DISSERTATION

IMPACT OF ENDOTHELIAL LIPASE ON STRUCTURAL AND FUNCTIONAL PROPERTIES OF SERUM AND HIGH-DENSITY LIPOPROTEIN

submitted by

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STATUTORY DECLARATION

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz“.

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DISCLOSURES

The content of the first part of the thesis has been published in a peer-reviewed journal and permission for re-print of the figures has been given with the Open-Access-Publication agreements of the publishing group. The second part of the thesis is unpublished.

The thesis is based on the following publication:

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All co-authors gave their consent to re-use data from the publication within this thesis. The following co-authors contributed to the data shown in the thesis:

- Sabine Kern performed the cholesterol efflux measurements shown in figures 5 A-C, 10 A-C, 12B, 13C, 17C and 18 A-C.
- Thomas O. Eichmann measured the lipid composition by mass spectrometry presented in figures 11, 15, 20, 24 and 27 C,D.
- Madalina Duta-Mare quantified LipG and LipC mRNA expression in figures 12A and 16.
- Gerhard Ledinski and Seth Hallström were responsible for measuring conjungated diene and MDA levels demonstrated in figures 29, 31, 33 and 36.
- Tobias Madl did SAXS analyses shown in figure. 23 D,E.
- Dagmar Kolb-Lenz visualized HDL particles by electron microscopy presented in figure 23B.
- Ruth Birner-Gruenberger carried out proteomics and data analysis shown in figure 35.

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LIST OF ABBREVIATIONS

A

AAPH	2,2'-aszobis 2-methyl-propanimidamide dihydrochloride
ABCA1	ATP binding cassette transporter A-1
ABCG1	ATP binding cassette transporter G-1
ACS	Acute coronary sydrome
Ad	Adenovirus
ANGPTL3	Angiopoetin-like 3
ApoA-I	Apolipoprotein A-I
apoB-DS	Apolipoprotein B-depleted serum
ATP	Adenosine triphosphate

B

BA	Bile acid
BSA	Bovine serum albumin

C

Ca^{2+}	Calcium
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CE	Cholesteryl ester
CEC	Cholesterol efflux capacity
CEOOH	Cholesteryl ester hydroperoxide
Cer	Ceramide
CETP	Cholesteryl ester transfer protein
CRP	C-reactive protein
Cu^{2+}	Copper
$CuCl_2$	Copper chloride
CVD	Cardiovascular disease

D

D_{max}	Maximal diameter
DMEM	Dulbecco's modified Eagle medium
DNPH	2,4-dinitrophenylhydrazine
DTT	Dithiothreitol

E

E-HDL	HDL containing ApoE
EL	Endothelial lipase
ELM	Endothelial lipase mutant
eNOS	Endothelial nitric oxide synthase

F

FA	Fatty acid
FC	Free cholesterol
FCS	Fetal calf serum
FPLC	Fast protein liquid chromatography

G

GPX	Glutathione peroxidase

H

h	Human
HDL	High-density lipoprotein
HepG2	Liver hepatocellular cells
HL	Hepatic lipase
HPLC	High performance liquid chromatography
HSPG	Heparin sulfate proteoglycan

I

ICAM-1	Intracellular adhesion molecule 1
IL	Interleukin
INF	Interferone
ISTD	Internal standard

L

LCAT	Lecithin:cholesterol acyltransferase
LDL	Low-density lipoprotein
LOOH	Lipid hydroperoxide
LPC	Lyso-phosphatidylcholine
LPE	Lyso-phosphatidylethanolamine
LPL	Lipoprotein lipase
LPS	Lipopolysachharide

M

MCP-1	Monocyte chemotactic protein 1
MDA	Malondialdehyde
Met	Methionine
miRNA	Micro RNA
MOI	Multiplicity of infection
MPO	Myeloperoxidase
MS	Mass spectrometry

N

NADPH	Nicotinamide adenine dinucleotide phosphate
NEFA	Non esterified fatty acid
NMR	Nuclear magnetic resonance
NO	Nitric oxide

O

oxLDL	Oxidized low-density lipoprotein

P

P(R)	Distance distribution
p.f.u	Plaque forming units
PAF-AH	Platelet activating factor acetylhydrolase
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PL	Phospholipid
PLOOH	Phospholipid hydroperoxide
PLTP	Phospholipid transfer protein
PON	Paraoxonase
PPARα	Peroxisome proliferation-activated receptor α
pPC	Proprotein convertase
PS	Penicillin/streptomycin (cell culture)
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride

R

RCT	Reverse cholesterol transport
RT	Room temperature

S

S.E.M.	Standard error of mean
S1P	Sphingosine-1-phosphate
SAA	Serum amyloid A
SAXS	Small-anlge X-ray scattering
shRNA	Short hairpin RNA
SM	Sphingomyelin
sPLA	Secretory phospholipase
SR-BI	Scavenger receptor class BI

T

TAG	Triacylglycerol
TC	Total cholesterol
TMP	1,1,3,3-tetramethoxypropane
TNF-α	Tumor necrosis factor α

V

VCAM-1	Vascular cell adhesion molecule 1
VLDL	Very-low density lipoprotein

MAIN INTRODUCTION

Atherosclerosis and as a consequence the development of coronary artery diseases (CAD) as well as its complications are still the leading causes of death worldwide [1]. As a treatment to reduce the risk of cardiovascular events, the administration of low-density lipoprotein (LDL) lowering drugs e.g. statins has been established for many years [2]. Nevertheless, 3 years of follow-up after an acute coronary syndrome (ACS), 20.4% of all CAD patients with an appropriate medical therapy including statins suffered from recurrent cardiovascular events [3]. Moreover, treated CAD patients with an increased level of high-density lipoprotein cholesterol (HDL-C) were more protected from major cardiovascular events than those with lower HDL-C [4]. This indicates that a decrease in HDL-C plasma levels or apolipoprotein A-I (ApoA-I) is associated with an increased risk of CAD. However, pharmacological cholesteryl ester transfer protein (CETP) inhibition could not confirm protective role of increased HDL-C plasma levels [5]. Recent studies showed that estimation of HDL particle number by nuclear magnetic resonance (NMR) lipoprotein analysis could be a better predictor for cardiovascular events compared to HDL-C plasma levels [6, 7]. Furthermore, the theory of HDL-C plasma levels and its relation to CAD is nowadays replaced by the hypothesis that numerous atheroprotective functions of HDL are responsible for the reduction of cardiovascular events [7].

1 HIGH-DENSITY LIPOPROTEIN (HDL)

Lipoproteins are lipid and protein containing complexes, which are responsible for solubilization of cholesterol, triacylglycerol (TAG) and other lipophilic molecules in blood. In addition, they regulate the transport of these molecules between peripheral tissues. The different classes of lipoproteins are characterized by their density, which is inversely correlated with the lipid content. HDL delineates the smallest (Stoke`s diameter = 5 to 17 nm) and most protein-rich particle with the highest density (>1.063 g/mL) [8].

1.1 STRUCTURE AND HETEROGENEITY

HDL is a very heterogeneous population of particles relating to size, shape, lipid and protein content as well as their functional properties.

The hydrophobic core is composed of cholesterol esters (CE), TAG and other soluble lipids surrounded by a surface monolayer of phospholipids (PL), free cholesterol (FC) and apolipoproteins (Apo). Apolipoproteins are responsible for the HDL assembly, structure and metabolism as well as mediate receptor interactions [8]. The most abundant HDL apolipoproteins are ApoA-I and ApoA-II. ApoA-I drives the initial step for HDL biosynthesis [9].

Based on which method is used, HDL particles can be separated into different subclasses. Using ultracentrifugation results in the following subfractions: HDL_2 (large, less-dense and lipid-rich) and HDL_3 (small, dense and protein-rich). These two subpopulations can be further divided into HDL_{2b} (10.6 nm), HDL_{2a} (9.2 nm), HDL_{3a} (8.4 nm), HDL_{3b} (8.0 nm) and HDL_{3c} (7.6 nm). According to size and charge, HDL can be fractionated into discoid precursor pre-β, α and pre-α particles. Furthermore, recent studies showed the differentiation of HDL into large, intermediate and small particles by NMR spectroscopy. Finally, based on apolipoprotein composition, HDL can be separated into LpA-I, LpaA-I:A-II or ApoE containing HDL. Analysis of HDL by proteomics revealed 50 to 75 HDL associated proteins. The distribution of these proteins depends on the different HDL subpopulations. Furthermore, HDL subclasses exhibit diverse lipid content. In detail, the ratio between FC:CE and SM:PC decreases with higher density from HDL_{2b} to HDL_{3c}. Since the classification of all these HDL subpopulations depends on which method is used, a novel HDL nomenclature has been established. Based on physical and chemical features, HDL is divided into five subclasses: very large, large, medium, small and very small particles [10, 11].

1.2 HDL BIOSYNTHESIS

The first step in HDL biosynthesis is the lipidation of ApoA-I, a 28 kDa protein, which is synthesized and secreted by the liver and intestine. ApoA-II, also synthesized by the liver, is only involved in the formation of a specific subpopulation of HDL. The ATP-binding cassette transporter A-1 (ABCA1) mediates the transport of FC and PLs from hepatocytes and enterocytes to lipid-free ApoA-I, forming lipid-poor ApoA-I. Subsequently, more FC and PLs are transported from peripheral cells via ABCA1, resulting in the formation of a nascent, disc-shaped HDL particle (pre-β HDL) [12–14]. During hydrolysis of TAG-rich lipoproteins (VLDL, LDL) by lipoprotein lipase (LPL), other lipids especially PLs and apolipoproteins are transferred to HDL, a process mediated by the phospholipid transfer protein (PLTP). Finally, the formation of mature HDL is driven by the action of lecithin:cholesterol acyltransferase (LCAT). Thereby, the HDL associated enzyme catalyzes the transfer of a fatty acid (FA) from PL to FC and produces CEs, whereby the hydrophobic lipid core of HDL is formed [15]. Recently it has been shown that the same process of HDL biogenesis with ApoE and ApoC-III instead of ApoA-I could be achieved in mice [16]. HDL biosynthesis is summarized in figure 1.

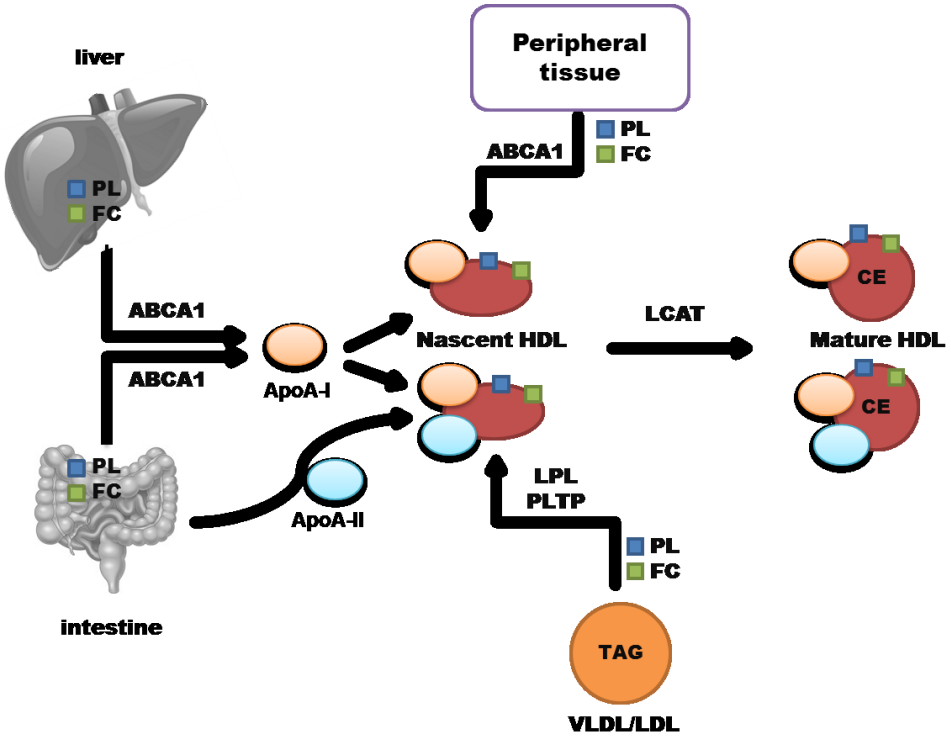

Figure 1: HDL biosynthesis pathway

Lipid-free ApoA-I is synthesized in the liver and intestine. Subsequently, ABCA1 transports PLs and FC to lipid-free ApoA-I. Additional lipids are transferred from TAG-rich lipoproteins as well as peripheral tissue to lipid-poor ApoA-I, forming the nascent HDL. LCAT esterifies FC to CE, which leads to the formation of mature HDL. ApoA-II is synthesized by the liver and initializes the formation of a HDL subfraction, containing both ApoA-I and ApoA-II.

1.3 HDL CATABOLISM

The catabolism of HDL can be divided first into the hepatic catabolism of HDL-C or second in the catabolism of lipid-poor ApoA-I by the kidneys or liver.

1.3.1 HDL CHOLESTEROL CATABOLISM

The liver is the major compartment, where HDL-C uptake takes place. The best known mechanism is the selective uptake by scavenger receptor class BI (SR-BI), which is characterized by the uptake of CE without degradation of HDL apolipoproteins and the release of small cholesterol-depleted HDL particles [15]. This two-step process includes the ApoA-I dependent binding to SR-BI and subsequent transfer of HDL-CE to the plasma membrane as well as the internalization of CE followed by hydrolysis of CE by neutral cholesterol esterases to FC. The generated FC is finally directly excreted into the bile or converted to bile acid (BA) before biliary excretion [8, 15].

Additionally, HDL catabolism can be regulated by CETP. In this pathway, CETP transfers TAG from TAG-rich lipoproteins to HDL in exchange for CE, resulting in the formation of TAG enriched and CE depleted HDL particles. Afterwards, CE is transported via VLDL/LDL to the liver and their binding to the LDL receptor leads to the uptake of CE, followed by conversion into FC and excretion into the bile [15, 17]. HDL particles enriched in TAG lose their stability and the affinity for ApoA-I. Subsequently, hepatic lipase (HL) uses these particles as a substrate, followed by size reduction and ApoA-I release [8].

1.3.2 HDL APOA-I CATABOLISM

The kidney and liver are known as major organs for HDL ApoA-I catabolism [9]. Regarding the evaluation of HDL-C and ApoA-I levels it is known, that the clearance/turnover rate of ApoA-I is more important than the ApoA-I production rate itself. In animals, one-third of ApoA-I is catabolized via kidneys, with the rest catabolized by the liver.

The pathway for ApoA-I catabolism by the kidneys starts in glomeruli by filtration of lipid-poor ApoA-I into primary urine, followed by cubilin/megalin-mediated reabsorption and catabolism in proximal renal tubular epithelial cells. Mature HDL is too large for renal catabolism and thus has to be modified into smaller HDL particles or lipid-poor ApoA-I as described in 1.3.1. Furthermore, the remodeling of HDL can be mediated by lipolytic enzymes (hepatic and endothelial lipase) [15, 17, 18]. The mechanism behind the hepatic ApoA-I catabolism is not well understood. Nevertheless, it has been shown that ApoE containing HDL particles bind to the LDL receptor and other ApoE receptors in the liver to promote HDL uptake. There are also some reports demonstrating the role of ATP synthase and the nucleotide G protein-coupled receptor P2Y13, involved in endocytosis of HDL particles [15]. HDL catabolism is summarized in figure 2.

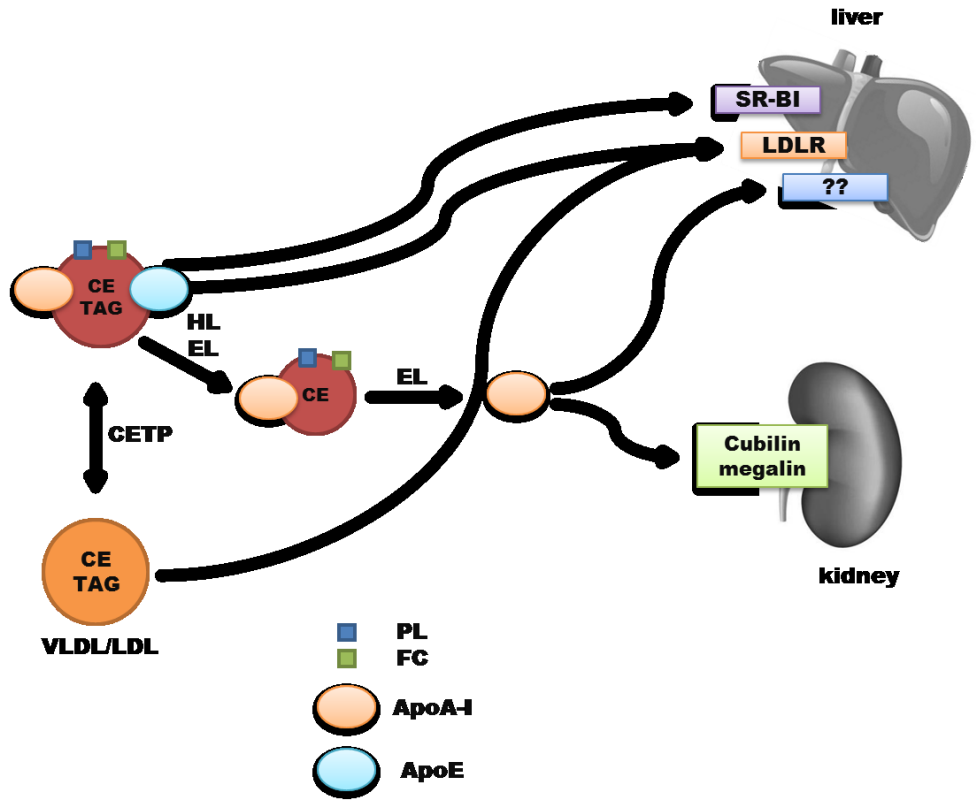

Figure 2: HDL and ApoA-I catabolism pathway

The HDL catabolism pathway is characterized by the uptake of CE and FC via SR-BI in the liver. Alternatively, CE that is transferred from HDL to TAG-rich lipoproteins via CETP can be taken up by the liver through the LDL receptor for biliary excretion. The ApoA-I catabolism starts with the filtration of lipid-poor ApoA-I at the glomerulus followed by degradation via the cubulin/megalin pathway. Lipid-poor ApoA-I is formed due to the action of HL and EL on mature HDL mediated by the transfer of TAG to HDL by CETP. Additionally, mature HDL can be catabolized in the liver through the binding of HDL ApoE to hepatic receptors.

1.4 PROTECTIVE FUNCTIONS OF HDL

After several years of testing pharmacological concepts aiming at increasing HDL-C plasma levels to reduce cardiovascular diseases (CVD), it has become clear that the functionality of HDL as well as the concentration of HDL particles but not HDL-CE content are associated with the development of atherosclerosis and CVD. Beneficial effects of HDL on the cardiovascular system is shown in figure 3 [10, 19].

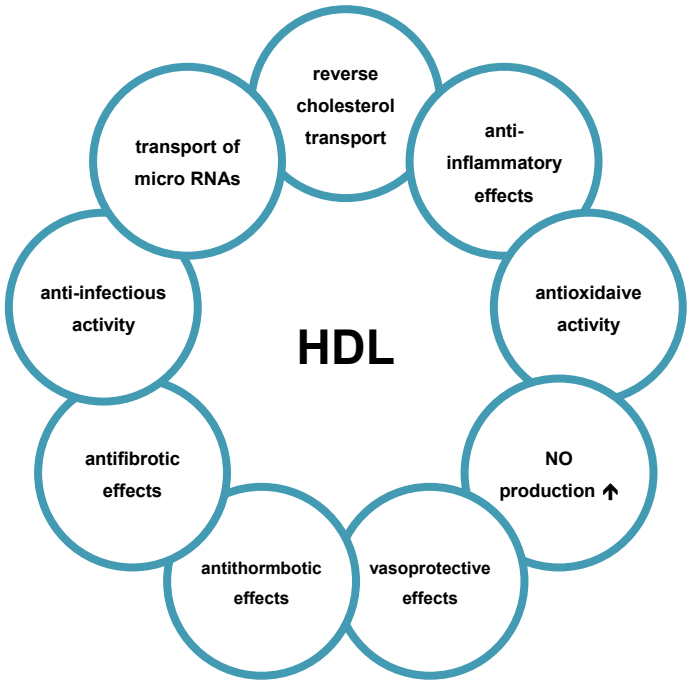

Figure 3: Atheroprotective functions of HDL

1.4.1 MACROPHAGE REVERSE CHOLESTEROL TRANSPORT

The reverse cholesterol transport (RCT) describes the removal of excess cholesterol from peripheral tissues and lipid-laden macrophages to the liver for biliary excretion [12]. This process was first described already in the 1960s [20]. In the first step of RCT, CE is hydrolyzed to FC followed by transfer of FC to mature HDL or lipid-poor ApoA-I, mediated by ABCG1 or ABCA1 transporters. ABCG1 promotes the cholesterol efflux to mature HDL and ABCA1 to lipid-poor ApoA-I [12]. In comparison to ABCG1, that transports only cholesterol to mature HDL, ABCA1 transfers both PLs and cholesterol to ApoA-I [21]. Subsequently, LCAT esterifies FC obtained from various cells, primarily macrophages. The final steps of RCT include the selective uptake of HDL-CE by the liver and excretion into bile, as described in 1.3 [12, 21].

1.4.2 ANTIOXIDATIVE CAPACITY

Many studies demonstrated the capacity of HDL to inhibit LDL oxidation as well as the production of lipid hydroperoxides (LOOH) and advanced products of oxidation. HDL shuttles both early and late products of lipid oxidation. It has been suggested that the ability of HDL to protect against LDL oxidation arise from the high content of antioxidants within the particle as well as the antioxidative capacity of ApoA-I and HDL associated enzymes. ApoA-I has the

ability to bind and remove LOOHs from LDL, followed by reducing LOOHs via its methionine (Met) residues forming lipid hydroxides and Met sulfoxides. Additionally, HDL associated enzymes such as paraoxonase (PON), platelet activating factor acetylhydrolase (PAF-AH), LCAT and glutathione peroxidase (GPX) contribute to the antioxidative capacity of HDL by preventing LDL oxidation and degrading its bioactive products [12, 16, 22]. In addition, low molecular weight antioxidants such as tocopherols and ubiquinols may contribute to the antioxidative capacity of HDL. However, due to the low content in HDL, the relative contribution is most probably negligible [23].

1.4.3 PROTECTION OF VASCULAR ENDOTHELIUM BY HDL

In addition to the promotion of RCT and the antioxidative capacity, HDL exert vascular protective effects by stimulating endothelial nitric oxide synthase (eNOS)-mediated NO production. Endothelial NO regulates vascular tone and structure, endothelial inflammatory response, platelet activity as well as proliferation of vascular smooth muscle cells [12]. Many studies confirmed that HDL facilitates eNOS expression and activity, followed by stimulation of endothelial NO production [24–27]. HDL mediated eNOS activation includes HDL binding to endothelial SR-BI, which triggers various cell signaling pathways including phosphoinositide (PI) 3-kinase, Akt and MAP-kinases leading to eNOS phosphorylation at serine residue 1177 and finally activation [26, 27]. Additionally, HDL associated lysophospholipids e.g. sphingosine-1-phosphate (S1P) promotes the eNOS-mediated NO production via S1P3 receptor [27]. It has been shown that the HDL associated enzyme PON1 plays a role in HDL mediated eNOS activation [24].

HDL exhibits a potent anti-inflammatory activity. By affecting endothelial sphingosine kinase and nuclear translocation of NF-κB [28], HDL diminishes the expression of pro-inflammatory adhesion molecules such as vascular cell adhesion molecule (VCAM)-1, intracellular adhesion molecule (ICAM)-1 and E-selectin [18, 22]. Furthermore, HDL attenuates endothelial apoptosis and promotes endothelial cell proliferation and migration [9, 16, 22].

1.4.4 OTHER ATHEROPROTECTIVE PROPERTIES OF HDL

HDL has also been shown to exhibit anti-thrombotic properties. The particle has the ability to inhibit platelet activation by modulating the activation of platelet-activating factor and cyclooxygenase-2. Additionally, it can reduce APC protein and thrombomodulin, resulting in a decreased formation of thrombin in endothelial cells [18]. The expression of endothelial cell thromboxane as well as the endothelial cell tissue factor is reduced whereas prostacyclin expression is increased by HDL that attenuates platelet aggregation [8].

Recent studies investigated the role of HDL in the transport of micro (mi) RNAs. HDL carried miRNAs positively influences the effects of HDL on endothelial cells, macrophages and other cell types, resulting in the protection of the vascular wall [29].

Furthermore, it has been reported that HDL inhibits the proliferation of hematopoietic stem cells, enhances the insulin secretion of pancreatic β-cells that prevents diabetes mellitus and has the ability to bind lipopolysachharides (LPS) as well as other pathogenic lipids [8, 9].

1.5 DYSFUNCTIONAL HDL

Under particular conditions HDL particles become dysfunctional and lose its atheroprotective biological functions. This phenomenon is caused by altered HDL composition (lipidome and proteome), structure and metabolism found in many diseases such as dyslipidemia, insulin resistance, inflammation, infection and CVD [30].

1.5.1 MODIFICATIONS OF APOA-I

Oxidative modification of ApoA-I by myeloperoxidase (MPO), a protein found in macrophages, monocytes and neutrophils in the atherosclerotic tissue, causes impaired HDL function. One mechanism behind is the oxidation of Met residues as well as chlorination of Tyr192 on ApoA-I. This leads to an impairment of ABCA1-dependet cholesterol efflux, LCAT activity and eNOS-mediated NO production. Increased levels of oxidized Met residues and chlorinated Tyr were found in HDL from patients with CAD and ACS compared to healthy subjects [12, 31]. Furthermore, it has been shown that the properties of HDL in stimulating endothelial repair are declined after modification by MPO [9]. Besides this, malondialdehyde (MDA) mediated modification of HDL causes lower ApoA-I concentrations, resulting in a decrease in ABCA1-dependent cholesterol efflux [32]. In patients with type 2 diabetes, high levels of oxidized FAs have been shown to impair the anti-inflammatory and antioxidative properties of HDL [21]. HDL isolated from diabetic patients was incapable in stimulating endothelial cell proliferation and migration due to an associated downregulation of SR-BI expression [33]. Additionally, oxidized HDL positively correlated with glucose levels indicating the relevance of glucose on oxidation [34]. Interestingly, HDL isolated from diabetic patients exhibited higher S1P levels causing protective effects on endothelial cells as a result of induced cyclooxygenase-2 expression levels and prostacyclin release [9].

1.5.2 INFLAMMATION: IMPACT ON HDL COMPOSITION AND FUNCTION

Acute inflammatory response massively alters HDL composition highlighted by increased TAG and decreased CE content. Accumulation of TAGs in HDL causes instability of HDL particles and accelerates ApoA-I catabolism [31]. Furthermore, decreased ApoA-I synthesis together with faster HDL catabolism and the replacement of ApoA-I by serum amyloid A (SAA) underlie the decreased ApoA-I content of HDL [35]. Besides affecting HDL composition, acute inflammatory response diminishes various HDL activities such as macrophage cholesterol efflux, protection against LDL oxidation or inhibition of adhesion molecule expression [9]. Under inflammatory conditions the loss of HDL associated enzymes such as PON and PAF-AH was observed indicating impaired antioxidative capacity of HDL [31]. Decreased PON1 activity in HDL of CAD patients was accompanied by decreased antioxidative capacity of HDL and increased MDA content of HDL. MDA enriched HDL by acting via LOX-1 receptor activates endothelial kinase C beta II (PKCbII) and in turn increases inhibitory eNOS phosphorylation resulting in decreased eNOS activity and NO production [12, 36].

1.5.3 VASCULAR ENDOTHELIUM

Several studies demonstrated the role of HDL proteome in HDL functionality [9]. For example, HDL isolated from patients after myocardial infarction exhibited markedly decreased ability to stimulate NO production and protect against endothelial cell apoptosis. This could be explained by changes in the HDL proteome including enrichment of SAA, complement C3 and C9 and ApoC-II as well as a decreased content of clusterin (ApoJ) and ApoA-IV [31]. Additionally, HDL from CAD or ACS patients was not able to inhibit endothelial cell apoptosis but stimulated proapoptotic pathways [36].

Taken together, remodeling of HDL composition, structure and metabolism can have both positive and negative effects on HDL functionality. Therefore, there is still a need to establish reproducible and specific assays to study the importance of HDL activities in atherogenesis, inflammation, infection and immunity.

2 ENDOTHELIAL LIPASE (EL)

Endothelial lipase (EL), encoded by the LIPG gene, was described for the first time from two independent groups in 1999 [37, 38]. EL emerged as a strong determinant of HDL-C levels as well as HDL metabolism [39]. Furthermore, it has been established that EL modulates HDL lipid composition and size, consequentially changing its functional properties [40–42].

2.1 STRUCTURE AND ACTIVITY

EL belongs together with LPL and HL to the TAG lipase gene family and exhibits molecular homology with LPL (45%) and HL (40%) [43]. The lid domain that is responsible for substrate specificity and forms the catalytic pocket of the enzyme distinguishes EL from LPL and HL. Additionally, it has a conserved catalytic triad as well as conserved lipoprotein and heparin binding sites. The lipase contains 482 amino acids and is synthesized as a 55 kDa protein [44]. Upon maturation EL is secreted into the media as a 68 kDa protein and can be cleaved into two fragments - N-terminal 40 kDa and C-terminal 28 kDa fragment - by proprotein convertases (pPCs) [45]. EL is mainly synthesized and secreted by endothelial cells. Additionally, it has been shown that EL is expressed in human placenta, thyroid gland, lung, liver, kidney, ovary, testis as well as macrophages [37]. In detail, EL expression e.g. in the liver was observed in endothelial cells and not hepatocytes [44]. After secretion, EL binds to cell surface heparin sulfate proteoglycans (HSPG), acting as a bridge between cells and lipoproteins thus promoting lipoprotein binding and uptake by underlying cells [46, 47].

