Pharmacological modulation of endothelial lipase - mediated HDL remodeling

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SVEUČILIŠTE U ZAGREBU
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Zagreb, 2017.

PSS studij: Klinička farmacija

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Rad ima 38 listova.

Preface

My thesis was done at the Institute for Molecular Biology and Biochemistry, Center of Molecular Medicine at Medical University of Graz.

My research was part of two main projects from PI Prof. dr.sc. Saša Frank, "Endothelial Lipase, high-density lipoprotein and endothelial dysfunction" and "Endothelial Lipase and HDL functionality: Studies in CAD patients".

I would like to thank dear Prof. Frank and my dear friends Snježana, Andrijana, Irene, Margarete and Therese for all their help, assistance and funny moments that we shared.

I also thank doc. dr. sc. Suzana Mimica Matanović, dr. med. for accepting mentoring.

Sažetak

Poznato je da su koncentracije kolesterola u lipoproteinu visoke gustoće (HDL) inverzno povezane s kardiovaskularnim rizikom. Farmakološki postupci s ciljem povećanja HDL-a, kojima bi se rizik smanjio, do sada nisu bili uspješni (inhibicija kolesterol ester transfer proteina; CETP). Nadalje, studije o HDL-u na pacijentima s dijabetesom, koronarnom arterijskom bolesti (CAD) te drugim upalnim bolestima povezanim s funkcionalnošću HDL-a dale su jasan dokaz da:

a) koncentracija HDL-a nije pouzdan prediktor kardiovaskularnog rizika

b) brojnost i veličina čestica HDL-a te

c) funkcionalnost HDL-a snažni su i nezavisni prediktori kardiovaskularnog rizika i smrtnosti.

Prijašnji i sadašnji eksperimenti s endotelnom lipazom (EL) jasno pokazuju da enzim svojom fosfolipaznom aktivnošću utječe na fosfolipide HDL-a. Primarno, na fosfatidillkolin (PC) čime dolazi do promjena u strukturi i funkcionalnosti HDL-a. Taj HDL nazvan je EL-HDL. EL- HDL je veličinom manji od kontrolnog HDL-a. Sadrži manje fosfatidilkolina ali više lizofosfatidilkolina (LPC). EL-HDL pokazuje slabiji afinitet vezanja za receptore (SR-BI; scavenger receptor class B type I), smanjenu mogućnost posredovanja pri transportu kolesterola iz makrofaga (efflux), smanjenu količinu i aktivnost enzima paraoksonaza-1 (PON-1) te promijenjenu aktivnost endotelne dušik oksid sintetaze (e-NOS) koja utječe na vazodilataciju posredovanu dušikovim monoksidom (NO).

Učinak endotelne lipaze na HDL remodeliranje proučavan je u *in vitro* modelu stanične kulture HepG2 stanica inficiranih adenovirusima za endotelnu lipazu (EL), kolesterol ester transfer protein (CETP) te lecitin-kolesterol-acil-transferazu (LCAT), uz pripadajuće kontrole, kako bismo dobili ekspresiju spomenutih enzima. Nakon inkubacije seruma zdravih dobrovoljaca s HepG2 stanicama uz odsutsvo/prisutstvo inhibitora CETP-a (torcetrapib) i LCAT-a (DTNB-a (5,5-ditiobis-2-nitrobenzoična kiselina)), HDL je izoliran iz svakog uzorka seruma. Uzorci HDL-a su promatrani s obzirom na veličinu i sastav (sveukupni kolesterol, trigliceridi, fosfolipidi, sadržaj PON1) te funkcionalnost (PON-1 aktivnost, eNOS inducibilnu aktivnost, kapacitet prijenosa kolesterola iz makrofaga). Metode korištene u eksperimentu su bazirane na radu sa staničnim kulturama, gel elektroforezi, Western blotu, dostupnim kitovima i metodama bojenja lipida i proteina.

Cilj eksperimenta je bio ispitati učinak CETP inhibitora (torcetrapiba) i LCAT inhibitora (DTNB-a) na veličinu, sastav i funkcionalnost EL-HDL-a.

Rezultati pokazuju da je EL-HDL dobar supstrat za oba enzima – CETP, LCAT. Modulacija enzimima i inhibitori utječu na lipidni sastav EL-HDL-a, dok je veličina ostala ista, ali i dalje manja od kontrolnog HDL-a. Funkcionalnost EL-HDL-a je uvijek smanjena u usporedbi s kontrolnim HDL-om. Zaključno, farmakološkom modulacijom *in vitro* nije došlo do poboljšanja, spoznato narušenih kardioprotektivnih mehanizama EL-HDL-a u usporedbi s kontrolnim HDL-om.

Summary

It is well established that HDL concentrations in plasma estimated by measuring cholesterol in HDL (HDL-cholesterol in clinical laboratory results, HDL-C = good cholesterol) is inversely related to cardiovascular risk. However, the failure of recent pharmacological attempts (inhibition of cholesterol ester transfer protein; CETP) to decrease cardiovascular events by increasing HDL-C levels, together with additional studies showing that HDL from patients with various pathologies (diabetes, CAD, inflammation) is dysfunctional, provided clear evidence that:

a) HDL-C levels are not reliable predictors of cardiovascular risk

b) HDL particle number and size as well as

c) HDL- functionality are strong and independent predictors of cardiovascular events and mortality.

Previous and current work clearly demonstrated that EL (endothelial lipase) by its phospholipase activity very potently cleaves HDL phospholipids, primarily phosphatidylcholine (PC), thereby generating structurally and functionally altered HDL, referred to as EL-HDL. EL-HDL is smaller in size, depleted in PC but enriched in lysophosphatidylcholine (LPC). It exhibits diminished binding to SR-BI (scavenger receptor class B type I), a reduced capacity to mediate cholesterol efflux from macrophages, decreased paraoxonase 1 (PON1)-content and altered capacity to induce endothelial NO (nitric oxide) production and to promote vasorelaxation.

Generation of modified HDL was achieved by incubation of healthy human pool serum with HepG2 cells transduced with adenoviruses encoding EL, CETP or LCAT. After isolation of modified HDL from serum (without and with inhibitors, after overexpression) following steps and methods were performed:

- Structural/compositional analysis of HDL (native gradient electrophoresis followed by Sudan staining and Coomassie staining, lipid composition analysis using kit measurements, PON1 Western Blots).
- Functionality assessment of HDL (HDL-associated PON1-activity, capacity of HDL to induce endothelial e-NOS activity and HDL cholesterol efflux capacity).

The aim was to examine the impact of CETP inhibitor – torcetrapib and LCAT inhibitor -DTNB, on EL-induced alterations in structural and functional properties of HDL.

Results showed that EL-modified HDL is a good substrate for CETP and LCAT. *In vitro*, both Torcetrapib and DTNB affected EL-HDL composition with no effect on the EL-HDL size. Furthermore, functionality of EL-HDL was decreased when compared to control HDL. *In vitro* pharmacological inhibition of CETP or LCAT activity failed to affect the impact of these enzymes on the functionality of EL-HDL.

Contents

1. Introduction

Atherosclerotic coronary artery disease (CAD) and its complications remain the leading cause of death in industrialized countries. In recent years, intensive lowering of low-density lipoprotein (LDL) cholesterol using statins has been established as an effective therapy to lower cardiovascular risk. However, the risk of major cardiovascular events in patients with CAD on optimal medical therapy, including statins, remains in the range of 20% after 3 years of follow-up after an acute coronary syndrome. Several lines of evidence have suggested that high-density lipoprotein (HDL) may act as an anti-atherogenic lipoprotein. HDL-targeted therapies are therefore intensely pursued as a potential novel anti-atherogenic strategy to reduce cardiovascular risk and are an important frontier of basic, translational and clinical cardiovascular research. Low high-density lipoprotein (HDL)-cholesterol levels are associated with an increased risk of coronary artery disease (CAD) and myocardial infarction, which has triggered the hypothesis that HDL, in contrast to low-density lipoprotein (LDL), acts as an anti-atherogenic lipoprotein. Moreover, experimental studies have identified potential anti-atherogenic properties of HDL, including promotion of macrophage cholesterol efflux and direct endothelial-protective effects of HDL, such as stimulation of endothelial nitric oxide production and repair, anti-apoptotic, anti-inflammatory and anti-thrombotic properties. Studies in gene-targeted mice, however, have also indicated that increasing HDL-cholesterol plasma levels can either limit (e.g. ApoA1; apolipoprotein A-I) or accelerate (e.g. SR-BI; scavenger receptor class B type I) atherosclerosis. Moreover, vascular effects of HDL have been observed to be heterogenous and are altered in patients with CAD or diabetes - ‘HDL dysfunction’ (1).

