity levels are expressed as arbitrary units (AU; 1 AU = 13.9 mmol/L/h, the level found in human reference plasma).

2.3. Separation of plasma lipoproteins and plasma lipid analysis

To determine plasma lipoprotein size distribution, pooled plasma from 6 to 10 mice was applied onto an FPLC system containing a Superdex 200 prepgrade and a Superose 6 prepgrade column connected in tandem. Columns were equilibrated and run in buffer containing 65 mmol/L sucrose, 225 mmol/L mannitol, 10 mmol/L Tris–HCl pH 8.1, 5 mmol/L EDTA, 0.02% NaN_3. Collected fractions were analysed for radioactivity and total cholesterol (TC) levels. TC was measured using a Wako kit.

2.4. Macrophage cell culture

To isolate peritoneal macrophages, mice were injected intraperitoneally with 2 mL of Bacto® Brewer Thioglycollate Medium (Difco). Three days later, mice were sacrificed and macrophages were isolated from peritoneal cavity.

For *in vitro* cholesterol efflux experiments, cells were plated onto 24-well dishes at a density of $1–2 \times 10^6$ cells/mL in Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 10% fetal calf serum (FCS) and 100 U/mL penicillin/streptomycin (Gibco), and incubated at 37 °C for 4 h. Acetylated LDL (AcLDL) was prepared as described [24]. DMEM containing 2% fat-free bovine serum albumin (BSA) (Sigma) and 50 μg/mL AcLDL, which was preincubated with 1 μCi/mL ^{3}H-cholesterol (Amersham) for 30 min, was added to the cells. After 30 h, medium was collected and radioactivity was counted. The labelled cells were washed and equilibrated for 10–14 h in DMEM containing 2% fat-free BSA, in the presence or absence of 0.3 mmol/L cAMP (Sigma).

For *in vivo* cholesterol efflux experiments, cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS, Gibco) and single cells were resuspended in DMEM medium containing 2% fat-free BSA, 10% Macrophage Colony-stimulating Factor (M-CSF) culture medium [25] and 25 μg/mL AcLDL, pre-incubated with 5 μCi/mL ^{3}H-cholesterol. The cells were kept in suspension at 37 °C for 20 h. After loading, cells were washed and equilibrated in DMEM containing 0.2% fat-free BSA for 2 h. Finally, cells were washed and resuspended in minimum essential medium.

2.5. In vitro cholesterol efflux from mouse peritoneal macrophages

Cholesterol efflux was initiated by incubating the ^{3}H-AcLDL-loaded macrophages in efflux medium consisting of DMEM, 0.2% fat-free BSA, and 50 μg/mL human HDL or 10 μg/mL lipid-free human apoAI (Calbiochem) as cholesterol acceptor. In every experiment, incubations without acceptor were included. Efflux medium was collected after 5 h, and used for liquid scintillation counting. After cells were washed with ice-cold DPBS, remaining cholesterol was extracted from the intact monolayers with isopropanol, and radioactivity was determined. Cells were lysed in 0.1 mol/L NaOH with 1% SDS, and cell proteins were quantified [26]. Relative cellular cholesterol efflux was calculated as the percentage of radioactivity in the medium relative to the total radioactivity in cells and medium, normalized for differences in cell proteins, and corrected for efflux of ^{3}H-tracer in the absence of acceptor particles. Relative efflux was determined in six mice per group, performed in triplo and expressed as percentage of radioactivity per milligram cell protein.

2.6. Cholesterol accumulation in macrophages

Radioactivity in labelled, AcLDL-loaded macrophages was quantified as described above. To determine cellular cholesterol content, peritoneal macrophages were incubated with 50 μg/mL AcLDL for 30 h after which lipids were extracted and total cholesterol (TC) content was determined in the extracts. Cells were lysed and proteins were quantified as described above.

Cholesterol accumulation in macrophages was expressed as the percentage of radioactivity recovered in cells per cell protein, or as the percentage of radioactivity per μg TC in the cells.

2.7. In vivo cholesterol efflux and RCT from mouse peritoneal macrophages

Per mouse, 0.5 mL medium with ^{3}H-AcLDL-loaded macrophages ($\sim 10 \times 10^6$ cells, 10×10^6 dpm) was injected intraperitoneally. Blood samples were collected at 6, 24 and 48 h after injection, and used for liquid scintillation counting. After 48 h, mice were bled and livers were removed. Plasma lipoprotein analyses were performed. Liver sections of approximately 0.2 g were dissolved overnight at 50 °C in Soluene-350, followed by treatment with isopropanol and hydrogen peroxide after which radioactivity was measured by liquid scintillation counting. Faeces were collected during these 48 h, weighed and stored at −20 °C until lipid extraction. Faeces were homogenized in a methanol–chloroform solution using an Ultra-Turrax, and subsequently faecal lipids were extracted [27]. ^{3}H-tracer levels in plasma, liver and faeces were expressed as percentages of injected ^{3}H dose.

Table 1
Plasma PLTP and lipids.

	apoAItg	wt	PLTPtg
TC	5.73 ± 0.29[a]	2.47 ± 0.07	1.18 ± 0.08[a,b]
HDL-C	3.56 ± 0.22[a]	1.43 ± 0.03	0.67 ± 0.04[a,b]
PLTP activity	2.76 ± 0.19[a]	1.92 ± 0.19	8.01 ± 0.61[a,b]

TC and HDL-C values are expressed in mmol/L, PLTP activity is expressed as arbitrary units. Values represent mean ± SEM.
[a] $p < 0.05$ compared to wild type mice.
[b] $p < 0.05$ compared to apoAI tg mice.

2.8. Statistics

All values are expressed as mean ± SEM. Differences were analyzed by two-sample Wilcoxon ranksum tests using Intercooled Stata 8.2/SE software (Stata Corporation, College Station, Texas USA).

3. Results

3.1. Macrophage ^{3}H-cholesterol efflux and RCT in PLTP transgenic mice

To elucidate the role of PLTP in cholesterol efflux and reverse cholesterol transport from macrophage to faeces *in vivo*, thioglycollate-elicited peritoneal macrophages derived from wild type mice were harvested and loaded with ^{3}H-labelled AcLDL, after which they were injected into the peritoneum of apoAI transgenic (apoAItg), PLTP transgenic (PLTPtg) and wild type (wt) recipient mice. Plasma PLTP activity levels in apoAItg mice were 1.4-fold higher than in wt mice, while overexpressing human PLTP in mice increased total plasma PLTP activity 4-fold. Total cholesterol (TC) and HDL-cholesterol (HDL-C) levels were strongly decreased in PLTPtg and increased in apoAItg mice, when compared to wt mice (Table 1). At 6, 24 and 48 h after injection of the mice with macrophages, a blood sample was taken and plasma ^{3}H-tracer levels were determined. As expected, ^{3}H-tracer was increased in plasma of apoAItg mice compared to wt mice, with a 2.6-fold increase after 48 h. In PLTPtg mice however, radioactive counts in plasma were 2-fold lower than in wt mice at all timepoints studied (Fig. 1A). At the end of the experiment, radioactivity was quantified in liver and in faeces, collected during the 48 h after injection of macrophages. Liver ^{3}H-tracer levels were not significantly different between the three groups (apoAItg: 6.56 ± 0.31 %ID; wt: 5.59 ± 0.24 %ID; PLTPtg: 5.17 ± 0.29 %ID). However, in PLTPtg mice, faecal excretion of ^{3}H-sterol was 22% and 40% lower than in wt and apoAItg mice, respectively (Fig. 1B). These results show that macrophage cholesterol efflux and reverse cholesterol transport are impaired in mice overexpressing PLTP.

To study whether the ^{3}H-labelled cholesterol, after efflux from macrophages, is equally distributed among lipoproteins in plasma as endogenous cholesterol, plasma samples taken after 48 h were pooled and subjected to gel filtration chromatography. FPLC analyses show that the profiles of macrophage-derived ^{3}H-cholesterol tracer and cholesterol mass in plasma lipoproteins tracked closely with each other (Fig. 2).

3.2. ^{3}H-cholesterol efflux from PLTP-overexpressing peritoneal macrophages to apoAI and HDL in vitro

To study the role of macrophage-PLTP in cholesterol efflux *in vitro*, peritoneal macrophages from PLTPtg and wt mice were loaded with ^{3}H-cholesterol-AcLDL, and equilibrated in the presence or absence of cAMP, which is known to further upregulate ATP-binding

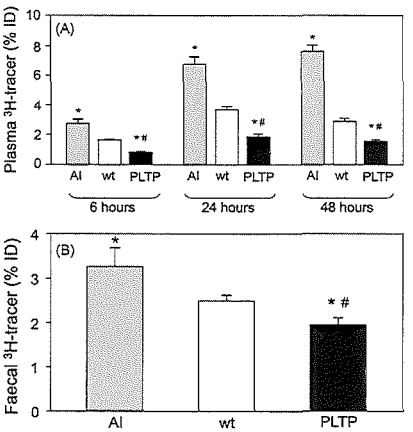

Fig. 1. Systemic PLTP in macrophage RCT *in vivo*. ApoAI transgenic (AI), wild type (wt), and PLTP transgenic (PLTP) mice were injected intraperitoneally with ^{3}H-cholesterol-labelled AcLDL-loaded wild type macrophages. ^{3}H-tracer was measured in plasma (A) and faeces (B). (A) Plasma ^{3}H-tracer at 6, 24, and 48 h after injection. Values are expressed as % injected dose (ID) ± SEM. $n = 6$–10 mice per group. *$p < 0.05$ versus wild type, #$p < 0.05$ versus apoAItg. (B) ^{3}H-tracer in faeces collected during 48 h after injection. Values are expressed as % injected dose (ID) ± SEM. $n = 6$–10 mice per group. *$p < 0.05$ versus wild type, #$p < 0.05$ versus apoAItg.

Cassette (ABC) transporter A1 [28]. PLTP activity found in culture medium of PLTPtg cells after 24 h was 10-fold higher compared to PLTP activity in medium of wt cells. Cholesterol accumulation in loaded macrophages, expressed as the amount of label per cell protein or per cellular cholesterol mass, was not different between wild type and PLTPtg macrophages (49 versus 47% ^{3}H/100 μg cell protein and 9.6 versus 8.9% ^{3}H/μg TC, for wt and PLTPtg cells, respectively). The efflux of ^{3}H-tracer to human apoAI or HDL was quantified (Fig. 3). As expected, treatment of the macrophages with cAMP increased cholesterol efflux to apoAI approximately 3-fold. Efflux of ^{3}H-cholesterol from PLTP-overexpressing macrophages to apoAI was slightly reduced in the absence of cAMP compared to wt macrophages, but when ABCA1 was further upregulated by cAMP-treatment, lipid efflux was not different between the two groups (Fig. 3A). When human HDL was used as acceptor, no difference in cholesterol efflux was seen between cells overexpressing PLTP and wt cells (Fig. 3B).

3.3. ^{3}H-cholesterol efflux and RCT from PLTP-overexpressing peritoneal macrophages in vivo

We further investigated the role of macrophage-PLTP in cholesterol efflux and RCT *in vivo*. Peritoneal macrophages derived from PLTPtg or wt mice were isolated, and loaded with ^{3}H-labelled AcLDL. Cells were injected intraperitoneally into wild type recipient mice. ^{3}H-tracer levels in plasma at 6, 24 and 48 h after injection were similar in both mouse groups (Fig. 4A). No significant difference in ^{3}H-tracer was found in liver (not shown) or faeces (Fig. 4B) of mice injected with PLTP-overexpressing macrophages compared to mice injected with wild type cells. These data indicate that elevation of macrophage-PLTP has no effect on cholesterol efflux and macrophage reverse cholesterol transport *in vivo*.