EL exhibits very low TAG lipase activity that is independent of ApoC-II. More pronounced is its phospholipase A1 activity, whereby it cleaves phosphatidylcholine (PC) on the sn-1 position liberating FAs and lyso-PCs (LPC) [43]. These lipolytic products partially remain in HDL, which are partially taken up by EL-overexpressing cells where they are used for biosynthesis of endogenous lipids [48].

2.2 REGULATION OF EL EXPRESSION AND ACTIVITY

EL expression and activity can be regulated by different factors and diseases. Inflammation and its related cytokine production of TNF-α and IL-1β enhance EL mRNA levels in human endothelial cells. Shear stress as well as cyclic stretch of the vasculature (biomechanical forces) also induced EL expression [49]. Furthermore, expression levels of the enzyme in macrophages are increased after LPS treatment due to the stimulation of toll-like receptor 4 [50]. Another study showed that hypertension and angiotensin II promote EL mRNA levels in vascular smooth muscle cells [51]. Human studies reported that EL mass positively correlates

with inflammatory markers such as CRP, IL-6 and sPLA2-IIa [52] and that a low dose of endotoxemia leads to a significant increase in EL [53]. Downregulation of EL expression and mass was observed after treatment of patients with statins [46]. A dimerisation of EL was found to be crucial for its activity [54]. EL activity has been found to be attenuated by pPCs, angiopoetin-like 3 (ANGPTL3), ApoA-II as well as protein glycosylation [46].

2.3 ROLE OF EL IN HDL METABOLISM

Testing the influence of EL activity on isolated lipoprotein fractions demonstrated a low activity against TAG-rich lipoproteins but a high one in hydrolyzing HDL lipids, particular PLs [43]. This suggests the impact of EL on HDL metabolism. Indeed, *in vivo* studies reported strong negative regulation of HDL-C levels by EL. Overexpression of human EL in LDL receptor deficient mice by adenovirus [38] as well as transgenic expression of human EL in mice [55] resulted in a reduction of HDL-C and ApoA-I levels. Maugeais et al. [56] observed a dose-dependent effect of EL overexpression on HDL metabolism in mice due to an increased phospholipase activity in post-heparin plasma as well as induced catabolic rate of injected radiolabeled mouse HDL in the liver and kidneys. Additionally, they observed reduced PL, CE and FC content in HDL but no changes in TAG levels [56]. These effects were mainly a consequence of the catalytic activity of EL and not its bridging function, because overexpression of lipolytic inactive EL did not result in any changes of HDL-C plasma levels in wild-type and ApoA-I transgenic mice [46, 57]. According to *in vivo* studies in mice, one recent study with transgenic rabbits with hepatic human EL overexpression also noticed decreased TC, PL, HDL-C, HDL-PL, ApoE and ApoA-I levels in plasma compared to controls [58]. TAG content was only significantly reduced in male transgenic rabbits. Interestingly, EL transgenic rabbits fed with high cholesterol diet developed lower hypercholesterolemia as well as less aortic and coronary atherosclerosis with a reduction in TC plasma levels, ApoB containing lipoproteins and HDL particles. This finding highlighted the antiatherogenic properties of EL [58]. Conversely, loss of EL function in mice demonstrated higher HDL-C, ApoA-I and PL levels in plasma, increased HDL particle size and delayed catabolic rate of HDL [55, 59, 60].

Related to *in vivo* mouse studies, one human study showed a positive correlation between small HDL particles and EL mass as well as a negative relationship between large HDLs and the EL mass levels in post-heparin plasma [61]. Studies in humans mainly focused on polymorphisms in the EL gene (LIPG), studying the impact of EL on HDL metabolism. The most common human variant Thr111Ile in LIPG was first described to be associated with increased HDL-C plasma levels, however this could not be confirmed in subsequent studies [62]. For

example, Edmondson et al [63] recently showed that there is no relationship between the Thr111Ile variant and HDL-C plasma levels in 3845 study participants. In addition, *in vitro* studies demonstrated a normal catalytic active EL of that variant [63]. Nevertheless, the Asn396Ser substitution in the LIPG gene was significantly associated with increased HDL-C levels. This was accompanied by an increase in HDL_2 and HDL_3 subfractions, enlarged HDL particles and increased ApoA-I levels [63, 64]. *In vitro* and *in vivo* analysis of the Asn396Ser variant demonstrated reduced lipase activity of that EL variant [63]. Several other LIPG polymorphisms were reported to be linked to altered HDL-C levels in humans [65–67], but there is still a need to demonstrate their relationship to atherosclerosis.

2.4 ROLE OF EL IN ATHEROSCLEROSIS

The role of EL in the development of atherosclerosis is still not well understood. Due to the fact that EL regulates HDL-C plasma levels negatively, someone can speculate that by inhibition of EL and subsequently raising HDL-C levels, atherosclerosis can be prevented. But the process is much more complex and it is still unclear whether EL promotes pro- or antiatherogenic effects. In one study the targeted inactivation of EL in $ApoE^{-/-}$ mice diminished atherosclerosis formation as well as enhanced HDL-C and VLDL/LDL cholesterol levels. Additionally, they found a decrease in the macrophage content within the atherosclerotic lesions [68]. In contrast, another report showed no effect of EL inactivation in $ApoE^{-/-}$ and LDL-receptor deficient mice on the prevention of atherosclerosis and macrophage content although HDL-C was increased [69]. Recently, quantification of aortic and coronary atherosclerosis in transgenic rabbits expressing human EL revealed a significant reduction of whole atherosclerotic lesions compared to controls [58]. Furthermore, it has been demonstrated that EL affects the catabolism of TAG-rich lipoproteins. While EL deficiency in mice resulted in the production of small dense LDL particles [70] and an increase in VLDL cholesterol levels [69], EL overexpression reduced the content of TAG-rich lipoproteins in plasma [57]. As described in 2.2, EL expression is enhanced by acute inflammation, a condition that is also important in the atherosclerosis development. On the one hand, it has been demonstrated that EL increases monocyte adhesion to the vessel wall [68] but on the other hand EL led to a reduced endothelial adhesion molecule expression by producing peroxisome proliferatior-activated receptor α (PPARα) ligands from HDL-PLs [46, 71]. Knockdown of EL in mice led to decreased monocyte adhesion on endothelial cells while EL transgenic mice showed increased adhesion [72]. Experiments with isolated macrophages from $EL^{-/-}$ mice or silencing of EL by shRNA resulted in low efficiency of LDL binding and uptake [73, 74]. Additionally, loss-of-function and gain-of-

function studies showed that EL in macrophages promotes LDL receptor mediated binding and uptake of LDL [74] as well as ApoA-I mediated cholesterol efflux [75]. These results indicate a bridging function of EL between monocytes and endothelial cells as well as lipoproteins and macrophages.

Several human studies demonstrated the involvement of EL in the development of atherosclerosis. For example, EL levels correlated with the content of macrophages within human atherosclerotic plaques. Within these plaques, EL expression was located in infiltrating cells as well as endothelial and smooth muscle cells [76]. Another study confirmed that EL mRNA and protein levels are present in atherosclerotic lesions between the necrotic core and the fibrotic cap. They also found an upregulation of EL mRNA expression during differentiation of monocytes into macrophages and downregulation during transformation of macrophages into foam cells [77]. Furthermore, Badellino et al. [61] observed that both pre-heparin and post-heparin EL mass levels are associated with coronary artery calcification scores as well as metabolic syndrome [61]. In a follow-up study, renal patients with cardiovascular events had higher serum EL levels compared to renal patients without any cardiovascular event [78]. Inhibition of EL in human macrophages by statins showed an inhibition of NF-κB as well as a decreased accumulation of oxysterols, contributing to the attenuated transformation of macrophages into foam cells [79].

Taken together, the role of EL in atherosclerosis remains inconclusive with several possible pro- and antiatherogenic mechanisms.

CHAPTER I

IMPACT OF ENDOTHELIAL LIPASE ON CHOLESTEROL EFFLUX CAPACITY OF SERUM AND HIGH-DENSITY LIPOPROTEIN

1 ABSTRACT

Despite the application of appropriate treatments, coronary artery diseases (CAD) are still the leading causes of death worldwide. Low high-density lipoprotein cholesterol (HDL-C) plasma levels are associated with a high risk of atherosclerosis. However, HDL exhibits numerous atheroprotective properties including the promotion of reverse cholesterol transport (RCT), a process in which excess cholesterol is delivered from the periphery back to the liver for biliary excretion. Endothelial lipase (EL) is a strong negative regulator of HDL plasma levels as well as a modulator of the structural and functional properties of HDL. The impact of EL on the cholesterol efflux capacity (CEC) of serum and isolated HDL has been analyzed previously yielding inconclusive results.

In the present study, the impact of EL on the composition and CEC of serum, apolipoprotein B-depleted serum (apoB-DS) and HDL isolated from EL-modified serum was investigated, using appropriate *in vitro* and *in vivo* approaches. EL overexpression *in vitro* was achieved by adenoviral transduction of cultured cells and in mice by adenoviral tail vein injection. CEC was analyzed in ^{3}H-cholesterol labeled J774 macrophages under basal conditions or with upregulation of ABCA1.

Incubation of human and mouse serum with EL overexpressing cells resulted in an enhanced CEC of serum and apoB-DS and decreased CEC of isolated HDL, accompanied by the formation of lipid-poor ApoA-I, alterations in the lipid composition as well as decreased size of human but not mouse HDL. In contrast, overexpression of human and mouse EL in mice impaired CEC of serum without altering CEC of isolated HDL. This was accompanied by reduced HDL-C and ApoA-I serum levels, markedly altered lipid composition of serum and HDL as well as unaltered HDL size.

The impact of EL on the CEC of serum and HDL reflects structural and compositional features as well as abundance of EL-modified HDL. Results of our study highlight the complexity of the interaction between EL and HDL, clearly showing the role of experimental models on the structural and functional properties of EL-modified HDL. Here we show that EL by acting on human and mouse serum *in vitro* results in accumulation of lipid-poor ApoA-I, a potent cholesterol acceptor. The relevance of these findings for human (patho)physiology needs to be addressed in future studies.

2 ZUSAMMENFASSUNG

Trotz modernen Behandlungskonzepten, stellt Atherosklerose und die damit verbundene Entstehung von verschiedenen Herzerkrankungen noch immer die häufigste Todesursache weltweit dar. Niedrige Konzentrationen von High-density Lipoprotein Cholesterin (HDL-C) im Blutplasma sind mit einem erhöhten Risiko an Atherosklerose zu erkranken, assoziiert. HDL weist verschiedene gefäßschützende Eigenschaften auf, einschließlich des Prozesses bei welchem überschüssiges Cholesterin von den peripheren Geweben mit Hilfe von HDL zur Leber für die biliäre Ausscheidung transportiert wird. Dieser Vorgang wird als „Reverser Cholesterin Transport (RCT)“ bezeichnet. Spezifisch führt dabei die Wirkung der endothelialen Lipase (EL) zu einer Senkung des HDL-C Plasmaspiegels. Darüber hinaus hat dieses Enzym die Fähigkeit sowohl die strukturellen als auch die funktionellen Eigenschaften des HDLs zu beeinflussen. Diverse Studien analysierten den Einfluss der EL auf die Cholesterin Efflux Kapazität (CEK) des Serums und isoliertem HDL, jedoch waren die Ergebnisse nicht aussagekräftig.

In der gegenwärtigen Studie wurde mit Hilfe von geeigneten *in vitro* und *in vivo* Experimenten, die Wirkung von EL auf die Zusammensetzung und die CEK von Serum, Apolipoprotein B-depletiertem Serum und isoliertem HDL untersucht. Demnach wurde EL mittels Adenovirus entweder *in vitro* in kultivierten Zellen oder *in vivo* in Mäusen überexprimiert. Die Analyse der CEK erfolgte mittels ^{3}H-Cholesterin markierten J774 Macrophagen unter basalen Bedingungen oder unter Hochregulierung des ABCA1 Transporters.

EL Überexpression *in vitro* in Zellen, welche mit Human- oder Mausserum inkubiert wurden, führte zu einer verbesserten CEK mit Serum und ApoB-DS, jedoch zu einer verringerten CEK mit isoliertem HDL. Dieses Resultat wurde sowohl durch die Entstehung von lipidarmen ApoA-I, als auch durch eine veränderte Lipidzusammensetzung und einer verminderten HDL Größe (nur Human) erzielt. Im Gegensatz zu den *in vitro* Ergebnissen, wurde durch Überexprimierung von Human und Maus EL in Mäusen eine Abnahme der CEK mit Serum und ApoB-DS, jedoch eine unveränderte CEK mit isoliertem HDL, beobachtet. Dies könnte sowohl durch eine starke Verringerung des HDL-C und ApoA-I Spiegels im Serum und eine veränderte Lipidzusammensetzung des Serums und HDLs als auch durch eine unveränderte HDL Größe des HDLs erklärt werden.

Der Einfluss von EL auf die CEK von Serum und HDL reflektiert sowohl die strukturellen und kompositionellen Eigenschaften als auch die Menge des EL modifizierten HDLs. Unsere Studie konnte demnach die komplexe Interaktion zwischen EL und HDL aufzeigen, indem der Einfluss von experimentellen Modellen auf die strukturellen und funktionellen Charakteristika des EL modifizierten HDLs gezeigt wurde. Es stellte sich heraus, dass die Wirkung von EL auf Human- und Mausserum *in vitro* zu einer Akkumulierung von lipid-armen ApoA-I, welches ein potenter Cholesterinakzeptor ist, führt. Nichtsdestotrotz sind weitere Untersuchungen erforderlich, um die Relevanz der Daten dieser Studie in Bezug auf die humane (Patho)physiologie zu prüfen.

3 INTRODUCTION

A big body of evidence demonstrated the relationship between HDL and cardiovascular health and disease. The most studied function of HDL is the ability to promote the RCT from macrophages. This process is described as the removal and transfer of excess cholesterol from the periphery back to the liver for biliary excretion [12] as described in the main introduction, chapter 1.4.1.

3.1 CHOLESTEROL EFFLUX PATHWAYS

Several pathways are responsible for the elimination of cholesterol from lipid-laden macrophages (foam cells) by HDL (table 1). The relative contribution of these pathways to the cholesterol efflux capacity from macrophages is different. In normal, non-lipid-laden macrophages, the passive/aqueous diffusion is the primary pathway, whereas in foam cells the pathway mediated by ABCA1 is the most common (table 1) [80]. The regulation of cholesterol efflux by HDL includes cell membrane transporters and plasma lipid acceptors as well as plasma proteins, enzymes and hepatic cellular receptors [81].

Table 1: Pathways by which cholesterol can be removed by HDL in non-lipid-laden macrophages

Pathway	Acceptor	Acceptor binding	Contribution
Passive diffusion	HDL_2 + HDL_3	No	35%
SR-BI	HDL_2 > HDL_3	Yes	9%
ABCG1	HDL_3	No	21%
ABCA1	Lipid-poor ApoA-I Lipid-poor ApoE	Yes	35%

3.1.1 PASSIVE/AQUEOUS DIFFUSION

The aqueous diffusion process is defined as a bidirectional flux of cholesterol among the cell membrane and HDL in the extracellular medium, regulated by the concentration of FC/PL ratio in the donor and acceptor. Therefore, cholesterol molecules are desorbed from the donor, followed by diffusion of these molecules through the aqueous phase until they crash with the acceptor, which finally absorbs the cholesterol. Desorption of cholesterol is increased by decreased PL content and reduced by a higher amount of sphingomyelin (SM) in the membrane. The rate of cholesterol efflux by aqueous diffusion depends on the concentration, structure and size of the acceptor particles [82, 83]. HDL particle size is not influencing this pathway, meaning that all HDL subclasses are equally good acceptors [84].

3.1.2 SR-BI MEDIATED CHOLESTEROL EFFLUX

SR-BI, an 82 kDa transmembrane protein, regulates the structure and composition of HDL. This protein is widely expressed by macrophages and in the liver [85]. SR-BI has the ability to promote bidirectional flux of FC between several cell types and HDL or other acceptors [86]. As describe in 1.3.1 (main introduction), HDL binds to hepatic SR-BI and mediates the cholesterol efflux due to the formation of a complex within a hydrophobic channel that allows the diffusion of cholesterol molecules [83]. SR-BI is an acceptor for apolipoproteins and HDL while the highest binding affinity was observed with large, mature HDL [87]. In contrast, the role of SR-BI in macrophage cholesterol efflux is not well understood. The development of atherosclerosis was increased after bone marrow transplantation from SR-BI deficient mice into LDL receptor [88] or ApoE [89] deficient mice. Overexpression of hepatic SR-BI led to enhanced macrophage cholesterol efflux while deletion of SR-BI resulted in decreased macrophage RCT [90]. Despite these observations, other *in vivo and in vitro* experiments showed no correlation between SR-BI and macrophage RCT [91–93].

3.1.3 CHOLESTEROL EFFLUX MEDIATED BY ABC TRANSPORTERS

ABC transporters are membrane proteins that are able to manage the cellular transport of several particles such as ions, lipids, cyclic nucleotides, peptides and proteins. They are equipped with membrane domains to facilitate the translocation of the substrate as well as cytoplasmic ABCs to regulate the transport by hydrolysis of ATP [94].

ABCA1 expression is detectable in liver, kidney, adrenal gland, intestine and CNS but also in lipid-loaded macrophages [95]. Low protein and mRNA levels of ABCA1 in macrophages are found under basal conditions, but loading of cholesterol triggers both and can be inverted by HDL mediated cholesterol efflux [96]. Expression of ABCA1 is induced by oxysterol mediated activation of LXR/RXR transcription factors, which bind to the ABCA1 promoter. These transcription factors contribute to the regulation of several genes that are involved in macrophage cholesterol efflux (ApoE, ABCA1, and ABCG1), cholesterol transport (LPL, CETP, ApoC), BA production of cholesterol (CYP7A) as well as metabolism and biliary or intestinal excretion (ABCG5 and ABCG8). Furthermore, ABCA1 mRNA levels in macrophages are upregulated by cAMP and downregulated by INF-γ and miRNAs [94, 97]. Studies with synthetic LXR agonists reported an upregulation of macrophage ABCA1 and ABCG1 levels as well as an increased cholesterol efflux *ex vivo* and macrophage RCT *in vivo* [98]. Application of synthetic LXR agonists in mice resulted in inhibitory effects in the progression of atherosclerosis without altered HDL-C plasma levels [99]. Other publications revealed that peroxisome prolifera-

tor-activated receptor (PPAR)-α and PPAR-γ agonists were able to promote cholesterol efflux *in vitro* by upregulating ABCA1 [100, 101], reduced foam cell accumulation and prevented atherosclerosis *in vivo* [102]. ABCA1 uses mainly lipid-poor ApoA-I as well as small dense HDL as acceptors [103]. Humans with loss-of-function mutations in ABCA1 (Tangier disease) exhibit markedly reduced HDL levels as well as accumulation of cholesterol in macrophages [104]. Similar results were observed in ABCA1 knockout mice [104]. In line with this, ABCA1 deficient macrophages exhibited massively decreased cholesterol efflux to lipid-poor ApoA-I [104, 105]. Additionally, transplantation of bone marrow from ABCA1 knockout mice resulted in an enhanced atherosclerosis development in acceptor mice despite normal HDL-C levels [105, 106]. In contrast, transplantation of bone marrow from ABCA1 overexpressing mice reduced atherosclerosis related pathology in acceptor mice [107]. In line with these observations significantly reduced *in vivo* RCT was found with ^{3}H-labelled ABCA1 deficient macrophages *in vivo* [91].

ABCG1 promotes macrophage cholesterol efflux, however in contrast to ABCA1 the preferred substrate is mature HDL [108, 109]. Similar to ABCA1, the upregulation of ABCG1 in macrophages is mediated by LXR activation and cholesterol loading [110]. ABCG1 deficient macrophages demonstrated decreased cholesterol efflux to mature HDL *ex vivo* as well as impaired RCT *in vivo* [91]. Interestingly, a reduced development of atherosclerosis was observed with ABCG1 deficient macrophages [111, 112]. This finding could be explained by a compensatory effect through upregulation of macrophage ABCA1 and ApoE [111] or increased sensitivity of macrophages to apoptosis induced by oxidized LDL (oxLDL) [112].

Both ABC transporters operate in a cooperative manner to facilitate RCT. ABCA1 transports lipids to lipid-poor ApoA-I to form mature HDL particles, which subsequently act as acceptors for cholesterol delivered by ABCG1 [113]. The additive effect of both transporters on cholesterol efflux was illustrated by simultaneous inactivation of both transporters in macrophages, which resulted in dramatically decreased cholesterol efflux capacity *ex vivo* as well as RCT *in vivo*. Inactivation of either ABCA1 or ABCG1 decreased cholesterol efflux, however to a much lesser extent compared to inactivation of both transporters [91]. In line with this, bone marrow transplantation from double knockout mice caused more severe atherosclerosis than transplantation from single knockout mice [114].

In summary, the cholesterol efflux capacity is regulated by the extracellular concentration and composition of HDL particles as well as by the activity of the ABC transporters.

3.1.4 REGULATION OF RCT BY APOE

In addition to lipid-poor ApoA-I, it has been established that HDL particles containing ApoE (E-HDL) contribute to the promotion of macrophage cholesterol efflux. ApoE is synthesized in the liver and macrophages. E-HDL has the ability to mediate cholesterol efflux from macrophages as well as the transfer of cholesterol to the liver. ApoE containing HDL particles are substrates for the ABCA1 or ABCG1 transporter and facilitate the removal of excess cholesterol from macrophages. Thereafter, LCAT on these particles converts FC to CE, followed by the delivery of cholesterol to the liver through binding to SR-BI or LDL receptor [81].

3.2 ROLE OF EL IN CHOLESTEROL EFFLUX

As described in the previous chapters, HDL plays an important role in regulating the cholesterol efflux from macrophages. The fact that EL modulates the composition and functionality of HDL prompted the hypothesis that EL influences the cholesterol efflux capacity of HDL. Several studies addressed the role of EL in cholesterol efflux, generating however contradictory results.

3.2.1 IMPACT OF EL ON THE MAIN RCT STEPS

In vitro studies showed an augmenting effect of EL overexpression on ABCA1 mediated cholesterol efflux from macrophages [75]. Inhibition of both lipolytic and bridging function of EL resulted in diminished macrophage cholesterol efflux capacity [75], indicating that both EL functions affect RCT. Yancey et al [115] reported that overexpression of EL in human ApoA-I transgenic mice enhanced the capacity of serum to act as a cholesterol acceptor effluxed by ABCA1 and decreased the capacity of serum to accept cholesterol effluxed via SR-BI [115]. In contrast to these results, another study showed abolished ABCA1 and reduced SR-BI mediated cholesterol efflux capacity in mice overexpressing EL as a consequence of profurin treatment [116]. Another study reported no correlation between EL and ABCA1 mediated cholesterol efflux but decreased SR-B1 mediated cholesterol efflux [117]. *Ex vivo* experiments with plasma and HDL from EL knockout mice demonstrated improved efflux compared to plasma and HDL from wild-type mice, with even more pronounced efflux capacity observed for both plasma and HDL from EL/HL double-knockout mice [70]. In contrast, Hara et al [41] observed no differences between HDL from EL knockout and wild-type mice [41].

The next RCT step comprises hepatic uptake of HDL-CE. *In vivo* and *in vitro* studies reported consistent results on the impact of EL in this process. Overexpression of EL in HepG2 cells promoted the binding of HDL to the cell surface and the selective uptake of HDL-CE, both processes being dependent on the catalytic activity as well as the bridging function of EL. In

line with impaired hepatic HDL-CE uptake using radiolabelled HDL_3 injected into EL knockout mice [70], SR-BI uptake was increased upon hepatic EL overexpression *in vivo* and *in vitro* [118, 119].

Finally, the last step in RCT is the secretion of cholesterol into bile. As a consequence of enhanced hepatic HDL-CE uptake, increased hepatic cholesterol content was found upon EL overexpression in mice but no changes were observed in biliary secretion [118, 119]. Furthermore, the mass fecal output was not influenced by EL overexpression in mice [118].

As described in the main introduction, chapter 2.1, EL cleavage of PCs results in the production of FAs and LPCs. It has been reported that LPCs have the ability to promote macrophage cholesterol efflux [120]. Qui et al [75] observed an enhanced LPC production by EL overexpression in macrophages. Furthermore, they found an increased and dose dependent effect of LPCs on the ApoA-I mediated cholesterol efflux via ABCA1 [75].

3.2.2 IMPACT OF EL ON *IN VIVO* RCT

The *in vivo* RCT comprises injection of ^{3}H-cholesterol labeled macrophages into acceptor mice followed by tracing ^{3}H-cholesterol in plasma, liver and feces. Increased ^{3}H-cholesterol plasma levels with no differences in hepatic radiolabeled cholesterol but increased fecal and biliary ^{3}H-cholesterol excretion were observed in EL deficient compared to wild-type mice [42]. The opposite was found upon EL overexpression in mice, namely lower ^{3}H-cholesterol plasma levels as well as less radiolabeled cholesterol in the liver, BAs and feces compared to wild-type mice [116].

Genetic inactivation of EL in humans demonstrated increased HDL-C plasma levels as well as enhanced cholesterol efflux capacity of apolipoprotein B-depleted serum (apoB-DS) [40].

4 THEORY AND HYPOTHESIS

Studies on the impact of EL on the cholesterol efflux capacity (CEC) of mouse serum provided conflicting results [115, 116]. So far the impact of EL overexpression on CEC of human serum has not been studied. Therefore, the present study has been designed to investigate the CEC of both human and mouse *in vitro* and *in vivo* EL modified serum (EL-serum), -apolipoprotein B-depleted serum (EL-apoB-DS) as well as HDL isolated from the modified serum.

5 MATERIAL AND METHODS

5.1 CELL CULTURE

HepG2 cells (ATCC®, HB-8065™) [121] were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM glutamine and 1% PS (100 U/mL penicillin, 100 µg/mL streptomycin). J774.2 macrophages (Sigma-Aldrich, Vienna, Austria; #85011428) [122] were cultured in RPMI1640 medium with 10% FCS.

5.2 PREPARATION OF HEPARIN MEDIA

Heparin media were prepared as described in our previous studies [45, 123].

5.3 *IN VITRO* GENERATED HUMAN AND MOUSE EL-MODIFIED AND EV-CONTROL SERUM

In vitro modification of human and mouse serum has been described previously [124]. After overnight fasting, human blood was collected from 8 healthy volunteers (4 females and 4 males). The local Ethics Committee of the Medical University of Graz approved all experimental protocols related to human volunteers (28-186 ex 15/16). From each volunteer a written instructed approval was obtained in compliance with Good Clinical Practice. To obtain serum, blood was incubated for 30 min at room temperature (RT), followed by centrifugation (3000 x g) at 4°C for 15 min. The serum was stored at -80°C.

For the experiments with mouse serum, non-fasted mice were anesthetized with sevorane (AbbVie, Vienna, Austria), followed by blood collection from the right ventricle. The Austrian Federal Ministry for Science and Research (BMWF-66.010/0133-II/3b/2012) approved all experimental animal protocols. Afterwards blood was incubated for 30 min at RT and centrifuged (3000 x g) at 4°C for 15 min, followed by storage of the serum at -80°C.

First, HepG2 cells were plated onto 60 mm dishes (2 x 10^6 cells/dish), followed by incubation for 24 h under standard conditions as described in 5.1. On the next day, cells were washed once with DMEM without FCS and infected with recombinant adenoviruses encoding human EL (hEL-Ad) or empty adenovirus, which contains no recombinant cDNA (EV-Ad) [123], with a multiplicity of infection of 20 (MOI 20) in DMEM without FCS for 2 h. Subsequently, the infection media was removed and cells were incubated in DMEM with 10% FCS for 20 h. In a final step, the infected cells were washed once with DMEM without FCS and incubated with 1.8 mL of 50% pooled human or mouse serum in DMEM without FCS per plate, for 8 h. Afterwards, the modified human or mouse serum was collected and centrifuged (1100 x g) for 3 min to remove cellular debris. The *in vitro* modified hEL-serum, hEV-serum, mEL-serum and

mEV-serum were further used for the preparation of appropriate apoB-DS or HDL as described in 5.5 and 5.6.

5.4 *IN VIVO* GENERATED EL-MODIFIED SERUM AND CONTROL EV SERUM

Male C57BL/6 mice (9-12 weeks old, non-fasted) were used for tail vein injection of EV-Ad, human EL-Ad or mouse EL-Ad [45, 123] with a concentration of 3.2×10^8 plaque forming units (p.f.u) in 100 µL PBS. After 48 h blood was taken from the right ventricle and serum was prepared as described in 5.3. The collected serum was used for the preparation of apoB-DS or HDL as described in 5.5 and 5.6. Mice were anesthetized by sevorane (AbbVie, Vienna, Austria) while they were injected via tail vein and punctured by right ventricle [124].

5.5 APOB-DEPLETION OF SERUM

ApoB-DS was achieved by incubating the mixture of 40 µL polyethylene glycol (20% in 200 mmol/L glycine buffer) and 100 µL mouse serum or 100 µL 50% human/mouse serum at RT for 20 minutes. Subsequently, the mixture was centrifuged (10.000 rpm, 30 min, 4°C) [125] and the supernatant was stored at -80°C for experiments.