These alterations in biological functions of HDL may need to be taken into account for HDL-targeted therapies and considering raising of HDL-cholesterol levels alone is likely not sufficient in this respect. It will therefore be important to further determine, which biological functions of HDL are critical for its anti-atherosclerotic properties, as well as how these can be measured and targeted (1). Initially, the concept that HDL may protect from CAD was suggested by numerous epidemiological studies, indicating that low plasma levels of HDL cholesterol or apoA1, the major protein component of HDL, are associated with an increased risk of CAD and CAD-related cardiovascular events in the general population (2). Studies have suggested that also in patients with CAD and very low LDL cholesterol levels on statin treatment, low plasma concentrations of HDL cholesterol remained associated with an increased risk of cardiovascular events. Low HDL cholesterol levels have also been suggested as a primary lipid abnormality in patients with premature CAD. However, low plasma HDL cholesterol levels may not generally be associated with accelerated atherosclerosis (3).

1.1. Biosynthesis of HDL

The first step of the biosynthesis of HDL entails the synthesis and secretion of apoA-I, the major protein constituent of HDL, by the liver and intestine. The second most abundant HDL protein, apoA-II, is synthesized only in the liver and its secretion results in the formation of a subclass of HDL particles containing apoA-I and apoA-II. Following secretion in the liver and intestine, lipid-poor apoA-I immediately acquires cholesterol and phospholipids, in particular from hepatocytes and enterocytes. The initial lipidation of apoA-I is primarily mediated by ABCA-1 (ATP-binding cassette transporter A-1) and results in the formation of nascent HDL. Nascent HDL acquires additional phospholipids and free cholesterol from extrahepatic tissues (1).

In addition, HDL acquires phospholipids and potentially apolipoproteins (such as apoC-III) during hydrolysis of triglyceride-rich lipoproteins. The transfer of phospholipid surface remnants from triglyceride-rich lipoproteins to HDL is mediated via the phospholipid transfer protein (PLTP). Phospholipids transferred via PLTP are not only an important structural component of the HDL surface, but also serve as substrate for the esterification of HDL-associated cholesterol. Moreover, PLTP promotes the fusion of smaller HDL3 particles and subsequent generation of larger HDL2 particles. During this process, lipid-poor apoA-I is shed off and can again undergo lipidation for regeneration of pre-beta-HDL. In transgenic animal models, increased systemic levels of PLTP have been shown to promote atherosclerosis in mice and rabbits. PLTP deficiency in atherosclerosis-prone mice results in decreased atherosclerotic plaque formation. These findings have been attributed, at least in part, to changes in apolipoprotein B (apoB) production and plasma cholesterol levels in these animals, since PLTP also stimulates hepatic secretion of VLDL (very low-density lipoprotein), possibly by phospholipidation of nascent apoB (1).

Following efflux of cholesterol and phospholipid transfer from peripheral tissues, a proportion of HDL-associated free cholesterol in plasma is esterified to cholesteryl ester by lecithin–cholesterol acyltransferase (LCAT). Although LCAT activity results in the formation of a hydrophobic core in HDL and thus is important for maturation of the HDL particle, recent studies in subjects with LCAT mutations and LCAT-transgenic mice have suggested that LCAT likely does not play a major role for efficient transport of cholesterol from peripheral tissues back to the liver. As described above, the findings with respect to the vascular phenotype of subjects with LCAT mutations are heterogenous (1).

Hepatic SR-BI (scavenger receptor class B type I) can selectively take up cholesteryl ester and unesterified cholesterol and thus directly deliver HDL-associated cholesterol to the liver. Alternatively, in humans and some other species, HDL cholesterol can be transferred to apoB-containing lipoproteins in exchange for triglycerides via the cholesteryl ester transfer protein (CETP) and subsequently be cleared by LDL receptor-mediated uptake of apoB-containing lipoproteins to the liver. In transgenic mice (that normally lack CETP) with CETP gene transfer, the transport of cholesterol from macrophages to the liver was increased. However, studies on the effect of CETP on development of atherosclerosis in mice have yielded mixed results. Whereas overexpression of CETP increased atherosclerosis in wild-type and hypercholesterolemic mouse, a decreased atherosclerotic plaque burden was observed in CETP transgenic hypertriglyceridemic mice and in CETP transgenic mice overexpressing LCAT, despite lower plasma HDL cholesterol levels in these animals. In rabbits (that are expressing CETP), inhibition of CETP by JTT-705 (known as dalcetrapib) attenuated atherosclerosis. Because CETP inhibition may change the composition of HDL particles and give rise to large HDL particles enriched in cholesterol esters, another study sought to characterize the ability of HDL from CETP-deficient subjects to mediate cholesterol efflux from macrophage foam cells. However, the cholesterol efflux potential of HDL isolated from human subjects with homozygous CETP deficiency was rather increased compared to normolipidemic control subjects. Importantly, whether pharmacological CETP inhibition by different CETP inhibitors affects other antiatherogenic properties of HDL in patients with CAD remains to be tested in future studies (1).

1.2. Molecular mechanisms mediating potential atheroprotective functions of HDL: alterations in cardiovascular disease

In recent years, several properties or functions of HDL have been identified that could exert anti-atherosclerotic effects. Besides promoting macrophage cholesterol efflux and RCT (reverse cholesterol transport), HDL has more recently been shown to exert direct potentially antiatherosclerotic effects on endothelial cells, such as the direct stimulation of endothelial nitric oxide production by HDL or endothelial anti-inflammatory effects and anti-oxidant effects. Recent evidence suggests that vascular effects of HDL are altered in patients with cardiovascular disease (1).

In recent years, it has become clear that HDL from healthy subjects can exert direct potential atheroprotective effects on endothelial cells and the understanding of the vascular effects of HDL considerably changed with the important observation that HDL may directly stimulate endothelial NO synthase (eNOS) mediated NO (nitric oxide) production as well as induce endothelium dependent, NO-mediated vasodilation via endothelial SR-BI (Fig.1) (4). Endothelial NO plays a crucial role in the regulation of vascular tone and structure and importantly, endothelial NO has been shown to exert a variety of atheroprotective effects in the vasculature, such as anti-thrombotic, anti-coagulant, anti-inflammatory and pro-fibrinolytic effects. In addition, experimental studies have consistently demonstrated the capacity of HDL to modify eNOS expression as well as activity and to stimulate endothelial NO production *in vitro* and *in vivo*. Several different mechanisms have been proposed to account for the endothelial NO-stimulating capacity of HDL (5). Early studies have suggested that HDL acts by preventing the detrimental effects of oxidized LDL on endothelial NO-synthase while a subsequent study by Yuhanna et al. suggested that

HDL can bind to endothelial SR-BI and thus directly stimulate eNOS-mediated NO production (6).

Exact mechanism of molecular pathway that activates eNOS in endothelial cells stimulated with HDL remains to be further elucidated (1).

1.3. Potential HDL-targeted therapies

According to a recent classification the HDL targeting drugs that are currently tested in preclinical or clinical studies are divided into four groups:

1) directly augmenting apoA-I levels (apoA-I infusions, up-regulators of endogenous apo A-I production),

2) indirectly augmenting apoA-I and HDL cholesterol levels (CETP inhibitors, niacin, endothelial lipase inhibitors),

3) mimicking the functionality of apoA-I (i.e. apoA-I mimetics),

4) enhancing RCT (liver receptor agonists, LCAT activators).

Most of these HDL-targeted drugs are still in preclinical or early clinical investigation being tested (7).

CETP inhibitors

The interest in pharmacological CETP inhibition as novel therapeutic approach to raise HDL cholesterol levels was kindled by the finding of elevated HDL cholesterol levels in Japanese families with genetic CETP deficiency. However, the first compound tested in large clinical trials, torcetrapib, did not to reduce the progression of carotid atherosclerosis in patients with familial hypercholesterolemia receiving statin treatment and was associated with progression of disease in the common carotid segment. Of note, these effects occurred despite a pronounced increase in

HDL cholesterol levels and a substantial decrease in LDL cholesterol and triglycerides (7). More importantly, in the **I**nvestigation of **L**ipid **L**evel **M**anagement to **U**nderstand its **I**mpact in **A**therosclerotic **E**vents (ILLUMINATE) study, a large clinical outcome trial characterizing the effects of torcetrapib on major cardiovascular events in patients at high cardiovascular risk who were receiving statins, torcetrapib therapy was associated with an increased risk of cardiovascular events and death from any cause. This was, at least in part, due to an increase in blood pressure, systemic aldosterone levels and alterations in serum electrolytes related to 'off-target' effects of the compound on aldosterone and cortisol synthesis in adrenal cortical cells (8). Moreover, torcetrapib administration to spontaneously hypertensive rats impairs endothelial NO production and increases production of reactive oxygen species as well as endothelin-1 in the endothelium, leading to endothelial dysfunction in these animals. Other CETP inhibitors were/are currently in clinical trials, i.e. dalcetrapib, anacetrapib and evacetrapib. In contrast to torcetrapib, none of these agents has been shown to alter blood pressure, electrolytes or serum aldosterone (9).