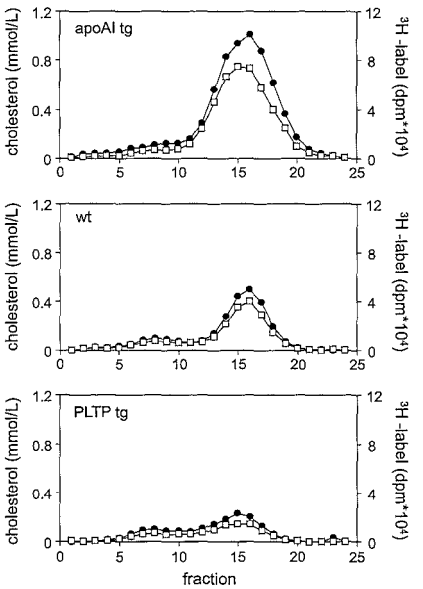

Fig. 2. Distribution of plasma cholesterol and ^{3}H-label among lipoprotein particles. At 48 h after injection, pooled plasma samples were subjected to FPLC. TC (filled circles) and ^{3}H-label (open squares) were quantified in the fractions. Values are mmol/L and disintegrations per minute (dpm) × 10^4, respectively, and represent the means of 6–10 mice.

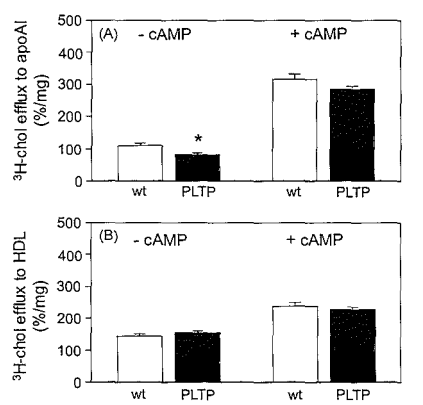

Fig. 3. Macrophage cholesterol efflux *in vitro*. Efflux of ^{3}H-labelled cholesterol from isolated peritoneal macrophages of wild type (wt) and PLTP transgenic (PLTP) to human apoAI (A) or HDL (B) was quantified *in vitro*, in the presence and absence of cAMP. Values are expressed as % cholesterol efflux per milligram cell protein ± SEM. $n = 6$ mice per group. $^*p < 0.05$ versus wild type.

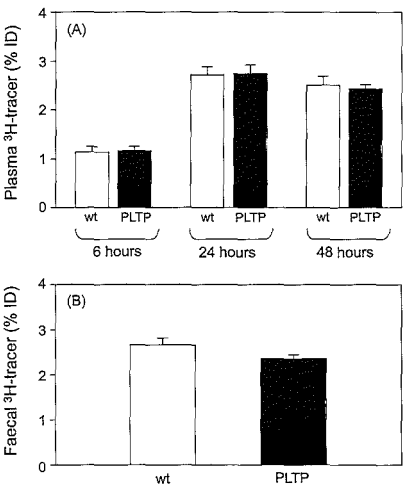

Fig. 4. Macrophage-PLTP in RCT *in vivo*. Wild type mice were injected intraperitoneally with ^{3}H-cholesterol-labelled AcLDL-loaded wild type (wt) or PLTP transgenic (PLTP) macrophages. ^{3}H-tracer was measured in plasma (A) and faeces (B). (A) Plasma ^{3}H-tracer at 6, 24, and 48 h after injection. Values are expressed as % injected dose (ID) ± SEM. $n = 7$–10 mice per group. $^*p < 0.05$ versus wild type (B) ^{3}H-tracer in faeces collected during 48 h after injection. Values are expressed as % injected dose (ID) ± SEM. $n = 6$–10 mice per group. $^*p < 0.05$ versus wild type.

4. Discussion

In the present study we demonstrate that (1) systemic overexpression of human PLTP in transgenic mice results in a decreased cholesterol efflux and reverse cholesterol transport from mouse peritoneal macrophages, and that (2) elevation of macrophage-PLTP does not affect macrophage cholesterol efflux.

PLTP is a key player in HDL metabolism. A facilitating role of PLTP in reverse cholesterol transport has been suggested, as (1) PLTP enhances the formation of preβ-HDL particles by converting HDL particles [4,5,18], (2) PLTP expression is up-regulated by activation of the nuclear liver X receptor (LXR), a transcription factor regulating the expression of several RCT genes, such as members of the ATP-binding cassette (ABC) transporter family ABCA1 and ABCG1 [11,20,29], and (3) PLTP might stimulate faecal bile acid excretion [30]. With regard to the role of PLTP in cellular cholesterol efflux and RCT, it is important to distinguish between local, cellular PLTP effects and systemic PLTP effects, which may influence atherogenesis differently. In the present study, we investigated the role of both systemic and cellular macrophage-PLTP in cholesterol efflux and RCT *in vivo*.

To study the effect of elevated systemic PLTP on RCT, we used a validated assay to trace labelled cholesterol from macrophage to faeces *in vivo*, as firstly reported by Zhang et al. [22]. In a later study by the same research group [23], parallel experiments were performed in which a direct comparison was made between either thioglycollate-elicited or bone marrow-derived macrophages from different mice. It was demonstrated that both types of macrophages are useful models to study macrophage cholesterol efflux and RCT *in vivo*. We found that elevation of plasma PLTP activity decreases RCT from thioglycollate-elicited mouse peritoneal macrophages, while

elevation of apoAI in apoAItg mice was used as a positive control showing increased macrophage RCT. RCT rates in our mice correlate with plasma HDL levels. It is likely that the effect of PLTP on cholesterol efflux and RCT can be attributed to the decreased plasma HDL levels, illustrating the importance of plasma acceptor concentration in the process of cholesterol efflux in these mouse models. However, it is important to notice that RCT rates are not always dependent on plasma HDL levels, as mice lacking scavenger receptor class B type I (SR-BI) have a reduced macrophage RCT despite higher plasma HDL levels [31], and wild type mice with ABCA1 or ABCG1 deficient macrophages have an impaired macrophage RCT, while plasma lipid levels are not altered [23]. Moreover, expression of cholesteryl ester transfer protein (CETP) in mice reduced HDL levels but promoted macrophage RCT [32]. Although PLTP and CETP share structural and functional characteristics, and overexpression *in vivo* of each protein results in a decrease of HDL levels, CETP stimulates macrophage RCT, while our present data show that PLTP reduces macrophage RCT. Studies in PLTP deficient mice illustrate that characteristics of HDL are just as important as its plasma concentration in physiological processes such as atherogenesis. PLTP deficient mice have reduced levels of HDL, with enhanced anti-inflammatory properties [33], and have decreased atherosclerosis [34]. The finding that apoAItg mice have an increased macrophage RCT *in vivo*, while these mice have significantly increased plasma PLTP activity levels, suggests that the impaired RCT found in PLTPtg mice is the result of an indirect action of PLTP through its effects on HDL metabolism, rather than a direct PLTP effect. To further investigate this hypothesis, it would be useful to investigate macrophage RCT using PLTP deficient mice.

PLTP is able to remodel HDL particles thereby enhancing the formation of preβ-HDL particles, which are efficient acceptors of cellular cholesterol *in vitro* [4,5,18]. Our current findings challenge the concept that preβ-HDL particles are important for reverse cholesterol efflux *in vivo*. Indeed, in a recent *in vivo* study, it was demonstrated that HDL is more critical in controlling RCT than lipid-poor apoAI [35].

In a previous study, PLTP was described as a positive regulator in faecal bile acid excretion in PLTP transgenic mice [30]. However, the levels of plasma PLTP activity required for this positive effect were high (15-fold higher than in wild type mice) compared with the PLTP activity in mice used in the present study (4-fold higher than in wild type mice). Moreover, the disposal of body cholesterol originating from all peripheral tissues was investigated, rather than cholesterol derived specifically from macrophages. Although the latter is only a very small portion of the total cholesterol pool, it is a very important source in the process of atherosclerosis. In a study with PLTP deficient mice, no difference in bile acid excretion was found, but these mice were shown to absorb less cholesterol [36]. We cannot exclude the possibility that intestinal cholesterol absorption is affected in our PLTPtg mouse model. A possible stimulation of intestinal cholesterol absorption could result in decreased faecal sterol excretion. Whether this is also the case for cholesterol originating from macrophages remains to be investigated.

Overall, the present data further support the hypothesis that elevated systemic PLTP activity levels are pro-atherogenic and its role in macrophage RCT may be an important contributor to atherosclerosis development.

Effects of systemic PLTP on atherosclerosis development however, may be different from the local cellular PLTP effects and its role in the vascular wall. In bone marrow transplantation studies, macrophage-specific PLTP deficiency was associated with an increased development of atherosclerosis in ApoE knockout [13] or LDLR knockout mice either with or without systemic PLTP expression [14,15], indicating an anti-atherogenic role of macrophage-PLTP. In another study however, an increased resistance to atherosclerosis was found in LDLR deficient mice with PLTP deficient macrophages [12], suggesting a pro-atherogenic role of macrophage PLTP. Although macrophage-PLTP contributed to plasma PLTP levels in all studies, the extent to which levels were increased varied significantly between different studies (up to 2-fold [12]), which could explain at least partly the different results in atherosclerosis development found between the groups. When transplanting bone marrow cells of PLTP overexpressing mice into LDLR knockout recipient mice, plasma PLTP activity levels were more than 3-fold higher and an increased susceptibility to diet-induced atherosclerosis was found [16]. The findings demonstrate that macrophage-specific PLTP might be either pro- or anti-atherogenic, depending on the experimental setup. Because macrophage-PLTP contributed significantly to plasma PLTP levels, it is hard to draw conclusions regarding local PLTP effects in the vessel wall and the exact involvement of these local cellular PLTP effects in atherosclerosis development. Suggested anti-atherogenic properties of macrophage-derived PLTP include (1) the stimulation of ABCA1-dependent cholesterol efflux [17,19], (2) the generation of preβ-HDL within the vessel wall [18], and (3) increasing cellular vitamin E content, resulting in lower oxidative stress and limiting LDL uptake by macrophages [21]. The possible role of these individual factors has not yet been addressed *in vivo*.

The contribution of PLTP to macrophage cholesterol efflux has been assessed in several *in vitro* studies. Exogenous human PLTP, added to macrophage cultures, enhanced cellular cholesterol efflux to HDL particles, by promoting cell-surface binding, interacting with and stabilizing ABCA1 [17]. In another study, macrophage cholesterol efflux to HDL was increased after preincubation of HDL with purified active PLTP, through the formation of preβ-HDL and large fused HDL particles, both efficient cholesterol acceptors *in vitro* [18]. However, as mentioned above, the role of cholesterol acceptor particles *in vivo* remains unclear. In studies using macrophages lacking PLTP, the role of endogenous PLTP in cellular cholesterol efflux has been investigated. Cholesterol efflux from thioglycollate-elicited PLTP deficient macrophages was not different compared to efflux from wild type macrophages, neither towards apoAI [20,21], nor towards HDL [15]. In non-elicited PLTP deficient macrophages, a decreased cholesterol efflux to apoAI and HDL particles has been observed, but only with cholesterol-loaded cells when cAMP was not added to the culture medium [19]. In the present study, we studied the involvement of intracellular human macrophage-PLTP in cholesterol efflux both *in vitro* and *in vivo*. Despite an increase in PLTP activity in culture medium, peritoneal macrophages from PLTP transgenic mice did not show differences in cholesterol efflux towards human HDL compared with wild type macrophages, in accordance with earlier findings [16]. We now report a slight but significant decrease in efflux towards apoAI in the absence of cAMP, but ABCA1-dependent cholesterol efflux after cAMP-treatment was not different. To study cellular cholesterol efflux and RCT *in vivo*, we injected cholesterol-loaded human wild type or PLTP transgenic macrophages into wild type mice. Injection of PLTP transgenic macrophages in mice did not affect plasma PLTP activity (data not shown), demonstrating that our method allows us to focus specifically on the cellular effects of macrophage-PLTP *in vivo*, without induction of systemic effects. No change in plasma tracer levels was found, suggesting that overexpressing human PLTP in macrophages has no effect on cholesterol efflux *in vivo*. Furthermore, the recovery of labelled cholesterol in liver or faeces from mice injected with PLTP transgenic macrophages was not significantly different from mice injected with wild type macrophages.