5.6 HDL ISOLATION FROM MODIFIED SERUM

One-step density gradient ultracentrifugation method was used for isolation of HDL from modified serum as previously described [124, 125]. Briefly, the density of serum was adjusted to 1.24 g/mL by potassium bromide and layered underneath a PBS solution with a density of 1.063 g/mL in long centrifuge tubes (16 x 76 mm; Beckman). Centrifugation was done at 330.000 x g for 6 h at 15°C (centrifuge: Beckman Optima L-80 ultracentrifuge, rotor: Sorvall T-1270). After the centrifuge step, the collected HDL was concentrated by Viva Spin Tubes (Sartorius, Vienna, Austria) and the potassium bromide was removed by gel filtration on Sephadex PD-10 columns (GE Healthcare, Munich, Germany). The isolated HDL was then stored at -80°C for further experiments.

5.7 FPLC OF *IN VITRO* AND *IN VIVO* MODIFIED SERUM

As described previously [124], FPLC was done on a Pharmacia FPLC system (Pfizer Pharma, Karlsruhe, Germany) and Supherose 6 column (Amersham Biosciences, Piscataway, NJ). 200 µL of modified serum was applied to the column and a buffer containing 10 mmol/L Tris-HCl, 1 mmol/L EDTA, 0.9% NaCl, and 0.02% NaN_3 (pH 7.4) was used for elution of lipoproteins in 0.5 mL fractions. In these fractions the total cholesterol (TC) concentrations were measured spectrophotometrically (Greiner Diagnostics AG, Bahlingen, Germany).

5.8 NMR SPECTROSCOPY

Modified human serum was produced as described in 5.3, except that EL-overexpressing or EV control HepG2 cells were incubated with 100% of human serum for 8 h. The analysis of EL-modified and EV-control samples were done by *AXINON*® *lipoFIT*® *–S100* test system (Numares Health, Regensburg, Germany) as described previously [126, 127].

5.9 CHOLESTEROL EFFLUX MEASUREMENT

J774 macrophages were plated onto 48-well plates (300.000 cells/well) and incubated with 1 µCi/mL [^{3}H]-cholesterol (Perkin Elmer, Boston, MA, USA) for 24 h. The upregulation of ABCA1 was done with 0.3 mmol/L 8-(4-chlorophenylthio)-cyclic AMP (Sigma, Darmstadt, Germany) in DMEM without FCS for 6 h. After labelling of cells with [^{3}H]-cholesterol, they were washed and incubated with 2.8% (v/v) of human or mouse serum, apoB-DS or with human or mouse HDL (2 µg HDL protein) or FPLC fractions (30%; v/v) for 4 hours. The radioactivity in the medium relative to total radioactivity in the medium and cells was calculated, which presents the total cholesterol efflux. To get the ABCA1-dependent cholesterol efflux, the ABCA1-independent efflux (without ABCA1 upregulation) was subtracted from the total efflux. The whole measurements were implemented in the presence of 2 µg/mL of the acyl coenzyme A cholesterol acyltransferase inhibitor Sandoz 58-035 (Sigma, Darmstadt, Germany).

5.10 NON-DENATURING GRADIENT GEL ELECTROPHORESIS AND WESTERN BLOTTING

Aliquots of serum (1.5 µL), apoB-DS (2 µL), HDL (10 µg) and FPLC fractions (15 µL) were diluted with native sample buffer (LifeTechnologies, Vienna, Austria) followed by electrophoresis on 4-16% non-denaturing polyacrylamide gels in a running buffer (Invitrogen, Vienna, Austria) at 125 V for 4 h at RT. Staining of gels was done with Sudan black (Sigma-Aldrich, Vienna, Austria) or with Coomassie Brillant Blue G250 after fixation with 10% sulfosalicylic acid for 30 min. Furthermore, gels were used for Western blot analysis. Therefore, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Carl Roth, Karlsruhe, Germany) at 400 mA for 75 min at 4°C, followed by blocking the membranes in 10% skim milk for 1h at RT. Subsequently, the membranes were incubated with ApoA-I antibody (Novus biological, NB100-65491, LOT 120810, dilution 1:3000, Littleton, CO, USA) against human ApoA-I or with *in house* generated ApoA-I antibody [128] (dilution 1:1000) against mouse ApoA-I at 4°C, overnight. After a washing step, the membranes were incubated with the appropriate secondary antibody (Dako, Vienna, Austria) for 1h at RT. The visualisation of protein signals

was done by incubation with Millipore Western Blotting Substrate (Millipore Corporation, Billerica, USA) using ChemiDoc system (Bio-Rad Laboratories, Vienna, Austria). All experiments were performed with the high molecular weight marker NativeMark (LifeTechnologies, Vienna, Austria) [124].

5.11 SDS-PAGE AND WESTERN BLOTTING

EL overexpression in HepG2 cells was analyzed in heparin media. Therefore 40 µL of heparin media was mixed with 6 x loading buffer [20% (w/v) glycerol, 5% (w/v) SDS, 0.15% (w/v) bromophenol blue, 63 mmol/L Tris-HCl, pH6.8 and 5% (v/v) ß-mercaptoethanol], and cooked for 10 min, 95°C. Subsequently, samples were loaded onto 10% SDS-PAGE gels and immunoblotted using EL specific antibody as described previously [45].

Aliquotes of serum (1.5 µL), apoB-DS (2 µL) and FPLC fractions (10 µL) were used for determination of human and mouse ApoA-I content. First, samples were mixed with 6 x loading buffer and boiled for 10 min, 95°C. Electrophoresis was done on 12% SDS-PAGE, followed by transferring the proteins onto PVDF membranes at 150 mA, 90 min. Then the membranes were blocked in 10% skim milk for 1h at RT and incubated overnight with mouse anti-ApoA-I antibody (Santa Cruz Biotechnology, sc-30089, LOT D2913, dilution 1:500, Heidelberg, Germany) or human anti-ApoA-I antibody (Novus biological, NB100-65491, LOT 120810, dilution 1:3000, Littleton, CO, USA) at 4°C. Thereafter, membranes were washed and incubated with appropriate secondary antibody (Dako, Vienna, Austria) for 1h at RT. For visualisation of proteins, the Millipore Western Blotting Substrate (Millipore Corporation, Billerica, USA) and ChemiDoc system (Bio-Rad Laboratories, Vienna, Austria) was used [124].

5.12 TARGETED LIPIDOMIC ANALYSIS

Extraction of total lipids of *in vivo* and *in vitro* samples (300 µg protein) was performed twice as described previously [129] using chloroform/methanol/water (2/1/0.6, v/v/v) containing 500 pmol butylated hydroxytoluene, 1% acetic acid, and 100 pmol of internal standards (ISTD, 17:0-17:0 PC, 19:0-19:0 PC, 17:0-17:0 PE, 17:0 FA, d18:1/17:0 Cer, 14:0-14:0 DG, 17:0 LPC, 17:0-17:0-17:0 TG, 15:0-15:0-15:0 TG, Avanti Polar Lipids) per each sample. This process was done under constant shaking for 60 min at RT. Subsequently, samples were centrifuged at 1,000 x *g* for 15 min at RT and the lower organic phase was collected. The remaining aqueous phase was mixed with 2.5 mL chloroform and the second extraction was performed as described above. The organic phases of double extraction were pooled together and dried under a stream of nitrogen. Samples were resolved in 200 µL methanol/2-propanol/water (6/3/1, v/v/v) for UPLC-TQ analysis. Chromatographic separation was modi-

fied after Knittelfelder et al. [130] using an AQUITY-UPLC system (Waters Corporation), equipped with a Kinetex EVOC18 column (2.1x50 mm, 1.7µm; Phenomenex) starting a 25 min gradient with 100% solvent A (MeOH/H2O, 1/1, v/v; 10mM ammonium acetate, 0,1% formic acid). Detection was done with a EVOQ Elite™ triple quadrupole mass spectrometer (Bruker) equipped with an ESI source. Analysis of lipid species was performed by selected reaction monitoring (PC: [MH]+ to m/z 184, 25eV, PE: [MH]+ to -m/z 141, 20eV, PI: [M-H]- to corresponding [FA]-, 50eV, LPC: [MH]+ to m/z 184, 22eV, LPE: [MH]+ to -m/z 141, 17eV, Cer: [MH]+ to m/z 264, 22eV, TG: [MNH4]+ to corresponding [DG-H2O]+, 22eV, DG: [MNH4]+ to [RCOO+58]+, 15eV, CE: [MNH4]+ to m/z 369, FC: [M-H2O]+, 0eV, FA: [M-H]-, 0eV, SM: [MH]+ to m/z 184, 23eV). Data acquisition was done by MS Workstation (Bruker). Data were normalized for recovery and extraction- and ionization efficacy by calculating analyte/ISTD ratios [124].

5.13 RNA ISOLATION AND QUANTITATIVE REAL-TIME PCR ANALYSIS

RNA isolation was performed with 50 mg liver using TriFast™ reagent according to the manufacturer's protocol (Peqlab, Erlangen, Germany). Thereafter, High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) was used for reverse transcription of two µg of total RNA. Quantitative real-time PCR was done on a Roche LightCycler 480 (Roche Diagnostics, Palo Alto, CA) using the GoTaq® qPCR MasterMix (Promega, Madison, WI). Samples were measured in duplicates and normalized to the expression levels of cyclophilin A. The $2^{-\Delta\Delta CT}$ method was performed to calculate the expression profiles and associated statistical parameters. Primers for mouse cyclophilin A (fw: CCATCCAGCCATTCAGTCTT; rev: TTCCAGGATTCATGTGCCAG) and mouse LipC (fw: CCATCCAGCCATTCAGTCTT ; rev: TTCCAGGATTCATGTGCCAG) were from Life Technologies (Vienna, Austria). Human LipG was done with Primer Assay QT00078967, purchased from Qiagen (Hilden, Germany).

5.14 STATISTICAL ANALYSIS

Represented data are means ± standard error of mean (S.E.M.). Differences between EV- and EL samples were analyzed by two-tailed unpaired *t*-test using Graph Pad Prism 5.0. Statistically significant differences between groups are indicated by *P*-values of < 0.05 (*), < 0.01 (**), or < 0.001 (***).

6 RESULTS

6.1 *IN VITRO* EL OVEREXPRESSION INCREASES CEC OF SERUM AND APOB-DS BUT DECREASES THAT OF ISOLATED HDL

Human (h) serum was incubated with human EL or EV (control) overexpressing HepG2 cells (Fig. 4), followed by using the modified serum, apoB-DS and isolated HDL for measurements of CEC. CEC was investigated in ^{3}H-cholesterol labeled J774 macrophages under basal conditions or with upregulation of ABCA1. The total CEC was significantly increased after incubation with human serum and apoB-DS but decreased with isolated HDL (Fig. 5A). The ABCA1-independent CEC was similar with hEL-serum and hEL-apoB-DS but reduced with hEL-HDL (Fig. 5B). Interestingly, hEL-serum and hEL-apoB-DS triggered ABCA1-dependent CEC, whereas there was no effect with isolated hEL-HDL (Fig. 5C) [124]. All results refer to respective EV as a control.

The significant increase of ABCA1-dependent CEC with hEL-serum and hEL-apoB-DS suggests that a formation of a potent component occurs during incubation of human serum with EL overexpressing cells. Furthermore, we conclude that mature HDL is influencing only the ABCA1-independent but not the ABCA1-dependent CEC.

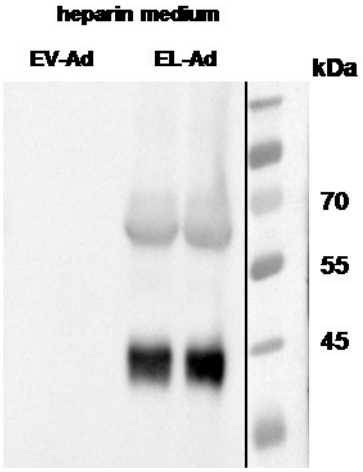

Figure 4: Western Blot analysis of human EL overexpression in HepG2 cells

Overexpression of EL was analyzed in heparin media of transduced HepG2 cells with EL adenovirus (EL-Ad) and EV-Ad by Western Blotting. This figure has been published previously in [124].

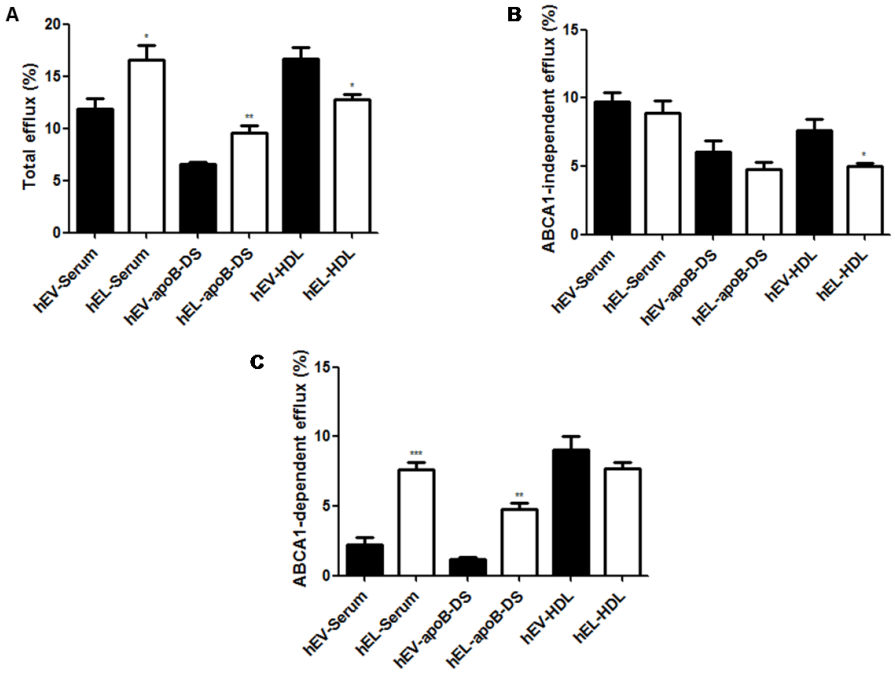

Figure 5: CEC of human modified EL-serum, EL-apoB-DS and hEL-HDL

A) Measurement of total CEC was done in ^{3}H-cholesterol labeled J774 macrophages after upregulation of ABCA1. B) ABCA1-independent efflux was determined without ABCA1 upregulation and C) ABCA1-dependent CEC was calculated by subtraction of the ABCA1-independent efflux from the total efflux. The cholesterol efflux was expressed as the radioactivity in the medium relative to total radioactivity in the medium and cells. Results are mean ± SEM of 5 independent modifications of pool-serum, each measured in duplicates and analyzed by two-tailed unpaired t-test. This figure has been published previously in [124]. *P < 0.05, **P < 0.01, ***P < 0.001

6.2 *IN VITRO* EL OVEREXPRESSION ALTERS SERUM LIPOPROTEIN AND LIPID CONCENTRATIONS

To examine the responsible factors for increased CEC of hEL-serum and decreased CEC of hEL-HDL, modified serum was first analyzed by NMR spectroscopy. Adenoviral overexpression of human EL *in vitro* demonstrated no differences in the total concentration of LDL particles but significantly reduced levels of large particles and increased levels of small LDLs compared to EV as a control (Fig. 6A). In contrast, the concentration of total HDL molecules were significantly lower in hEL-serum compared to control, consisting of significantly lower amounts of large and higher amounts of small HDL particles (Fig. 6B) [124]. Furthermore, a decrease in total-C, LDL-C, HDL-C and TAG levels in modified EL-serum compared to EV-serum was observed (Fig. 6C).

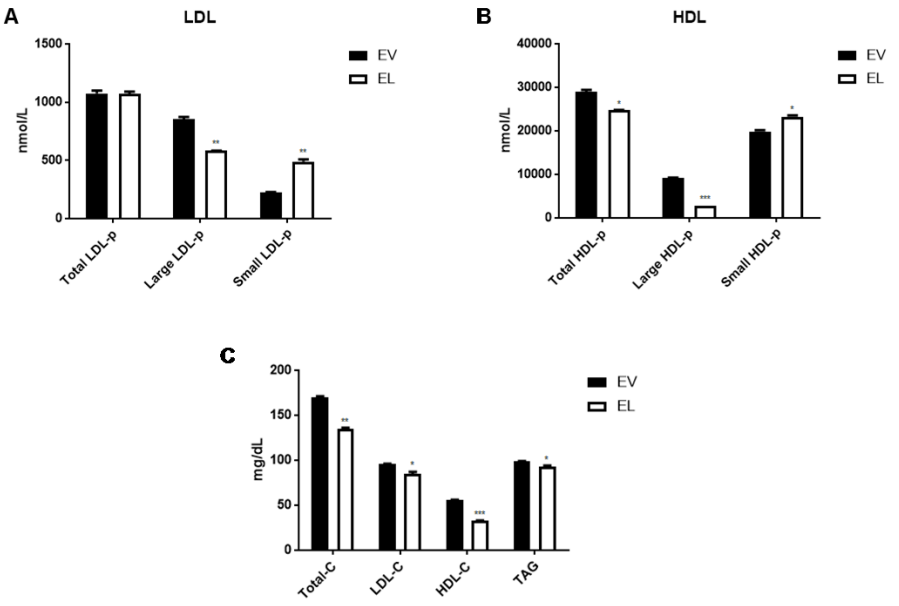

Figure 6: EL overexpression in HepG2 cells alters serum lipid and lipoprotein content

Analysis of lipoprotein and lipid concentration in modified EV- and EL-serum was done with NMR spectroscopy. Results are mean ± SEM of 2 independent modifications of pool-serum and analyzed by two-tailed unpaired t-test. Part of this figure has been published previously in [124]. *P < 0.05, **P < 0.01, ***P < 0.001

6.3 *IN VITRO* EL OVEREXPRESSION CAUSES HDL SIZE REDUCTION

To analyze the size of HDL, non-denaturing gradient gel electrophoresis followed by Sudan black or Coomassie blue staining was performed. Staining of modified serum and apoB-DS by Sudan black revealed decreased HDL particle size upon EL overexpression compared to control (Fig. 7 A,B) [124]. To confirm this result, we performed non-denaturing gradient gel electrophoresis and subsequent Sudan black and Coomassie blue staining as well as ApoA-I immunoblotting of HDL isolated from modified serum. Results shown in Fig. 7 C-E confirmed the decreased size of hEL-HDL compared to hEV-HDL, both isolated from respective modified serum (Fig. 7 C-E) [124].

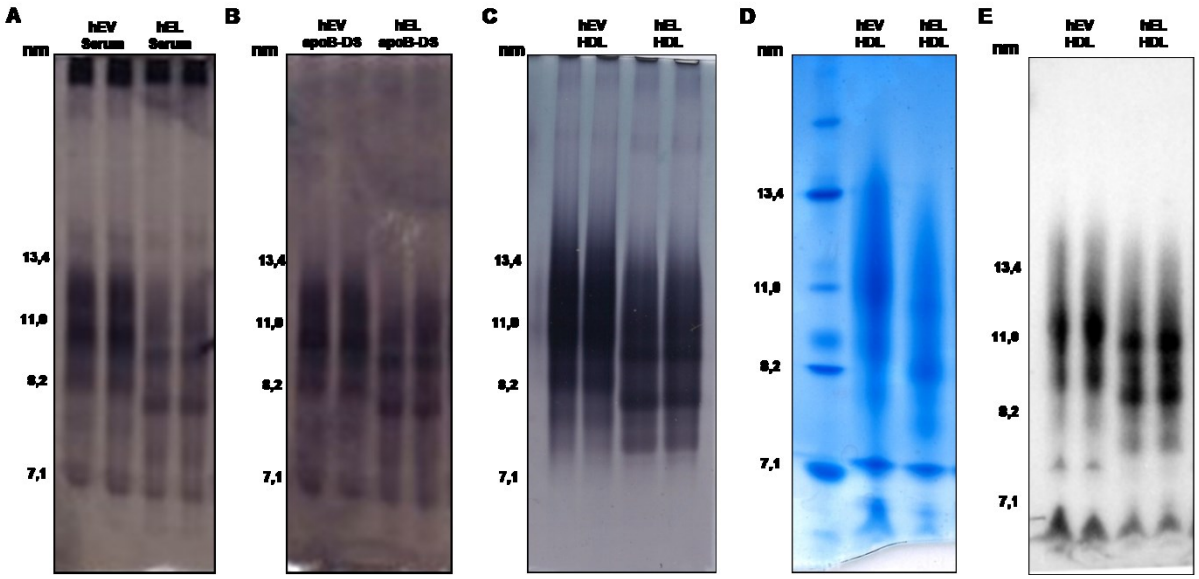

Figure 7: Modification of human serum by EL *in vitro* resulted in decreased HDL size

Non-denaturing polyacrylamide electrophoresis was performed by loading aliquots of human (h) EV-serum or EL-serum (1.5 µL), hEV-apoB-DS or hEL-apoB-DS (2 µL) and hEV-HDL or hEL-HDL (10 µg protein) onto 4-16% gels. Afterwards, gels were stained with Sudan Black (A-C) and Coomassie blue (D) or Western Blotting against ApoA-I (E) was done. Protein size corresponds to the high molecular weight marker bands on the gels or membrane. Results are representative of 5 different modifications of the pool-serum from 8 donors. This figure has been published previously in [124].

6.4 *IN VITRO* EL OVEREXPRESSION RESULTS IN LIPID-POOR APOA-I FORMATION

It has been established that lipid-poor ApoA-I is the main acceptor for ABCA1-dependent cholesterol efflux [108]. Therefore, to examine whether EL overexpression generates lipid-poor ApoA-I, the modified serum and apoB-DS were analyzed using non-denaturing gradient gel electrophoresis with subsequent ApoA-I immunoblotting. Interestingly, Western Blotting against ApoA-I identified a remarkable band smaller than 7.1 nm in hEL-serum as well as hEL-apoB-DS but not in respective controls (Fig. 8 A,B) [124]. The prominent bands were not detected when isolated HDL was analyzed (Fig. 8E) [124]. These results indicate the production of lipid-poor ApoA-I upon EL modification of human serum *in vitro*.

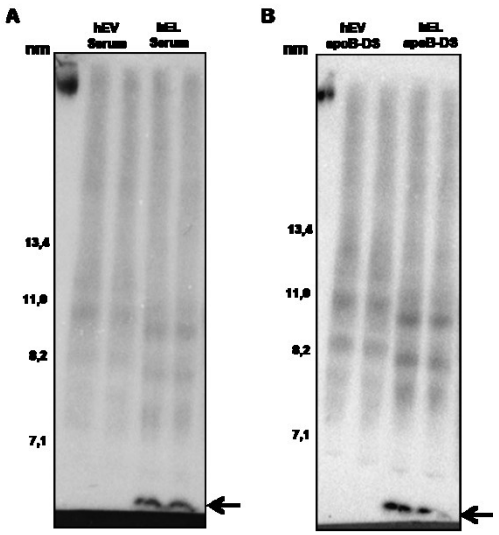

Figure 8: Lipid-poor ApoA-I is generated upon EL modification of human serum

Non-denaturing polyacrylamide electrophoresis was performed onto 4-16% gels followed by Western Blotting against ApoA-I. Protein size corresponds to the high molecular weight marker bands on the membranes. The position of lipid-poor ApoA-I is indicated by the arrows. Results are representative of 5 different modifications of the pool-serum from 8 donors. This figure has been published previously in [124].

6.5 LIPID-POOR APOA-I CONTRIBUTES TO THE INCREASED CEC OF EL-MODIFIED SERUM

To establish that the formation of lipid-poor ApoA-I is responsible for the increased CEC of hEL-serum and hEL-apoB-DS [124], the modified serum was fractionated by FPLC (Fig. 9A). Immunoblotting against ApoA-I was performed to detect HDL (ApoA-I) positive fractions (Fig. 9B).

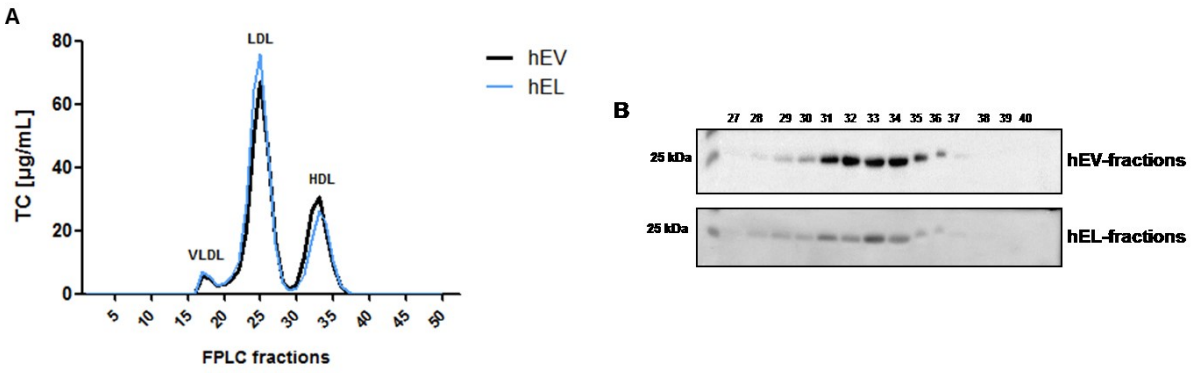

Figure 9: FPLC and Western Blot against ApoA-I of modified serum

(A) Human modified serum was separated by FPLC and (B) FPLC fractions 27 – 40 were analyzed by SDS-PAGE and ApoA-I Western Blot. Protein size corresponds to the protein marker bands on the membranes. Results are representative of 2 different modifications of the pool-serum. This figure has been published previously in [124].

After identification of the ApoA-I positive fractions, CEC of each single fraction was determined to demonstrate the contribution of lipid-poor ApoA-I. As expected, the EL serum fractions 30 and 31 that represent mature HDL without lipid-poor ApoA-I (Fig. 10D) showed significantly lower total CEC compared to EV fractions (Fig. 10A) [124]. In contrast, the EL fractions 33 – 37 containing significantly more lipid-poor ApoA-I compared to control fractions (Fig. 10D), exhibited significantly higher total as well as ABCA1-dependent CEC compared to corresponding EV-fractions (Fig. 10 A,B) [124]. The ABCA1-independent CEC was similar between EV and EL serum fractions (Fig. 10C) [124].

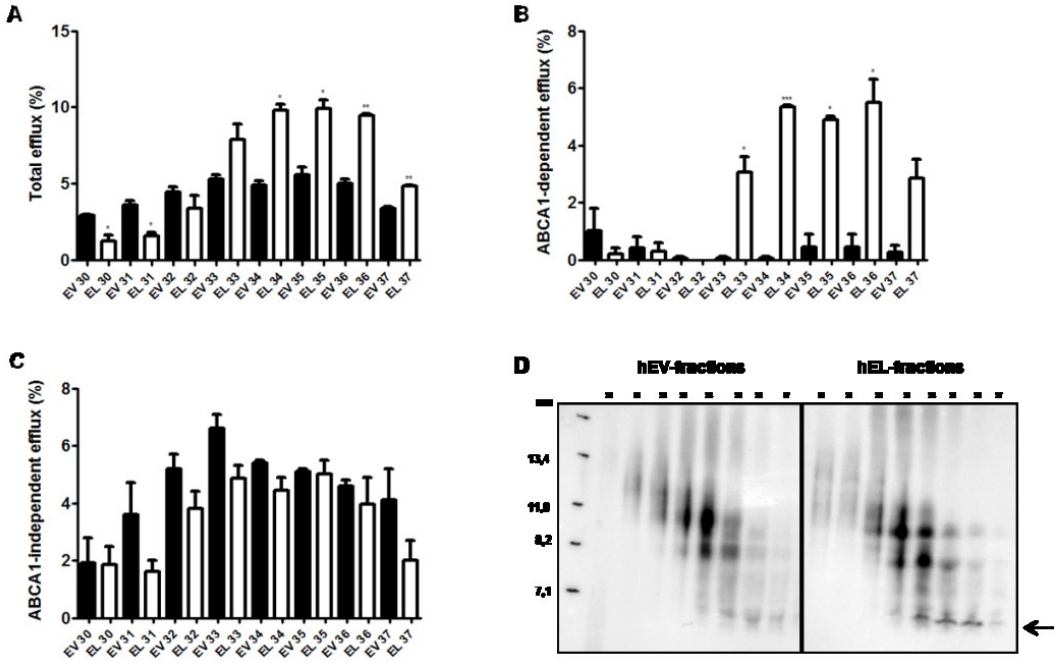

Figure 10: CEC and ApoA-I Western Blot of FPLC fractions of modified serum

(A) Total CEC, (B) ABCA1-dependent CEC, (C) ABCA1-independent CEC and (D) Non-denaturing polyacrylamide electrophoresis followed by ApoA-I Western Blot of FPLC fractions of hEV-serum and hEL-serum. Protein size corresponds to the high molecular weight marker bands on the membranes. The position of lipid-poor ApoA-I is indicated by the arrow (C). Shown is a representative experiment out of 2 independent modifications, both giving similar results. Results are mean ± SEM of one modification, each measured in duplicates and analyzed by two-tailed unpaired t-test. This figure has been published previously in [124]. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

6.6 LIPID COMPOSITION OF HUMAN EL SERUM

Overexpression of EL *in vitro* resulted in alterations of CEC and HDL size as well as the production of lipid-poor ApoA-I. As previously reported, the lipid composition can influence the HDL functionality [131]. Therefore, the lipid composition of EL-modified serum, apoB-DS and isolated HDL were assessed by mass spectrometry (MS). EL overexpression significantly reduced phosphatidylcholine (PC) (Fig. 11A), phosphatidylethanolamine (PE) (Fig. 11B), phosphatidylinositol (PI) (Fig. 11C) and TAG (Fig. 11D) levels in serum, apoB-DS and HDL. As supposed, the LPC content was increased in hEL-serum, hEL-apoB-DS and hEL-HDL compared to respective controls (Fig. 11E). Furthermore, increased levels of lysophosphatidylethanolamine (LPE) (Fig. 11F) and FC (Fig. 11G) were only observed in isolated hEL-HDL. CEs were unchanged in all conditions (Fig. 11H). Finally, the ceramide (Cer) and SM content was reduced in hEL-HDL compared to control (Fig. 11 I,J) [124].