1.4. Heterogenity of the HDL proteome and HDL lipidome

Recent proteomics studies have identified between 28 and 67 HDL-associated proteins using liquid chromatography–electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) (10). Interestingly, these proteomics studies of HDL have found not only proteins with well-characterized roles in lipid metabolism and antioxidant properties of HDL, but also a number of proteins involved in the acute phase response, complement regulation and proteinase inhibition. Of note, the protein composition of HDL may differ between healthy subjects and patients with

CAD, suggesting a potential remodeling of the HDL particle in these patients. A combined statin and niacin therapy partially reversed changes in the HDL proteome observed in patients with CAD, yielding a protein composition that resembled that in apparently healthy age- and sex-matched control subjects. However, whether changes in the HDL proteome are of relevance for the altered vascular effects of HDL in patients with CAD needs to be examined in future studies. Posttranslational modifications of apoA-I have been shown to impair the cholesterol efflux capacity of HDL (Fig 1). In particular, myeloperoxidase (MPO), a hypochlorous acid (HOCl)-generating enzyme that is enriched in human atheroma, can modify apoA-I and induce chloro- and nitrotyrosine formation as well as methionine oxidation. Modification of HDL by MPO leads to a profound impairment of the cholesterol efflux capacity of HDL and an impaired capacity of HDL to stimulate endothelial NO production. Besides MPO-mediated oxidation, modification of HDL by malondialdehyde, an advanced lipid oxidation product, has been shown to impair the cholesterol efflux capacity of HDL *in vitro*, suggesting that lipid oxidation itself can induce alterations of HDL-associated proteins (1).

Considerably less is known about the HDL lipidome. It has been demonstrated that HDL is enriched in phosphatidylcholine, lysophosphatidylcholine, phosphatidyl-ethanolamine and phosphatidylethanolamine-based plasmalogens, when compared to other lipoprotein fractions in plasma (11). However, a comprehensive and detailed profiling of the molecular lipid species in HDL including information on the type of fatty acids and the position where they are attached to the glycerol backbone is not yet available. Furthermore, it remains unknown whether the HDL-associated lipid profile differs in patients with stable CAD or acute coronary syndrome. Interestingly, it has been demonstrated that the phosphatidylcholine species composition of HDL

influences its anti-inflammatory activity, suggesting that the lipid composition might be an important determinant of HDL functionality. However, this needs to be tested in more detail in future studies (1).

1.5. Role of PON1 for lipid oxidation and impaired endothelial protective effects of HDL in patients with CAD

PON1 (paraoxonase-1) is an HDL-associated esterase/lactonase that has been shown to protect against lipid peroxide formation in LDL and HDL. The activity of HDL-associated PON1 was profoundly impaired in HDL from patients with CAD as compared to HDL from age and gender-matched healthy subjects. Inhibition of HDL-associated PON1, as observed in patients with CAD, led to an increase of MDA-lysine adducts in HDL that subsequently activated endothelial protein kinase C beta II (PKCbII) via the endothelial LOX-1 receptor (lectin-like oxidized LDL receptor 1) (Fig.1) (12).

An important open question in the field remains which alterations of the atheroprotective functions of HDL are related to adverse clinical cardiovascular outcome in CAD patients. It is presently unknown which potential atheroprotective function of HDL, or which particular protein or lipid marker in HDL, may be suited best to assess the antiatherogenic capacity of HDL. It seems likely that only raising HDL with vasoprotective properties can be expected to exert beneficial cardiovascular effects (1).

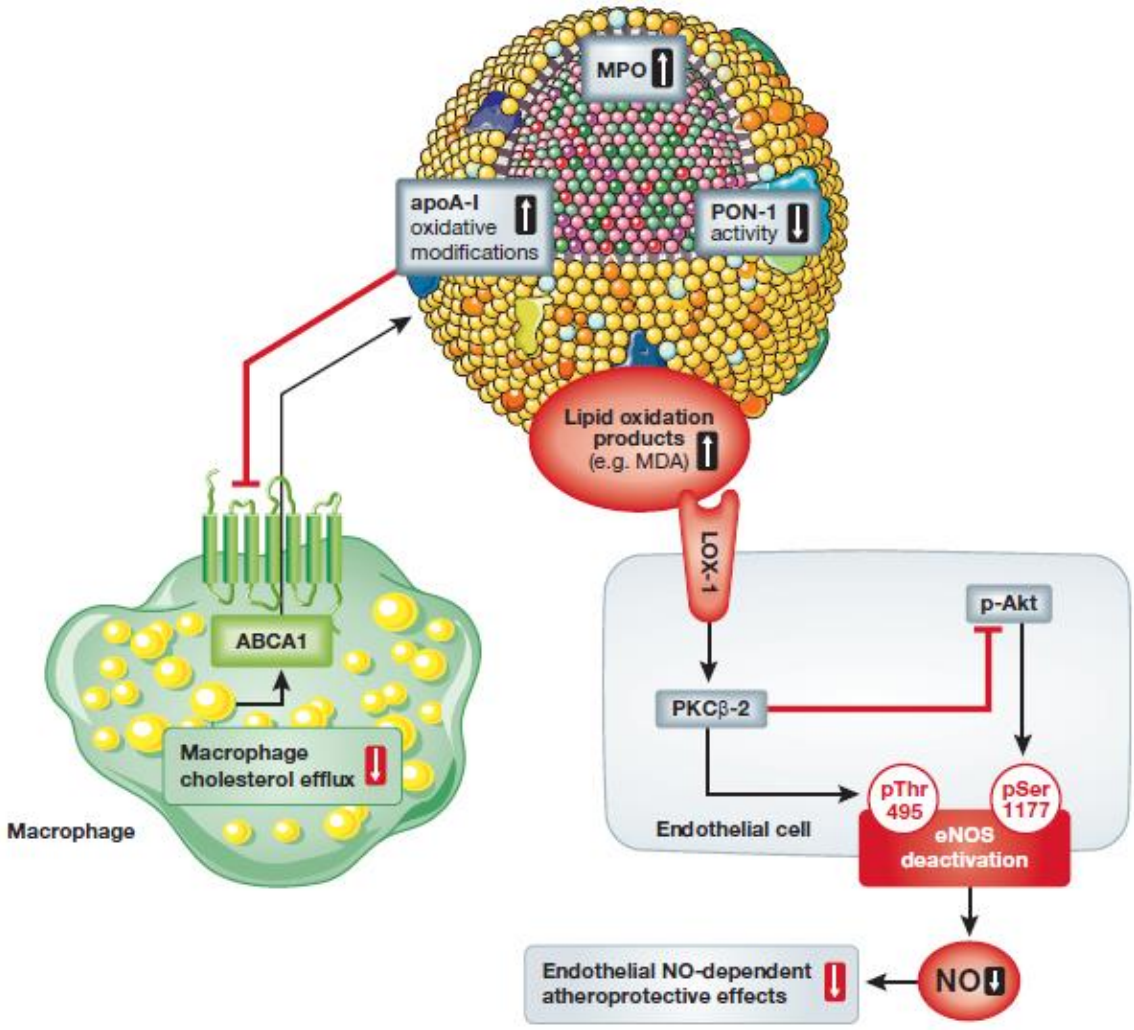


Figure 1. Alterations of the atheroprotective effects of HDL in patients with CAD.

Modification of apoA-I by MPO has been shown to impair the macrophage cholesterol efflux capacity of HDL. More recently, accumulation of MDA in HDL from patients with CAD due to an impaired HDL-associated PON1 activity has been observed to stimulate activation of endothelial PKCbeta-II via the LOX-1 receptor. PKCbeta-II activation by HDL from patients with CAD inhibited Akt-dependent phosphorylation of eNOS at serine residue 1177 and increased the inhibitory eNOS phosphorylation at threonine 495, leading to reduced endothelial NO production (1).