In conclusion, macrophage cholesterol efflux was not affected by elevation of macrophage-PLTP, suggesting that the atherogenicity of human PLTP in mice is not influenced by a local effect on macrophage cholesterol efflux. Systemic PLTP however, decreased

macrophage RCT, contributing to the pro-atherogenic potential of PLTP in transgenic mice. Our findings demonstrate that lowering high plasma PLTP activity levels could be of therapeutic benefit for the prevention or treatment of atherosclerosis.

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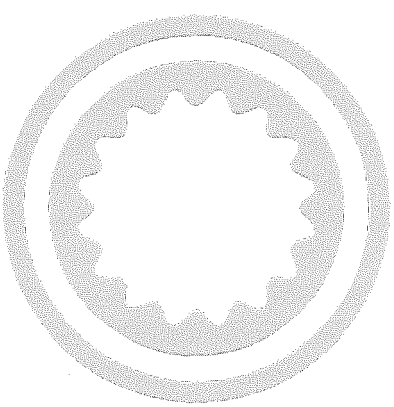
References

[1] Wang X, Rader DJ. Molecular regulation of macrophage reverse cholesterol transport. Curr Opin Cardiol 2007;22:368–72.
[2] Tall AR. Cholesterol efflux pathways and other potential mechanisms involved in the athero-protective effect of high density lipoproteins. J Intern Med 2008;263:256–73.
[3] Tall AR, Krumholz S, Olivecrona T, Deckelbaum RJ. Plasma phospholipid transfer protein enhances transfer and exchange of phospholipids between very low density lipoproteins and high density lipoproteins during lipolysis. J Lipid Res 1985;26:842–51.
[4] Jauhiainen M, Metso J, Pahlman R, et al. Human plasma phospholipid transfer protein causes high density lipoprotein conversion. J Biol Chem 1993;268: 4032–6.
[5] Tu AY, Nishida HI, Nishida T. High density lipoprotein conversion mediated by human plasma phospholipid transfer protein. J Biol Chem 1993;268: 23098–105.
[6] van Haperen R, van Tol A, Vermeulen P, et al. Human plasma phospholipid transfer protein increases the antiatherogenic potential of high density lipoproteins in transgenic mice. Arterioscler Thromb Vasc Biol 2000;20:1082–8.
[7] Jiang X, Francone OL, Bruce C, et al. Increased prebeta-high density lipoprotein, apolipoprotein AI, and phospholipid in mice expressing the human phospholipid transfer protein and human apolipoprotein AI transgenes. J Clin Invest 1996;98:2373–80.
[8] van Haperen R, van Tol A, van Gent T, et al. Increased risk of atherosclerosis by elevated plasma levels of phospholipid transfer protein. J Biol Chem 2002;277:48938–43.
[9] O'Brien KD, Vuletic S, McDonald TO, et al. Cell-associated and extracellular phospholipid transfer protein in human coronary atherosclerosis. Circulation 2003;108:270–4.
[10] Desrumaux CM, Mak PA, Boisvert WA, et al. Phospholipid transfer protein is present in human atherosclerotic lesions and is expressed by macrophages and foam cells. J Lipid Res 2003;44:1453–61.
[11] Laffitte BA, Joseph SB, Chen M, et al. The phospholipid transfer protein gene is a liver X receptor target expressed by macrophages in atherosclerotic lesions. Mol Cell Biol 2003;23:2182–91.
[12] Vikstedt R, Ye D, Metso J, et al. 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;27:578–86.
[13] Liu R, Hojjati MR, Devlin CM, Hansen IH, Jiang XC. Macrophage phospholipid transfer protein deficiency and ApoE secretion: impact on mouse plasma cholesterol levels and atherosclerosis. Arterioscler Thromb Vasc Biol 2007;27:190–6.
[14] Valenta DT, Bulgrien JJ, Bonnet DJ, Curtiss LK. Macrophage PLTP is atheroprotective in LDLr-deficient mice with systemic PLTP deficiency. J Lipid Res 2008;49:24–32.
[15] Valenta DT, Ogier N, Bradshaw G, et al. Atheroprotective potential of macrophage-derived phospholipid transfer protein in low-density lipoprotein receptor-deficient mice is overcome by apolipoprotein AI overexpression. Arterioscler Thromb Vasc Biol 2006;26:1572–8.
[16] van Haperen R, Samyn H, Moerland M, et al. Elevated expression of phospholipid transfer protein in bone marrow derived cells causes atherosclerosis. PLoS ONE 2008;3:e2255.
[17] Oram JF, Wolfbauer G, Vaughan AM, Tang C, Albers JJ. Phospholipid transfer protein interacts with and stabilizes ATP-binding cassette transporter A1 and enhances cholesterol efflux from cells. J Biol Chem 2003;278: 52379–85.
[18] Vikstedt R, Metso J, Hakala J, et al. Cholesterol efflux from macrophage foam cells is enhanced by active phospholipid transfer protein through generation of two types of acceptor particles. Biochemistry 2007;46:11979–86.
[19] Lee-Rueckert M, Vikstedt R, Metso J, et al. Absence of endogenous phospholipid transfer protein impairs ABCA1-dependent efflux of cholesterol from macrophage foam cells. J Lipid Res 2006;47:1725–32.
[20] Cao G, Beyer TP, Yang XP, et al. Phospholipid transfer protein is regulated by liver X receptors *in vivo*. J Biol Chem 2002;277:39561–5.
[21] Ogier N, Klein A, Deckert V, et al. Cholesterol accumulation is increased in macrophages of phospholipid transfer protein-deficient mice: normalization by dietary alpha-tocopherol supplementation. Arterioscler Thromb Vasc Biol 2007;27:2407–12.
[22] Zhang Y, Zanotti I, Reilly MP, et al. Overexpression of apolipoprotein A-I promotes reverse transport of cholesterol from macrophages to feces *in vivo*. Circulation 2003;108:661–3.
[23] Wang X, Collins HL, Ranalletta M, et al. Macrophage ABCA1 and ABCG1, but not SR-BI, promote macrophage reverse cholesterol transport in vivo. J Clin Invest 2007;117:2216–24.
[24] Moerland M, Samyn H, van Gent T, et al. Atherogenic, enlarged, and dysfunctional HDL in human PLTP/apoA-I double transgenic mice. J Lipid Res 2007;48:2622–31.
[25] Sklar MD, Tereba A, Chen BD, Walker WS. Transformation of mouse bone marrow cells by transfection with a human oncogene related to c-myc is associated with the endogenous production of macrophage colony stimulating factor 1. J Cell Physiol 1985;125:403–12.
[26] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[27] Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911–7.
[28] Oram JF, Lawn RM, Garvin MR, Wade DP. ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages. J Biol Chem 2000;275:34508–11.
[29] Tontonoz P, Mangelsdorf DJ. Liver X receptor signaling pathways in cardiovascular disease. Mol Endocrinol 2003;17:985–93.
[30] Post SM, de Crom R, van Haperen R, van Tol A, Princen HM. Increased fecal bile acid excretion in transgenic mice with elevated expression of human phospholipid transfer protein. Arterioscler Thromb Vasc Biol 2003;23:892–7.
[31] Zhang Y, Da Silva JR, Reilly M, et al. Hepatic expression of scavenger receptor class B type I (SR-BI) is a positive regulator of macrophage reverse cholesterol transport *in vivo*. J Clin Invest 2005;115:2870–4.
[32] Tanigawa H, Billheimer JT, Tohyama J, et al. Expression of cholesteryl ester transfer protein in mice promotes macrophage reverse cholesterol transport. Circulation 2007;116:1267–73.
[33] Yan D, Navab M, Bruce C, Fogelman AM, Jiang XC. PLTP deficiency improves the anti-inflammatory properties of HDL and reduces the ability of LDL to induce monocyte chemotactic activity. J Lipid Res 2004;45:1852–8.
[34] Jiang XC, Qin S, Qiao C, et al. Apolipoprotein B secretion and atherosclerosis are decreased in mice with phospholipid-transfer protein deficiency. Nat Med 2001;7:847–52.
[35] Wang MD, Kiss RS, Franklin V, et al. Different cellular traffic of LDL-cholesterol and acetylated LDL-cholesterol leads to distinct reverse cholesterol transport pathways. J Lipid Res 2007;48:633–45.
[36] Liu R, Iqbal J, Yeang C, et al. Phospholipid transfer protein-deficient mice absorb less cholesterol. Arterioscler Thromb Vasc Biol 2007;27:2014–21.

Chapter 11

Summary and future perspectives



11 Summary and future perspectives

11.1 Summary

PLTP has been identified as an important player in lipoprotein metabolism and the development of atherosclerosis. However, the exact role of PLTP in these processes is far from resolved. We provided further insight into the atherogenic properties of PLTP using a broad variety of experimental setups. First of all, we developed new mouse models to study the role of PLTP in lipoprotein metabolism and atherogenesis: one model with conditional expression of human PLTP, and one model with overexpression of an inactive mutant form of human PLTP. In **chapter 2** we explored the features of a mouse model with a tetracycline-dependent conditional induction of human PLTP expression. This tetracycline-responsive mouse model provides the opportunity to study the interference of acute changes in PLTP expression in a pathological situation or in a metabolic pathway. This situation varies largely from conventional PLTP transgenic mouse models, in which the effect of the genetic modification is present life-long, resulting in metabolic adaptation to this modification. The tetracycline-responsive mouse model that we used is preferable over an adenovirus-mediated overexpression system, as expression of the gene of interest is better controlled, easily reversible, not mainly liver-located, and without reduction of transgene expression over time. Our mouse model proved to be feasible for induction of an acute and easily reversible elevation of PLTP activity. We used our model to study the effects of an acute elevation of PLTP activity on lipoprotein metabolism. The increase in PLTP activity resulted in a decrease in plasma cholesterol and phospholipids, which was explained by a decrease in plasma HDL levels. The decrease in HDL levels could not be explained by changes in RNA expression levels of SR-BI, apoB or ABCA1, suggesting that the decline in HDL is a direct effect of PLTP. An advantage of this inducible mouse model is that it provides a stable and controlled elevation of PLTP activity, making it suitable for examination of the effects of acutely induced long-term overexpression of human PLTP, which we did in the follow-up study. In this study, described in **chapter 3**, we used the mouse model with conditional induction of human PLTP expression to investigate the effects of PLTP expression on lipoprotein metabolism under diet-induced hyperlipidemic conditions, and on pre-existing atherosclerosis. We observed that an acute increase in plasma PLTP activity had major effects on lipoprotein metabolism. HDL levels were strongly decreased upon expression of human PLTP, in accordance with the findings reported in chapter 2. In addition, we discovered that acute induction of human PLTP largely increased plasma triglyceride levels, and inhibited the catabolism of apoB-containing lipoproteins. Literature shows that, under certain conditions, the secretion of apoB-containing lipoproteins by the liver is stimulated by overexpression of PLTP, but as

far as we are aware, a PLTP-dependent increase of plasma triglyceride levels and inhibition of the catabolism of apoB-containing lipoproteins has never been demonstrated before. Apparently, the metabolic effects of an acute increase in PLTP activity differ from the effects of the life-long increased plasma PLTP activity levels in human PLTP transgenic mice. The increased VLDL levels in the conditional PLTP mice could not be explained by an increase in VLDL production, as triglyceride secretion rate by the liver was not affected by overexpression of PLTP. However, the removal of VLDL from the circulation was delayed, with a strongly decreased uptake of cholesterol in the livers of these mice. The decreased VLDL clearance in human PLTP expressing mice could be partly explained by a decrease in plasma LPL activity. Furthermore, we studied the effect of increased PLTP activity on pre-existing atherosclerotic lesions. The unfavourable lipoprotein profile observed in this mouse model resulted in a strong increase in atherosclerotic lesion area when Western diet was stopped and PLTP activity was induced. In addition, acute expression of PLTP resulted in the development of unstable lesions, as determined by lesion macrophage and collagen content. This study supplies new evidence that high systemic PLTP expression unfavourably affects the condition of the cardiovascular system, not only by decreasing plasma HDL levels, but also by inhibition of VLDL catabolism.