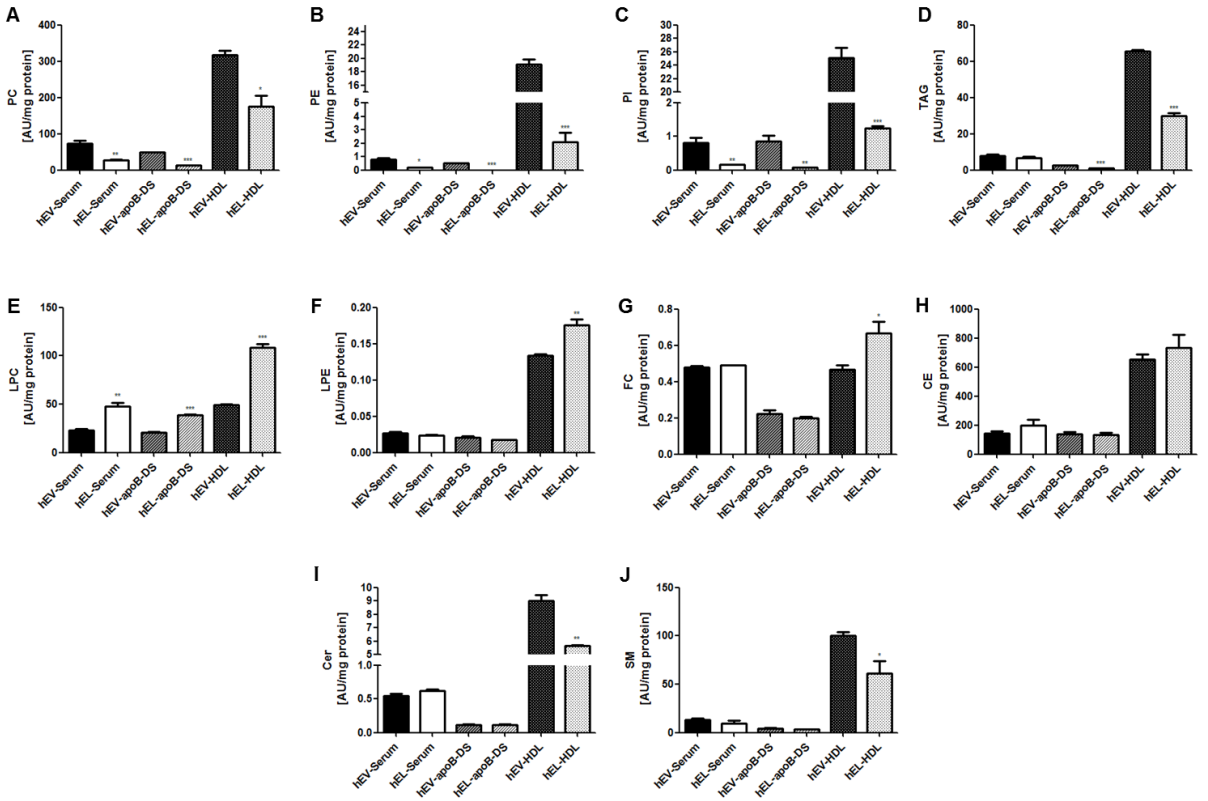

Figure 11: Lipid composition of hEL-serum, hEL-apoB-DS and hEL-HDL

Lipids from hEV-serum, hEL-serum, hEV-apoB-DS, hEL-apoB-DS, hEV-HDL and hEL-HDL (corresponding to 300 µg serum or HDL protein) were extracted and (A) PC, (B) PE, (C) PI, (D) TAG, (E) LPC, (F) LPE, (G) FC, (H) CE, (I) Cer and (J) SM were analyzed by MS. Results are mean ± SEM of 3 independent modifications of the pool-serum. This figure has been published previously in [124]. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

6.7 OVEREXPRESSION OF HUMAN EL IN MICE REDUCED CEC OF SERUM AND APOB-DS WHEREAS CEC OF HDL IS UNALTERED

In vitro results showed an increased CEC of hEL-serum and hEL-apoB-DS due to the formation of lipid-poor ApoA-I, whereas CEC of hEL-HDL was decreased. To clarify whether EL causes the same change *in vivo*, mice were injected with human EL or control (EV) adenovirus (Fig. 12A), followed by preparation of mouse (m) serum, apoB-DS and HDL for CEC measurements. CEC was examined in ^{3}H-cholesterol labeled J774 macrophages with upregulation of ABCA1. The CEC of mEL-serum and mEL-apoB-DS was significantly lower compared to EV as a control, whereas CEC of isolated HDL was unchanged between the two groups (Fig. 12B) [124].

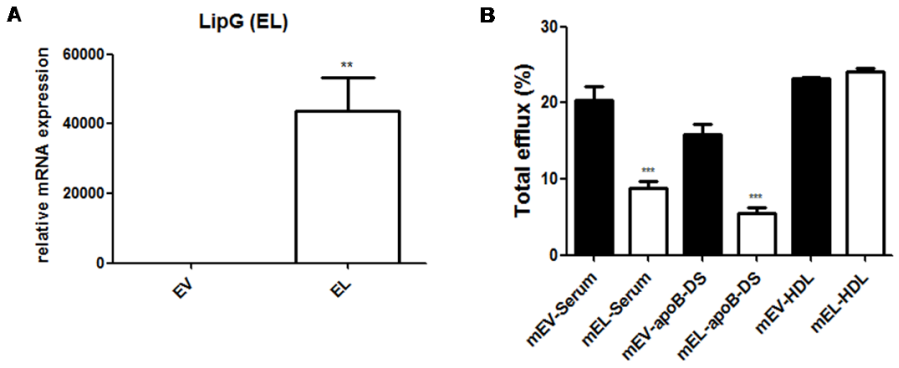

Figure 12: EL overexpression *in vivo* impairs CEC of serum and apoB-DS

A) Hepatic hEL mRNA expression levels. Total RNA was isolated from the liver from 4 EV and 5 EL-transduced mice, followed by examination of LipG (hEL) mRNA expression levels. B) Measurement of total CEC was done in ^{3}H-cholesterol labeled J774 macrophages after upregulation of ABCA1. The cholesterol efflux was expressed as the radioactivity in the medium relative to total radioactivity in the medium and cells. Results are mean ± SEM of 2 independent *in vivo* modifications with 12 EL-Ad and 4 EV-Ad-transduced mice per each modification, measured twice in duplicates and analyzed by two-tailed unpaired t-test. This figure has been published previously in [124]. $^{**}P < 0.01$, $^{***}P < 0.001$

6.8 CEC OF *IN VIVO* MODIFIED SERUM AFTER FPLC

To confirm the observed unaltered CEC of isolated mouse HDL upon EL overexpression and to exclude the contribution of a possible contamination with LDL upon ultracentrifugation, the *in vivo* generated modified serum was fractionated by FPLC (Fig. 13A). Each FPLC fraction (27 – 40) was analyzed by SDS-PAGE and subsequent ApoA-I Western Blot (Fig. 13B). Simi-

lar CEC of pooled EL and EV fractions 27 – 40 (Fig. 13C) confirmed the lack of the impact of EL overexpression on CEC of HDL [124].

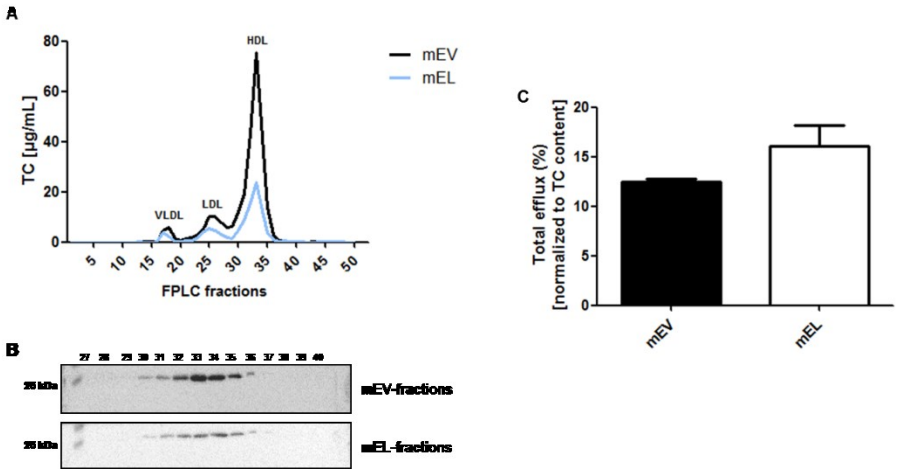

Figure 13: CEC and ApoA-I Western Blot of FPLC fractions of modified mouse serum

A) FPLC fractions of modified mouse serum were analyzed by B) SDS-PAGE and ApoA-I Western Blot. C) Total CEC of pooled fractions (27 – 40) was determined in J774 macrophages after ABCA1 upregulation and normalized to TC content of the pooled FPLC fractions. Protein size annotations refer to protein marker bands on the membranes. Results are mean ± SEM of 2 *in vivo* modifications, with 12 EL-Ad and 4 EV-Ad -transduced mice per each modification. Cholesterol efflux capacity of pooled fractions of each modification was measured twice in duplicates and analyzed by unpaired t-test. This figure has been published previously in [124].

6.9 *IN VIVO* EL OVEREXPRESSION DECREASES HDL LEVELS WITHOUT ACCUMULATION OF LIPID-POOR APOA-I

To clarify the discrepancies between results obtained *in vitro* and *in vivo* regarding the impact of EL overexpression on CEC of serum and HDL, we first examined the impact of EL overexpression *in vivo* on HDL abundance and size. Human EL overexpression in mice caused a 64% reduction in serum HDL-C levels compared to controls (73 ± 8 mg/dL vs. 26 ± 11 mg/dL). HDL-C reduction was confirmed by FPLC (Fig. 13A). Additionally, HDL signals obtained by non-denaturing gradient gel electrophoresis with subsequent Sudan black staining (Fig. 14A) and ApoA-I signals determined by Western Blot (Fig. 14B) were reduced in mEL-serum and mEL-apoB-DS compared to controls. However, analysing the HDL size by non-denaturing gradient gel electrophoresis with subsequent Sudan black or Coomassie staining revealed no alterations by EL overexpression (Fig. 14 C,D). Interestingly, in contrast

to the *in vitro* studies, there was no generation of lipid-poor ApoA-I upon *in vivo* modification of mouse serum by EL (Fig. 14E) [124].

Based on these results, we concluded that the reduced CEC of mEL-serum and mEL-apoB-DS is caused by the EL mediated HDL depletion and the absence of lipid-poor ApoA-I.

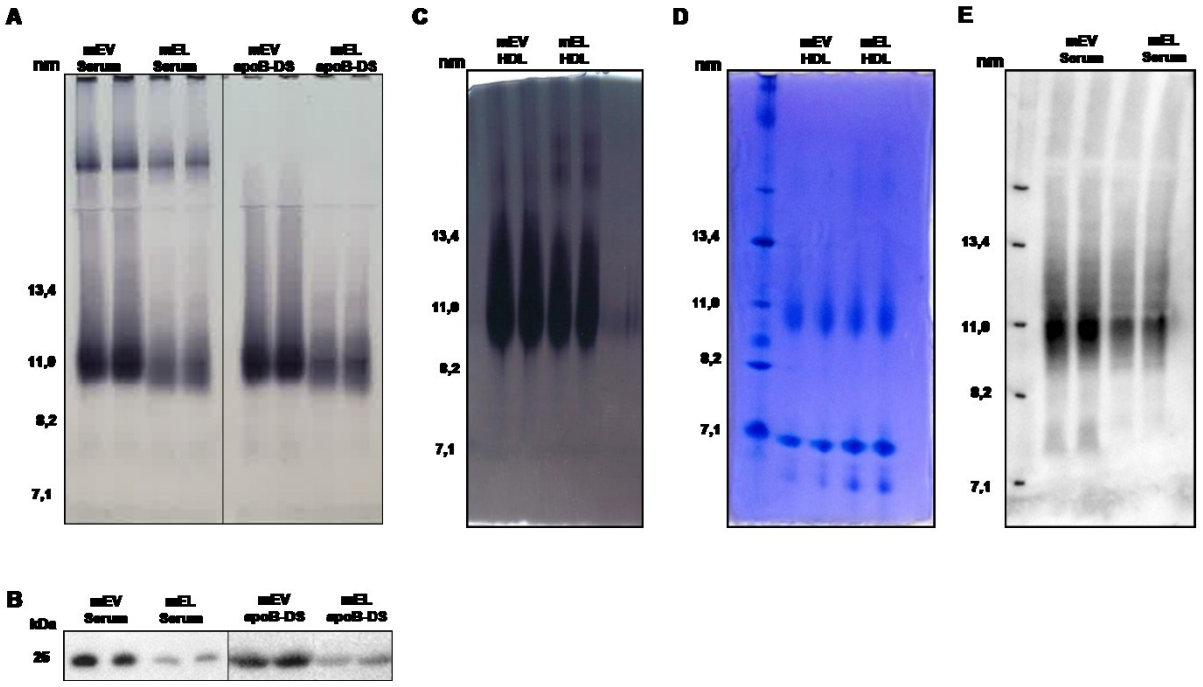

Figure 14: EL overexpression in mice depletes HDL

Non-denaturing polyacrylamide electrophoresis was performed by loading aliquots of mouse (m) EV-serum or mEL-serum (1.5 µL), mEV-apoB-DS or mEL-apoB-DS (2 µL) and mEV-HDL or mEL-HDL (10 µg protein) onto 4-16% gels. Afterwards, gels were stained with Sudan Black (A,C) and Coomassie blue (D) or ApoA-I Western Blot was done (E). B) SDS-PAGE and ApoA-I Western Blot of modified serum and apoB-DS. Protein size corresponds to the high molecular weight or protein marker bands on the gels or membranes. Results are representative of 2 different *in vivo* modifications with 12 EL-Ad and 4 EV-Ad -transduced mice per each modification. This figure has been published previously in [124].

6.10 *IN VIVO* EL OVEREXPRESSION ALTERS LIPID COMPOSITION OF SERUM AND HDL

To explain in more detail the impaired CEC of mouse serum and unchanged CEC of isolated mouse HDL, the lipid composition of EL modified serum, apoB-DS and isolated HDL was analyzed by MS. EL overexpression in mice significantly reduces PC (Fig. 15A) and PI (Fig. 15C) levels in mEL-serum and mEL-apoB-DS. Interestingly, PE (Fig. 15B) and TAG

(Fig. 15D) content were significantly increased in mEL-serum and mEL-HDL. Furthermore, a decrease in LPC (Fig. 15E), LPE (Fig. 15F), CE (Fig. 15G), FC (Fig. 15H), Cer (Fig. 15I) and SM (Fig. 15J) was observed in mEL-serum and mEL-apoB-DS. In contrast, mEL-HDL exhibited diminished LPC (Fig. 15E), LPE (Fig. 15F), FC (Fig. 15H) and Cer (Fig. 15I) content, whereas CE (Fig. 15G) and SM (Fig. 15J) were unaltered [124]. All results refer to respective EV controls.

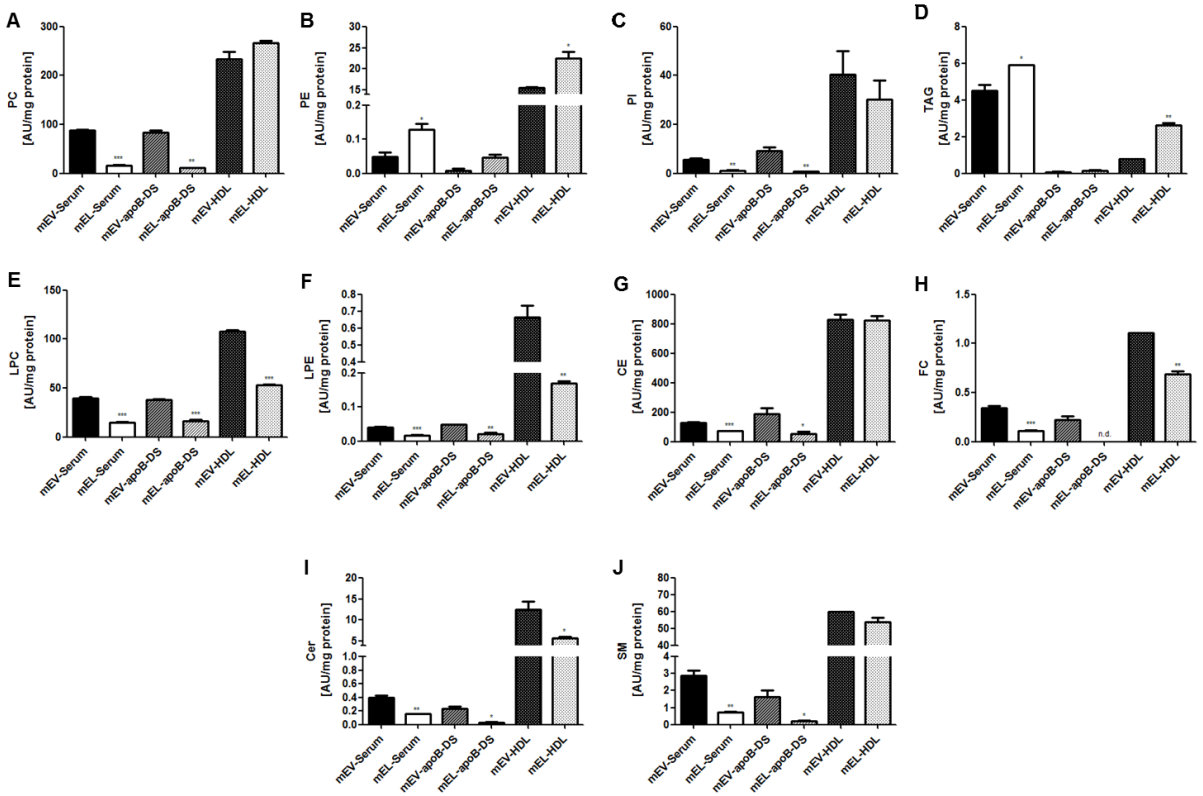

Figure 15: *In vivo* EL overexpression modulates lipid composition of serum, apoB-DS and HDL

Lipids from mouse (m) EV-serum, mEL-serum, mEV-apoB-DS, mEL-apoB-DS, mEV-HDL and mEL-HDL (corresponding to 300 µg serum or HDL protein) were extracted and (A) PC, (B) PE, (C) PI, (D) TAG, (E) LPC, (F) LPE, (G) CE, (H) FC, (I) Cer and (J) SM were analyzed by MS. Results are mean ± SEM of 3 measurements for each serum, apoB-DS and HDL obtained in 2 independent *in vivo* modifications, with 12 EL-Ad- and 4 EV-Ad-transduced mice per each modification. This figure has been published previously in [124]. *$P < 0.05$, **$P < 0.01$, **$P < 0.001$

6.11 EL OVEREXPRESSION DOES NOT INFLUENCE HL EXPRESSION

In consideration of a major role of HL in HDL metabolism due to the conversion of large HDL particles into small ones as well as the formation of lipid-poor ApoA-I [132], the impact of EL overexpression on LipC (HL) expression levels in the liver was investigated. The hepatic LipG (EL) mRNA levels (Fig. 16A) were significantly increased whereas the LipC mRNA concentrations (Fig. 16B) in the liver were unaltered upon human EL overexpression *in vivo [124]*.

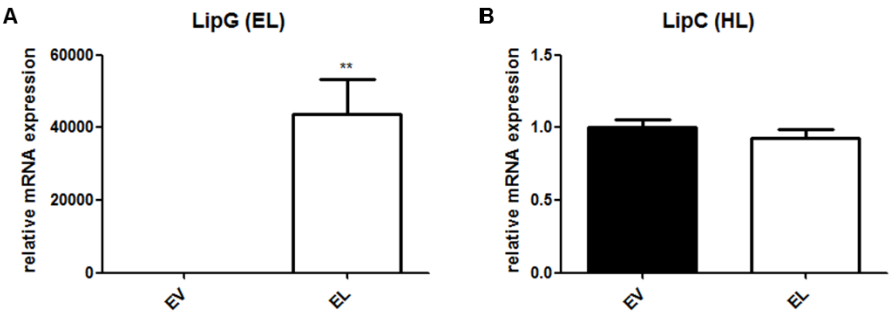

Figure 16: mRNA levels of LipC (HL) are not changed by EL overexpression *in vivo*

4 EV- and 5 EL-transduced mice were injected with adenovirus encoding h EL, followed by isolation of total RNA from the liver. mRNA expression levels of (A) LipG (hEL) and (B) LipC (HL) were determined. mRNA expression was analyzed in duplicates by real-time PCR and normalized to cyclophilin A expression as a reference gene. Expression profiles and associated statistical parameters were determined by the $2^{-\Delta\Delta Ct}$ method. This figure has been published previously in [124]. $^{**}P < 0.01$

6.12 OVEREXPRESSION OF MOUSE EL *IN VIVO* IMPAIRED CEC AND DECREASED HDL LEVELS WITHOUT ALTERING HDL SIZE

To rule out the possibility that results obtained *in vivo* do not reflect solely the features of human EL, we overexpressed mouse EL using adenovirus, followed by measuring CEC of modified serum, apoB-DS and isolated HDL. Overexpression of mouse EL, as found for human EL, depleted HDL-C levels (Fig. 17A) and reduced CEC of serum and apoB-DS compared to controls (Fig. 17C). Additionally, neither HDL size (Fig. 17 A,B) nor CEC (Fig. 17C) of isolated HDL were altered by overexpression of mouse EL [124].

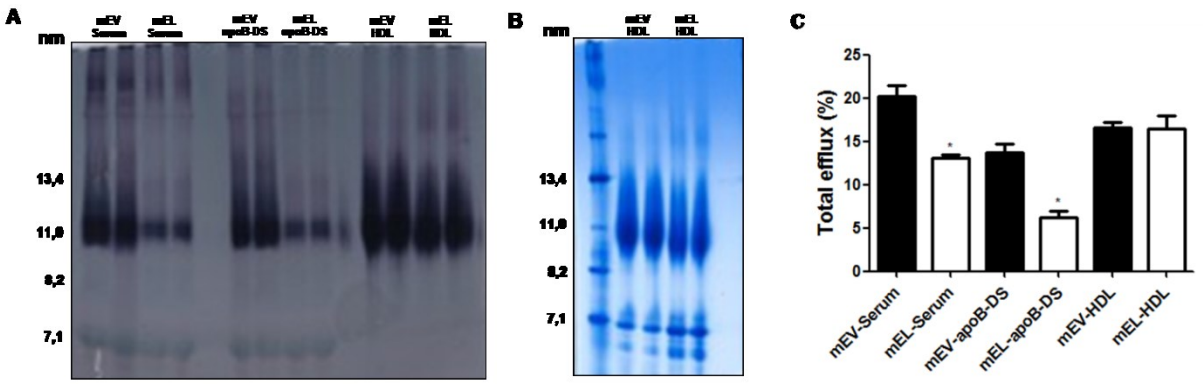

Figure 17: Impact of mouse EL overexpression *in vivo* on CEC, HDL levels and size of serum, apoB-DS and isolated HDL

A) Non-denaturing gradient gel electrophoresis with subsequent Sudan black staining of serum, apoB-DS and isolated HDL. B) Non-denaturing gradient gel electrophoresis with subsequent Coomassie blue staining of isolated HDL C) Total CEC was measured in J774 macrophages upon upregulation of ABCA1. Results are mean ± SEM of 2 independent *in vivo* modifications, with 12 mEL-Ad and 4 EV-Ad -transduced mice per each modification. Cholesterol efflux capacity of the modified serum, apoB-DS and HDL from each *in vivo* modification was measured twice in duplicates. This figure has been published previously in [124]. *$P < 0.05$

6.13 *IN VITRO* MODIFICATION OF MOUSE SERUM ENHANCES CEC OF SERUM AND APOB-DS BUT DECREASES CEC OF ISOLATED HDL

To establish whether the discrepancies concerning the impact of EL on CEC *in vitro* and *in vivo* are caused by species specific properties of human and mouse serum or by the utilisation of different experimental models, namely *in vivo* vs. *in vitro*, we incubated mouse serum with EL overexpressing cells *in vitro*. Compared to mEV controls, the total as well as the ABCA1-dependent CEC of mEL-serum and mEL-apoB-DS were increased, whereas CEC of mEL-HDL was decreased (Fig. 18 A,B). While the ABCA1-independent CEC of mEL-serum and mEL-apoB-DS were unaltered, that of mEL-HDL was reduced compared to EV-controls (Fig. 18C) [124].

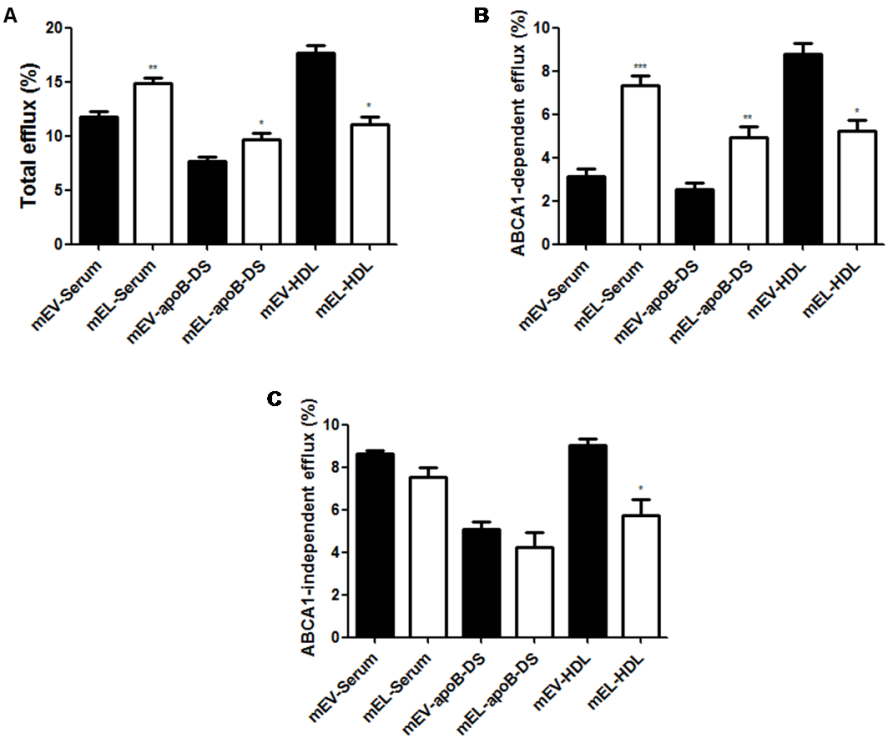

Figure 18: CEC of serum, apoB-DS and isolated HDL upon EL modification of mouse serum *in vitro*

A) Total CEC, B) ABCA1-dependent CEC and C) ABCA1-independent CEC of serum, apoB-DS and isolated HDL upon incubation of mouse serum with human EL overexpressing cells and controls. Results are mean ± SEM of 2 independent modifications of the mouse pool serum, each measured twice in duplicates and analyzed by two-tailed unpaired t-test. This figure has been published previously in [124]. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

6.14 *IN VITRO* EL MODIFICATION OF MOUSE SERUM GENERATES LIPID-POOR APOA-I WITHOUT AFFECTING HDL SIZE

To assess whether incubation of mouse serum with EL overexpressing cells results in generation of lipid-poor ApoA-I and alters HDL size, serum, apoB-DS and isolated HDL were analyzed by non-denaturing gradient gel electrophoresis followed by Sudan black and Coomassie blue staining or ApoA-I immunoblotting. Similar to *in vitro* studies with human serum, the formation of lipid-poor ApoA-I was observed by incubation of mouse serum with EL overexpressing cells compared to EV controls (Fig. 19C). Furthermore, there was no alteration in HDL size and abundance in mEL-serum, mEL-apoB-DS and isolated mEL-HDL compared to respective EV controls (Fig. 19 A,B) [124].

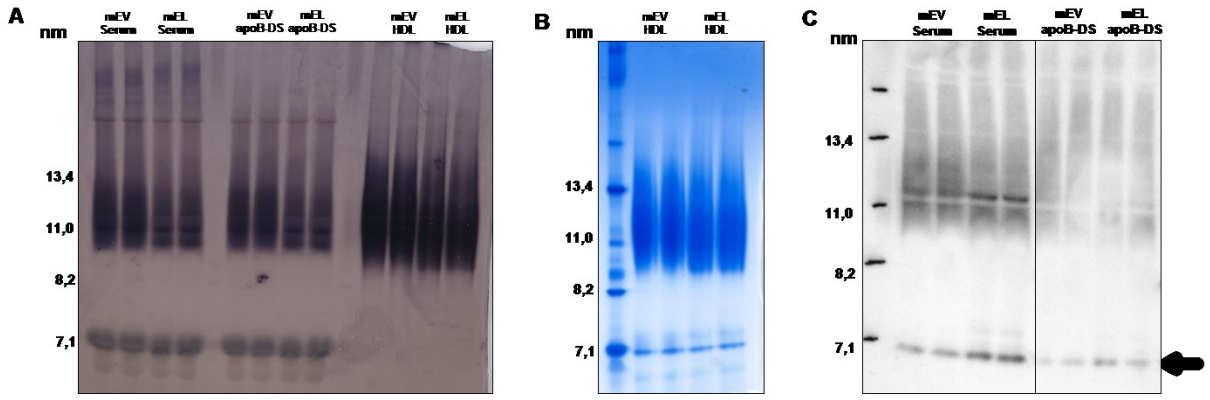

Figure 19: *In vitro* EL modification of mouse serum produces lipid-poor ApoA-I whereas HDL size is not affected

Aliquots of modified serum (1.5 µL), apoB-DS (2 µL) and isolated HDL (10 µg protein) were electrophoresed on 4-16% non-denaturing polyacrylamide gels followed by (A) Sudan black and (B) Coomassie staining or (C) Western Blotting against ApoA-I. Protein size corresponds to the high molecular weight or protein marker bands on the gels or membranes. The position of lipid-poor ApoA-I is indicated by the arrow (C). Results are representative of 2 different modifications of the mouse pool-serum. This figure has been published previously in [124].