1.6. HDL remodeling

Remodeling and catabolism of HDL is the result of interactions of HDL with cell receptors and other membrane and plasma proteins including hepatic lipase (HL), endothelial lipase (EL), phospholipid transfer protein (PLTP), cholesteryl ester transfer protein (CETP), apolipoprotein M (apoM), scavenger receptor class B type I (SR-BI), ATP-binding cassette transporter G1 (ABCG1), the F1 subunit of ATPase (Ecto F1-ATPase), and the cubulin/megalin receptor. Following synthesis by the liver and the intestine, HDL is remodeled by various plasma proteins and is subsequently catabolized in the plasma by cell receptors and other plasma proteins (Fig. 2.) (13).

HDL remodeling affects the structure and metabolic turnover of HDL and generates a dynamic mixture of discrete HDL subfractions that vary in size, shape, apolipoprotein, and lipid composition and functions. Remodeling of HDL by the action of hepatic lipase (HL) and endothelial lipase (EL) involves hydrolysis of residual triglycerides and some phospholipids of HDL leads to the conversion of HDL2 to HDL3 and preβ-HDL and accelerates the catabolism of HDL. Portion of the cholesteryl esters formed by the actions of LCAT can be transferred to VLDL/IDL/LDL by the cholesteryl ester transfer protein (CETP). The phospholipid transfer protein (PLTP) can transfer the phospholipids from VLDL/IDL to the HDL particle during lipolysis to generate HDL2 and can also convert HDL3 particles to HDL2 and preβ-HDL. HDL-binding proteins/receptors or transporters have been documented at all steps of HDL metabolism and involve the SR-BI, which is mostly expressed in hepatocytes, macrophages, and steroidogenic tissues and mediates selective CE uptake by the cells and tissues and cholesterol efflux; the ABCG1, which mediates cholesterol efflux. HDL is first remodeled in the circulation and subsequently catabolized by cells and tissues. Hepatic lipase (HL) and endothelial lipase (EL) are two plasma lipases

playing an important role in HDL remodeling. HL and EL have specificity primarily for phospholipids and to a lesser extend for triglycerides of apoB containing lipoprotein remnants and large HDL. Recent study demonstrated that targeted inactivation of both HL and EL in mice promoted macrophage-to-feces RCT (reverse cholesterol transport) and enhanced HDL antioxidant properties. HL-deficient patients have elevated plasma concentrations of cholesterol in the HDL and β-VLDL and increased concentration of triglycerides and phospholipids in the LDL and HDL. Analyses carried out in complete and partial HL-deficient subjects as well as in normotriglyceridemic and hypertriglyceridemic controls suggested that HL activity is important for physiologically balanced HDL metabolism. However, the presence of HL may not be necessary for normal HDL-mediated reverse cholesterol transport process and is not associated with pro-atherogenic changes in HDL composition and metabolism (13).

Endothelial lipase (EL) is an enzyme with phospholipase activity (mostly PLA1 activity) with additional weak triglyceride lipase activity. Overexpression of EL in mice markedly decreased plasma HDL cholesterol and apoA-I levels, had a modest effect on apoB-containing lipoproteins, and increased 2.5–3-fold the uptake of the HDL by the kidney and the liver. In contrast, the EL deficiency in mice increased HDL cholesterol levels and reduced atherosclerosis in the background of apoE/mice. Overexpression of EL in mice markedly decreased plasma HDL cholesterol and apoA-I levels and had a modest effect on apoB-containing lipoproteins. Furthermore, the HDL phospholipid and cholesteryl ester content decreased, while HDL triglyceride content increased and the free cholesterol content remained unaltered. Fast protein liquid chromatography analysis and agarose gel electrophoresis showed that the expression of EL resulted in the generation of small preβ-HDL particles. Data

support a model in which EL-mediated phospholipid hydrolysis of HDL destabilizes the particle, resulting in the shedding of poorly lipidated apoA-I from the particle surface, which are preferentially cleared by the kidneys and via increased selective uptake by SR-BI. Several genetic EL variants have been reported to be associated with plasma HDL-C levels, and genome-wide association studies have shown that single-nucleotide polymorphisms (SNPs) are associated with plasma HDL-C levels. However, the relationship of genetic variation in the EL locus with the risk for coronary artery disease remains uncertain. A newer study showed that carriers of an EL mutant characterized by complete loss of function had significantly higher plasma HDL cholesterol levels compared to carriers having partial loss-of-function mutations (13).

1.7. HDL cargo

Along proteins, variety of lipids are also carried by HDL, and some of them are or can be transformed to potent bioactive molecules. Furthermore, HDL carries and transports fat soluble vitamins, steroid hormones, carotenoids, as well as numerous more polar metabolites such as heneicosanoic acid, pentitol, and oxalic acid which were found to be significantly correlated with insulin resistance. It has been reported that HDL also transports small RNAs, including microRNAs, tRNA-derived RNA fragments, and RNase P-derived RNA fragments. How all this protein and lipid decoration affects HDL metabolism and HDL particle function remain to be studied (14).

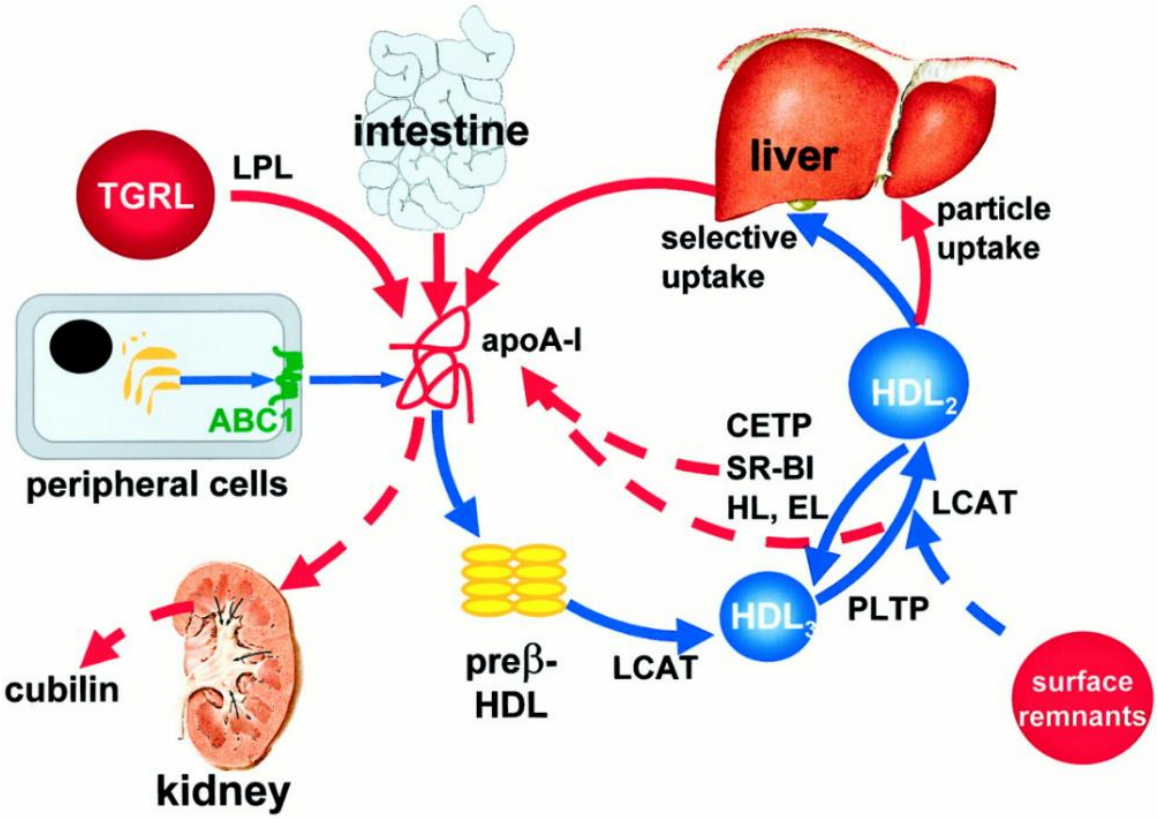


Figure 2. HDL remodeling.

Mature HDL3 and HDL2 are generated from lipid-free apoA-I or lipid-poor pre-β1-HDL as the precursors. These precursors are produced as nascent HDL by the liver or intestine or are released from lipolysed VLDL and chylomicrons or by interconversion of HDL3 and HDL2. ABC1-mediated lipid efflux from cells is important for initial lipidation; LCAT-mediated esterification of cholesterol generates spherical particles that continue to grow on ongoing cholesterol esterification and PLTP-mediated particle fusion and surface remnant transfer. Larger HDL2 particles are converted into smaller HDL3 particles on CETP-mediated export of cholesteryl esters from HDL onto apoB-containing lipoproteins, on SR-BI–mediated selective uptake of cholesteryl esters into liver and steroidogenic organs, and on HL- and EL-mediated hydrolysis of phospholipids. HDL lipids are catabolized either separately from HDL proteins (by selective uptake or via CETP transfer) or together with HDL proteins (ie, via uptake through as-yet-unknown HDL receptors or apoE receptors). The conversion of HDL2 into HDL3 and the PLTP-mediated conversion of HDL3 into HDL2 liberated lipid-free or poorly lipidated apoA-I. A part of lipid-free apoA-I undergoes glomerular filtration in the kidney and tubular reabsorption through cubilin. Blue arrows represent lipid transfer processes, and red arrows represent protein transfer processes. TGRL indicates triglyceride-rich lipoproteins (14).