Although many different mouse models have been used to study the role of PLTP in atherosclerosis development, until now, studies in mice focused on the absence of PLTP protein (PLTP knockout mice) or on the overexpression of PLTP (PLTP transgenic mice). PLTP displays phospholipid transfer activity, which has been well described *in vitro*. However, the importance of this lipid transfer activity *in vivo* is not completely understood. In human plasma, two forms of PLTP have been described. Besides the catalytically active form, also a low-active form with unknown function exists. In order to provide more insight into the physiological role of PLTP transfer activity and PLTP mass, we generated a new mouse model with overexpression of a human PLTP protein with a mutation at the site responsible for the lipid transfer activity of the protein. This mutant PLTP was still able to associate with HDL, but lacked phospholipid transfer activity. In **chapter 4**, we compared the effects of overexpression of the mutant and normal human PLTP transgene in mice with only one functional allele for the LDL receptor, thereby obtaining a plasma lipoprotein profile more similar to that in humans. We demonstrated that the presence of phospholipid transfer activity is essential for the PLTP-mediated modulation of plasma HDL levels and preβ-HDL formation. Also stimulation of hepatic VLDL secretion appeared to be dependent on the phospholipid transfer activity of PLTP. Importantly, after feeding the mice a cholate-enriched high fat diet, atherosclerosis development was only increased in mice expressing the active form of human PLTP. These results demonstrate that active PLTP is essential for the increased atherogenesis induced by PLTP.

Next, we studied whether the functioning of PLTP would be altered by the presence of other important proteins in lipoprotein metabolism: human apoAI, CETP, or hepatic lipase. Because PLTP is a lipid transfer protein, its action is mainly determined by the concentration gradient of available substrates, and therefore sensitive to environmental changes. As a consequence, the absence or presence of certain proteins that act in close relationship with PLTP might strongly influence the action of human PLTP. Although mouse models have provided important mechanistical insight in human lipoprotein metabolism and atherogenesis, it is important to note that distinct differences exist between murine and human lipid metabolism (see Table 2, Chapter 1.2). In order to partly overcome these differences, we assessed the effects of increased PLTP expression in two 'humanized' mouse models: the human apoAI transgenic and the human CETP transgenic mouse. In **chapter 5**, we described the effects of increased human PLTP expression in a human apoAI transgenic background. Human apoAI is the natural carrier for human PLTP that is bound to HDL. We studied the effect of PLTP overexpression on different HDL properties (particle size, density, and functionality), and on atherosclerotic lesion development. Overexpression of the human PLTP gene in a human apoAI transgenic mouse line resulted in decreased total plasma cholesterol and phospholipid levels, which were caused by decreased HDL levels. In addition, the action of human PLTP together with human apoAI resulted in the formation of a large cholesteryl ester-rich subpopulation of HDL particles with LDL density, and apoAI as the main apolipoprotein. Overexpression of human PLTP may not only decrease plasma HDL levels and change HDL composition, but it could also unfavourably affect the functionality of the remaining HDL particles. Therefore, we measured the cholesterol efflux capacity of the PLTP-enlarged HDL and found that it was decreased compared to HDL from control mice. Also when mice were crossed into an atherosclerosis-prone genetic background, HDL isolated from the human PLTP-expressing mice was less efficient in promoting cholesterol efflux from cultured macrophages. This may explain, at least partly, the observation that the development of atherosclerotic lesions was increased in human PLTP overexpressing mice. In conclusion, PLTP may act as a pro-atherogenic factor not only by decreasing plasma HDL levels, but also by changing HDL particle composition and functionality, strengthening the hypothesis that high plasma PLTP activity levels are harmful for cardiovascular health.

In **chapter 6** we described the effects of PLTP in another humanized mouse model, the human CETP transgenic mouse. In earlier studies of our group, it was demonstrated that the presence of CETP does not influence the effect of PLTP on atherogenesis. In the study described in chapter 6, we investigated the effect of overexpression of human PLTP on atherosclerosis development in male and female mice. It is well-known that male mice are less susceptible to diet-induced atherosclerosis than female mice. Although there is no clear explanation for this difference between genders, we used it as a starting point

to elucidate the atherogenic mechanism behind PLTP overexpression. We studied which of two well-known pro-atherogenic PLTP effects, either the stimulation of hepatic VLDL secretion or the lowering of plasma HDL levels, is the most important factor in explaining the atherogenic potential of PLTP. No difference was observed in the rate of hepatic VLDL secretion between male and female mice. In contrast, HDL levels were significantly lower in female mice than in male mice. Therefore, we concluded that the decrease in plasma HDL has a more substantial contribution to the atherogenicity of elevated PLTP levels than the stimulation of hepatic VLDL secretion. However, the gender-related difference in atherosclerosis should not too easily be ascribed completely to the changes in the levels of HDL. We did not study differences in lipoprotein distribution, cellular cholesterol efflux, apolipoproteins, lipolytic enzymes, qualitative HDL properties or other factors that may contribute to the gender differences in atherosclerosis susceptibility. These experiments do not provide certainty whether PLTP influences atherogenicity solely at the level of plasma lipoproteins or also locally at the level of the vessel wall. However, we can conclude that PLTP overexpression enhances the gender differences in atherosclerosis development.
Cross-breeding of transgenic animals is not only a good approach to generate a 'humanized' mouse model in which the role of PLTP can be studied, but it is also an interesting way to study a possible functional interaction between PLTP and other proteins. Hepatic lipase is such a protein that might affect the function of PLTP. Hepatic lipase is a lipolytic enzyme, involved in the conversion of VLDL into LDL, and in HDL remodelling, two pathways in which also PLTP is involved. We investigated the possibility of a functional relationship between PLTP and hepatic lipase, as described in **chapter 7**. First of all, we investigated the effect of overexpression of PLTP on hepatic lipase activity by comparing wild type mice with human PLTP transgenic mice. We found that in transgenic animals, the binding of hepatic lipase to cellular proteoglycans was increased. This may be explained by the PLTP-mediated reduction of plasma HDL levels, as hepatic lipase is preferably bound to circulating HDL in mice. Furthermore, a possible functional relationship between both proteins was studied by crossing PLTP transgenic mice into a hepatic lipase deficient background. Plasma PLTP activity in either wild type or in PLTP transgenic mice was not affected by hepatic lipase deficiency. Moreover, hepatic lipase was shown not to be required for the PLTP-dependent increase in hepatic triglyceride secretion or decrease in plasma HDL levels. These results indicate that hepatic lipase and PLTP act independently. Importantly, we demonstrated that elevated PLTP activity lowered plasma HDL levels by stimulating HDL plasma turnover and increasing the uptake of HDL cholesteryl esters by the liver.

In another set of experiments, described in **chapter 8**, we focussed on the relationship between PLTP expression and plasma HDL levels. In ABCA1 deficient mice, we

investigated the effect of reduced HDL levels on PLTP gene expression, secretion and plasma distribution. PLTP mRNA expression in liver and macrophages was not different in ABCA1 deficient mice compared to wild type mice. Also PLTP secretion by cultured ABCA1 deficient macrophages was not decreased. As neither a decreased PLTP gene expression nor a decrease in PLTP secretion could explain the reduced plasma PLTP levels observed in ABCA1 deficient mice, we hypothesized that the explanation may be at the systemic level instead of the cellular level. We showed that the phospholipid transfer activity levels of PLTP, secreted from macrophages, decreased when serum from ABCA1 deficient mice was present in the culture medium, compared to medium with serum from wild type mice. Macrophage-derived PLTP activity appeared to be controlled by the HDL concentration in the culture medium. From these findings, we suggested that PLTP could be stabilised by HDL particles, and that the low plasma PLTP activity levels found in ABCA1 deficient mice are the result of an indirect effect of the absence of ABCA1 through reduction of HDL levels, rather than a direct cellular effect of ABCA1 on PLTP. In addition, we discovered that the distribution of PLTP among lipoprotein particles was totally changed in ABCA1 deficient mice. PLTP was found associated with HDL particles in wild type mice, whereas in ABCA1 deficient mice PLTP was associated with VLDL and LDL particles.
To further elucidate the effect of HDL on PLTP expression and distribution, we used a different approach, as described in the second part of chapter 8. We studied the effect of LXR activation and subsequent alteration of the lipoprotein profile on PLTP expression and distribution in different mouse lines with varying plasma HDL and PLTP levels. Wild type, ABCA1 deficient, apoE deficient and PLTP transgenic mice were treated with a synthetic LXR ligand. LXR treatment resulted in an upregulation of PLTP gene expression in all mouse models. Plasma PLTP activity levels were increased, but the extent of LXR-mediated plasma PLTP induction appeared to be related to plasma HDL level. Importantly, PLTP activity was found at the position of HDL particles in all mouse models, except in ABCA1 deficient mice, likely because HDL was virtually absent. These results demonstrate that HDL metabolism may regulate plasma PLTP activity and distribution.

In the last part of this thesis, we focussed on the functionality of macrophage-derived PLTP, in addition to systemic PLTP. Hepatic and plasma lipoprotein metabolism have always played a major role in the investigation of atherogenesis. However, more recently, increasing attention has been paid to inflammatory and local processes in the vessel wall. In earlier chapters, we reported the effect of elevated systemic PLTP levels on lipoprotein metabolism and atherosclerosis development in mice. However, the macrophage is a key component of the atherosclerotic lesion and PLTP has been shown to colocalise with foam cells in atherosclerotic lesions. Recent studies focused on the role of macrophage-derived PLTP in the development of atherosclerosis using the technique of bone marrow transplantation.

It has not been clarified yet how macrophage-derived PLTP within the artery wall may influence the development of atherosclerosis. Recent publications showed conflicting results, suggesting either a pro-atherogenic or an anti-atherogenic role of macrophage-derived PLTP. To elucidate the contribution of elevated levels of macrophage-derived PLTP to plasma phospholipid transfer activity and atherosclerosis development, we performed bone marrow transplantation experiments (**chapter 9**). Transplantation of bone marrow cells from PLTP transgenic mice resulted in elevated plasma PLTP activity levels in the LDLR-deficient acceptor mice compared to mice transplanted with wild type bone marrow cells. As a result of increased plasma PLTP activity levels, plasma levels of HDL cholesterol decreased, and plasma levels of non-HDL cholesterol increased. Transplantation of PLTP overexpressing bone marrow cells resulted in increased atherosclerosis development. Most likely, the effects on plasma lipoproteins account for the observed enhanced atherosclerosis in mice transplanted with PLTP transgenic bone marrow. However, there might also be a local effect of macrophage-derived PLTP in the vascular wall, as PLTP was abundantly present within the atherosclerotic lesions. The results from our study, using an alternative mouse model and different techniques compared to the experiments performed by other research groups, do not resolve the apparent discrepancies between earlier studies. However, our results indicate that the presence of PLTP in human atherosclerotic lesions contributes to the pathological process and that PLTP in atherosclerotic lesions may be a valuable target for therapy.