6.15 *IN VITRO* EL MODIFICATION OF MOUSE SERUM ALTERS LIPID COMPOSITION OF SERUM AND HDL

To study the impact of EL on the lipid composition as well as its relationship to the altered CEC of mouse serum, the modified serum, apoB-DS and isolated HDL were analyzed by MS. EL overexpression significantly reduced PC levels in serum but not in apoB-DS and HDL (Fig. 20A). PE content was significantly decreased in mEL-serum and mEL-HDL whereas it was unchanged in mEL-apoB-DS (Fig. 20B). Additionally, less PI levels were found in mEL-serum, mEL-apoB-DS and mEL-HDL (Fig. 20C). An increase of TAG content was observed in mEL-serum, whereas it was diminished in mEL-apoB-DS and mEL-HDL (Fig. 20D). Furthermore, EL overexpression increased LPC levels in mEL-serum and mEL-HDL (Fig. 20E) as well as decreased LPE content in mEL-HDL (Fig. 20F). The CE concentrations were decreased in mEL-apoB-DS (Fig. 20G) and FC was increased in mEL-serum and mEL-HDL (Fig. 20H). Decreased Cer levels (Fig. 20I) and increased SM levels were observed in mEL-serum (Fig. 20J) [124]. All results refer to EV as a control.

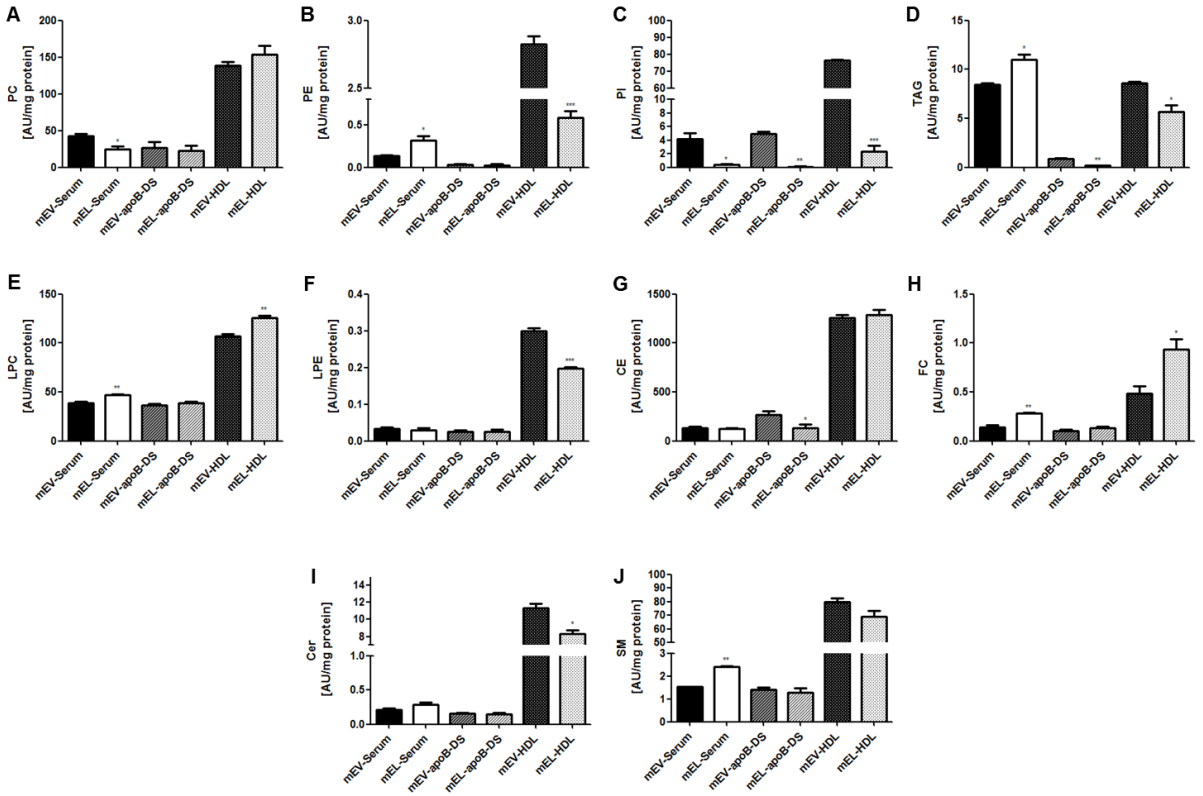

Figure 20: Lipid composition of *in vitro* modified mouse serum, apoB-DS and isolated HDL

Lipids from mEV-serum, mEL-serum, mEV-apoB-DS, mEL-apoB-DS, mEV-HDL and mEL-HDL (corresponding to 300 µg serum or HDL protein) were extracted and A) PC, (B) PE, (C) PI, (D) TAG, (E) LPC, (F) LPE, (G) CE, (H) FC, (I) Cer and (J) SM were analyzed by MS. Results are mean ± SEM of 3 measurements for each condition obtained in 2 independent *in vitro* modifications of pooled mouse serum. This figure has been published previously in [124]. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

7 DISCUSSION

The roles of EL expression in the development of atherosclerosis and regulation of HDL metabolism as well as in promoting cholesterol efflux have been established for many years. However, reports focused on the impact of EL on the CEC of serum and HDL *in vitro* and *in vivo* presented inconclusive data. Additionally, studies on the influence of EL overexpression on the CEC of human serum have not been provided yet. According to that, the present study was performed to investigate the importance of EL overexpression *in vitro* and *in vivo* on CEC of serum, apoB-DS as well as isolated HDL.

To this end, human EL overexpressing HepG2 cells were incubated with either human or mouse serum followed by measuring CEC in ^{3}H-cholesterol labeled J774 macrophages under basal conditions or with upregulation of ABCA1. In both cases, treatment with human and mouse serum, an enhanced total as well as ABCA1-dependent CEC were observed with EL-serum and EL-apoB-DS whereas the CEC of isolated EL-HDL was significantly attenuated [124]. Several studies reported that small, dense HDL subpopulations as well as lipid-poor ApoA-I are the major acceptors in promoting the ABCA1-dependent cholesterol efflux [103, 133, 134]. In accordance to these reports, a pronounced generation of lipid-poor ApoA-I was observed with human as well as mouse serum and apoB-DS upon *in vitro* EL overexpression [124]. In addition, a previous study demonstrated that EL overexpression establishes a negative relationship between ABCA1-dependent cholesterol efflux and the serum PL/ApoA-I ratio [115], pointing toward the importance of EL generated cholesterol acceptor, lipid-poor ApoA-I. Another publication showed the augmentation of ABCA1-dependent cholesterol efflux due to the production of LPCs by EL overexpression [75]. These findings support the observed increased CEC of *in vitro* EL-modified serum in the present study [124], accompanied by a decrease in PLs as well as an increase in LPCs and lipid-poor ApoA-I. Additionally, the reported effects of HL on HDL metabolism by generating smaller HDL particles and lipid-poor ApoA-I, followed by promoting cholesterol efflux [132] can be excluded given that in the present study HL mRNA levels were not increased upon EL overexpression [124].

In the present study, EL action on HDL generated small, dense, PL depleted and LPC enriched HDL particles with significantly reduced CEC [124]. The decreased CEC of EL-modified HDL can be explained by a reduced affinity for SR-BI [117] on the one hand and by PL depletion on the other hand. Indeed, a previous study demonstrated a positive relationship between serum PL/ApoA-I ratio and SR-BI mediated cholesterol efflux during EL overexpression [115]. Furthermore, studies have found a strong relationship between HDL asso-

ciated FC, SM and Cer content and the CEC of HDL. In detail, enrichment of FC, SM and Cer in HDL particles decreases the fluidity of the surface PLs and therefore impairs CEC. However, due to its affinity for cholesterol, increased levels of SM in HDL might augment CEC capacity of HDL [133, 135, 136]. In the present study, the FC content was significantly increased in both human and mouse *in vitro* generated EL-HDL [124], providing a possible explanation for the observed reduction in CEC. The increased FC content in EL-HDL can be explained by increased cholesterol efflux from HepG2 cells due to EL induced formation of LPCs [75] during modification of HDL *in vitro*.

It is well established that the HDL-PL content influences CEC of HDL [137–139]. In detail, enrichment of serum [137] or HDL [138] with PCs led to an enhanced ABCA1-independent [137] or SR-BI mediated [138] cholesterol efflux of both serum and HDL [137, 138]. In contrast, isolated HDL from CAD patients had decreased PL content accompanied by a decreased CEC [139]. In the present study, EL-HDL generated *in vitro* by modification of both mouse and human serum exhibited decreased CEC. The decreased CEC was accompanied by a reduced PE and PI content whereas PC reduction was only observed in human EL-HDL [124]. These observations strongly suggest that not the most abundant PL species PC, but the minor PL species PI and PE affect the capacity of HDL to promote cholesterol efflux. In addition, it has been shown that the content of another minor PL species namely phosphatidylserine (PS) largely determines the CEC of HDL, despite its markedly lower content compared to PC [140]. In the present study the HDL associated PS content could not be identified because of technical limitations. It is conceivable, however that the PS reduction contributes, at least in part, to the reduced CEC of EL-modified HDL.

In contrast to the augmenting effect of EL on the CEC of serum found *in vitro*, the CEC of mouse serum and apoB-DS was significantly reduced by *in vivo* overexpression of human as well as mouse EL [124]. The major effect of EL overexpression was a strong HDL depletion without accumulation of lipid-poor ApoA-I, the latter due to an efficient clearance of lipid-poor ApoA-I via kidneys in mice [46, 56]. This indicates that the decreased CEC of the *in vivo* generated EL-serum and -apoB-DS is a consequence of an EL mediated acceleration of HDL catabolism as well as the fast renal clearance of lipid-poor ApoA-I.

In contrast to reduced CEC of *in vitro* EL-modified HDL, the CEC of *in vivo* EL-modified HDL was not significantly altered and was similar to control HDL [124]. Considering, that the content of PL species and FC influences the HDL mediated efflux capacity, the unaltered CEC of *in vivo* EL-modified HDL could be explained by an unaltered PC and PI content when

compared to control HDL. Interestingly, the FC and Cer content were decreased in *in vivo* EL-modified HDL [124], however, the related possible improvement of CEC was not detected.

We observed a significant augmentation of PE levels in the *in vivo* generated EL-modified serum and HDL as well as in the *in vitro* EL-modified serum [124]. These findings were unexpected, because previous studies established PE as substrate for EL [141] and overexpression of EL in mice caused a prominent reduction in PE plasma levels [142]. Presently it is not clear whether the degree or duration of EL overexpression underlie the discrepancy between the findings observed in the present and previous studies.

A particularly interesting finding of the present study is the difference between human and mouse HDL in terms of the HDL particle size upon action of EL. While human HDL became smaller, the size of mouse HDL was unaltered by the action of EL [124]. A possible explanation for the observed difference could be the decreased PC content in human but not mouse EL-modified HDL. Because the TAG content was decreased in both human and mouse EL-modified HDL generated *in vitro*, and because mouse EL-HDL exhibited unaltered and human EL-HDL decreased size, the alterations in the TAG content seem not to underlie the size differences of human and mouse EL-modified HDL. To eliminate the possibility of species specificity and the failure of human EL to cleave mouse HDL, mouse EL was overexpressed in mice followed by determination of HDL size. Also in this case, as upon overexpression of human EL, no alteration in HDL size was detectable [124].

Keeping in mind the role of HL in HDL metabolism [132], the augmentation of TAG content in HDL upon EL overexpression in mice could be due to an attenuated cleavage of HDL associated TAG by HL. However, HL mRNA levels were not affected by EL overexpression [124].

The findings of the present study [124] regarding EL-mediated generation of lipid-poor ApoA-I and thereby accompanied increase in CEC are in agreement with one report showing that EL suppression in different cell types results in a significantly decreased ApoA-I mediated cholesterol efflux [75]. The same group observed the opposite effect by overexpression of EL or addition of exogenous EL [75].

In the present study, overexpression of mouse or human EL in mice resulted in an impaired CEC of serum [124]. This fits in with results reported by Jin and colleagues, who demonstrated that overexpression of profurin in mice [116], which blocks the cleavage and inactivation of EL, causes a reduction in ABCA1 as well as SR-BI mediated cholesterol efflux [116]. In contrast, serum of EL overexpressing human ApoA-I transgenic mice exhibited

markedly increased ABCA1-dependent cholesterol efflux [115]. A possible cause for the observed discrepant results could be the impact of human ApoA-I, which in contrast to mouse ApoA-I might have longer half-life and slower catabolic rate, thus allowing accumulation of lipid-poor ApoA-I in plasma upon EL overexpression. Unfortunately, in that study, the serum ApoA-I content was not reported [115]. In contrast to the impact of EL on mouse serum, the CEC of HDL isolated from EL overexpressing mice was similar to control HDL, which fits in with a similar CEC of HDL from EL knockout and wild-type mice [41]. In contrast, Brown et al [70] observed an enhanced CEC of HDL from EL knockout mice compared to HDL from wild-type mice [70].

Taken together, an impaired CEC of serum from EL overexpressing mice observed in the present study as well as in a previous study [59], indicate a negative correlation between EL activity and CEC of serum. The negative relationship between EL and CEC is corroborated by the results showing an increased CEC of serum from individuals with partial or complete loss-of-function mutations in the EL gene [40] as well as an enhanced CEC of serum from EL deficient mice [42, 70]. In the present study, however, the action of EL on serum *in vitro* augmented CEC of serum due to the production of a potent cholesterol acceptor, namely lipid-poor ApoA-I. This indicates that by acting on serum, EL generates cholesterol acceptor(s), which are not operative *in vivo* in mice due to the rapid catabolism and renal removal. It remains to be determined whether EL overexpression in human serum *in vivo* generates lipid-poor ApoA-I, and whether the generated lipid-poor ApoA-I accumulates in serum and augments CEC of serum or is rapidly catabolized without affecting CEC.

8 CONCLUSION

The results obtained in this study indicate that the formation and accumulation of lipid-poor ApoA-I upon EL-mediated modification of serum *in vitro* is responsible for the enhanced ABCA1-dependent CEC of EL-modified serum. The decreased CEC of HDL isolated from serum modified by EL *in vitro* most likely reflects structural and functional alterations induced by EL. In contrast, overexpression of EL *in vivo* resulted in an impaired CEC of serum due to HDL depletion and the lack of lipid-poor ApoA-I accumulation. Further investigations are necessary to examine whether EL overexpression in humans *in vivo* by modulating HDL size, composition and structure affects the CEC of serum.

The impact of EL overexpression *in vitro* and *in vivo* on CEC of serum and isolated HDL is summarized in figure 21.

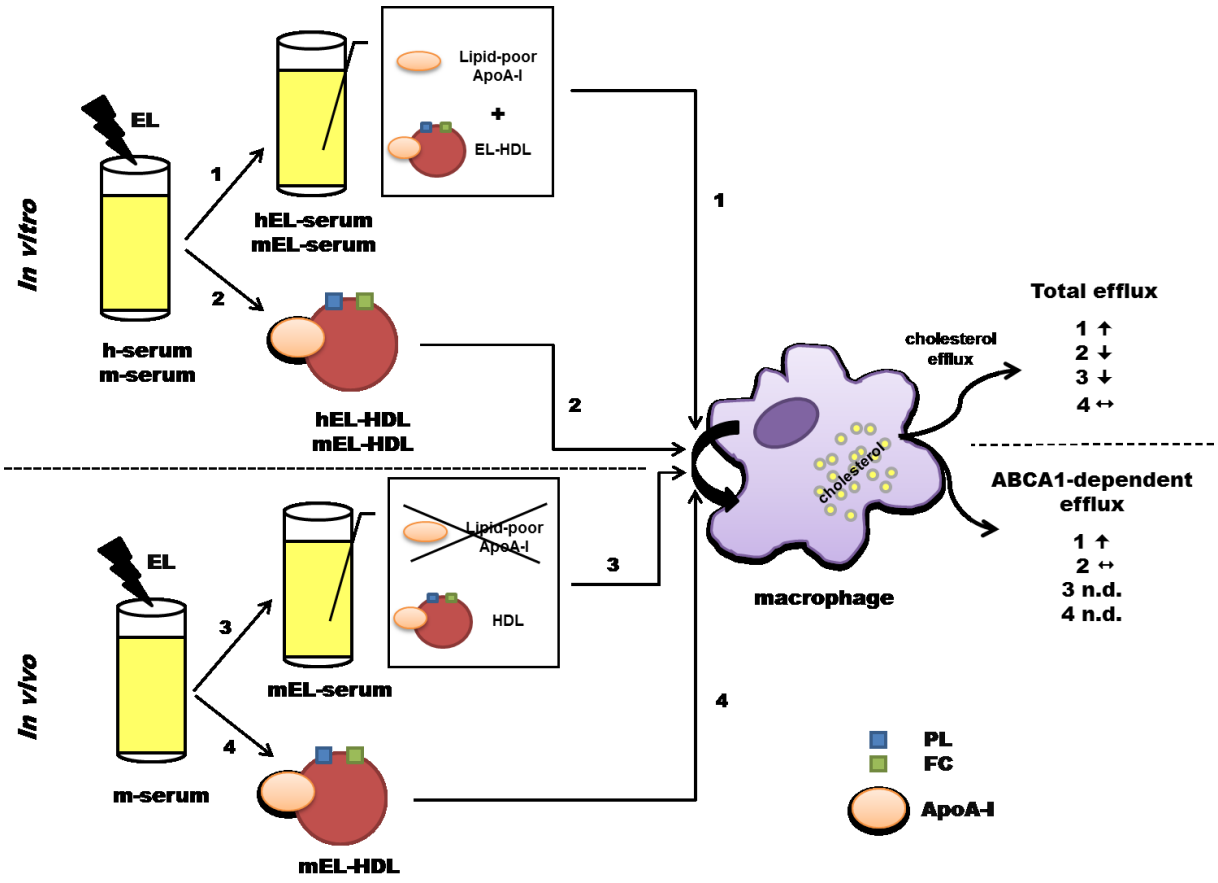

Figure 21: CEC of *in vitro* and *in vivo* EL-modified serum and HDL

EL overexpression *in vitro* was achieved by adenoviral transduction of cultured cells and in mice (*in vivo*) by adenoviral tail vein injection. CEC was analyzed in ^{3}H-cholesterol labeled J774 macrophages under basal conditions or with upregulation of ABCA1. ***In vitro*:** EL overexpressing cells were incubated with human (h) or mouse (m) serum to generate respective EL-modified serum and EL-HDL. 1) *In vitro* modified hEL-serum and mEL-serum contained lipid-poor ApoA-I as well as EL-modified HDL, and exhibited increased total and ABCA1-dependent efflux. 2) HDL isolated from EL-modified serum exhibited decreased total efflux and unaltered ABCA1-dependet efflux. ***In vivo*:** 3) The generation of mEL-serum upon EL overexpression in mice resulted in an impaired CEC due to the depletion of HDL and the lack of lipid-poor ApoA-I. 4) Total CEC of HDL isolated from *in vivo* modified serum was not altered by EL overexpression. Not determined (n.d.)

CHAPTER II

ENDOTHELIAL LIPASE AUGMENTS ANTIOXIDATIVE CAPACITY OF HIGH-DENSITY LIPOPROTEIN

1 ABSTRACT

The capacity of HDL to protect LDL from oxidation is determined by the composition and size of HDL particles as well as the relative abundance and antioxidative activity of associated proteins such as ApoA-I, PON1, LCAT and PAF-AH. We have shown previously that EL by acting on HDL massively alters structure, composition and cholesterol efflux capacity of EL-modified HDL (EL-HDL). Therefore, we hypothesized that the antioxidative capacity of HDL is modulated by EL. Previous studies reported inconclusive data regarding the antioxidative capacity of HDL isolated from EL deficient mice. The present study has been designed to investigate the relationship between composition, structure and the antioxidative capacity of *in vitro* generated EL-HDL.

EL-HDL and control HDL were generated by incubation of isolated HDL with EL overexpressing or control HepG2 cells. Modified HDL was purified by ultracentrifugation followed by FPLC. The antioxidative capacity of HDL was determined by measuring its capacity to attenuate $CuCl_2$ induced LDL oxidation and MDA formation in the presence or absence of a methionine blocker “chloramine T”. Proteomic analysis was used to examine the rate of oxidation of ApoA-I methionine residues. The content of HDL associated enzymes was examined by Western Blotting and arylesterase activity with a photometric assay using phenyl acetate as substrate. HDL size was analyzed by native gel electrophoresis, SAXS analysis and electron microscopy, while the lipid composition was determined by mass spectrometry.

Compared to control, EL-HDL had significantly increased antioxidative capacity reflected by a prolonged lag phase and less MDA accumulation. HDL particles were smaller in size and had altered lipid composition upon EL overexpression. Interestingly, despite a better antioxidative activity, EL-HDL had reduced PON1 content and activity as well as decreased LCAT and PAF-AH mass. Incubation of HDL with cells overexpressing enzymatically inactive EL resulted in an increased HDL-PON1 content and activity, indicating a bridging function of EL and the importance of EL lipolytic activity in displacing PON1 from HDL. Small EL-HDL particles showed higher antioxidative capacity compared to large EL-HDL particles, whereby the smallest particles exhibited the lowest PON1 content. Modification of HDL with EL in the presence of bovine serum albumin yielded EL-HDL with markedly lower content of lipolytic products and profoundly decreased antioxidative capacity. The rate of ApoA-I Met136 oxidation was higher in EL-HDL compared to control HDL, indicating a higher antioxidative activity of that residue in EL-HDL compared to control HDL. Chloramine T abolished the capacity of

EL-HDL and control HDL to protect LDL from oxidation, whereby the oxidation rate of EL-HDL was significantly lower compared to control HDL.

In conclusion, the EL induced alteration in HDL size, structure and lipid composition, in particular the enrichment of lipolytic products in EL-HDL, augment the antioxidative capacity of EL-HDL, independent of HDL associated enzymes.

2 ZUSAMMENFASSUNG

Die schützende Wirkung des HDLs in Bezug auf die Inhibierung der LDL Oxidation wird sowohl auf die Zusammensetzung und Größe des Partikels als auch auf die relative Menge und antioxidative Aktivität von assoziierten Enzymen wie ApoA-I, PON1, LCAT und PAF-AH zurückgeführt. Es konnte bereits im Vorfeld gezeigt werden, dass die Wirkung von EL auf HDL eine Veränderung der Struktur, der Zusammensetzung und der Cholesterin Efflux Kapazität des EL modifierten HDLs (EL-HDL) verursacht. Demnach wurde die Hypothese aufgestellt, dass die antioxidative Aktivität des HDLs durch EL beeinflusst wird. Studien, welche sich mit der Wirkung von isoliertem HDL von EL defizienten Mäusen auf die Inhibierung der LDL Oxidation beschäftigten, konnten bislang keine aussagekräftigen Resultate liefern. Im Zuge dessen wurde in der gegenwärtigen Studie die Relation zwischen Zusammensetzung und Struktur zu der antioxidativen Kapazität von *in vitro* generiertem EL-HDL untersucht.

EL-HDL und Kontroll-HDL wurden durch die Inkubation von isoliertem HDL mit EL überexprimierenden oder Kontrollzellen generiert. Danach erfolgte die Aufreinigung des modifizierten HDLs mittels Ultrazentrifuge und FPLC. Die antioxidative Wirkung des HDLs wurde sowohl durch die Analyse der Kapazität, die $CuCl_2$ induzierte LDL Oxidation zu dämpfen, als auch die Akkumulierung von MDA in der An- oder Abwesenheit des Methionin Blockers „Chloramin T“ bestimmt. Die Untersuchung der Oxidationsrate der ApoA-I Met-Reste erfolgte mit Hilfe von proteomischer Techniken. Der Anteil an HDL assoziierten Enzymen konnte mittels Western Blotting analysiert werden. Die Arylesterase Aktivität wurde durch einen photometrischen Assay mit Hilfe von Phenylacetat als Substrat bestimmt. Die Untersuchung der HDL Größe erfolgte durch die Kombination von nativer Gelelektrophorese, SAXS Analysen und Elektronenmikroskopie. Die Lipidzusammensetzung wurde mit Massenspektrometrie analysiert.

Im Vergleich zur Kontrolle, konnte eine signifikant erhöhte antioxidative Wirkung von EL-HDL festgestellt werden, welche sich in einer verlängerten Lag Phase und reduzierten MDA Akkumulation widerspiegelte. Durch die Überexprimierung von EL entstanden kleinere HDL Partikel mit veränderter Lipidzusammensetzung. Von Bedeutung ist, dass trotz der verbesserten antioxidativen Kapazität, sowohl ein verminderter PON1 Gehalt und Aktivität als auch eine reduzierte LCAT und PAF-AH Menge nachgewiesen werden konnte. Die Verwendung von enzymatisch inaktiver EL führte zu einer Anreicherung des PON1 Gehaltes und dessen Aktivität, wodurch auf eine „Bridging“ Funktion von EL und die Wichtigkeit der lipolytischen EL

Aktivität für die Verdrängung von PON1 am HDL Partikel hingewiesen werden konnte. Kleine EL-HDL Partikel zeigten im Vergleich zu großen eine höhere antioxidative Wirkung, wobei die kleinsten Partikel den niedrigsten PON1 Gehalt aufwiesen. Die Entfernung von lipolytischen Produkten, welche durch EL generiert werden, führte zu einer geringeren ausgeprägten antioxidativen Kapazität von EL-HDL. Im Vergleich zur Kontrolle konnte im EL-HDL eine erhöhte Oxidationsrate von ApoA-I Met136 beobachtet werden. Dies deutet auf eine höhere antioxidative Aktivität dieser Aminosäure im EL-HDL, im Vergleich zur Kontrolle, hin. Chloramin T hob die antioxidative Wirkung sowohl von EL-HDL als auch von Kontroll-HDL auf, wobei die Oxidationsrate von EL-HDL im Vergleich zum Kontroll-HDL signifikant geringer war.

Zusammenfassend konnte der Einfluss von EL auf die antioxidative Wirkung von HDL, auf eine EL induzierte Veränderung der HDL Größe und Lipidzusammensetzung, insbesondere durch die Anreicherung von Spaltprodukten in EL-HDL, zurückgeführt werden. HDL assoziierte Enzyme spielen demnach keine Rolle in der Förderung der antioxdativen Kapazität des HDLs.

3 INTRODUCTION

The incidence of atherosclerosis and cardiovascular diseases is associated with environmental and genetic risk factors e.g. age, gender, cigarette smoking, obesity, hypertension, diabetes mellitus and serum cholesterol levels, which causes accumulation and subsequent oxidation of LDL in the arterial wall [143]. It has been reported, that HDL protects LDL against oxidation and absorbs the products of lipid oxidation [144, 145]. Additionally, it has been demonstrated that HDL particles transport plasma LOOHs in atherosclerotic animal models [146] as well as in humans [144].

3.1 OXIDATION OF LDL

The hypothesis that LDL oxidation takes part in the development of atherosclerosis includes the ability of oxLDL to 1) recruit monocytes into the intima of the vascular wall, 2) inhibit the capability of macrophages to exit the intima, 3) increase the uptake of LDL by macrophages leading to the generation of foam cells and 4) be cytotoxic resulting in damage of endothelial integrity [143].

The procedure of LDL oxidation occurs in two phases [147, 148]. The initial step of *in vitro* LDL oxidation forms the so called minimally oxLDL, which can be still recognized by the LDL receptor. This type of oxLDL is characterized by the modification of LDL lipids without or minimal alterations in ApoB-100, the main protein on LDL [143]. It has been shown *in vitro* that this stage leads to the production of MCP-1 in smooth muscle and endothelial cells [149], accompanied by the recruitment of inflammatory cells [150]. These recruited cells produce inflammatory cytokines, chemokines and adhesion molecules, which promote LDL oxidation. In this stage, more LDL lipids are oxidized as well as the ApoB-100 is modified, resulting in the recognition of oxLDL by scavenger receptors on macrophages to generate foam cells [151]. Additionally oxLDL influences other biological features contributing to the formation of atherosclerosis including stimulation of monocyte binding to endothelial cells as well as induction of growth factors, collagen production, platelet adhesion and aggregation [152]. Within the artery wall, LDL oxidation can be driven non-enzymatically by transition metal ions, hemin or many other oxidants. Furthermore, several enzymes such as lipooxygenase, MPO, NADPH oxidase and NOS can facilitate the formation of oxLDL [151]. These enzymes are able to generate reactive chlorine, nitrogen and oxygen species in terms of reactive one-electron or two-electron oxidants, whereby oxidations driven by lipooxygenase and MPO products are the most relevant for LDL oxidation *in vivo* [153].

Studies showed that LDL oxidation occurs in the arterial wall and not in the circulation because lipoprotein lipids in the serum are defended from oxidation by antioxidants such as α-tocopherol [143, 147].

3.2 THE ROLE OF HDL IN LDL OXIDATION

HDL has the ability to protect LDL and other lipoproteins against oxidative stress, which is induced by reactive one-electron or two-electron oxidants [154, 155]. In detail, HDL lipid and protein components can be modified by oxidants resulting in the generation of lipid and protein radicals. Subsequently, oxidation products are accumulating in the HDL particle, which leads to the oxidation of further HDL components [143]. Therefore, HDL has the ability to defend lipid and protein moieties of LDL against free radical oxidation and prevents the accumulation of oxidation products such as LOOH, short-chain oxPLs and aldehydes within the LDL particle [146, 156].