2. Objective

Our previous and current work clearly demonstrated that EL by its phospholipase activity very potently cleaves HDL phospholipids, primarily phosphatidylcholine (PC), thereby generating structurally and functionally altered HDL, referred to as EL-HDL. EL-HDL is smaller in size, depleted in PC but enriched in lysophosphatidylcholine (LPC) and free fatty acids. It exhibits diminished binding to SR-BI, a reduced capacity to mediate cholesterol efflux from macrophages, decreased paraoxonase 1 (PON1)-content and altered capacity to induce endothelial NO production and to promote vasorelaxation (15-19).

In the present study, we aimed to examine the impact of CETP and LCAT as well as their inhibitors, torcetrapib and DTNB, respectively, on structural and functional properties of EL-HDL.

3. Methods and materials

Sequential Modification of HDL:

1. Transduction of HepG2 cells with empty-adenovirus (EV) (1μL/mL) used as a control, and EL adenovirus (0.35 μL/mL).

2. Serum modification: Human pool-serum (50%; diluted 1:1 with cell culture medium) was incubated with EL-overexpressing cells or EV-control cells in the presence of 4% BSA (bovine serum albumin) for 16 h, followed by collection of serum.

3. Collected serum was incubated with EV-control or HepG2 cells transduced with adenovirus (3μL/mL) encoding CETP or LCAT enzymes without or with inhibitors in the presence of 4% BSA for 12 h, followed by collection of serum.

4. Isolation of HDL from serum modified with EL, CETP or LCAT, by ultracentrifugation.

Structural/compositional analysis of modified HDL with

1. Native gradient (4-15%) gel electrophoresis followed by Sudan staining (neutral lipids): enables to see the alterations in HDL size.

2. Native gradient gel electrophoresis followed by PON1 Western blot: enables to see distribution of PON1 on HDL particles of various sizes.

3. Kit measurement for determination of lipid composition

Functionality assessment of modified HDL by

1. HDL-associated PON1-activity measurement.

2. measurement of capacity to induce e-NOS-inducing activity.

3. measurement of cholesterol efflux capacity.

Serum modification and Cell culture

Human serum was collected after overnight fasting from 10 healthy subjects (6 females and 4 males). 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. Pooled serum was stored at -80°C or used immediately for further experiments in cell culture.

All cell types were cultured in humidified atmosphere of 5% CO2/95% air at 37°C. HepG2 cells (ATCC®, HB-8065TM) were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) (all Gibco, Life Technologies). Cell culture medium was supplemented with penicillin G sodium sulfate (100 units/ml), streptomycin sulfate (100 mg/ml), and amphotericin B (2.5 mg/ml) (all Gibco, Life Technologies).

HepG2 cells were plated onto 60 mm dishes. After 24 h, cells were washed once with DMEM without FCS and were infected, separately, with recombinant adenoviruses encoding human EL, CETP and LCAT in DMEM without FCS for 2 h. After removal of infection media cells were incubated with fresh DMEM containing 10% fetal calf serum (FCS) for 24 h. Afterwards, cells were washed once with DMEM without FCS, followed by incubation of 1.8 ml of 50 % pooled human serum in DMEM without FCS, with EV- or EL-transduced cells for 16 h. After incubation, the serum was collected and spun at 1100 x g for 3 min to remove cellular debris and further incubated with CETP- or LCAT-transduced cells in the absence or presence of respective inhibitors for further 12 h. HDL was isolated from modified serum by ultracentrifugation.

Human endothelial cell line EA.hy926 (ATCC, Wesel, Germany) was cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum

(FBS) and 1% HAT Media Supplement (all Gibco, Life Technologies). Cell culture medium was supplemented with penicillin G sodium sulfate (100 units/ml), streptomycin sulfate (100 mg/ml), and amphotericin B (2.5 mg/ml) (all Gibco, Life Technologies).

Macrophages J774.2 (#85011428, Sigma-Aldrich, Vienna, Austria;) were maintained in RPMI1640 medium with 10% FCS.

Cells were sub-cultured using 0.025% trypsin/0.01% EDTA.

HDL isolation

HDL was isolated by a one-step density gradient ultracentrifugation method using long centrifuge tubes (16 x 76 mm; Beckman). Briefly, the density-adjusted serum (1.24 g/ml with KBr) was layered underneath a KBr-density solution (1.063 g/ml). Samples were centrifuged at 330,000 x g for 6 h (centrifuge: Beckman Optima L-80 ultracentrifuge, rotor: Sorvall T-1270). Thereafter, the collected HDL was concentrated by Viva Spin Tubes (Sartorius, Vienna, Austria), desalted by gel filtration on Sephadex PD-10 columns (GE Healthcare, Munich, Germany) and used directly for compositional/functional analysis or stored at -80°C for the same purpose.

Native gels

Aliquots of HDL (10 µg) (without and with inhibitors) were electrophoresed on 4-15% non-denaturing polyacrylamide gels upon dilution with native sample buffer (Bio-Rad Laboratories, Vienna, Austria). The HiMark™ Pre-stained Protein Standard was used as standard for size determination. (Thermo Fischer Scientific, Germany). Electrophoresis was done in a running buffer (Invitrogen, Vienna, Austria) at 125V for 4 h at room temperature. Gels were stained with Sudan black (Sigma-Aldrich,

Vienna, Austria) or were fixed with 10% sulfosalicylic acid for 30 min and then stained with Coomassie Brilliant Blue G250 (Sigma-Aldrich, Vienna, Austria).

Western blots

From HDL samples (without and with inhibitors), PON1-content was analyzed in aliquots of HDL (10 µg protein). The high molecular weight marker Native Mark (Life Technologies, Vienna, Austria) was used as standard. Samples were supplemented with 6 x loading buffers, boiled for 10 min and electrophoresed on 12% SDS-PAGE for 55 minutes at 175 V. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Carl Roth, Karlsruhe, Germany) with blotting buffer (Tris, Glycine, EDTA, sodium azide) at 150 mA at 4°C for 90 min. Membranes were blocked at RT in 10% powder milk for 1h followed by overnight incubation at 4°C with PON1 antibody (Abcam-ab24261, Cambridge, UK).

After washing and incubation with appropriate secondary antibody (Dako, Vienna, Austria) protein signals were visualized by incubation with Millipore Western Blotting Substrate (Millipore Corporation, Billerica, USA) using ChemiDoc system (Bio-Rad Laboratories, Vienna, Austria). Expression levels PON1 of HDL samples (without or with inhibitors) were examined. Western blot signals were quantified using ImageJ (version 2006.02.01). Afterwards, signals were illustrated in Graph Pad Prism (version 5.0) as AUC under the signals.

Kit measurement

Commercially available kits for the determinations of total cholesterol (TC), triglycerides (TG) and phospholipids (PL) were purchased from Sigma Aldrich, Vienna, Austria). The amount of lipid component was normalized to 10 µg of protein

and quantitated by measuring the absorbance (490nm) on the Anthos microplate-reader 2001 from Anthos Labtec Instruments GmbH (Salzburg, Austria) for each sample of HDL.

PON1 (aryl esterase)-activity

Ca2+-dependent aryl esterase activity of HDL-associated PON1 was determined with a photometric assay using phenyl acetate. Each HDL sample (1 µg protein) was added to 200 µl buffer containing 100 mmol/L Tris, 2 mmol/L CaCl2 (pH 8.0), and 1 mmol/L phenyl acetate in a 96-well quartz glass plate (Hellma, Baden, Germany). The rate of hydrolysis of phenyl acetate was monitored by the increase of absorbance at 270 nm, and readings were taken every 15 seconds at room temperature to generate a kinetic plot. The slope from the kinetic chart was used to determine the increase in fluorescence per minute. Enzymatic (aryl esterase) activity was calculated with the Beer-Lambert Law from the molar extinction coefficient of 1310 mol×L−1×cm−1 for phenyl acetate.

eNOS-inducing activity measurement

Intracellular conversion of L-[^{3}H] arginine into L-[^{3}H] citrulline was measured as a direct indicator of eNOS-inducing activity. Briefly, EA.hy926 cells grown in 6-well plates were washed and incubated at 37°C with 50 mM Tris buffer, pH 7.4, containing 100 mM NaCl, 5 mM KCl, 1 mM MgCl2, 3 mM CaCl2, 5% (vol/vol) FBS, L-[2,3-3H]arginine (~106 dpm) and 60 µM LPC 18:1 or PBS (vehicle). Reactions were terminated after 15 min by washing the cells with chilled Tris buffer (50 mM, pH 7.4), containing 100 mM NaCl, 5 mM KCl, 1 mM MgCl2 and 0.1 mM EGTA. After lysis of the cells with 0.01 N HCl, an aliquot was removed for determination of incorporated

radioactivity. To the remaining sample, 200 mM sodium acetate buffer (pH 13.0) containing 10 mM L-citrulline was added (final pH ~5.0), and L-[^{3}H] citrulline separated from L-[^{3}H] arginine by cation exchange chromatography.