In **chapter 10,** we described the role of PLTP in reverse cholesterol transport from macrophages, making the distinction between elevation of systemic PLTP and elevation of macrophage-PLTP. Cholesterol efflux from macrophages in the arterial wall and subsequent transport to the liver and intestine for excretion may prevent cellular lipid accumulation, foam cell formation, and the development of atherosclerosis. Until recently, knowledge on the physiological importance of reverse cholesterol transport and the role of key proteins in this process was based on findings from *in vitro* experiments or on methods quantifying reverse cholesterol transport from peripheral cells in the entire body. The complex network of plasma and cellular proteins involved in reverse cholesterol transport made it difficult to translate the results obtained *in vitro* to the situation *in vivo*, and to draw conclusions about reverse cholesterol transport originating specifically from macrophages, also called macrophage reverse cholesterol transport. The development of a new method to measure macrophage reverse cholesterol transport provided us the opportunity to study systemic as well as macrophage-specific effects of PLTP in this process *in vivo*. By injecting lipid-loaded wild type macrophages into wild type or PLTP transgenic mice, we demonstrated that cholesterol efflux and reverse cholesterol transport from these cells are impaired when systemic PLTP is elevated, most likely explained by the PLTP-mediated lowering of plasma HDL. However, elevation of macrophage-PLTP did not affect cholesterol efflux

and reverse cholesterol transport *in vivo*, as shown by injection of macrophages from wild type or PLTP transgenic mice into wild type mice. Therefore we hypothesize that increased systemic PLTP levels enhance the development of atherosclerosis by decreasing the rate of macrophage reverse cholesterol transport.

Taken together, we demonstrated the pro-atherogenic potential of elevated systemic PLTP levels in many different mouse models. By using a variety of new approaches, we confirmed the major effect of PLTP on HDL metabolism, resulting in an increased susceptibility to atherosclerosis development. In addition, we reported effects of PLTP on VLDL metabolism that have not been described before, and we described the effect of macrophage-derived PLTP on lipoprotein metabolism, macrophage reverse cholesterol transport and atherogenesis. Overall, the main findings of our studies can be summarized as follows:

1) PLTP overexpression, causing elevated systemic PLTP activity levels, results in an increased susceptibility to atherosclerosis in mice. This effect of PLTP is observed independently of the transgenic mouse model that is used: both ubiquitous and tissue- or cell-specific expression, as well as permanent and acute expression of PLTP, results in increased atherosclerosis development; 2) Overexpression of PLTP in mice results in decreased plasma HDL levels, caused by enhanced HDL catabolism. Also this effect of PLTP is observed independently of the PLTP transgenic mouse model that is used. Under certain circumstances, elevated expression of PLTP not only affects plasma HDL levels, but also HDL composition and functionality, as demonstrated in human apoAI transgenic mice; 3) Systemic elevation of PLTP in mice impairs macrophage reverse cholesterol transport, which is most likely due to the reduced plasma HDL levels. Elevation of macrophage-PLTP does not affect cholesterol efflux and macrophage reverse cholesterol transport; 4) Elevation of PLTP activity might affect apoB-containing lipoprotein metabolism. PLTP-induced enhancement of VLDL production and impairment of the catabolism of apoB-containing lipoproteins are not unambiguous, and depend on mouse model, genetic background, gender, age, fasting state, and experimental set-up; 5) The atherogenicity of elevated PLTP levels can be explained mainly by the systemic effects of PLTP on lipoprotein metabolism. In our mouse models, potential local macrophage-specific anti-atherogenic effects of PLTP are of minor importance compared to the systemic pro-atherogenic effects; 6) Phospholipid transfer activity is essential for the effects of PLTP on lipoprotein metabolism and atherogenesis. In our mouse model, there is no evidence for a function of the inactive form of PLTP; 7) HDL metabolism regulates plasma PLTP expression and distribution. Under normal conditions, PLTP is found associated with HDL particles. When HDL particles are virtually absent, PLTP levels are strongly reduced and PLTP is found associated with other lipoprotein particles.

11.2 Future perspectives

Atherosclerosis is the leading cause of death in the developed world, and predicted to be the main cause of death worldwide by the year 2020 (Fonarow, 2007). The current treatment of atherosclerotic disease is based on targeting classical risk factors by pharmacological intervention (antihypertensive and lipid-lowering drugs) in combination with lifestyle advice (low-fat diet, smoking cessation, physical activity). As hypercholesterolemia is considered as the main risk factor for development of atherosclerosis, the most commonly used pharmacotherapy for prevention, treatment and control of atherosclerosis consists of lipid-lowering drugs. During the last 20 years, the use of drugs to lower plasma LDL levels, such as statins, achieved a strong reduction in cardiovascular risk (Kapur and Musunuru, 2008). However, statin therapy only reduces cardiovascular events worldwide by about one-third (Tall, 2008), while further spreading of Western lifestyle results in an ever increasing prevalence of atherosclerosis. Obviously, there is an urgency for the development of additional pharmacotherapies to treat atherosclerosis in a more efficient way.

Because the inverse relationship between plasma HDL-cholesterol level and the prevalence of atherosclerotic cardiovascular disease is well-established, an emerging target of therapy is the level of plasma HDL. HDL particles have an important anti-atherogenic function, by exerting anti-inflammatory, anti-oxidative, and antithrombotic effects (Joy and Hegele, 2008; Kontush *et al.*, 2008). In addition, HDL serves as an acceptor particle for cellular cholesterol, thereby promoting the efflux of lipids from the vessel wall, which is the first step in reverse cholesterol transport and important in the prevention of cellular cholesterol accumulation. Currently available drugs to increase HDL-cholesterol levels are nicotinic acid (niacin) and fibric acid derivates (fibrates). Niacin is an effective drug to increase HDL-cholesterol, but a main limitation of niacin treatment is the high frequency of side effects, which makes the drug less suitable for long-term therapy. Fibrates are weak PPAR-α agonists that are used to raise HDL-cholesterol levels. In several clinical trials, fibrate treatment resulted in a reduction of cardiovascular morbidity and a reduced progression of atherosclerosis (Hausenloy and Yellon, 2008; Joy and Hegele, 2008). However, the outcomes of clinical trials with fibrates vary, and the potential of this drug class to induce adverse effects when given in combination with statins, limit the use of fibrates as cardioprotective agents. These difficulties demonstrate the demand for alternative HDL-raising strategies.

Inhibition of elevated levels of systemic PLTP could be an approach to increase HDL levels and reduce atherosclerosis. This hypothesis is supported by the experiments described in this thesis, in which we evaluated the role of PLTP in lipoprotein metabolism and atherosclerosis development in genetically modified mouse models. PLTP could serve as a potential therapeutic target for treatment of atherosclerotic disease by raising plasma HDL levels. However, it is not certain whether inhibition of PLTP activity is the right approach

to increase plasma HDL level and inhibit atherogenesis. Not only PLTP overexpressing mice, but also homozygous PLTP knockout mice have decreased plasma HDL levels, obviously caused by different mechanisms. In mice overexpressing PLTP, PLTP-mediated redistribution of lipids among lipoprotein populations probably causes instability of HDL particles, leading to an accelerated catabolism (Föger *et al.*, 1997). In PLTP deficient mice, the transfer of lipids to HDL is arrested, thereby preventing the formation of mature HDL particles (Qin *et al.*, 2000). Apparently, complete inhibition of plasma PLTP activity will not necessarily be a useful therapy to increase plasma HDL levels. It is important to realise that besides an important role in HDL metabolism, PLTP is also involved in other processes (of which some may not have been discovered yet) that will be disturbed by complete inhibition of PLTP activity. One process in which PLTP is involved is the transfer of vitamin E from lipoproteins to tissues. PLTP deficiency enhances the accumulation of vitamin E in apoB-containing lipoproteins, resulting in a decreased susceptibility of these lipoproteins to oxidative modification and a reduced atherosclerosis development (Jiang *et al.*, 2001; Jiang *et al.*, 2002). However, vitamin E transfer by PLTP is also an important process in preventing oxidative damage in the brain, and PLTP deficiency could be of major pathophysiological relevance in neurodegenerative diseases (Desrumaux *et al.*, 2005).

The inhibition of plasma CETP activity as a therapy to raise HDL levels serves as an example for the practical problems that could be encountered when a comparable therapeutic approach would be chosen for PLTP. In extensive animal studies and preclinical trials, CETP inhibition by monoclonal antibodies or synthetic compounds demonstrated clinical efficacy on plasma lipid levels by markedly increasing HDL-cholesterol (Vourvouhaki and Dedoussis, 2008). In addition, CETP inhibition might protect against atherosclerosis by improving the anti-inflammatory and antioxidative properties of HDL (Kastelein, 2007). However, CETP could also have an anti-atherogenic effect, as introduction of CETP in mice enhanced macrophage reverse cholesterol transport (Tanigawa *et al.*, 2007). The effect of CETP expression on atherosclerosis susceptibility in animal models is highly dependent on the level of CETP expression and on the genetic and metabolic context (Parini and Rudel, 2003). A large clinical trial with the CETP inhibitor torcetrapib (in combination with atorvastatin) in patients with coronary artery disease was terminated as a result of excessive mortality in the active treatment group relative to the placebo group, despite a significant increase in HDL level (Barter *et al.*, 2007). Unlike other CETP inhibitors, torcetrapib treatment was associated with a moderate increase in blood pressure, indicating that this inhibitor has off-target adverse effects that are probably unrelated to its HDL-raising action (Kastelein, 2007; Kontush *et al.*, 2008). However, the unexpected failure of torcetrapib highlighted important unresolved questions in the field of HDL research. There has been considerable speculation that the strategy of CETP inhibition might lead to formation of dysfunctional HDL. HDL particles that are formed upon CETP inhibition

could be less efficient in stimulating cellular cholesterol efflux and reverse cholesterol transport, and may be pro-inflammatory and pro-oxidant (Singh *et al.*, 2007; Tall *et al.*, 2008). In conclusion, both HDL quantity and HDL quality should be taken into account when developing and evaluating pharmacological HDL-raising strategies.

The example of torcetrapib raises the question whether increasing HDL levels by all means, and under any condition, would be of therapeutic profit for the treatment of atherosclerosis. It is important to note that the pool of HDL particles is structurally and functionally diverse, consisting of many highly dynamic particle subpopulations that differ in their atheroprotective capacity (Rye *et al.*, 2008). The existence of a mutant form of apoAI that causes very low HDL levels with moderate hypertriglyceridemia, but that is not associated with an increased risk for cardiovascular disease, demonstrates the importance to consider HDL as a heterogeneous pool of particles (Chiesa and Sirtori, 2003). In a large intervention trial, subjects with new cardiovascular events had significantly lower levels of α-HDL, and significantly higher levels of preβ-HDL compared to event-free subjects, further supporting a functional distinction between different HDL subpopulations (Asztalos *et al.*, 2008). By the experiments described in this thesis, we demonstrated that under certain conditions PLTP affects HDL level, composition and functionality. However, before PLTP activity can be considered with certainty as a therapeutic target for treatment of atherosclerotic disease, the exact physiological role of PLTP should be established in more detail.

PLTP is a lipid transfer protein and as a consequence its action may depend on fluctuations in plasma lipid levels. Experiments investigating the effects of hyperlipidemic conditions on the action of PLTP could provide important new physiological insights. We demonstrated that overexpression of PLTP in mice results in enhanced HDL catabolism and decreased plasma HDL levels, independently of the PLTP transgenic mouse model that is used. In contrast, the effects of elevated PLTP activity on apoB-containing lipoprotein metabolism (i.e. enhancement of VLDL production and impairment of the catabolism of non-HDL particles) are not unambiguous. Therefore, the role of PLTP in the metabolism of apoB-rich lipoproteins should be investigated more thoroughly. In our mouse models, PLTP-mediated changes in apoB-containing lipoprotein metabolism were dependent on various parameters such as genetic background and gender. The influence of fasting state, background, gender, and age could greatly influence the action of PLTP, and attention should be paid to these issues when assessing the influence of PLTP in the process of atherogenesis.