3.2.1 MECHANISMS OF HDL MEDIATED PROTECTION AGAINST LDL OXIDATION

The HDL mediated protection against LDL oxidation includes the removal of LOOH from LDL or cells. In fact, Zerrad-Saadi et al [157] reported the transfer of PL hydroperoxides (PLOOH) as well as CE hydroperoxides (CEOOH) from LDL to HDL upon co-incubation of oxLDL and HDL *in vitro,* depending on the surface lipid rigidity and ApoA-I Met residues [157]. Oxidized lipids exhibit higher surface activity compared to non-oxidized ones, whereby they are more exposed to the aqueous phase at the lipoprotein surface [158]. The transfer of oxidized lipids from LDL to HDL can occur either spontaneously or mediated by transfer proteins. Spontaneous transfer between membranes was first demonstrated by using photodynamically peroxidized erythrocyte ghosts as LOOH donors and unilamellar liposomes as acceptors [159]. The same group showed the possibility of spontaneous translocation of LOOHs between photoperoxidized erythrocyte membranes and LDL [160]. However, previous studies demonstrated the transfer of LOOHs between lipoproteins regulated by transfer proteins such as CETP. Accumulation of small amounts of LOOH was observed upon incubation of native HDL with oxLDL in the absence of CETP. In contrast, the same setup in the presence of purified CETP resulted in a 7.5- to 10-fold increase of accumulated LOOHs in the HDL particle [161, 162]. Lipid-free ApoA-I has also the ability to promote the removal of LOOHs from LDL [163]. In addition, *in vivo* oxidized CEs within the HDL particle were removed by the liver for biliary excretion to a higher extent than non-oxidized CEs. This hepatic uptake pathway was mediated by parenchymal cells, confirmed also in *in vitro* experiments [164]. Another report showed that SR-BI is responsible for the uptake of oxidized CE from HDL in SR-BI transfect-

ed Chinese hamster ovary cells [165]. Taken together, LOOHs from LDL are transferred to HDL and cleared by the SR-BI mediated hepatic uptake of HDL and excreted into bile.

3.2.2 COMPONTENTS OF HDL CONTRIBUTING TO ITS ANTIOXIDATIVE CAPACITY

Following the transfer of oxidized lipids from LDL to HDL, HDL associated LOOHs are subsequently inactivated. This inactivation occurs in a redox reaction, in which hydroperoxides are reduced to hydroxides by the action of HDL proteins [157].

Apolipoproteins

ApoA-I, the most abundant apolipoprotein in HDL, plays a crucial role in the antioxidative activity of HDL. It has the ability to bind and remove LOOHs of LDL *in vitro* as well as *in vivo*, after injection into mice or infusion in humans [166]. The ability of ApoA-I to reduce LOOHs in its inactive forms is regulated by the oxidation of its Met residues 112 and 148 [167]. This reaction causes the generation of Met sulfoxides that can be converted back to Met by Met sulfoxide reductase [168]. Additionally to Met residues, ApoA-I associated cysteine [169] and histidine [170] residues contribute to its antioxidative activity. Indeed, reconstituted HDL that contains only ApoA-I and PLs efficiently delayed lipid peroxidation of LDL, to a degree similar to that of HDL_3 [157]. Navab et al [163] demonstrated that removal of oxidized lipids from LDL by ApoA-I, renders LDL resistance to oxidation by vascular cells. Furthermore, injection of ApoA-I into humans and mice rendered isolated LDL resistance to oxidation initiated by incubation with human artery wall cells [163].

Besides the contribution of ApoA-I in preventing LDL from oxidation, also other HDL associated (apolipo)proteins such as **ApoM** [171], **ApoE** [172], **clusterin** (ApoJ) [173], **ApoA-II** [167, 168, 174] and **ApoA-IV** [175] support the HDL mediated protection against LDL oxidation. For instance, isolated HDL from ApoM transgenic mice delayed LDL oxidation induced by Cu^{2+} or 2,2'-azobis 2-methyl-propanimidamide dihydrochloride (AAPH), due to its capacity to bind oxPLs [171]. Treatment of mice or HepG2 cells with mildly oxLDL resulted in increased levels of ApoJ, a protein capable of preventing LOOH formation in LDL [173]. Garner et al [167, 168] demonstrated the potential role of ApoA-II in reducing oxidized lipids into its inactive forms [167] and the role of Met residues on ApoA-II in this process [168]. In contrast, HDL isolated from mice injected with ApoA-II failed to protect LDL form oxidation by artery wall cells [163]. Additionally, overexpression of human ApoA-II in mice facilitated atherosclerosis development due to an enhanced accumulation of oxLDL and decreased antioxidative capacity of HDL [174].

Enzymatic components

The anti-atherogenic actions of HDL are also regulated by several enzymatic components, including PAF-AH, GPX, LCAT and PON1, which are known to hydrolyze proinflammatory oxPLs and decompose bioactive products of LDL oxidation [22, 156].

PON1, a 43 kDa protein [176], is specifically expressed in the liver and released to the blood stream, where it associates with HDL particles [177]. The activity of PON1 is stabilized by ApoA-I and ApoJ [178–180]. The enzyme is a lactonase/esterase that requires Ca^{2+} for its activity. Moreover, it reveals peroxidase-like activity and hydrolyses organophosphate substrates such as paraxon [181, 182]. Various human studies demonstrated a correlation between serum PON1 activity and the risk of CVD [183]. *Ex vivo* experiments suggested the capacity of PON1 to hydrolyze lipid peroxides within human carotid and coronary lesions [182]. Inhibition of all PON1 activities, including lactonase, paraoxonase and arylesterase activity, was observed under oxidative conditions and was accompanied by diminished ability to protect LDL from oxidation [184]. *In vivo* studies in mice showed, that deficiency of PON1 leads to a higher incidence to develop atherosclerosis [185, 186], characterized by enhanced oxidative stress in macrophages [187] and accumulation of oxidized lipids in LDL [188]. In contrast, overexpression of human PON1 in atherosclerotic mouse models prevented atherosclerosis development and macrophage oxidative stress accompanied by a decreased LDL oxidation [189–191]. Furthermore, adenoviral overexpression of PON1 in ApoE knockout mice ameliorated oxidative stress in the vascular wall and enhanced endothelial function [192]. Isolated HDL from transgenic PON1 overexpressing mice augmented the ability of HDL to inhibit LDL oxidation [190], whereas HDL from mice lacking PON1 showed no longer this effect [186]. Furthermore, purified PON1 decreased the accumulation of oxPLs in LDL thus protecting LDL from oxidation [166]. By contrast, some studies described a PON1 independent antioxidative activity of HDL [157, 167, 193].

PAF-AH, a serine esterase, is associated in humans predominantly with LDL and to a minor extent with HDL [194, 195]. The enzyme has the ability to hydrolyze the acetyl group on the sn-2 position of PAF [196] as well as PLOOHs within oxLDL generating LPC and FFA hydroperoxides, which are transported to HDL and reduced to hydroxides by ApoA-I [197]. LDL oxidation induced by Cu^{2+} was inhibited by the action of PAF-AH [198]. Pre-treatment of HDL with a PAF-AH inhibitor resulted in a decreased capacity of HDL to protect against LDL oxidation and enrichment of HDL with purified PAF-AH restored this effect [199]. Furthermore, HDL associated PAF-AH activity was increased in human ApoA-I overexpressing ApoE knockout

mice, whereby oxidative stress in plasma, ICAM and VCAM expression as well as recruitment of monocytes into the arterial wall were reduced [200]. Another study demonstrated that overexpression of PAF-AH in mice using adenovirus reduced oxLDL plasma levels and HDL associated PAF-AH attenuated foam cell formation and promoted macrophage cholesterol efflux [201]. In contrast, pre-treatment of HDL with a PAF-AH inhibitor showed no effect on the HDL mediated inactivation of LOOHs in oxLDL and on the accumulation of conjungated dienes in LDL [157]. Human studies revealed a positive correlation between PAF-AH plasma levels and the risk of cardiovascular events [202, 203]. However, a recent study showed that high PAF-AH plasma levels are associated with increased risk of cardiac death in patients with stable CAD, while HDL associated PAF-AH lowers this risk [204].

LCAT that is mainly responsible for cholesterol esterification in HDL biogenesis facilitates also the antioxidative capacity of HDL. Indeed, inhibition of LCAT demonstrated increased levels of LDL oxidation products in plasma while purified LCAT was able to hydrolyze these products [205]. The accumulation of LOOHs and conjungated dienes in LDL was prevented upon incubation of LDL with purified human LCAT. The enzyme was able to inhibit spontaneous as well as Cu^{2+} induced LDL oxidation [206, 207]. Both studies showed the importance of one serine residue in inhibiting LDL oxidation. Overexpression of human LCAT by adenovirus in mice resulted in a reduced LDL oxidation in the circulation as well as in a decreased accumulation of oxLDL and macrophages in the aortic arch [208]. In contrast, Zerradi-Saadi et al [157] reported no contribution of LCAT on HDL mediated inactivation of LOOH in oxLDL as well as in accumulation of conjungated dienes in LDL [157].

Finally, alterations in the HDL lipidome can influence the antioxidative capacity of HDL [131, 209].

HDL lipidome

The composition of HDL lipids affects the antioxidative capacity of HDL, mainly by modulating the chemical and physical characteristics of HDL surface lipids [131]. The lipid surface rigidity of HDL is especially influenced by the content of Cer, SM, FC as well as saturated and monounsaturated FAs. Reduced amounts of these lipids increase the fluidity of the surface lipid monolayer [157]. Indeed, the transfer of PLOOHs from LDL to HDL correlates with the fluidity of the surface PL monolayer [157, 160]. Reconstituted HDL with an elevated surface lipid fluidity demonstrated an enhanced ability in protecting LDL from oxidation as well as an increased transfer rate of PLOOHs from LDL to HDL [157]. Additionally, an augmented transfer rate of LOOHs from membranes to HDL was observed [160]. Besides these findings, also

the composition of the HDL lipid core can influence its antioxidative capacity. The ratio of CE to TAG in the HDL core alters the conformation of ApoA-I [210, 211] and modifications of these lipids changes the net surface charge of HDL [212]. Keeping in mind that ApoA-I plays a central role in the antioxidative capacity of HDL, these modulations of the HDL lipid core might affect the transfer of LOOHs from LDL to HDL. A recent study demonstrated that TAG enrichment of HDL_{3a} and HDL_{3b} leads to a significantly reduced ability to prevent against AAPH induced LDL oxidation [213]. Furthermore, HDL is a carrier of lipophilic antioxidants, especially tocopherols, contributing to a minor extent to its property in inactivating LOOHs [23, 144, 168].

3.2.3 HDL HETEROGENEITY AND ANTIOXIDATIVE CAPACITY

HDL heterogeneity influences its structure, metabolism and biological properties [35]. HDL associated apolipoproteins, enzymes and lipids that contribute to its antioxidative activity are not equally distributed among HDL subpopulations [209]. Indeed, ApoA-I content correlates with HDL size and density whereby small, dense HDL_3 exhibits the highest amount [135]. In addition, an enrichment of LCAT, PAF-AH, PON1 as well as ApoA-IV was observed in small, dense HDL particles compared to large ones [214, 215]. Studies demonstrated a higher capacity to protect against Cu^{2+} and AAPH induced LDL oxidation with small, dense and protein-rich HDL particles [215, 216], including the transfer and inactivation of LOOHs from LDL to HDL as well as the action of ApoA-I Met residues in reducing oxidized lipids into their inactive forms [157]. One explanation for these observations could be that the surface fluidity of small HDL particles is increased due to a decreased SM and FC content, resulting in a higher capacity to incorporate exogenous molecules such as oxidized lipids [156, 217]. Second, relative enrichment of ApoA-I in small, dense HDL particles [135] might facilitate the reduction of LOOHs into hydroxides [157]. Furthermore, alterations in the ApoA-I conformation can occur due to a lower lipid content in HDL_3 compared to HDL_2 [209]. Conformational changes in ApoA-I lead to a higher exposure to the aqueous phase [210], thereby promoting the action of Met residues in inactivating LOOHs. Finally, Davidson et al [218] found a unique protein distribution in isolated HDL subpopulations from normolipidaemic patients, whereby HDL associated proteins such as ApoJ, ApoM, SAA4 and PON positively correlated with the protection against AAPH induced LDL oxidation *in vitro* [218].

Taken together, small HDL particles exert better antioxidative capacity due to 1) enrichment of ApoA-I, 2) enrichment of HDL associated enzymes, 3) higher lipid surface fluidity and 4) alterations in ApoA-I conformation.

3.3 THE ROLE OF EL IN THE ANTIOXIDATIVE CAPACITY OF HDL

The fact that EL affects HDL plasma levels, HDL size and composition prompted the investigations on the impact of EL on the antioxidative capacity of HDL. First, Hara et al [41] examined the effect of HDL from EL knockout mice on Cu^{2+} induced LDL oxidation. This group observed no differences in delaying LDL oxidation between HDL from $EL^{-/-}$ compared to wild-type mice. Interestingly, plasma activities of PAF-AH and PON1 were increased in EL deficient mice, but HDL associated activities of both enzymes were similar to wild-type mice [41]. One study confirmed these results by analyzing the antioxidative and anti-inflammatory activities of HDL from patients with partial or complete loss-of-function mutations in the EL gene [40]. HDL from these patients showed no significant differences in affecting superoxide production in endothelial cells measured by electron spin resonance spectroscopy as well as TNF-α induced VCAM1 expression levels in endothelial cells compared to HDL from healthy controls [40]. In contrast, one report described a higher ability of HDL from EL deficient mice to protect against Cu^{2+} induced LDL oxidation compared to HDL from wild-type mice. In that study, the antioxidative effects of HDL positively correlated with both the HDL PL/total protein and ApoA-I/total protein ratio [42]. Furthermore, EL deficiency resulted in higher total plasma and HDL bound PAF-AH activities, with no influence on HDL associated PON1 activity [42].

4 THEORY AND HYPOTHESIS

Overall, the antioxidative capacity of HDL and the relative contribution of HDL-associated ApoA-I and enzymes such as PON1, LCAT and PAF-AH to the antioxidative capacity of HDL are largely determined by the composition and size of HDL particles [209]. This, together with the pronounced EL mediated alterations in HDL structure and composition [45, 119] strongly suggest altered antioxidative capacity of EL-modified HDL (EL-HDL). Inconclusive data were observed studying the impact of EL deficiency on the capacity of HDL to prevent LDL oxidation [41, 42]. Therefore, the present study has been designed to investigate the relationship between composition, structure and antioxidative capacity of *in vitro* generated EL-HDL.

5 MATERIAL AND METHODS

5.1 CELL CULTURE

HepG2 cells (ATCC®, HB-8065™) [121] were cultured in DMEM containing 2 mM glutamine, 1% PS (100 U/ml penicillin, 100 µg/ml streptomycin) and 10% fetal calf serum (FCS).

5.2 PREPARATION OF HEPARIN MEDIA

Heparin media were prepared as described in chapter I, 5.2.

5.3 HDL ISOLATION FROM HUMAN PLASMA

HDL was obtained from human plasma by a one-step density gradient ultracentrifugation method, as described previously [124, 125, 219] and in chapter I, 5.6.

5.4 PREPARATION OF EL-HDL AND CONTROL EV-HDL

HepG2 cells (2 x 10^6 cells/dish) were plated onto 60 mm dishes and incubated under standard conditions as described in 5.1. After 24 h, cells were washed ones with DMEM without FCS and infected with MOI 10 of adenovirus encoding human EL (EL-Ad), human EL mutant (ELM-Ad) or empty adenovirus containing no recombinant cDNA (EV-Ad) [45] with or without co-infection (MOI 20) of adenovirus encoding human PON1 (Vector Biosystems Inc., Malvern, PA, USA) in DMEM without FCS for 2 h. After removal of infection media, cells were incubated with fresh DMEM supplemented with 10% FCS for 28 h. Thereafter, cells were washed ones with DMEM without FCS and each plate was incubated under cell culture conditions with 2 mg isolated HDL protein, re-suspended in 1.8 mL DMEM without FCS, in the absence or presence of 4% (final concentration) of non-esterified fatty acid (NEFA)-free bovine serum albumin (BSA) (Sigma-Aldrich, Vienna, Austria) for 16 h. After incubation, the media were collected and spun at 1100 x g for 3 min to remove cellular debris. The modified HDL was re-isolated from the media by one-step density gradient ultracentrifugation, concentrated, desalted and stored at -80°C.

5.5 NON-DENATURING GRADIENT GEL ELECTROPHORESIS

10 µg of modified HDL were electrophoresed on 4-20% non-denaturing polyacrylamide gels (Bio-Rad Laboratories, Vienna, Austria). Before loading, samples were diluted with native sample buffer (LifeTechnologies, Vienna, Austria). Electrophoresis was done in a running buffer (0.09 mol/L Tris, 0.08 mol/L boric acid, 3 mmol/L EDTA, pH 8.3) at 125 V for 3.5 h at RT. Fixation of gels was done with 10% sulfosalicylic acid for 30 min and then stained with

Coomassie Brillant Blue G250. As a standard, the high molecular weight marker NativeMark (LifeTechnologies, Vienna, Austria) was applied [124].

5.6 LIPIDOMIC ANALYSIS

Analysis of the HDL lipid composition was performed as described in chapter I, 5.13.

5.7 SAXS (SMALL-ANGLE X-RAY SCATTERING)

SAXS data were recorded on an in-house SAXS instrument (SAXSess mc2, Anton Paar, Graz, Austria) equipped with a Kratky camera, a sealed X-ray tube source and a two-dimensional Princeton Instruments PI•SCX:4300 (Roper Scientific) CCD detector. The scattering patterns were determined with a 90 min exposure time (540 frames, each 10 seconds) for several solute concentrations in the range from 0.5 to 1.0 mg/mL. Radiation damage was excluded based on a comparison of individual frames of the 90 min exposures, where no changes were detected. A range of momentum transfer of $0.012 < s < 0.63$ Å^{-1} was covered ($s = 4\pi \sin(\theta)/\lambda$, where 2θ is the scattering angle and $\lambda = 1.5$ Å is the X-ray wavelength).

All SAXS data were analyzed with the package ATSAS (version 2.5). The data were processed with the SAXSQuant software (version 3.9), and desmeared using the programs GNOM and GIFT [220, 221]. The forward scattering [I(0)], the radius of gyration [Rg], the maximum dimension [D_{max}] and the inter-atomic distance distribution functions [P(R)] were computed with the program GNOM and GIFT. The masses of the solutes were evaluated by comparison of the forward scattering intensity with that of a human serum albumin reference solution (molecular mass 69 kDa) and using Porod's law. To generate *ab initio* shape models, a total number of 50 models were calculated using the program DAMMIF [222, 223] and aligned, and averaged using the program DAMCLUST.

5.8 NEGATIVE STAIN ELECTRON MICROSCOPY FOR HDL VISUALIZATION

Glow discharged carbon coated copper grid were used. The grids were placed on 100 µL of lipoprotein solution on Parafilm for 1 min. The specimens were washed three times with 200 µL water on Parafilm. Samples were blotted with filter paper and placed on a drop of 2% aqueous uranyl acetate solution for 1 min, blotted with filter paper and air dried at RT [224]. Specimens were examined with an FEI Tecnai G 2 equipped with an ultrascan 1000 ccd camera (Gatan).

5.9 FPLC OF MODIFIED HDL

In brief, an ÄKTA pure FPLC System (GE Healthcare, Munich, Germany) equipped with a Superdex 200 Increase 10/300 column (GE Healthcare, Munich, Germany) was used with PBS as running buffer. After loading, HDL samples were separated with a constant flow of 0.5 mL/min and fractionation was done in a range of 9 to 13.5 mL elution volume with 0.5 mL per fraction. The software UNICORN (GE Healthcare, Munich, Germany) was used for analysis.

5.10 SDS-PAGE AND WESTERN BLOTTING

Analysis of EL overexpression was done as described in chapter I, 5.11 [45, 124]. Expression levels of human PON1 in transduced HepG2 cells were examined by SDS-PAGE after lysing cells with RIPA buffer (Thermo Fisher Scientific, Schwerte, Germany) followed by protein estimation with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Schwerte, Germany).

HDL-associated proteins (10 µg) and cell lysates (30 µg) were separated by 12% SDS-PAGE at 175 V for 90 min. Separated proteins were transferred to PVDF membrane (Carl Roth, Karlsruhe, Germany) with blotting buffer (Tris, Glycine, EDTA, sodium azide) at 150 mA for 90 min. Membranes were blocked at RT in 10% skim milk for 2 h followed by overnight incubation at 4°C with antibodies specific for: human PON1 (Abcam, ab24261, Cambridge, UK), human ApoA-I (Novus biological, NB100-65491, Littleton, CO, USA), human LCAT (Novus biological, Littleton, CO, USA), human PAF-AH (Cayman chemical, 160603, Ann Arbor, MI, USA), α-tubulin (Cell signaling technology, 11H10, Leiden, Netherlands) and albumin (Abcam, ab83465, Cambridge, UK). After washing and incubation with appropriate secondary antibody (Dako, Vienna, Austria) protein signals were visualized by incubation with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Schwerte, Germany) using ChemiDoc system (Bio-Rad Laboratories, Vienna, Austria).

5.11 ARYLESTERASE ACTIVITY

Ca^{2+}-dependent arylesterase activity of EV-HDL and EL-HDL was determined with a photometric assay using phenyl acetate as substrate as described previously [219].

5.12 LDL PREPARATION AND OXIDATION

Human LDL was isolated from fasted normolipidemic plasma as described previously [225]. LDL was dialyzed against PBS (pH 7.40) without EDTA and diluted to a final concentration of 0.3 mg ApoB/mL. Oxidation was performed by addition of a $CuCl_2$ solution giving a final con-

centration of 10 µmol/L. After 4 hours, the oxidation was stopped with EDTA (200 µmol/L) and the LDL was dialysed against PBS (GIBCO, Life technologies, pH 7.40).

5.13 KINETIC OF LIPOPROTEIN OXIDATION (CONJUGATED DIENES FORMATION)

Copper-induced formation of conjugated dienes was examined as described [226]. Briefly, $CuCl_2$ (2 µM) was added to EV-HDL or EL-HDL or to LDL in the absence or presence of different concentrations of EV-HDL or EL-HDL and the formation of conjugated dienes was continuously monitored at 234 nm with a spectrophotometer (Hitachi U-2000) at 37°C using 1 cm quartz cuvette. For determination of the lag phase, a simple sigmoid model was used. The mathematical properties of the model were determined using the software package MATHEMATICA version 11 (Wolfram Research Inc., Champaign, IL, USA) and then implemented in a Microsoft EXCEL sheet for convenient usage.

5.14 MDA ANALYSIS

MDA content was measured in samples described in 4.15. after termination of conjugated diene formation, using HPLC method described by Pilz et al [227] after derivatization with 2,4-dinitrophenylhydrazine (DNPH). The slight modifications of the method have been reported [228]. Briefly, for alkaline hydrolysis of protein bound MDA, 25 µL of 6 mol/L sodium hydroxide was added to 0.125 mL of the samples (1.5 mL Eppendorf tubes) and incubated at 60° (Eppendorf heater) for 30 min. The hydrolyzed sample was deproteinized with 62.5 µL 35% (v/v) perchloric acid. 125 µL supernatant obtained after centrifugation (14000 g; 2 min) were mixed with 12.5 µL DNPH solution and incubated for 10 min. This reaction mixture, diluted derivatized standard solutions (0.625 nmol/mL – 10 nmol/mL) and reagent blanks were injected into the HPLC system (injection volume: 40 µL). The MDA standard was prepared by dissolving 25 µL 1,1,3,3-tetramethoxypropane (TMP) in 100 mL bidistilled H_2O (stock solution: 1 mmol/L). The hydrolysis was performed with 200 µL TMP stock solution in 10 mL 1% sulfuric acid and incubation for 2h at RT [229].The resulting MDA standard of 20 nmol/mL were further diluted with 1% sulfuric acid to the final concentrations. The DNPH derivates (hydrazones) were isocratically separated on a 5-µm ODS hypersil column (150x4.6 mm) guarded by a 5 µm ODS hypersil column (10x4.6 mm; Uniguard holder) with a mobile phase consisting of a 0.2% (v/v) acetic acid solution (bidistilled water) containing 50% acetonitrile (v/v) at a flow rate of 0.8 mL/min. The HPLC separations were performed with an L-2200 autosampler (injection volume: 40 µL), a L-2130 HTA pump a L-2450 diode array detector (all:

VWR Hitachi Vienna; Austria). Detector signals (absorbance at 310 nm) were recorded and program EZchrom Elite (VWR) was used for data requisition and analysis.

5.15 OXIDATION OF HDL MET RESIDUES

Blocking of Met residues was done as described previously [157]. Shortly, FPLC-purified EV-HDL and EL-HDL (1.5 mg protein/mL) were incubated at 4°C for 1 h with 2mM chloramine T (Sigma Aldrich, Vienna, Austria), which oxidizes protein Met residues into corresponding sulfoxides [23] and modifies free SH groups [230]. Chloramine T was removed by gel filtration on Sephadex PD-10 columns (GE Healthcare, Munich, Germany).

5.16 ANALYSES OF APOA-I MET RESIDUES BY PROTEOMICS

$CuCl_2$ oxidized LDL (50 µg) was incubated with EV-HDL or EL-HDL (both 50 µg) for 30, 75 and 120 min at 37°C, followed by stopping the reaction with acetone [224] and detection of oxidized ApoA-I Met residues by proteomics. For tryptic digest, 50 µg of HDL protein was precipitated with 4 volumes of acetone at -20°C overnight, solubilised in 50 µl 100 mM ammonium bicarbonate, reduced with 50 µl of 10 mM DTT for 20 min by shaking at 550 rpm at 56°C and alkylated with 50 µl of 60 mM iodoacetamide by shaking at 550 rpm at RT for 15 min. Protein was digested by adding 1 µg modified trypsin (Promega) and shaking overnight at 550 rpm at 37°C. The resulting peptide solution was acidified by adding 3 µl of 5% formic acid. 250 ng of the digest was injected and concentrated on the enrichment column (C18, 5 µm, 100 Å, 5 x 0.3 mm) for 2 min using 0.1% formic acid as isocratic solvent at 5 µl/min flow rate. The column was then switched in the nanoflow circuit and the sample was loaded on the nanocolumn, Acclaim PepMap RSLC nanocolumn (C18, 2 µm, 100 Å, 500 x 0.075 mm), at a flow rate of 250 nl/min at 60°C and separated using the following gradient: solvent A: water, 0.1% formic acid; solvent B: acetonitrile, 0.1% formic acid; 0-2min: 4% B; 2-90 min: 4-25% B; 90-95 min: 25-95% B, 96-110 min: 95% B; 110-110.1 min: 4% B; 110.1-125 min: 4% B. The sample was ionized in the nanospray source equipped with stainless steel emitters (Thermo Fisher Scientific, Vienna, Austria) and analyzed in a Thermo Orbitrap velos pro mass spectrometer in positive ion mode by alternating full scan MS (m/z 300 to 2000, 60000 resolution) in the ICR cell and MS/MS by CID of the 20 most intense peaks in the ion trap with dynamic exclusion enabled. The LC-MS/MS data were analyzed by searching the human Swiss-Prot protein database containing all common contaminants with Proteome Discoverer 1.4 (Thermo Fisher Scientific) and Mascot 2.4.1 (MatrixScience, London, UK). Carbamidomethylation on cysteine was entered as fixed and oxidation on methionine and sulfonation at cysteine as variable modification. Detailed search criteria were used as follows: trypsin; max. missed

cleavage sites: 2; search mode: MS/MS ion search with decoy database search included; precursor mass tolerance +/- 10 ppm; product mass tolerance +/- 0.7 Da; Label free quantitation was done with Proteome Discoverer 1.4 (Thermo Fisher Scientific).

5.17 STATISTICAL ANALYSIS

Data are represented as means ± standard error of mean (S.E.M.). Differences between EV- and EL samples were assessed by two-tailed unpaired *t*-test between or one-way ANOVA using Graph Pad Prism 5.0. Statistically significant differences between groups are indicated by *P*-values of < 0.05 (*), < 0.01 (**), or < 0.001 (***).

6 RESULTS

6.1 EL ALTERS STRUCTURE AND SIZE OF HDL

To study the impact of EL on the antioxidative capacity of HDL, we prepared EL-modified HDL and control HDL by incubation of isolated HDL with EL- and EV-overexpressing HepG2 cells (Fig. 22).

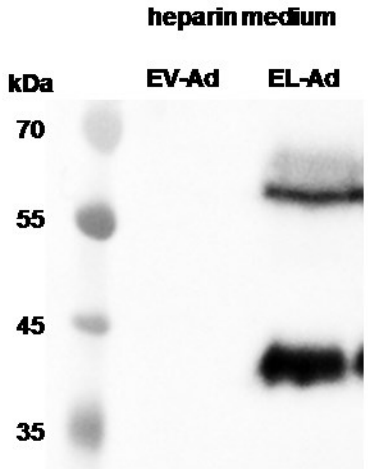

Figure 22: Western Blot analysis of human EL overexpression in HepG2 cells

Overexpression of EL was analyzed in heparin media of transduced HepG2 cells with EL adenovirus (EL-Ad) and EV-Ad by Western blotting. Protein size annotations refer to protein marker bands on the membrane.

First, we examined the size and structure of EL-HDL and control EV-HDL. As revealed by native gradient gel electrophoresis and subsequent protein staining EL-HDL particles were smaller in size compared to EV-HDL (Fig. 23A). This was further confirmed by electron microscopy, showing greater abundance of small (0 nm – 10 nm) and lower abundance of large (11 nm – 15 nm) particles in EL-HDL compared to EV-HDL (Fig. 23 B,C). The impact of EL on size and structure of HDL was determined by SAXS analysis showing a mean diameter of 133 Å for EL-HDL compared to 150 Å for EV-HDL (Fig. 23D). Furthermore, compared to EV-HDL, an increased distance distribution P(R) between 20 and 40 Å was observed for EL-HDL, indicating decreased lipid content in EL-HDL. In addition, compared to EV-HDL, EL-HDL exhibited decreased maximal spatial dimension, exemplified by reduced density distribution R > 80 Å and maximal diameter (D_{max}), respectively (Fig. 23E).