Cholesterol efflux capacity measurement

J774 macrophages plated on 48-well plates (300.000 cells/well) were labeled with 1 µCi/mL [^{3}H]-cholesterol (Perkin Elmer, Boston, MA, USA) for 24 hours. To upregulate ABCA1, the cells were stimulated with serum-free DMEM containing 0.3 mmol/L 8-(4-chlorophenylthio)-cyclic AMP (Sigma, Darmstadt, Germany) for 6 hours. After labeling, the cells were washed and the [3H]-cholesterol efflux was determined by incubating the cells with 2.8% of HDL samples (2 µg HDL protein) for 4 hours. The cholesterol efflux was expressed as the radioactivity in the medium relative to total radioactivity in the medium and cells. All steps were performed in the presence of 2 µg/mL of the acyl coenzyme A cholesterol acyltransferase inhibitor Sandoz 58-035 (Sigma, Darmstadt, Germany).

Inhibitors

Torcetrapib and DTNB (5,5-dithio-bis-2-nitrobenzoic acid) were purchased from Sigma Aldrich (Vienna, Austria) and diluted in ethanol. The end concentrations of inhibitors were 10 µM and 2 mM, respectively

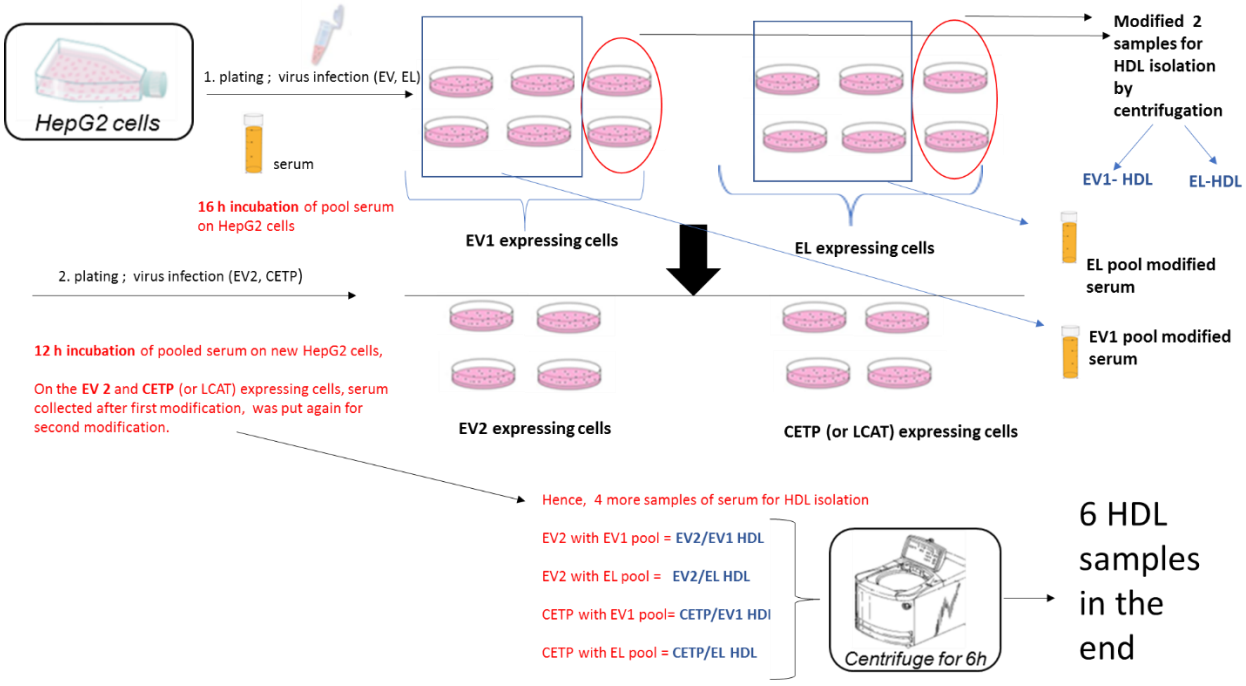


Figure 3. Schematic view of the experiment.

Pooled serum (with HDL) was kept on EL overexpressing HepG2 cells for 16 h. Afterwards, following 12h incubation with CETP- or LCAT- overexpressing HepG2 cells without or with torcetrapib or DTNB, HDL was isolated from every condition yielding 6 different HDL samples.

4. Results

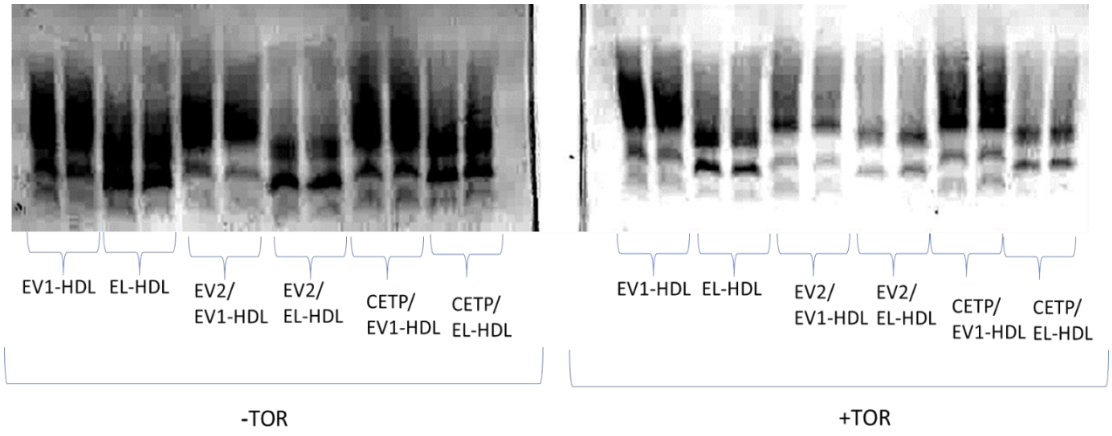


Figure 4. Native gel Sudan Black staining of lipids. Samples are in duplicates without (left) and with (right) an inhibitor torcetrapib.

CETP enhanced the intensity of Sudan staining of EV-HDL and EL-HDL both in the absence and presence of torcetrapib as compared to respective EV-controls (Fig. 4.). CETP and torcetrapib had no impact on size of either EV-HDL or EL-HDL (Fig. 5.).

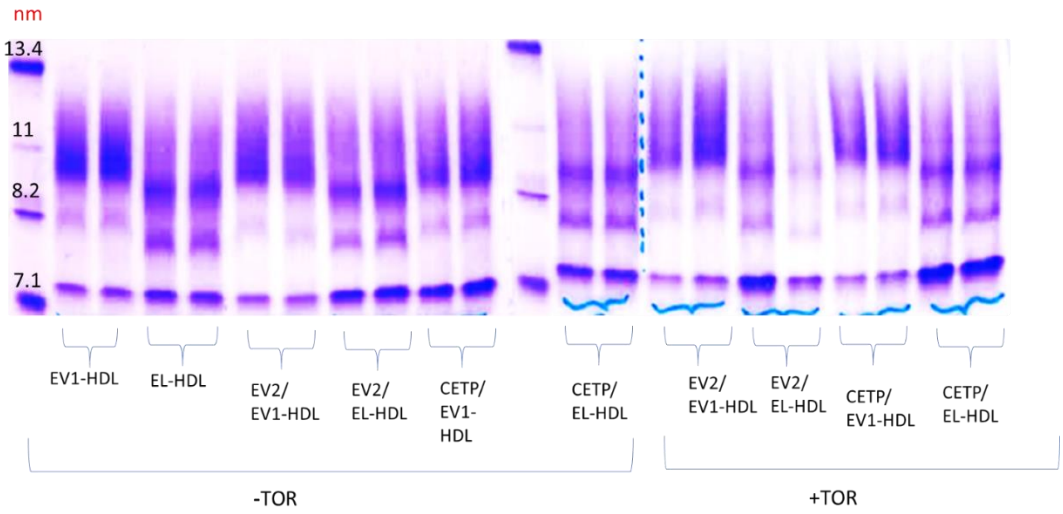


Figure 5. Native gel Coomassie staining of proteins. Samples are in duplicates without (left) and with (right) torcetrapib.