The hypothesis that strongly elevated systemic levels of PLTP are pro-atherogenic has been confirmed by the experimental work as presented in this thesis. However, the effect of moderate changes in plasma PLTP activity remains uncertain. For this purpose, evaluation of the effect of PLTP inhibition in less extreme mouse models is needed. This is supported by the fact that in humans there are no reports of PLTP deficiency or extremely high PLTP activity levels. Although variations in PLTP levels have been associated with different

pathophysiological conditions in human subjects (summarized in chapter 1), it is hard to identify the exact contribution of PLTP to the pathology of atherosclerosis. Studies in some less extreme mouse models have been performed, such as mice with only one functional PLTP allele or mice with only 29% elevated PLTP levels (Jiang *et al.*, 1996; Jiang *et al.*, 1999). These models did not display any difference in lipoprotein metabolism compared to wild type mice. However, in these models, potential local PLTP effects may be of significant importance in the process of atherogenesis, and should therefore be studied in detail. Bone marrow transplantation studies with PLTP deficient macrophages revealed conflicting results, so that the local effects of PLTP on atherogenesis remain unclear (Liu *et al.*, 2007; Valenta *et al.*, 2008; Valenta *et al.*, 2006; Vikstedt *et al.*, 2007). Also our bone marrow transplantation study did not elucidate the specific effects of local PLTP expression on atherosclerosis development, as the contribution of macrophage-derived PLTP to plasma phospholipid transfer activity was substantial and resulted in altered plasma lipoprotein levels. Various bone marrow transplantation studies demonstrated that macrophage-specific expression of a gene of interest can alter the susceptibility to atherosclerosis, without changing the plasma lipoprotein profile (Aiello *et al.*, 2002; Nong *et al.*, 2003; Out *et al.*, 2006; Overton *et al.*, 2007). Therefore, it would be interesting to develop a strategy to study the local effects of increased macrophage-PLTP, without the interference of systemic PLTP effects (i.e. decreased plasma HDL levels). Potentially, this approach might lead to the identification of peripheral PLTP instead of systemic PLTP as a useful target for treatment of cardiovascular disease.

We demonstrated that elevation of macrophage-PLTP expression does not alter macrophage reverse cholesterol transport in mice. However, it is obvious that other local macrophage-specific effects of PLTP could play an important role in the process of atherogenesis. For example, peripheral PLTP could influence the inflammatory status of atherosclerotic lesions, as PLTP deficiency clearly alters the systemic inflammatory response (Schlitt *et al.*, 2005; Shelly *et al.*, 2008). At the moment, it is unclear whether PLTP is directly involved in the inflammatory response, or whether inflammation is observed secondary to PLTP-induced effects on lipoprotein metabolism and atherogenesis. As atherosclerosis can be considered as an inflammatory disease, it is important to study the involvement of PLTP in this process in more detail, both on the systemic and the cellular level.

In conclusion, an elevated systemic PLTP level may be a promising target for treatment of atherosclerosis in the future. However, a better understanding of the (patho)physiological effects of PLTP is essential. Before tissue- or cell-specific targeting of PLTP can be considered as a potential therapy, it is crucial to investigate whether local effects of cellular PLTP are beneficial or harmful.

11.3 References

Aiello, R.J., Brees, D., Bourassa, P.A., Royer, L., Lindsey, S., Coskran, T., Haghpassand, M., and Francone, O.L. (2002). Increased atherosclerosis in hyperlipidemic mice with inactivation of ABCA1 in macrophages. Arterioscler Thromb Vasc Biol *22*, 630-637.

Asztalos, B.F., Collins, D., Horvath, K.V., Bloomfield, H.E., Robins, S.J., and Schaefer, E.J. (2008). Relation of gemfibrozil treatment and high-density lipoprotein subpopulation profile with cardiovascular events in the Veterans Affairs High-Density Lipoprotein Intervention Trial. Metabolism *57*, 77-83.

Barter, P.J., Caulfield, M., Eriksson, M., Grundy, S.M., Kastelein, J.J., Komajda, M., Lopez-Sendon, J., Mosca, L., Tardif, J.C., Waters, D.D., *et al.* (2007). Effects of torcetrapib in patients at high risk for coronary events. N Engl J Med *357*, 2109-2122.

Chiesa, G., and Sirtori, C. R. (2003). Apolipoprotein A-I(Milano): current perspectives. Curr Opin Lipidol *14*, 159-163.

Desrumaux, C., Risold, P.Y., Schroeder, H., Deckert, V., Masson, D., Athias, A., Laplanche, H., Le Guern, N., Blache, D., Jiang, X.C., *et al.* (2005). Phospholipid transfer protein (PLTP) deficiency reduces brain vitamin E content and increases anxiety in mice. Faseb J *19*, 296-297.

Föger, B., Santamarina-Fojo, S., Shamburek, R.D., Parrot, C.L., Talley, G.D., and Brewer, H.B., Jr. (1997). Plasma phospholipid transfer protein. Adenovirus-mediated overexpression in mice leads to decreased plasma high density lipoprotein (HDL) and enhanced hepatic uptake of phospholipids and cholesteryl esters from HDL. J Biol Chem *272*, 27393-27400.

Fonarow, G.C. (2007). The global burden of atherosclerotic vascular disease. Nat Clin Pract Cardiovasc Med *4*, 530-531.

Hausenloy, D.J., and Yellon, D.M. (2008). Targeting residual cardiovascular risk: raising high-density lipoprotein cholesterol levels. Heart *94*, 706-714.

Jiang, X., Francone, O.L., Bruce, C., Milne, R., Mar, J., Walsh, A., Breslow, J.L., and Tall, A.R. (1996). Increased prebeta-high density lipoprotein, apolipoprotein AI, and phospholipid in mice expressing the human phospholipid transfer protein and human apolipoprotein AI transgenes. J Clin Invest *98*, 2373-2380.

Jiang, X.C., Bruce, C., Mar, J., Lin, M., Ji, Y., Francone, O.L., and Tall, A.R. (1999). Targeted mutation of plasma phospholipid transfer protein gene markedly reduces high-density lipoprotein levels. J Clin Invest *103*, 907-914.

Jiang, X.C., Qin, S., Qiao, C., Kawano, K., Lin, M., Skold, A., Xiao, X., and Tall, A.R. (2001). Apolipoprotein B secretion and atherosclerosis are decreased in mice with phospholipid-transfer protein deficiency. Nat Med *7*, 847-852.

Jiang, X.C., Tall, A.R., Qin, S., Lin, M., Schneider, M., Lalanne, F., Deckert, V., Desrumaux, C., Athias, A., Witztum, J.L., and Lagrost, L. (2002). Phospholipid transfer protein deficiency protects circulating lipoproteins from oxidation due to the enhanced accumulation of vitamin E. J Biol Chem *277*, 31850-31856.

Joy, T., and Hegele, R.A. (2008). Is raising HDL a futile strategy for atheroprotection? Nat Rev Drug Discov *7*, 143-155.

Kapur, N.K., and Musunuru, K. (2008). Clinical efficacy and safety of statins in managing cardiovascular risk. Vasc Health Risk Manag *4*, 341-353.

Kastelein, J.J. (2007). Refocusing on use of cholesteryl ester transfer protein inhibitors. Am J Cardiol *100*, n47-52.

Kontush, A., Guerin, M., and Chapman, M.J. (2008). Spotlight on HDL-raising therapies: insights from the torcetrapib trials. Nat Clin Pract Cardiovasc Med *5*, 329-336.

Liu, R., Hojjati, M.R., Devlin, C.M., Hansen, I.H., and Jiang, X.C. (2007). Macrophage phospholipid transfer protein deficiency and ApoE secretion: impact on mouse plasma cholesterol levels and atherosclerosis. Arterioscler Thromb Vasc Biol *27*, 190-196.

Nong, Z., Gonzalez-Navarro, H., Amar, M., Freeman, L., Knapper, C., Neufeld, E.B., Paigen, B.J., Hoyt, R.F., Fruchart-Najib, J., and Santamarina-Fojo, S. (2003). Hepatic lipase expression in macrophages contributes to atherosclerosis in apoE-deficient and LCAT-transgenic mice. J Clin Invest *112*, 367-378.

Out, R., Hoekstra, M., Hildebrand, R.B., Kruit, J.K., Meurs, I., Li, Z., Kuipers, F., Van Berkel, T.J., and Van Eck, M. (2006). Macrophage ABCG1 deletion disrupts lipid homeostasis in alveolar macrophages and moderately influences atherosclerotic lesion development in LDL receptor-deficient mice. Arterioscler Thromb Vasc Biol *26*, 2295-2300.

Overton, C.D., Yancey, P.G., Major, A.S., Linton, M.F., and Fazio, S. (2007). Deletion of macrophage LDL receptor-related protein increases atherogenesis in the mouse. Circ Res *100*, 670-677.

Parini, P., and Rudel, L. L. (2003). Is there a need for cholesteryl ester transfer protein inhibition? Arterioscler Thromb Vasc Biol *23*, 374-375.

Qin, S., Kawano, K., Bruce, C., Lin, M., Bisgaier, C., Tall, A.R., and Jiang, X. (2000). Phospholipid transfer protein gene knock-out mice have low high density lipoprotein levels, due to hypercatabolism, and accumulate apoA-IV-rich lamellar lipoproteins. J Lipid Res *41*, 269-276.

Rye, K.A., Bursill, C.A., Lambert, G., Tabet, F., and Barter, P. J. (2008). The metabolism and anti-atherogenic properties of HDL. J Lipid Res.

Schlitt, A., Liu, J., Yan, D., Mondragon-Escorpizo, M., Norin, A. J., and Jiang, X. C. (2005). Anti-inflammatory effects of phospholipid transfer protein (PLTP) deficiency in mice. Biochim Biophys Acta *1733*, 187-191.

Shelly, L., Royer, L., Sand, T., Jensen, H., and Luo, Y. (2008). Phospholipid transfer protein deficiency ameliorates diet-induced hypercholesterolemia and inflammation in mice. J Lipid Res *49*, 773-781.

Singh, I.M., Shishehbor, M.H., and Ansell, B.J. (2007). High-density lipoprotein as a therapeutic target: a systematic review. Jama *298*, 786-798.

Tall, A.R. (2008). Cholesterol efflux pathways and other potential mechanisms involved in the athero-protective effect of high density lipoproteins. J Intern Med *263*, 256-273.

Tall, A.R., Yvan-Charvet, L., Terasaka, N., Pagler, T., and Wang, N. (2008). HDL, ABC transporters, and cholesterol efflux: implications for the treatment of atherosclerosis. Cell Metab *7*, 365-375.

Tanigawa, H., Billheimer, J.T., Tohyama, J., Zhang, Y., Rothblat, G., and Rader, D.J. (2007). Expression of cholesteryl ester transfer protein in mice promotes macrophage reverse cholesterol transport. Circulation *116*, 1267-1273.

Valenta, D. T., Bulgrien, J. J., Bonnet, D. J., and Curtiss, L. K. (2008). Macrophage PLTP is atheroprotective in LDLr-deficient mice with systemic PLTP deficiency. J Lipid Res *49*, 24-32.

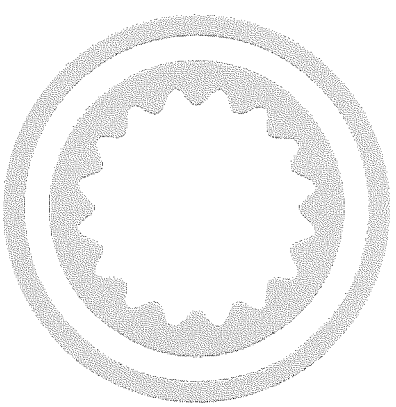
Valenta, D.T., Ogier, N., Bradshaw, G., Black, A. S., Bonnet, D.J., Lagrost, L., Curtiss, L.K., and Desrumaux, C.M. (2006). Atheroprotective potential of macrophage-derived phospholipid transfer protein in low-density lipoprotein receptor-deficient mice is overcome by apolipoprotein AI overexpression. Arterioscler Thromb Vasc Biol *26*, 1572-1578.