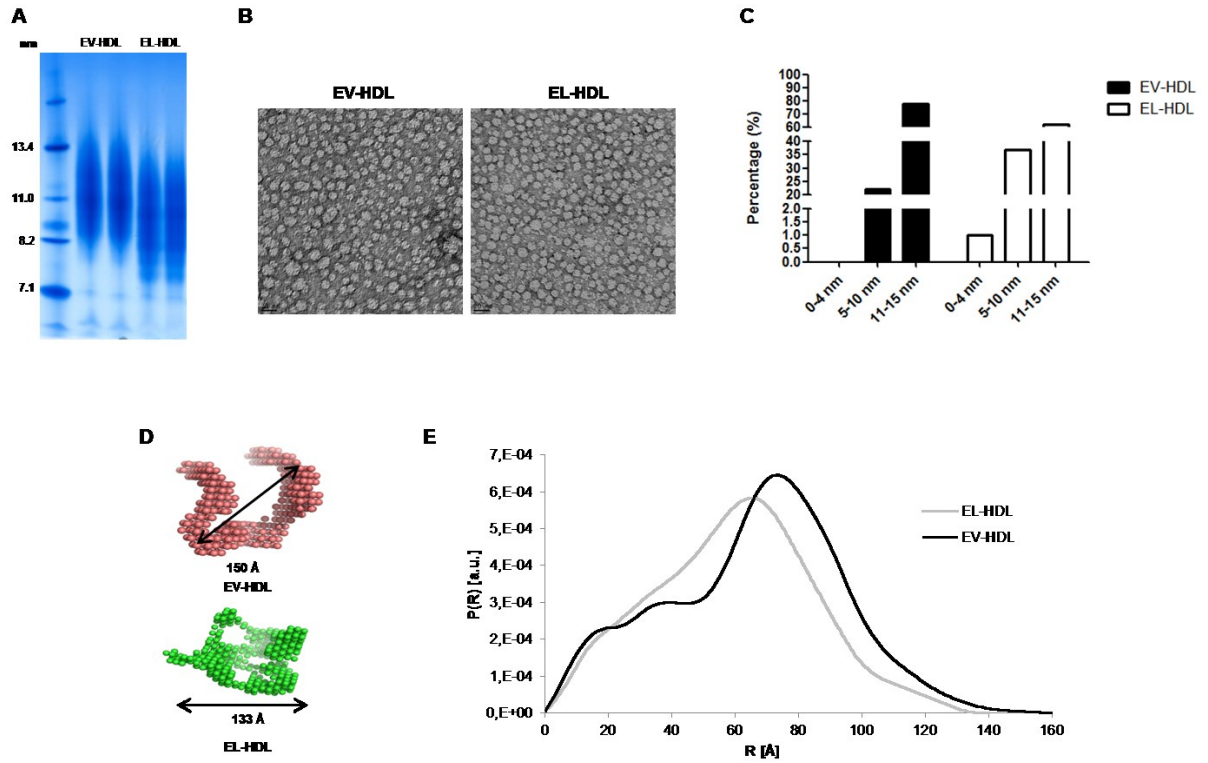

Figure 23: EL overexpression alters HDL size and lipid content

A) EV-HDL and EL-HDL (10 µg protein) were electrophoresed on 4-20% non-denaturing polyacrylamide gels followed by Coomassie Brillant Blue staining. Protein size annotations refer to protein marker bands on the gel B) Electron micrographs for EV- and EL-HDL. Particle window size was 20 nm. C) Calculated particle size distribution. The number of analyzed particles was n=1999 for EV-HDL and n=1336 for EL-HDL, shown as percentage. D) Ab initio shape models and E) Pairwise distance distribution function of modified HDL generated by SAXS analysis.

6.2 EL-HDL EXHIBITS ALTERED LIPID COMPOSITION

Next, the lipid composition of purified EV-HDL and EL-HDL was examined. As revealed by MS analysis, the levels of PC (Fig. 24A), PE (Fig. 24B), PI (Fig. 24C) and TAG (Fig. 24D) were significantly decreased and the levels of LPC (Fig. 24E), LPE (Fig. 24F), FA (Fig. 24G), FC (Fig. 24H) and Cer (Fig. 24J) were significantly increased in EL-HDL compared to EV-HDL. CE (Fig. 24I) and SM (Fig. 24K) content were similar in EL-HDL and EV-HDL.

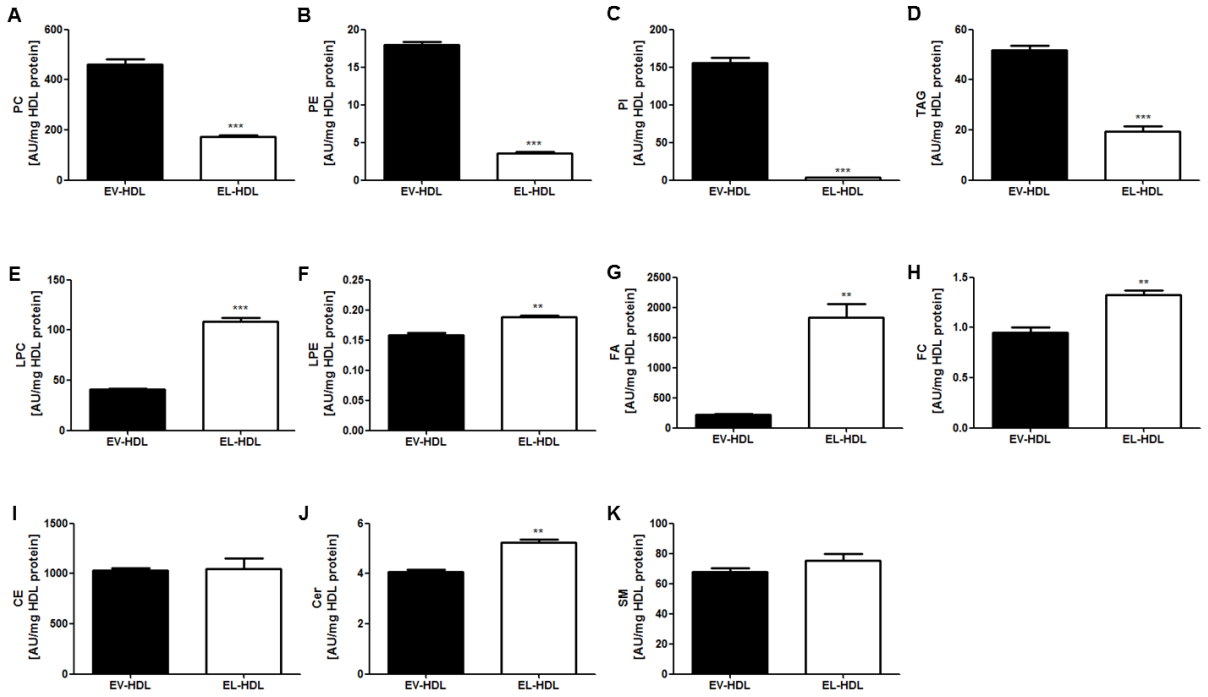

Figure 24: Lipid composition of purified EV-HDL and EL-HDL

Lipids from EV-HDL and EL-HDL (300 µg protein) purified by ultracentrifugation followed by FPLC were extracted and A) PC, (B) PE, (C) PI, (D) TAG, (E) LPC, (F) LPE, (G) FA, (H) FC, (I) CE, (J) Cer and (K) SM were analyzed by MS. Results are mean ± SEM of 3 independent modifications of human HDL. The differences between EV-HDL and EL-HDL were analyzed by two-tailed unpaired t-test between EV- and EL-HDL. $^{**}P < 0.01$, $^{***}P < 0.001$

6.3 HDL ASSOCIATED ENZYMES (PON1, LCAT AND PAF-AH) ARE DECREASED IN EL-HDL

Considering established antioxidative activities of HDL associated PON1, LCAT and PAF-AH [209], the mass and activity of these enzymes was determined. Interestingly, PON1 content (Fig. 25A) and its arylesterase activity (Fig. 25B) were significantly decreased in EL-HDL compared to EV-HDL, irrespective of HDL modification was done in the absence (w/o PON) or presence (w/ PON) of PON1 co-overexpression (Fig. 25 A,B). PON1 co-overexpression significantly increased the PON1 content and its arylesterase activity in EV- and EL-HDL (Fig. 25 A,B). Furthermore, LCAT and PAF-AH content were significantly decreased in EL-HDL compared to control (Fig. 25 C,D)

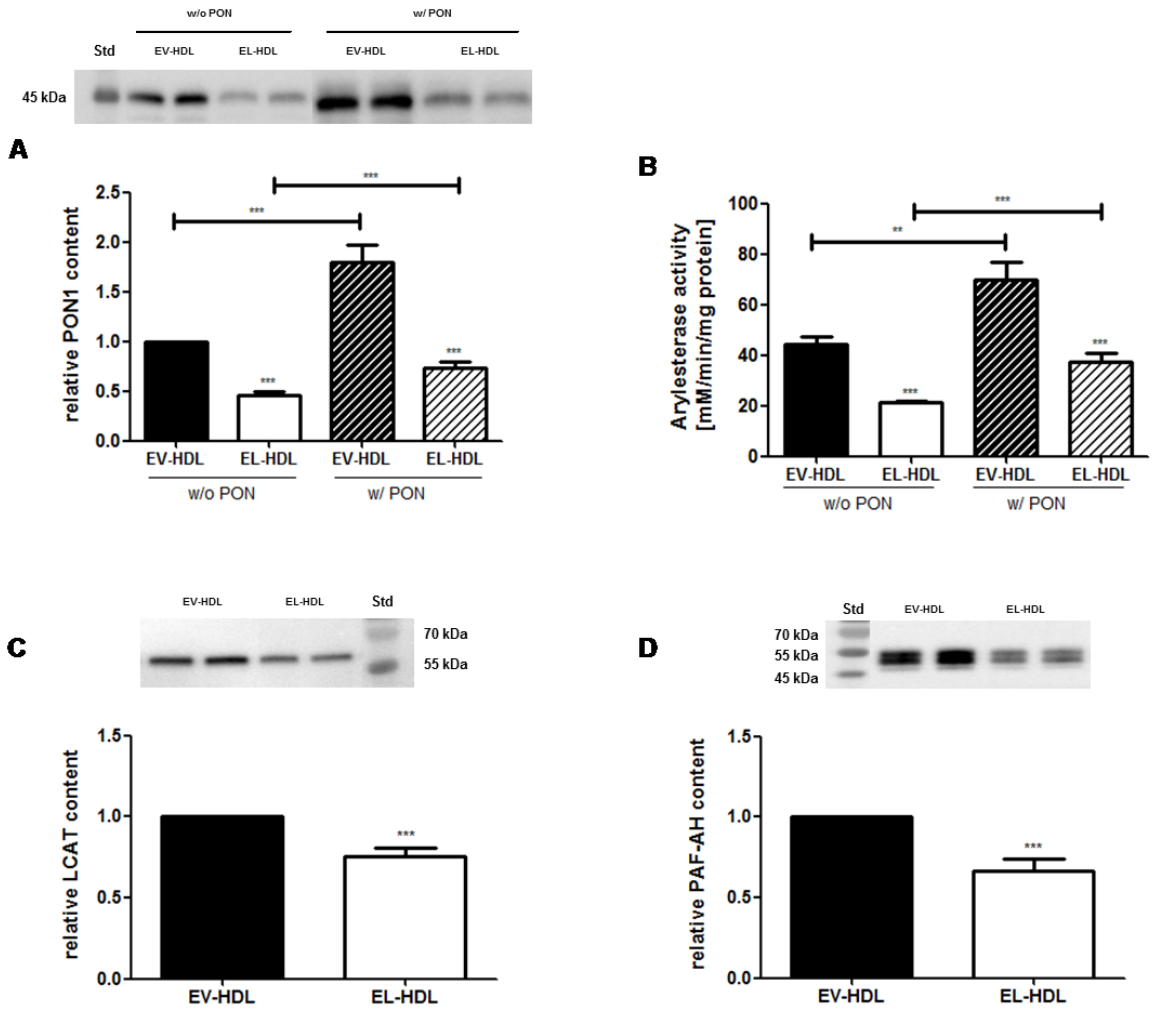

Figure 25: PON1, LCAT and PAF-AH are decreased in EL-HDL

A) Representative PON1 Western Blot of modified HDL (10 µg protein) without (w/o) or with (w/) PON1 co-overexpression. B) HDL associated PON1 arylesterase activity of modified EV-HDL and EL-HDL w/o or w/ PON1 co-overexpression. C) Representative LCAT Western Blot of modified HDL (10 µg protein). D) Representative PAF-AH Western Blot of modified HDL (10 µg protein). Protein size corresponds to the protein marker bands on the membranes. Densitometric analysis was done with Image Lab software. EV-HDL was set to 1 and all other conditions were normalized to EV-HDL. Results in A,C,D are mean ± SEM of 4 independent modifications of human HDL, each loaded in duplicates and analyzed by Image Lab software (Bio-Rad) and two-tailed unpaired t-test. Results in B are mean ± SEM of 5 independent modifications of human HDL, each measured in duplicates and analyzed by two-tailed unpaired t-test. **P < 0.01, ***P < 0.001

6.4 EL MEDIATED LIPOLYSIS IS RESPONSIBLE FOR DECREASED PON1 CONTENT

To address the impact of EL enzymatic activity on PON1 content and arylesterase activity, isolated HDL was incubated with HepG2 cells overexpressing enzymatically inactive EL (ELM) in the absence (w/o) or presence (w/) of PON1 co-overexpression. In the absence of PON1 co-overexpression, ELM-HDL exhibited significantly increased PON1 content and slightly but not significantly increased arylesterase activity compared to EV-HDL (Fig. 26 A,B). In the presence of PON1 co-overexpression, the PON1 content and arylesterase activity were similar in ELM-HDL and EV-HDL (Fig. 26 A,B). PON1 co-overexpression significantly increased the PON1 content in EV-HDL and slightly but not significantly increased that in ELM-HDL compared to respective samples generated in the absence of PON1 co-overexpression (Fig. 26A). In line with the PON1 content, the arylesterase activity was significantly increased upon PON1 co-overexpression in EV-HDL but not in ELM-HDL compared to samples generated in the absence of PON1 co-overexpression (Fig. 26B).

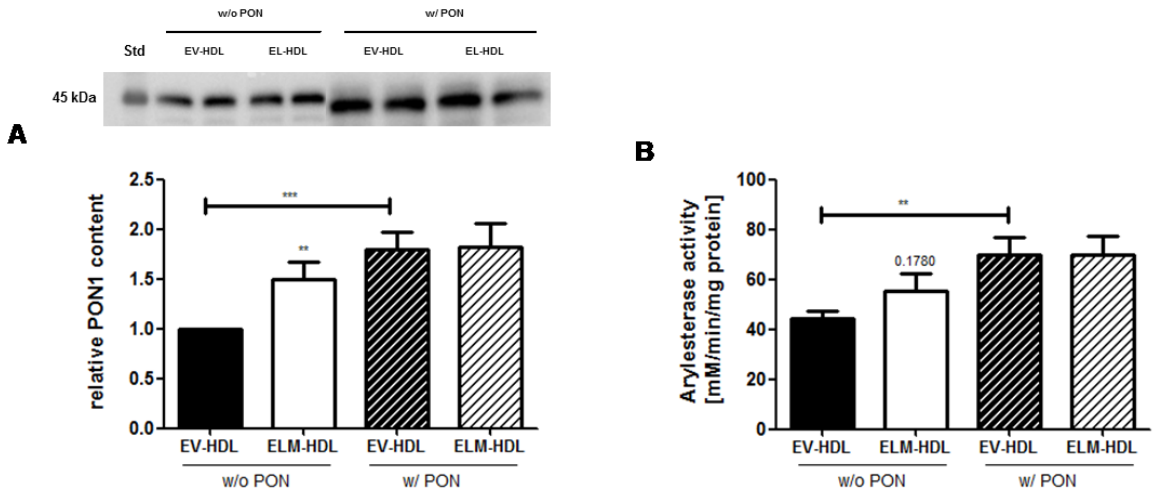

Figure 26: Enzymatically inactive EL overexpression increases PON1 content of HDL

A) Representative PON1 Western Blot of modified HDL (10 µg protein) without (w/o) or with (w/) PON1 co-overexpression. Protein size corresponds to the protein marker bands on the membrane. Densitometric analysis was done with Image Lab software. EV-HDL was set to 1 and all other conditions were normalized to EV-HDL. B) HDL associated PON1 arylesterase activity of modified EV- and ELM-HDL w/o or w/ PON1 co-overexpression. Results in A are mean ± SEM of 4 independent modifications of human HDL, each loaded in duplicates and analyzed by Image Lab software (Bio-Rad) and two-tailed unpaired t-test. Results in B are mean ± SEM of 5 independent modifications of human HDL, each measured in duplicates and analyzed by two-tailed unpaired t-test. **P < 0.01, ***P < 0.001

6.5 PON1 CONTENT AND ACTIVITY ARE NOT AFFECTED BY EL GENERATED LIPOLYTIC PRODUCTS

To investigate the impact of EL generated lipolytic products (LPCs and FFAs) on HDL associated PON1 content and activity, HDL modification was performed in the absence or presence of 4% BSA without or with co-overexpression of PON1, followed by removal of BSA bound lipolytic products by ultracentrifugation.

As expected, the LPC and FA content was significantly reduced in EV-HDL and EL-HDL generated in the presence of BSA compared to HDL modification without BSA (Fig. 27 C,D). However, similarly as found with material generated in the absence of BSA (Fig. 25) the PON1 content (Fig. 27A) and arylesterase activity (Fig. 27B) were significantly decreased in EL-HDL compared to EV-HDL, ruling out the role of EL generated lipolytic products in the reduction of HDL PON1 content and activity.

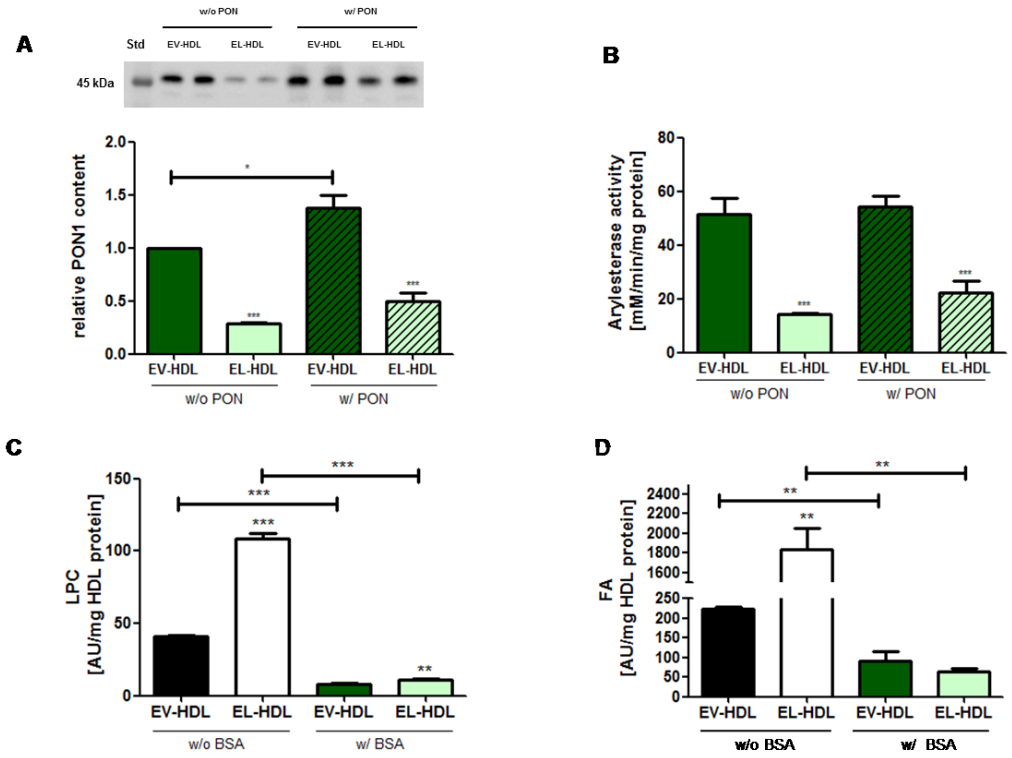

Figure 27: HDL-PON1 is not affected by EL generated lipolytic products

HDL modification was done in the presence of 4% BSA to remove lipolytic products. A) Representative PON1 Western Blot of modified HDL (10 µg protein) without (w/o) or with (w/) PON1 co-overexpression. Protein size corresponds to the protein marker bands on the membrane. Densitometric analysis was done with Image Lab software. EV-HDL was set to 1 and all other conditions were normalized to EV-HDL. B) HDL associated PON1 arylesterase activity of modified EV- and EL-HDL w/o or w/ PON1 co-overexpression. C,D) LPC and FA content without (w/o) or with (w/) addition of 4% BSA during HDL modification analyzed by MS. Results in A are mean ±

SEM of 4 independent modifications of human HDL, each loaded in duplicates and analyzed by Image Lab software (Bio-Rad) and two-tailed unpaired t-test. Results in B are mean ± SEM of 5 independent modifications of human HDL, each measured in duplicates and analyzed by two-tailed unpaired t-test. Results in C are mean ± SEM of 3 independent modifications of human HDL and analyzed by two-tailed unpaired t-test. *P < 0.05, **P < 0.01, ***P < 0.001

6.6 EL MODIFIED HDL EXHIBITS INCREASED CAPACITY TO PROTECT LDL FROM OXIDATION

After performing a thorough structural and compositional characterization of EL-HDL and EV-HDL, we examined the capacity of those HDL particles following purification by ultracentrifugation and FPLC (Fig. 28), to protect LDL from $CuCl_2$ induced oxidation as well as their resistance to copper induced oxidation. Compared to EV-HDL, EL-HDL exhibited higher anti-oxidative capacity exemplified by a significantly longer lag phase (Fig. 29 A,B,C) and significantly lower MDA formation (Fig. 29D) at all tested HDL concentrations. The lag phase could not be estimated for copper induced auto-oxidation of EV-HDL and EL-HDL, but the kinetics in Fig. 29 A and B clearly shows slower formation of conjungated dienes in EL-HDL compared to EV-HDL. Furthermore, significantly lower MDA levels in EL-HDL compared to EV-HDL (Fig. 29D) were observed, suggesting an increased oxidation resistance of EL-HDL.

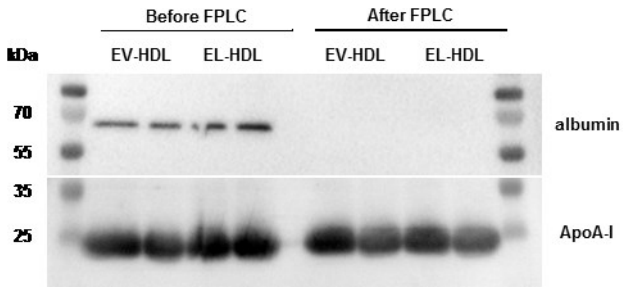

Figure 28: Albumin and ApoA-I Western Blot before and after purification of HDL

EV-HDL and EL-HDL were purified by ultracentrifugation followed by FPLC. Albumin and ApoA-I content in HDL were analyzed by SDS-PAGE and Western Blotting. Size annotations refer to protein marker bands on the membranes.

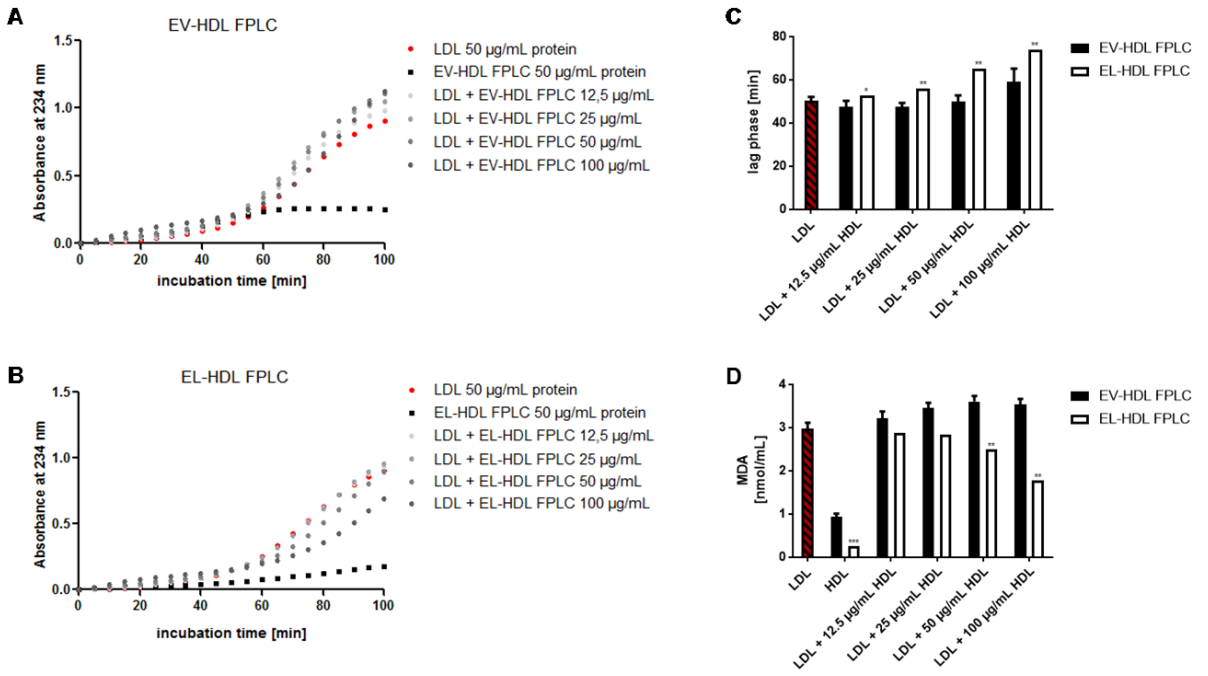

Figure 29: EL augments antioxidative capacity of HDL

Human LDL was oxidized by 2 µM $CuCl_2$ and the formation of conjungated dienes was monitored in the absence or presence of increasing concentrations of FPLC purified HDL at 234 nm. A,B) Representative kinetic graphs of LDL oxidation. C) For all concentrations, the lag phase was calculated. D) MDA levels were measured after termination of LDL oxidation by HPLC. Results are mean ± SEM of 4 independent modifications of human HDL and analyzed by two-tailed unpaired t-test between EV-HDL and EL-HDL for each concentration. *P < 0.05, **P < 0.01, ***P < 0.001

6.7 LIPOLYTIC PRODUCTS AFFECT THE ANTIOXIDATIVE ACTIVITY OF HDL

Because the action of EL on HDL massively increases the content of lipolytic products in HDL, we examined whether accumulated lipolytic products affect antioxidative capacity of HDL. For this purpose, HDL modification was performed in the presence of 4% BSA, followed by HDL purification by ultracentrifugation and FPLC to achieve a complete removal of BSA and BSA bound lipolytic products (Fig. 30).

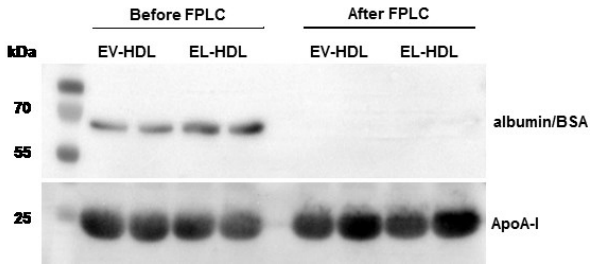

Figure 30: Albumin and ApoA-I Western Blot before and after purification of HDL

EV-HDL and EL-HDL modified in the presence of 4% BSA were purified by ultracentrifugation followed by FPLC. Albumin/BSA and ApoA-I content in HDL were analyzed by SDS-PAGE and Western Blotting. The antibody used for albumin/BSA detection was tested and recognizes human albumin as well as BSA (not shown). Size annotations refer to protein marker bands on the membranes.

Interestingly, although the duration of the lag phase was still significantly longer and the amount of generated MDA significantly lower in the presence of EL-HDL compared to EV-HDL (Fig. 31), the magnitude of the difference between EV-HDL and EL-HDL regarding duration of lag phase and MDA accumulation were markedly diminished upon removal of lipolytic products (compare Fig. 31 with Fig. 29). Accordingly, the results clearly show that the capacity of EL-HDL to protect LDL from oxidation was markedly attenuated upon removal of lipolytic products. For a better visualization to what extent the lipolytic products contribute to the antioxidative capacity of HDL, we calculated the lag phase- and the MDA-ratios, respectively of EV-HDL and EL-HDL. As shown in Fig. 32A, the lag phase-ratio of EV-HDL and EL-HDL was significantly higher at each HDL concentration for HDLs lacking lipolytic products (w/ BSA) compared to HDL containing lipolytic products (w/o BSA). This indicates shortening of the lag phase in incubations containing EL-HDL devoid of lipolytic products. Correspondingly, the MDA-ratios of EV-HDL and EL-HDL were lower (statistically significant at the two highest HDL concentrations) in the absence of lipolytic products (w/ BSA) compared to HDLs containing lipolytic products (w/o BSA), indicating increased accumulation of MDA in incubations with EL-HDL devoid of lipolytic products (Fig. 32B).

These results indicate that EL generated lipolytic products that accumulate in EL-HDL contribute but not fully explain higher capacity of EL-HDL to protect LDL from oxidation compared to EV-HDL. In contrast to diminished capacity of EL-HDL to protect LDL from oxidation, the capacity of EL-HDL to counteract auto-oxidation (in the absence of LDL) was not significantly affected by the removal of lipolytic products; MDA ratio of EV-HDL and EL-HDL was similar for HDLs containing or not lipolytic products (Fig. 32).