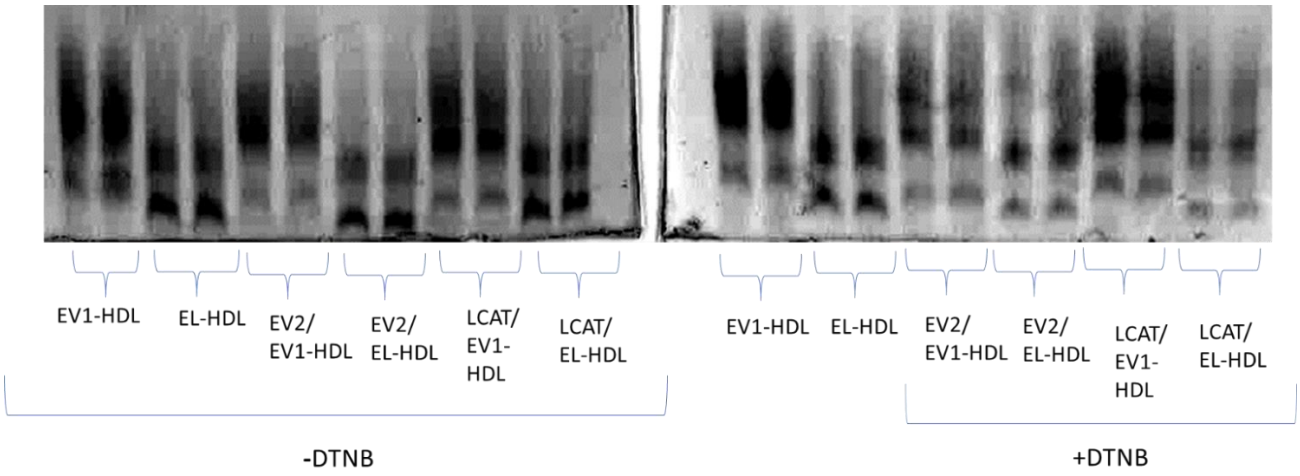


Figure 6. Native gel Sudan Black staining of lipids. Samples are in duplicates without (left) and with (right) an inhibitor DTNB.

LCAT overexpression enhanced the intensity of EL-HDL Sudan staining (compared to EV2/EL-HDL) in the absence of DTNB (Fig. 6.). In the presence of DTNB the Sudan staining of LCAT/EL-HDL was weaker compared to EV2/EV1-HDL control (Fig. 6.). Neither LCAT nor DTNB affected size of EV-HDL or EL-HDL (Fig. 7.).

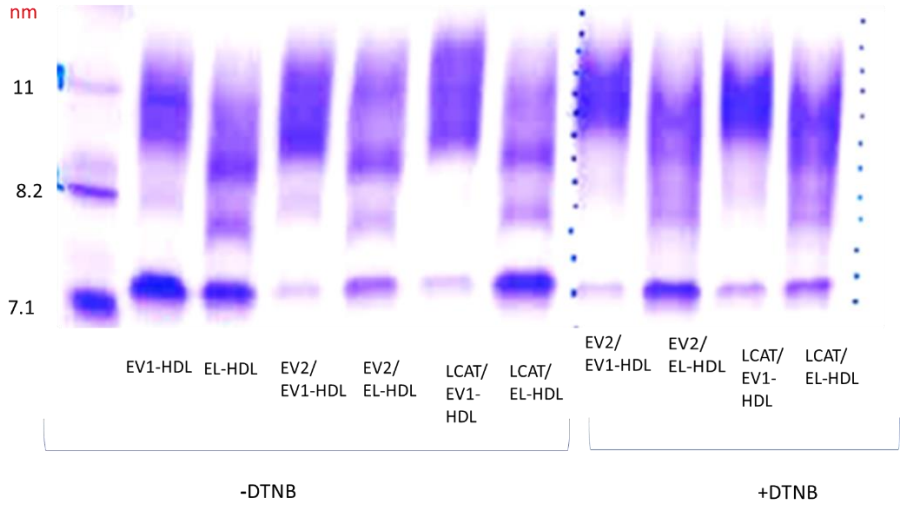


Figure 7. Native gel Coomassie staining of proteins. Samples are without (left) and with (right) an inhibitor DTNB.

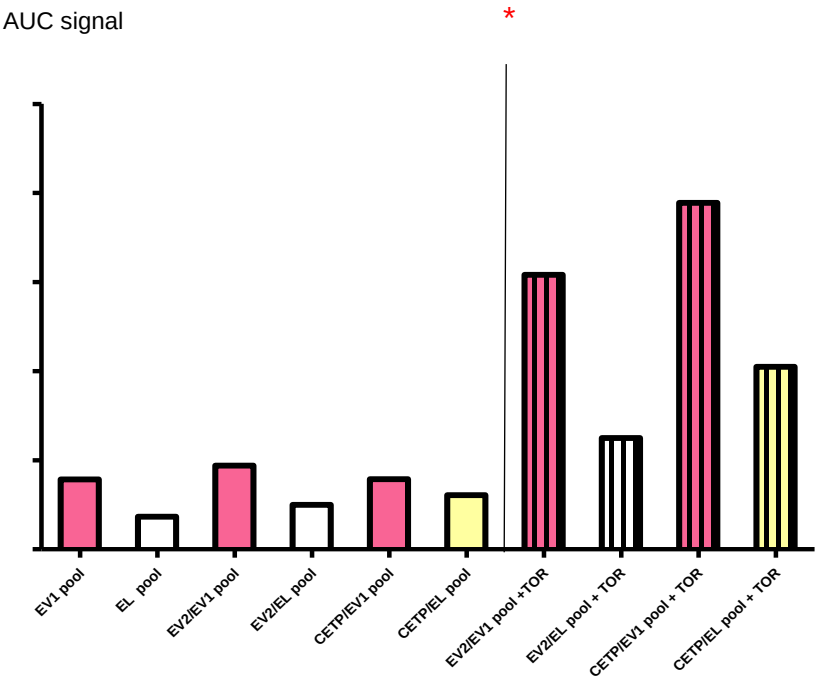


Figure 8. Western blots signals of PON1 without and with an inhibitor torcetrapib.

EL-HDL samples showed decreased PON1-content. Torcetrapib (Fig. 8.) and DTNB (Fig. 9.) did not positively affect PON1-content of EL-HDL (yellow). **Line is separation for Western blots done separately.*

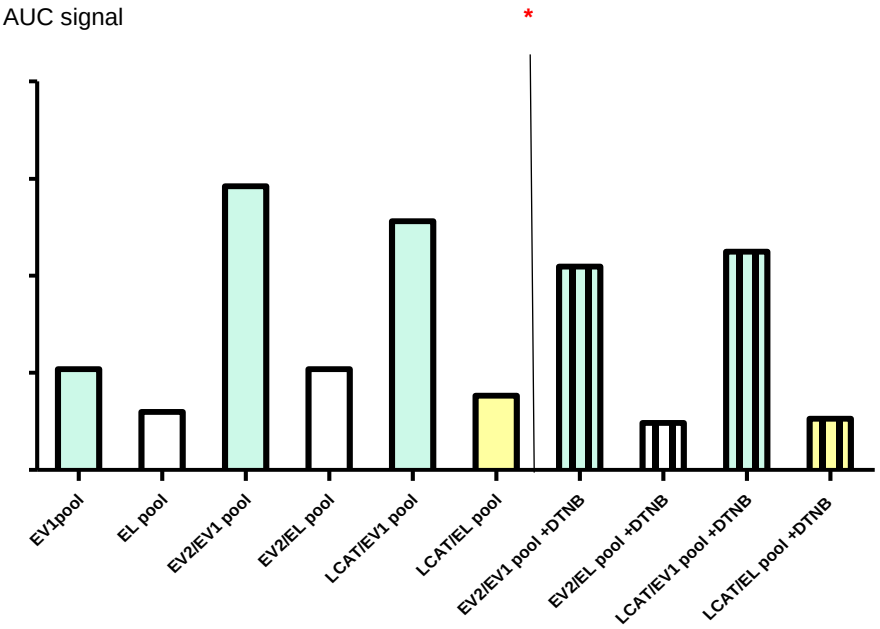
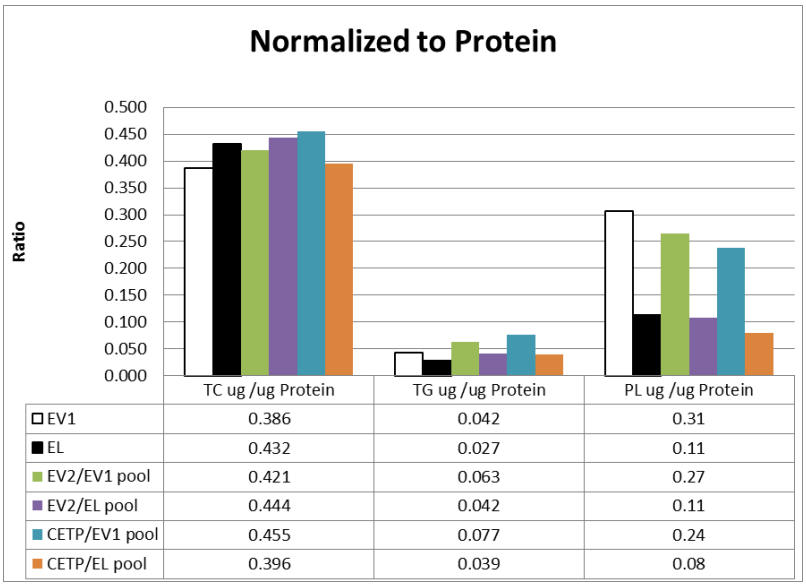