Vikstedt, R., Ye, D., Metso, J., Hildebrand, R.B., Van Berkel, T.J., Ehnholm, C., Jauhiainen, M., and Van Eck, M. (2007). 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 *27*, 578-586.

Vourvouhaki, E., and Dedoussis, G.V. (2008). Cholesterol ester transfer protein: a therapeutic target in atherosclerosis? Expert Opin Ther Targets *12*, 937-948.

Chapter 12

Nederlandse samenvatting



12 Nederlandse samenvatting

Aderverkalking, of atherosclerose, is een ziekte van de grote en middelgrote slagaders, gekenmerkt door de ophoping van vetten in de vaatwand en gepaard gaand met ontstekingsreacties. Hierdoor vermindert de elasticiteit van het bloedvat en ontstaat er vaatvernauwing. Een belangrijke risicofactor voor het ontstaan van aderverkalking is hyperlipidemie, verhoogde concentraties van vetten in het bloed. In het begin van de jaren tachtig van de vorige eeuw werd in menselijk bloed een eiwit ontdekt dat in staat was om fosfolipiden, een specifiek type vet, te transfereren. Dit eiwit werd "phospholipid transfer protein" (PLTP) genoemd. De precieze functie van PLTP in het lichaam was, en is nog steeds, niet volledig opgehelderd. Experimenten met proefdieren, voornamelijk muizen, hebben aangetoond dat PLTP een rol speelt in het ontstaan van aderverkalking. Dit zou voor een groot deel verklaard kunnen worden door het effect van PLTP op de stofwisseling van de zogenaamde lipoproteïnen. Lipoproteïnen zijn complexen van vetten (waaronder cholesterol en fosfolipiden) en eiwitten, die het transport van deze vetten in de bloedbaan mogelijk maken. Lipoproteïnen kunnen in het laboratorium worden gescheiden naar dichtheid, bijvoorbeeld zeer lage dichtheid (very low-density lipoprotein, VLDL), lage dichtheid (low-density lipoprotein, LDL), en hoge dichtheid (high-density lipoprotein, HDL). De bloedspiegels van de verschillende soorten lipoproteïnen zijn nauw verbonden met het ontstaan van aderverkalking. Zo worden VLDL en LDL als schadelijk beschouwd voor hart en vaten ("slecht cholesterol"), terwijl het hebben van een hoge HDL-spiegel als beschermend wordt gezien ("goed cholesterol").

De afgelopen 15 jaar is de rol van PLTP uitgebreid onderzocht door middel van experimenten *in vitro* (letterlijk "in glas", d.w.z. bijvoorbeeld in een reageerbuis) en *in vivo* (in proefdieren of in mensen). Dit heeft tot waardevolle inzichten geleid, maar verschillende vragen zijn tot op heden onopgelost. Aan de ene kant heeft PLTP eigenschappen die als 'pro-atherogeen' aangemerkt kunnen worden, m.a.w. die het ontstaan van aderverkalking zouden kunnen stimuleren. Zo veroorzaakt een verhoogde transferactiviteit van PLTP in het bloed een daling van de HDL-spiegel. Verder stimuleert PLTP de productie van VLDL. Ook zou het direct de ontstekingsreactie en oxidatie van lipoproteïnen kunnen beïnvloeden. Aan de andere kant bezit PLTP ook enkele 'anti-atherogene' eigenschappen, die beschermend zouden zijn en het proces van aderverkalking tegengaan. PLTP stimuleert de vorming van een bepaald soort HDL dat heel efficiënt vetten uit cellen kan opnemen (het zogenaamde preß-HDL). Op deze manier zou de ophoping van vetten in cellen in de vaatwand geremd kunnen worden. Het feit dat PLTP blijkbaar zowel pro- als anti-atherogene eigenschappen bezit, maakt het moeilijk om de precieze rol van PLTP in de ontwikkeling van aderverkalking vast te stellen. Gebruik makend van de muis als onderzoeksmodel hebben wij geprobeerd de rol

van PLTP in de stofwisseling van lipoproteïnen en het ontstaan van aderverkalking verder te verduidelijken. De verschillende experimentele benaderingen die hiervoor gebruikt zijn, en de resultaten die hiermee verkregen zijn, staan beschreven in dit proefschrift.
Door middel van genetische manipulatie hebben we twee nieuwe muismodellen ontwikkeld: één model met conditionele expressie van humaan PLTP, waarbij het eiwit op elk gewenst moment tot expressie gebracht kan worden, en één model met expressie van een inactieve vorm van humaan PLTP, welke niet in staat is om fosfolipiden te transfereren. Deze modellen onderscheiden zich van de muismodellen die in het verleden zijn gebruikt in die zin dat in die modellen voornamelijk sprake was van continu verhoogde expressie van actief humaan PLTP. Door gebruik te maken van het conditionele muismodel hebben we het pro-atherogene effect van tijdelijke expressie van PLTP op al aanwezige aderverkalking aangetoond. Met het tweede muismodel hebben we de noodzaak van de transferactiviteit van PLTP voor de pro-atherogene eigenschappen van het eiwit laten zien.
Verder hebben we bestudeerd of het functioneren van PLTP beïnvloed wordt door de aan- of afwezigheid van andere eiwitten met een belangrijke rol in de stofwisseling van lipoproteïnen: humaan apoAI, CETP, en leverlipase. Hoewel muismodellen belangrijk mechanistisch inzicht kunnen leveren in fysiologische processen in de mens, is het belangrijk te beseffen dat metabole verschillen tussen muis en mens kunnen leiden tot onjuiste interpretatie van resultaten. Om deze verschillen voor een deel te compenseren hebben we de rol van humaan PLTP in twee 'gehumaniseerde' muismodellen bestudeerd: muizen met humaan apoAI en muizen met humaan CETP. ApoAI is het oppervlaktemolecuul op HDL waar PLTP aan bindt. CETP is een eiwit dat structureel en functioneel nauw verwant is aan PLTP. In beide modellen hebben we pro-atherogene eigenschappen van PLTP aangetoond. Verder hebben we onderzocht of er een functionele interactie bestaat tussen PLTP en leverlipase, een eiwit dat betrokken is in verschillende fysiologische processen waar ook PLTP een rol in speelt. Uit dit onderzoek concludeerden we dat de functie van PLTP niet beïnvloed wordt door de aan- of afwezigheid van leverlipase.
Dat PLTP een belangrijke rol speelt in de stofwisseling van HDL is algemeen bekend. PLTP bindt aan HDL, waarna het HDL omzet tot zowel grotere als kleinere HDL-deeltjes. In muizen zonder ABCA1, een lipide-transporteiwit in het celmembraan, worden extreem lage HDL-spiegels in het bloed gevonden, in combinatie met een verlaagde PLTP-activiteit. We hebben een mogelijk causaal verband tussen lage HDL-spiegels en lage PLTP-activiteit in het bloed bestudeerd. Ook in andere muismodellen hebben we vastgesteld dat verlaagde HDL-spiegels in het bloed het niveau en de distributie van PLTP kunnen beïnvloeden.

Hoewel de stofwisseling van vetten in de lever en het bloed altijd centraal stonden in het onderzoek naar de ontwikkeling van aderverkalking, wordt er de laatste jaren steeds meer aandacht besteed aan lokale ontstekingsprocessen in de vaatwand. Het belangrijkste celtype

dat verantwoordelijk is voor de vetophoping in de wand van door aderverkalking aangetaste bloedvaten is de macrofaag. PLTP en met vet geladen macrofagen (schuimcellen) komen op dezelfde plek in de vaatwand voor. Daarom hebben we de rol van PLTP afkomstig uit macrofagen in de ontwikkeling van aderverkalking onderzocht, gebruik makend van de beenmergtransplantatie-techniek. Hierbij werd het beenmerg van muizen geïnactiveerd d.m.v. bestraling, waarna beenmergcellen geïsoleerd uit donormuizen met humaan PLTP werden geïnjecteerd in de bloedbaan van de bestraalde muizen. Hiermee hebben we aangetoond dat PLTP afkomstig uit macrofagen in grote mate bijdraagt aan de PLTP-concentratie in het bloed, en een pro-atherogene werking heeft.
In het laatste deel van dit proefschrift hebben we de rol van PLTP in het 'omgekeerde cholesteroltransport' van macrofaag naar faeces in de muis onderzocht. Het omgekeerde cholesteroltransport bestaat uit de efflux van cholesterol uit macrofagen in de vaatwand, en het daaropvolgende transport van cholesterol naar de lever en darmen voor uitscheiding via de faeces. Dit proces voorkomt de stapeling van vetten in macrofagen, dus de vorming van schuimcellen, en daarmee de ontwikkeling van aderverkalking. Met behulp van een methode om *in vivo* het omgekeerde cholesteroltransport uit macrofagen te meten hebben we aangetoond dat verhoogde PLTP-spiegels in het bloed een remmende werking hebben op het omgekeerde cholesteroltransport. Het proces wordt echter niet beïnvloed door verhoogde expressie van PLTP uitsluitend in macrofagen.

Door middel van de in dit proefschrift beschreven experimenten hebben we aangetoond dat in muizen een verhoging van de PLTP-activiteit in het bloed een pro-atherogene werking heeft, m.a.w. het stimuleert de ontwikkeling van aderverkalking. Onze belangrijkste bevindingen kunnen als volgt worden samengevat:
1) De overexpressie van humaan PLTP, resulterend in een verhoogde PLTP-activiteit in het bloed, versterkt de ontwikkeling van aderverkalking in muizen. Dit effect van PLTP wordt waargenomen onafhankelijk van het muismodel dat wordt bestudeerd: zowel alomtegenwoordige als weefselspecifieke expressie van PLTP, en zowel permanente als acute expressie van PLTP resulteert in een versterkte ontwikkeling van aderverkalking.
2) Verhoging van PLTP-activiteit in muizen leidt tot een verlaagde HDL-spiegel in het bloed. Ook dit effect van PLTP wordt waargenomen onafhankelijk van het muismodel dat wordt gebruikt. Onder bepaalde omstandigheden beïnvloedt een verhoogde PLTP-spiegel in het bloed niet alleen de HDL-spiegel, maar ook de samenstelling en functionaliteit van HDL.
3) Verhoging van PLTP in bloed remt het omgekeerde cholesteroltransport uit macrofagen naar de faeces, wat hoogstwaarschijnlijk verklaard kan worden door de verlaagde HDL-spiegel in het bloed. Verhoging van macrofaag-specifiek PLTP heeft geen effect op de efflux van cholesterol en het omgekeerde cholesteroltransport uit macrofagen.

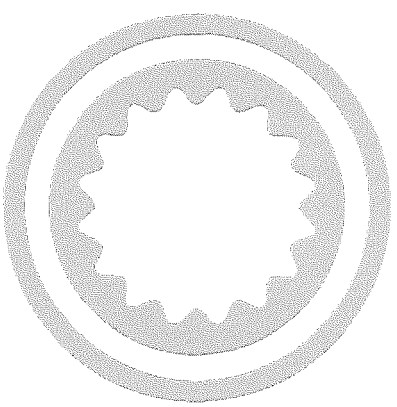
4) Verhoging van PLTP-activiteit kan de stofwisseling van VLDL beïnvloeden. De stimulatie van VLDL-productie en de remming van VLDL-afbraak is niet eenduidig, maar afhankelijk van muismodel en experimentele opzet.
5) Het stimulerende effect van een verhoogde PLTP-spiegel op de ontwikkeling van aderverkalking kan voornamelijk verklaard worden door de effecten van PLTP aanwezig in het bloed op de stofwisseling van lipoproteïnen. In onze muismodellen zijn de potentieel anti-atherogene lokale effecten van macrofaag-specifiek PLTP minder sterk dan de pro-atherogene effecten in het bloed.
6) Fosfolipide-transferactiviteit is essentieel voor de effecten van PLTP op de stofwisseling van lipoproteïnen en het ontstaan van aderverkalking.
7) HDL reguleert de expressie en distributie van PLTP in het bloed.