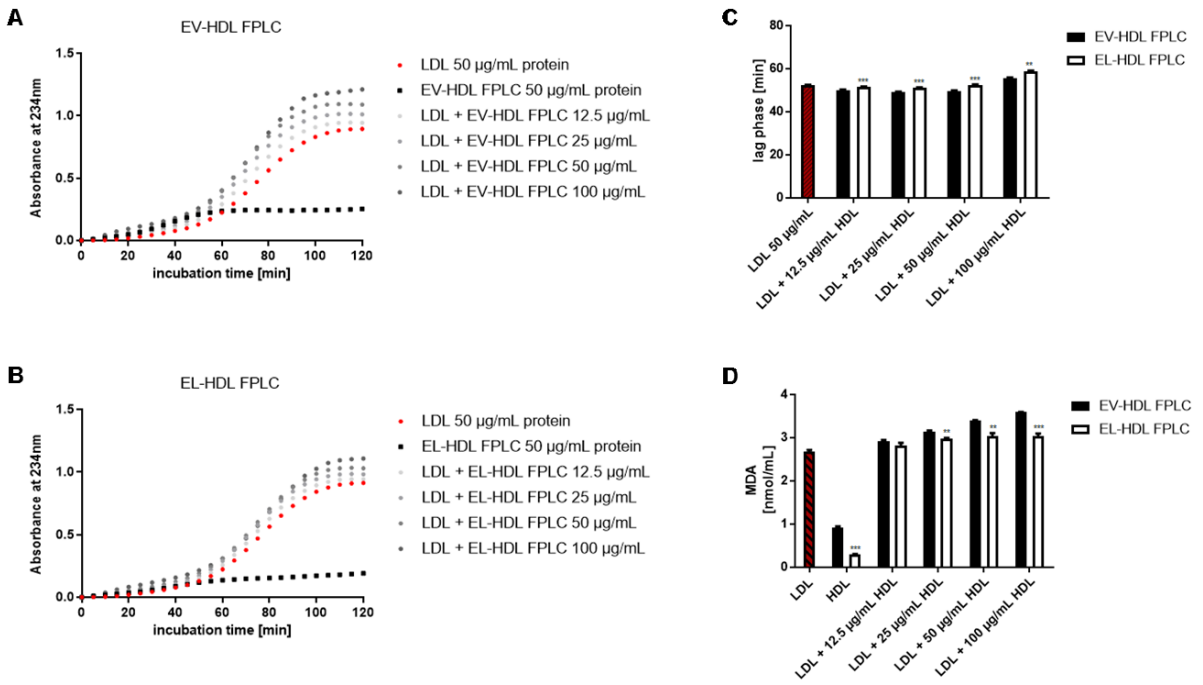

Figure 31: EL generated lipolytic products affect antioxidative capacity of HDL

EL and control overexpressing HepG2 cells were incubated with HDL in the presence of 4% BSA to generate modified EV-HDL and EL-HDL. To remove residual BSA bounded LPCs and FFAs, modified HDL was purified by ultracentrifugation, followed by FPLC. Human LDL was oxidized by 2 µM $CuCl_2$ and the formation of conjugated dienes was monitored in the absence or presence of modified HDL in different concentrations at 234 nm. A,B) Representative kinetic graphs of LDL oxidation. C) For all concentrations, the lag phase was calculated. D) MDA levels were measured after termination of LDL oxidation by HPLC. Results are mean ± SEM of 4 independent modifications of human HDL and analyzed by two-tailed unpaired t-test between EV- and EL-HDL for each concentration. **P<0.01, ***P < 0.001

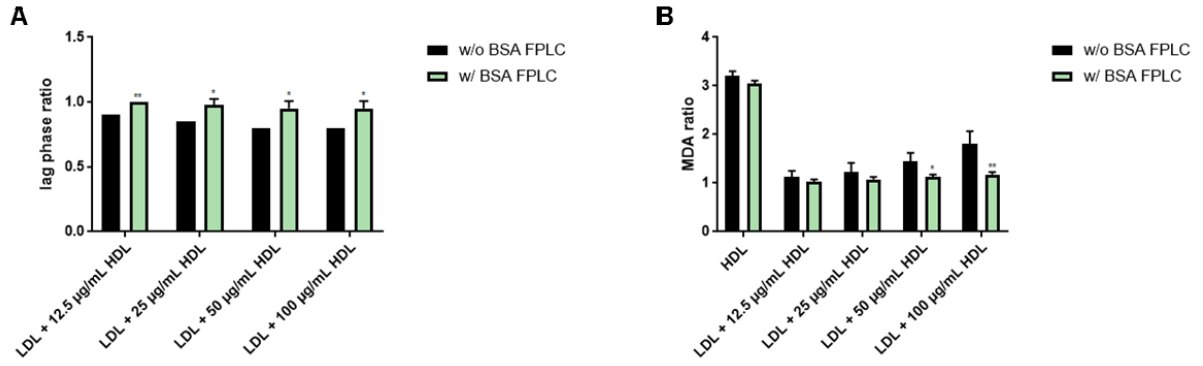

Figure 32: Lag phase (A) and MDA (B) ratios of EV-HDL and EL-HDL containing or not lipolytic products

The lag phase and MDA ratios of EV-HDL and EL-HDL calculated for experiments shown in Fig. 27 (w/o BSA) were compared with those from experiment Fig. 29 (w/ BSA). *P < 0.05, **P < 0.01

6.8 REVERSE RELATIONSHIP BETWEEN SIZE AND ANTIOXIDATIVE CAPACITY OF SIZED EL-HDL PARTICLES

Previous studies reported a size dependent antioxidative capacity of HDL showing that small HDL particles protect better against LDL oxidation compared to large ones [209]. Due to these findings and the fact that lipolytic products do not fully explain the better antioxidative capacity of EL-HDL, we examined whether particle size affects HDL antioxidative capacity in our experimental model. To test this, we prepared small, medium and large EL-HDL (Fig. 33A), using FPLC and used those differently sized EL-HDL particles to test their resistance to $CuCl_2$ induced oxidation and the capacity to delay LDL oxidation and MDA formation. We observed a trend of a reverse relationship between HDL particle size and the duration of the lag phase (Fig. 33B) as well as a trend for a link between HDL particle size and MDA accumulation (Fig. 33C). Statistical significance between small and large particles to delay LDL oxidation was only observed when $CuCl_2$ induced LDL oxidation was measured in the presence of 25 µg/mL HDL (Fig. 33B). In contrast to EL-HDL, differently sized EV-HDL did not show any trend of relationship between HDL particle size and antioxidative capacity (data not shown). Furthermore, interestingly the smallest EL-HDL particles exhibited the lowest PON1 content (Fig. 34), providing additional proof that PON1 is not responsible for the better antioxidative capacity of EL-HDL.

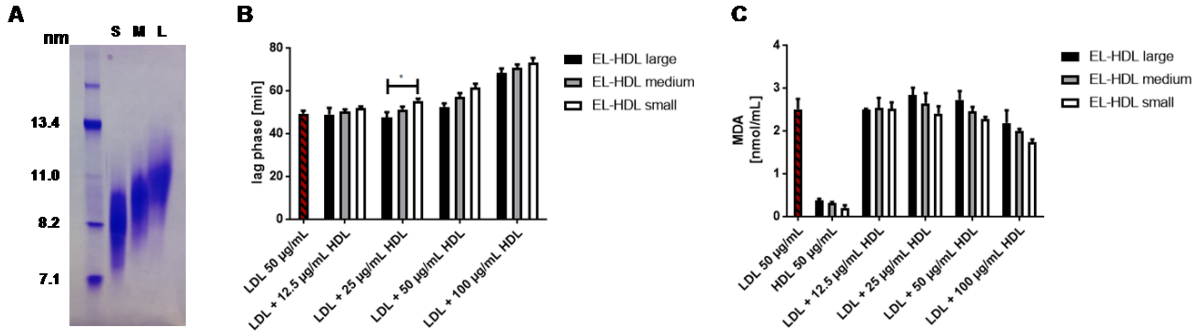

Figure 33: Relationship between EL-HDL size and antioxidative capacity

The modified EL-HDL was purified and separated into three size groups by FPLC. A) These three groups, S (small), M (medium) and L (large) EL-HDLs (10 µg protein) were electrophoresed on 4-20% non-denaturing polyacrylamide gels followed by Coomassie Blue staining. B) Human LDL was oxidized by 2 µM $CuCl_2$ and the formation of conjungated dienes was monitored in the absence or presence of separated EL-HDL in different concentrations at 234 nm. For all conditions, the lag phase was calculated. C) MDA levels were measured after termination of LDL oxidation by HPLC. Results are mean ± SEM of 3 independent modifications of human HDL and analyzed by one-way ANOVA. *P < 0.05

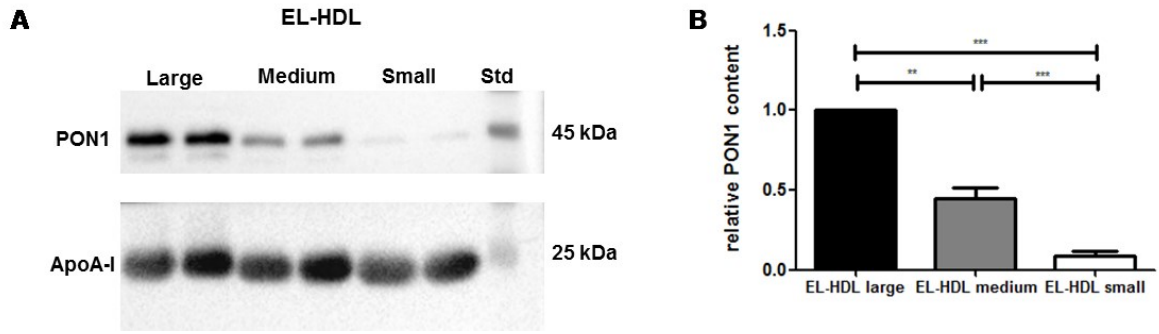

Figure 34: Small EL-HDL exhibits the lowest PON1 content

The modified EL-HDL was purified and separated into three size groups by FPLC followed by Western Blotting against PON1 and ApoA-I. Protein size annotations refer to protein marker bands on the membranes. Densitometric analysis was done with Image Lab software. EL-HDL large was set to 1 and others were normalized to EL-HDL large. Results are mean ± SEM of 3 independent modifications of human HDL, each loaded in duplicates and analyzed by Image Lab software (Bio-Rad) and two-tailed unpaired t-test. ***P* < 0.01, ***P < 0.001

6.9 THE RATE OF APOA-I MET RESIDUE OXIDATION UPON EXPOSURE TO OXLDL IS DIFFERENT BETWEEN EV-HDL AND EL-HDL

Previous studies have established that HDL size and structure influence the conformation of HDL associated proteins, primarily ApoA-I, thereby affecting exposition and accessibility of antioxidant Met residues and free SH groups [209, 210]. Therefore, we investigated the contribution of ApoA-I Met residues to the antioxidative capacity of EL-HDL. For this purpose, purified EV-HDL and EL-HDL were incubated in the absence or presence of $CuCl_2$ oxidized LDL up to 120 min at 37°C, followed by quantification of ApoA-I Met sulfoxide levels using proteomics. The levels of ApoA-I Met136 sulfoxides were significantly higher (Fig. 35A) in EL-HDL compared to EV-HDL after 120 min. incubation of HDL with oxLDL. In contrast, the levels of Met172 sulfoxides (Fig. 35B) as well as of other ApoA-I Met sulfoxides (data not shown) were similar in EV-HDL and EL-HDL. Incubation of EV-HDL and EL-HDL in the absence of oxLDL resulted in very low and similar levels of ApoA-I Met136 and Met172 sulfoxides in EV-HDL and EL-HDL (data not shown).

Based on these results, we concluded that the ApoA-I Met136 residues in EL-HDL exhibit higher antioxidative activity compared to those residues in EV-HDL, which at least in part may contribute to a higher antioxidative capacity of EL-HDL.

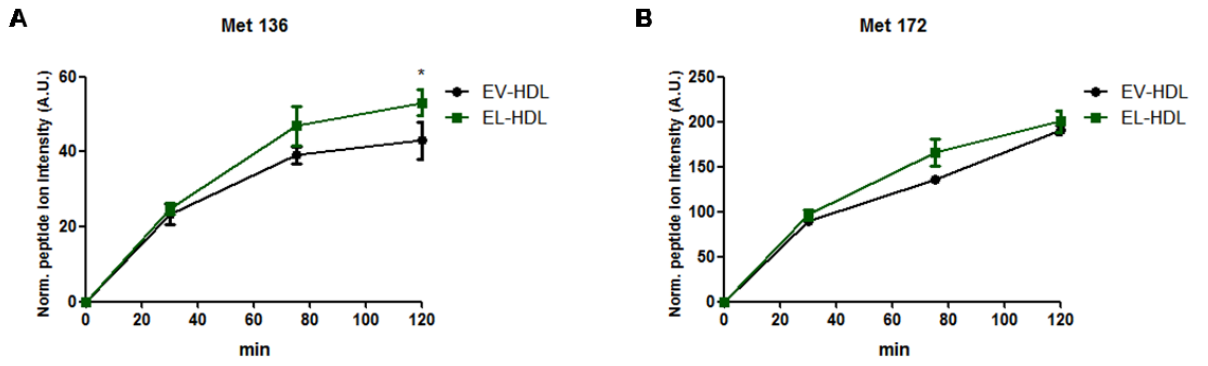

Figure 35: ApoA-I Met residue 136 is more oxidized in EL-HDL compared to EV-HDL

$CuCl_2$ oxidized LDL (50 µg) was incubated with EV-HDL or EL-HDL (50 µg) for 30, 75 and 120 min at 37°C, followed by stopping the reaction with acetone. ApoA-I Met sulfoxides were measured by proteomics. Results are mean ± SEM of 3 independent modifications of human HDL and analyzed by two-tailed unpaired t-test between EV- and EL-HDL for each time-point. *P < 0.05

6.10 CHLORAMINE T SENSITIVE MECHANISMS ARE NOT RESPONSIBLE FOR THE DIFFERENT ANTIOXIDATIVE CAPACITY OF EV-HDL AND EL-HDL

To examine whether more reactive Met residues (and free SH groups) may explain the better antioxidative capacity of EL-HDL, we examined the antioxidative capacity of EV-HDL and EL-HDL following chloramine T mediated oxidative inactivation of Met residues and free SH groups [23, 230]. To determine the efficiency of chloramine T treatment, EV-HDL and EL-HDL were incubated with different concentrations of chloramine T (500 µM, 1 mM, 1.5 mM and 2 mM) followed by analysing the Met sulfoxide levels by proteomics. The Met sulfoxide levels increased in a dose-dependent manner. In detail, using a concentration of 500 µM approximately 75-80% of all Met residues in ApoA-I were oxidized, while a concentration of 2 mM chloramine T oxidized 85-90% of all ApoA-I Met residues. Modification with 2 mM of chloramine T was used for further experiments.

Chloramine T abolished the capacity of EV-HDL and EL-HDL to protect LDL from oxidation exemplified by shorter lag phases (Fig. 36 A,B,C) and higher MDA levels (Fig. 36D) in the presence of EV-HDL and EL-HDL, compared to LDL alone. Interestingly, however the duration of the lag phase in the presence of EL-HDL was still significantly longer (Fig. 36C) and the levels of MDA were significantly lower (Fig. 36D) at each HDL concentration compared to EV-HDL. Furthermore, the oxidation of EV-HDL and EL-HDL in the absence of LDL was not affected by chloralmine T (Fig. 36D), exemplified by a similar rate of MDA formation and a

similar difference between EV-HDL and EL-HDL regarding MDA content in the samples without (Fig. 29D) and with chloramine T (Fig. 36D).

From these results we concluded that: 1) chloramine T sensitive components of HDL largely contribute to the capacity of EV-HDL and EL-HDL to protect LDL from oxidation, 2) do not affect the capacity of EV-HDL and EL-HDL to counteract auto-oxidation in the absence of LDL, and 3) importantly, chloramine T sensitive mechanisms are not responsible for the difference between EV-HDL and EL-HDL regarding their capacity to protect LDL from oxidation.

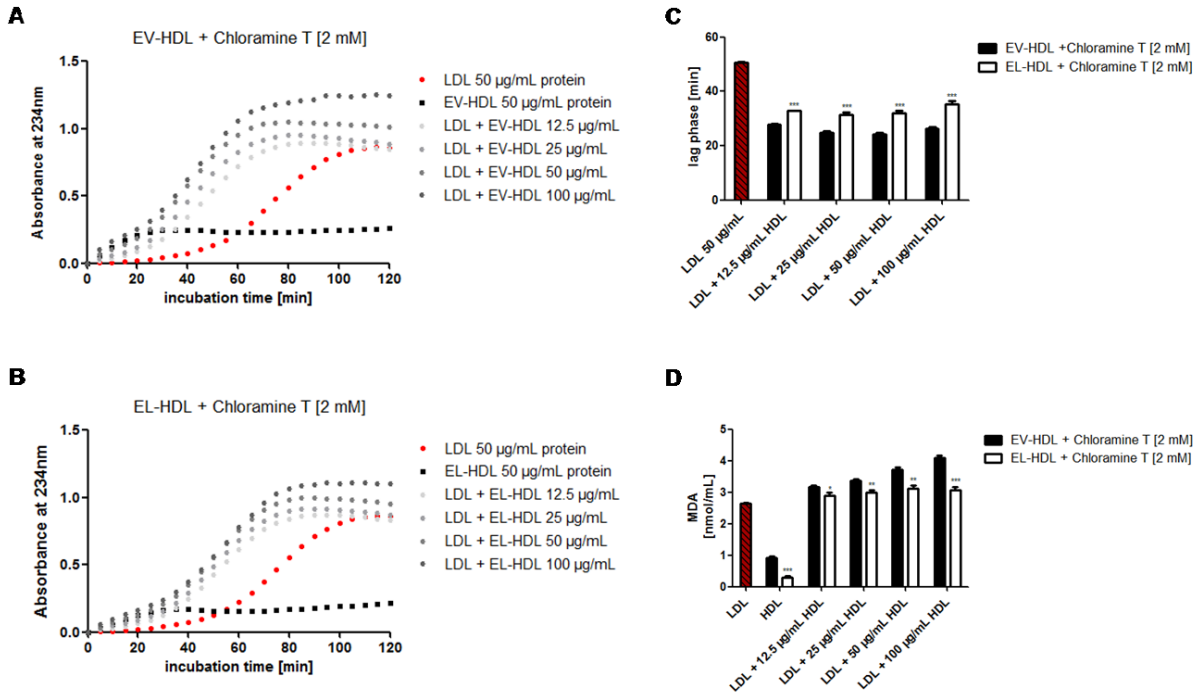

Figure 36: Chloramine T sensitive antioxidative activity affects the antioxidative capacity of modified HDL

ApoA-I Met resides and free SH groups of purified EV- and EL-HDL (1.5 mg/mL) were oxidized by chloramine T (2 mM) for 1h at 4°C, followed by monitoring the formation of conjungated dienes at 234 nm. A,B) Representative kinetic graphs of LDL oxidation. C) For all concentrations, the lag phase was calculated. D) MDA levels were measured after termination of LDL oxidation by HPLC. Results are mean ± SEM of 2 independent modifications of human HDL, measured in duplicates and analyzed by two-tailed unpaired t-test between EV- and EL-HDL for each time-point. *P < 0.05, **P < 0.01, ***P < 0.001

7 DISCUSSION

It has been established that the capacity of HDL to protect LDL from oxidation *in vivo* and *in vitro* is determined by structural features and composition of HDL [209]. Reports that focused on the ability of HDL from EL deficient mice in delaying LDL oxidation and its relationship to HDL associated enzymes showed inconclusive data [41, 42]. Due to the fact that EL alters structure and composition of HDL [45, 142], the present study was designed to examine the impact of EL overexpression *in vitro* on the antioxidative properties of HDL.

To test the hypothesis that EL affects the antioxidative activity of HDL, HDL was modified by incubation with EL overexpressing cells followed by monitoring the resistance of EL-modified HDL to $CuCl_2$ induced oxidation as well as its capacity to delay the initiation of LDL oxidation (conjungated dienes formation) and to attenuate the formation of LOOH decomposition product, MDA. Quantification of LOOH generated in the propagation phase of LDL oxidation was not measured in the present study.

Interestingly, EL modification of HDL *in vitro* markedly enhanced the antioxidative capacity of HDL, reflected in a prolonged initiation of LDL oxidation (lag phase) and a decreased MDA accumulation. This result contrasts with an increased antioxidative capacity of HDL isolated from EL deficient mice [42]. It remains to be investigated whether the similar impact of EL overexpression and EL deficiency on the capacity of HDL to protect LDL from oxidation can be explained by species specific differences or discrepancies between *in vivo* and *in vitro* approaches. However, a better antioxidative capacity of HDL from EL deficient mice was only observed in one [42] but not in another study [41].

Previous studies have demonstrated that small dense HDL particles reveal higher antioxidative properties than larger, less dense particles [209]. It has been described that the better antioxidative capacity of small HDL particles is determined by several mechanisms and molecular components, including the enrichment in PON1, LCAT, PAF-AH [215] as well as the relative accumulation of ApoA-I [135]. Furthermore, a decrease in HDL particle size causes conformational changes in ApoA-I leading to a better exposure and antioxidative activity of ApoA-I Met residues [209, 210]. Additionally, an altered lipid composition and packing of surface lipids which facilitate insertion of exogenous molecules including LOOH was observed in small HDL particles [217].

A striking compositional feature of EL-HDL was enrichment with lyso-PLs and FAs, whose removal upon incubation with BSA and purification of HDL by ultracentrifugation and

FPLC significantly attenuated the antioxidative capacity of EL-HDL pointing toward an important role of lipolytic products generated by EL in the antioxidative capacity of HDL. This positive effect of LPC enrichment observed in the present study may be due to LPC induced increase in HDL surface fluidity [231] and in turn decreased packing of surface lipids accompanied by augmented capacity of HDL to incorporate oxidation products including LOOH. Further experiments are required to test whether enrichment of EL-HDL, prepared in the presence of BSA and accordingly devoid of lyso-PLs, with LPC restores the antioxidative capacity of EL-HDL.

The fact that EL overexpression mediates the generation of smaller HDL particles could partially explain the better antioxidative capacity of EL-HDL in this study. Indeed, we fractionated EL-HDL by FPLC into small, medium and large particles and found a clear trend for an inverse relationship between EL-HDL size and its antioxidative capacity. Interestingly, this was not the case with small, medium and large EV-HDL particles which all showed completely similar antioxidative capacity without any trend of relationship with HDL particle size (not shown). This finding suggests that not (only) the size but possibly the composition of differently sized particles, such as a different rate of enrichment with lipolytic products, affects the antioxidative capacity of HDL particles.

Furthermore, if HDL particle number outperforms HDL mass regarding antioxidative capacity, application of HDL on a protein basis, as was the case in our assays, might additionally explain the higher antioxidative capacity of EL-HDL; due to its smaller size EL-HDL is more abundant in terms of particle number compared to larger and therefore less abundant EV-HDL particles.

In contrast to the findings that small dense HDL particles are enriched in HDL associated enzymes [177, 209, 215], the PON1 content and activity as well as the LCAT and PAF-AH amounts were markedly decreased in EL-HDL. Aviram et al [182, 184, 232, 233] have shown that free SH groups of PON1 protect HDL [232] and LDL [233] from oxidation. However, the smallest EL-HDL particles isolated by FPLC demonstrated the highest antioxidative capacity and the lowest PON1 content arguing against PON1 and PON1 associated SH groups being responsible for the augmented antioxidative capacity of EL-HDL. Considering the role of HDL-PLs in the association of PON1 with HDL [234, 235], the reduced PL content and size of EL-HDL could be an explanation for the decreased PON1 content in EL-HDL. Most recent data showed that the specific PCs in the human carotid plaque have the ability to interact with PON1 and exert a positive effect on its activity [236]. The increase in FC content of EL-HDL

could be also responsible for the reduced PON1 activity, in line with results from a previous study demonstrating a significant reduction in HDL associated PON1 activity in the presence of non-esterified cholesterol [237]. In the present study, overexpression of enzymatically inactive EL resulted in an increased HDL-PON1 content, indicating that EL by its bridging function, similarly as found for SR-BI [238] augments loading of HDL with PON1, thus highlighting the importance of EL lipolytic activity in displacing PON1 from HDL.

As shown in our present and previous studies, EL by acting on HDL generates various LPCs and FFAs causing enrichment of HDL with lipolytic products [45, 142]. Considering this and results from another study reporting decreased LPC content in the population of HDL particles containing PON1 [239] suggests that increased LPC content of EL-HDL might be responsible for decreased PON1. However, a reduced PON1 content and activity were also observed in EL-HDL after the removal of LPCs by BSA, arguing against the responsibility of accumulated LPCs for the decreased PON1 content in EL-HDL.

It is well documented that the antioxidative capacity of HDL is regulated by the abundance and conformation of ApoA-I, whereby inactivation of LOOHs by HDL is driven by ApoA-I Met residues [157]. Indeed, incubation of EV-HDL and EL-HDL with oxLDL resulted in significantly higher oxidation rate of ApoA-I Met136 in EL-HDL compared to EV-HDL, suggesting a more pronounced antioxidative activity of Met136 in ApoA-I of EL-HDL. As described previously [209], this finding could be explained by an altered conformation of ApoA-I, due to an EL induced reduction in particle size as well as an altered lipid content and composition, which may facilitate the exposure of that Met residues to the aqueous phase resulting in its augmented antioxidative capacity. Interestingly, despite the fact that chloramine T abolished the capacity of both EV-HDL and EL-HDL to protect LDL from oxidation, the difference in the duration of the lag phase and MDA accumulation between EV-HDL and EL-HDL were not affected by chloramine T. These findings indicate that chloramine T sensitive mechanisms largely contribute to the overall antioxidative capacity of EV-HDL and EL-HDL but are not responsible for better antioxidative capacity of EL-HDL.

The removal of LOOHs from LDL for subsequent reduction to inactive hydroxides by Met residues of ApoA-I is negatively affected by the enrichment of HDL surface lipids with FC, SM or Cer [209]. Accordingly, accumulation of FC and Cer within EL-HDL, possibly as a consequence of an enhance cholesterol efflux from HepG2 cells triggered by EL generated LPCs during HDL modification [75], contrasts with the observed increased antioxidative capacity of EL-HDL. However, considering results obtained by structural modeling of lipoprotein

particles [217], one can speculate that the pronounced EL induced perturbations in HDL size and composition caused by EL promote redistribution of CE to the HDL surface, leading to disturbed packing of surface lipids, which in turn promotes an efficient insertion of LOOHs (despite increased FC and Cer) and conceivably facilitates displacement of PON1. Besides, a recent study demonstrated a negative correlation between the TAG content of HDL and HDL antioxidative capacity [213]. Therefore, the decreased TAG levels in EL-HDL could be another explanation for the increased antioxidative activity.

Taken together, EL modification of HDL *in vitro* generates small HDL particles with altered lipid and protein composition and augmented antioxidative capacity. While alterations in the abundance and activity of HDL associated proteins such as ApoA-I, PON1, LCAT and PAF-AH do not contribute to the better antioxidative capacity of EL-modified HDL, decreased particle size and altered lipid composition, in particular enrichment with EL generated lipolytic products seems to underlie augmented antioxidative capacity of EL-modified HDL.

8 CONCLUSION

The results obtained in this study indicate that the EL mediated decrease in HDL particle size and alteration in lipid composition, especially the enrichment of lipolytic products, are responsible for the better antioxidative capacity of EL-modified HDL. Alterations in the abundance and activity of HDL associated proteins such as ApoA-I, PON1, LCAT and PAF-AH do not contribute to the better antioxidative capacity of EL-modified HDL.

The importance of EL overexpression *in vitro* on the structure, composition and antioxidative capacity is summarized in figure 37.

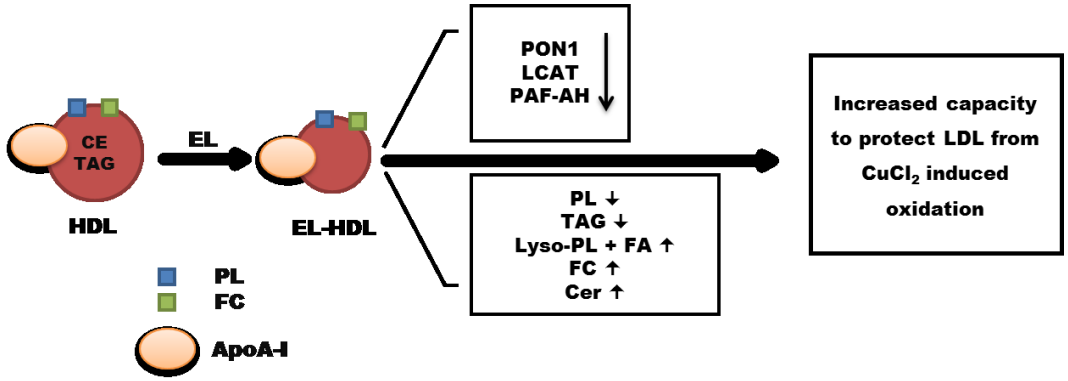

Figure 37: EL overexpression *in vitro* enhances the capacity of HDL to protect against LDL oxidation
EL-HDL was generated by incubation of isolated HDL with EL overexpressing cells. EL-HDL is smaller in size with a decreased PL and TAG content as well as increased Lyso-PL, FA, FC and Cer levels. EL overexpression resulted in a reduced abundance and activity of HDL associated proteins such as PON1, LCAT and PAF-AH. The capacity of EL-modified HDL to protect LDL from $CuCl_2$ induced oxidation as well as the resistance to copper induced oxidation was enhanced, reflected in a prolonged lag phase and less MDA accumulation.

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