De resultaten van de experimenten zoals beschreven in dit proefschrift tonen aan dat PLTP een mogelijk therapeutisch doel voor de behandeling van aderverkalking zou kunnen zijn. In onze muismodellen resulteerden verhoogde PLTP-spiegels in het bloed in een daling van de HDL-spiegels en een sterkere ontwikkeling van aderverkalking. HDL heeft een anti-atherogene functie, wat verklaard wordt door de anti-inflammatoire, anti-oxidatieve, en anti-thrombotische effecten van HDL. Verder kan HDL de efflux van cholesterol uit cellen in de vaatwand stimuleren en daarmee de ophoping van cholesterol in de vaatwand voorkomen. Vele onderzoeken hebben een sterke correlatie aangetoond tussen verlaagde HDL-spiegels in het bloed en een verhoogd risico op hart- en vaatziekten. Remming van PLTP zou een mogelijke therapie kunnen zijn voor de behandeling van aderverkalking via een verhoging van de HDL-spiegel in het bloed. Het is echter nog onduidelijk of remming van PLTP ook daadwerkelijk de juiste aanpak is voor dit doeleinde. Alhoewel een hoge PLTP-activiteit resulteerde in lage HDL-spiegels in onze muismodellen, toont de literatuur dat ook PLTP-deficiënte muizen verlaagde HDL-spiegels hebben. Een volledige remming van PLTP lijkt daarom geen geschikte therapeutische optie. Verder speelt PLTP niet alleen een grote rol in het metabolisme van HDL, maar is het ook betrokken bij diverse andere (deels onbekende) processen. Remming van PLTP zou deze processen ernstig kunnen verstoren. Verder is het niet bekend of de remming van PLTP zal leiden tot de vorming van functioneel HDL. Niet alleen de absolute stijging van het HDL-niveau door PLTP-remming, maar ook de kwaliteit van het HDL (om inflammatie en oxidatie tegen te gaan, en om het omgekeerde cholesteroltransport te stimuleren) zal bestudeerd moeten worden. Het is van belang rekening te houden met het feit dat er een grote structurele en functionele diversiteit in HDL bestaat, met belangrijke verschillen in de mate van bescherming tegen aderverkalking. Voordat PLTP-activiteit daadwerkelijk als therapeutisch doel beschouwd kan worden voor de behandeling van aderverkalking, zal daarom eerst de exacte fysiologische rol van PLTP vastgesteld moeten worden. Hierbij moet gedacht worden aan een uitgebreid

onderzoek naar de rol van PLTP in de stofwisseling van VLDL en LDL, waarbij ook de invloed van leeftijd, geslacht, genetische achtergrond, en gevaste of gevoede toestand van de proefdieren nauwkeurig vastgesteld dient te worden.
De hypothese dat een sterk verhoogde systemische PLTP-spiegel het ontstaan van aderverkalking stimuleert, is bevestigd door het experimentele werk dat beschreven is in dit proefschrift. Het effect van kleine veranderingen in PLTP-activiteit is echter nog onduidelijk, en zou onderzocht moeten worden in minder extreme muismodellen. Deze stelling wordt gesteund door het feit dat er in mensen geen meldingen zijn van volledige afwezigheid van PLTP, of van extreem hoge PLTP-spiegels. Ook lokale effecten van PLTP zouden wel eens van belang kunnen zijn voor de ontwikkeling van aderverkalking, en deze effecten zouden daarom meer in detail bestudeerd moeten worden. Hierbij kan bijvoorbeeld gedacht worden aan de rol van PLTP in het ontstekingsproces in de vaatwand. Op het moment is het onbekend of PLTP direct de ontstekingsreactie beïnvloedt, of dat ontsteking secundair is aan effecten van PLTP op de stofwisseling van lipoproteïnen en de ontwikkeling van aderverkalking.

Samengevat kan gesteld worden dat een verhoogde PLTP-spiegel in het bloed een veelbelovend therapeutisch doel zou kunnen zijn voor de toekomstige behandeling van aderverkalking. Hiervoor is echter een beter begrip van de (patho-)fysiologische effecten van PLTP noodzakelijk. Voordat weefsel- of celspecifieke aanpak van PLTP beschouwd kan worden als potentiële therapie, is het van cruciaal belang eerst vast te stellen of lokale effecten van cellulair PLTP beschermend of juist schadelijk zijn.

Curriculum Vitae



Curriculum Vitae

Matthijs Moerland, 12 September 1980, Rotterdam

2007-	Senior Clinical Scientist Department of Vascular Medicine Centre for Human Drug Research, Leiden
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Publications

1. Elevation of systemic PLTP, but not macrophage-PLTP, impairs macrophage reverse cholesterol transport in transgenic mice. Samyn H, **Moerland M**, Van Gent T, Van Haperen R, Grosveld F, Van Tol A, De Crom R. *Atherosclerosis. 2008;Epub ahead of print.*
2. Plasma phospholipid transfer activity is essential for increased atherogenesis in PLTP transgenic mice: a mutation-inactivation study. Samyn H, **Moerland M**, Van Gent T, Van Haperen R, Metso J, Grosveld F, Jauhiainen M, Van Tol A, De Crom R. *J Lipid Res. 2008;49:2504-2512.*
3. Elevated expression of phospholipid transfer protein in bone marrow derived cells causes atherosclerosis. Van Haperen R, Samyn H, **Moerland M**, Van Gent T, Peeters M, Grosveld F, Van Tol A, De Crom R. *PLoS ONE. 2008;3:e2255.*
4. Acute elevation of plasma PLTP activity strongly increases pre-existing atherosclerosis. **Moerland M**, Samyn H, Van Gent T, Van Haperen H, Dallinga-Thie G, Grosveld F, Van Tol A, De Crom R. *Arterioscler Thromb Vasc Biol. 2008;28:1277-1282.*
5. Ubiquitous expression of the rtTA2S-M2 inducible system in transgenic mice driven by the human hnRNPA2B1/CBX3 CpG island. Katsantoni EZ, Anghelescu NE, Rottier R, **Moerland M**, Antoniou M, de Crom R, Grosveld F, Strouboulis. J. *BMC Dev Biol. 2007;27:108-117.*
6. Atherogenic, enlarged, and dysfunctional HDL in human PLTP/apoA-I double transgenic mice. **Moerland M**, Samyn H, Van Gent T, Jauhiainen M, Metso J, Van Haperen R, Grosveld F, Van Tol A, De Crom R. *J Lipid Res. 2007;48:2622-2631.*
7. Inducible expression of phospholipid transfer protein (PLTP) in transgenic mice: acute effects of PLTP on lipoprotein metabolism. **Moerland M**, Anghelescu N, Samyn H, Van Haperen R, Van Gent T, Grosveld F, Van Tol A, De Crom R. *Transgenic Res. 2007;16:503-515.*
8. Sex differences in atherosclerosis in mice with elevated phospholipid transfer activity are related to decreased plasma high density lipoproteins and not to increased production of triglycerides. Lie J, **Moerland M**, Van Gent T, Van Haperen R, Scheek L, Sadeghi-Niaraki F, De Crom R, Van Tol A. *Biochim Biophys Acta. 2006;1761:1070-1077.*
9. Endothelial binding of recombinant tissue plasminogen activator: quantification in vivo using a recirculatory model. Kemme MJ, Schoemaker RC, Burggraaf J, Van der Linden M, Noordzij M, **Moerland M**, Kluft C, Cohen AF. *J Pharmacokinet Pharmacodyn. 2003;30:3-22.*

Curriculum Vitae

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1997-2001	Student Medical Biochemistry University of Antwerp, Belgium **Internship** (Erasmus Exchange Program): Department of Experimental Microbiology LUMC, Leiden Prof. dr. E.J.H.J. Wiertz
1996-1997	AFS Intercultural Exchange Student, Pilar, Paraguay
1990-1996	Bisschoppelijk Lyceum, Roeselare, Belgium

Publications

1. Elevation of systemic PLTP, but not macrophage-PLTP, impairs macrophage reverse cholesterol transport in transgenic mice. **Samyn H**, Moerland M, Van Gent T, Van Haperen R, Grosveld F, Van Tol A, De Crom R. *Atherosclerosis. 2008;Epub ahead of print.*
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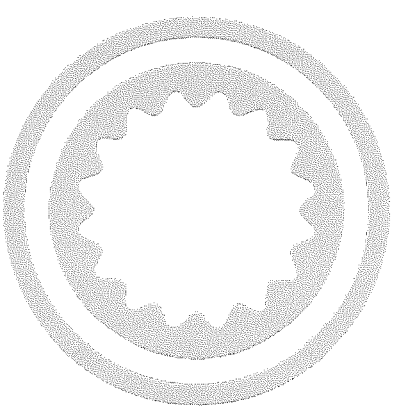
7. Inhibiting cyclin-dependent kinase/cyclin activity for the treatment of cancer and cardiovascular disease. Ivorra C, **Samyn H**, Edo MD, Castro C, Sanz-González SM, Déez-Juan A, Andrés V. *Curr Pharm Biotechnol. 2003;4:21-37.*

Dankwoord



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Matthijs, ons boekje is mooi geworden! Dat was zonder al jouw positieve energie en optimisme nooit gelukt. Bedankt om in mij te geloven! We hebben de laatste tijd afgezien, maar nu is het tijd om te lachen. Want samen lachen we toch het hardst!

Hannelore

Erasmus MC
Universitair Medisch Centrum Rotterdam

PhD Portfolio Summary

Summary of PhD training and teaching activities

Name PhD student: Matthijs Moerland Erasmus MC Department: Cell Biology Research School: MGC	PhD period: December 2002-December 2006 Promotor(s): Prof. F.G. Grosveld Supervisor: Dr. M.P.G. de Crom	
1. PhD training		
	Year	**Workload (Hours/ECTS)**
In-depth courses (e.g. Research school, Medical Training)		
- Master of Molecular Medicine	2002-2003	
- Transgenesis, gene targeting and gene therapy	2002	
- Radiation course (level 5B)	2003	
- Scientific English (Oxford Higher Examination)	2003	
International conferences		
- International Lipoprotein Club (Tutzing, Germany)	2003	
- Scandinavian Society for Atherosclerosis Research (Humlebæk, Denmark)	2005-2006	
- International Vascular Biology Meeting (Noordwijkerhout, The Netherlands)	2006	
Seminars and workshops		
- Dutch Atherosclerosis Society (Ermelo)	2003-2006	
- HDL symposium COEUR (Rotterdam)	2006	
- Dutch Lipoprotein Club	2003-2006	
- MGC symposia and workshops (Lille, Leuven, Oxford)	2003-2005	
- Weekly seminars and monthly guest speakers (Erasmus MC)	2002-2006	
Didactic skills		
- Student supervision	2004-2005	
2. Teaching activities		
	Year	**Workload (Hours/ECTS)**
- Student supervision	2004-2005	

Erasmus MC
Universitair Medisch Centrum Rotterdam

PhD Portfolio Summary

Summary of PhD training and teaching activities

Name PhD student: Hannelore Samyn	PhD period: November 2002-March 2007
Erasmus MC Department: Cell Biology	Promotor(s): Prof. F.G. Grosveld
Research School: MGC	Supervisor: Dr. M.P.G. de Crom

1. PhD training		
	Year	**Workload (Hours/ECTS)**
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- Master of Molecular Medicine	2002-2003	
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- Radiation course (level 5B)	2003	
- Scientific English (Oxford Higher Examination)	2003	
- Experimental animal course (Art. 9)	2003	
International conferences		
- Ernst Klenk Symposium (Cologne, Germany)	2003	
- HLCS Hormones and Genome Conference (Ulm, Germany)	2003	
- International Lipoprotein Club (Tutzing, Germany)	2004	
- Scandinavian Society for Atherosclerosis Research (Humlebæk, Denmark)	2005-2006	
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- Student supervision	2004-2006	