Figure 9. Western blots signals of PON1 without and with an inhibitor DTNB.

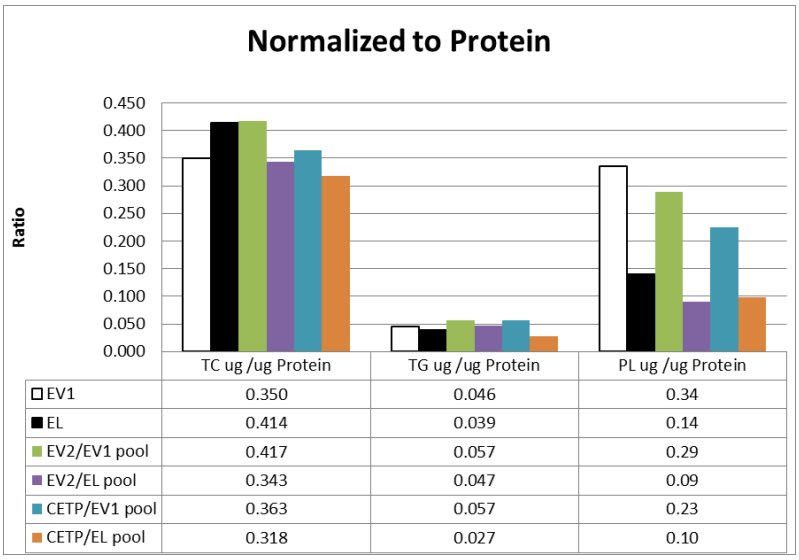
**Line is separation for Western blots done separately.*



	TC ug /ug Protein	TG ug /ug Protein	PL ug /ug Protein
EV1	0.386	0.042	0.31
EL	0.432	0.027	0.11
EV2/EV1 pool	0.421	0.063	0.27
EV2/EL pool	0.444	0.042	0.11
CETP/EV1 pool	0.455	0.077	0.24
CETP/EL pool	0.396	0.039	0.08

Figure 10. Kit measurement of lipid composition (TC=total cholesterol, TG=triglycerides, PL=phospholipids) of samples without torcetrapib.

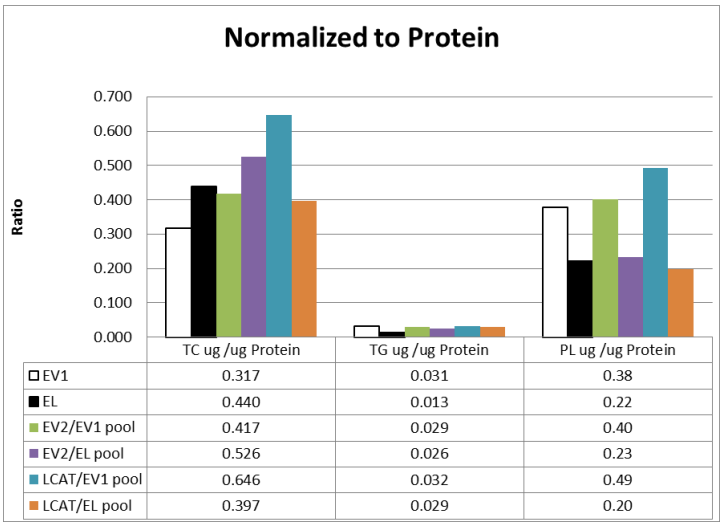
EL-HDL samples showed increase in total cholesterol and decrease in triglycerides and phospholipids (Fig. 10.). EL-HDL modified with CETP overexpression (orange) had decreased total cholesterol (Fig. 10.).



	TC ug /ug Protein	TG ug /ug Protein	PL ug /ug Protein
EV1	0.350	0.046	0.34
EL	0.414	0.039	0.14
EV2/EV1 pool	0.417	0.057	0.29
EV2/EL pool	0.343	0.047	0.09
CETP/EV1 pool	0.363	0.057	0.23
CETP/EL pool	0.318	0.027	0.10

Figure 11. Kit measurement of lipid composition (TC=total cholesterol, TG=triglycerides, PL=phospholipids) of samples with torcetrapib (green, purple, blue and orange).

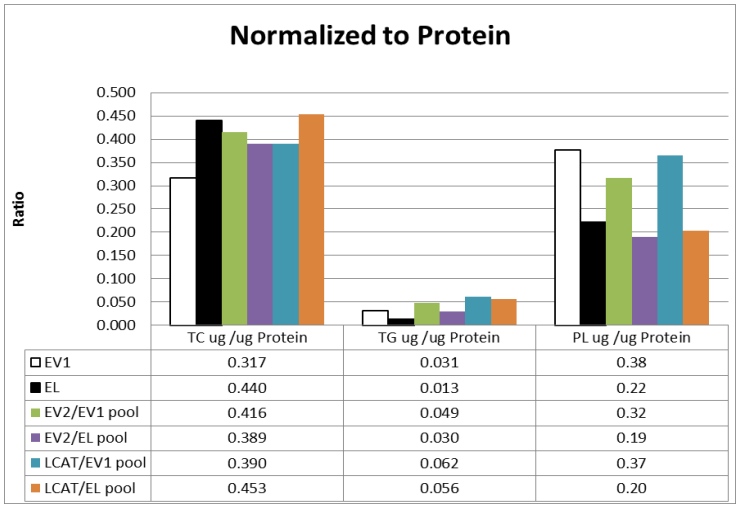
Sample (EV2/EL; purple) had decreased total cholesterol, which was also visible in blue and orange samples. Sudan bands of CETP/EL with torcetrapib (Fig. 4.) were also less dark (sample CETP/EL), indicating lower cholesterol ester content. This suggests lower impact of torcetrapib on the composition of CETP/EL samples.



	TC ug /ug Protein	TG ug /ug Protein	PL ug /ug Protein
EV1	0.317	0.031	0.38
EL	0.440	0.013	0.22
EV2/EV1 pool	0.417	0.029	0.40
EV2/EL pool	0.526	0.026	0.23
LCAT/EV1 pool	0.646	0.032	0.49
LCAT/EL pool	0.397	0.029	0.20

Figure 12. Kit measurement of lipid composition (TC=total cholesterol, TG=triglycerides, PL=phospholipids) of samples without DTNB.

EL-HDL samples showed increased total cholesterol and decreased triglycerides and phospholipids. Decrease of total cholesterol was observed in EL-HDL following modification with LCAT (orange) (Fig.12.).



	TC ug /ug Protein	TG ug /ug Protein	PL ug /ug Protein
EV1	0.317	0.031	0.38
EL	0.440	0.013	0.22
EV2/EV1 pool	0.416	0.049	0.32
EV2/EL pool	0.389	0.030	0.19
LCAT/EV1 pool	0.390	0.062	0.37
LCAT/EL pool	0.453	0.056	0.20

Figure 13. Kit measurement of lipid composition (TC=total cholesterol, TG=triglycerides, PL=phospholipids) of samples with DTNB (green, purple, blue and orange).

Sample (EV2/EL; purple) had decreased total cholesterol. Sample LCAT/EL (orange) had increased total cholesterol (Fig. 13.), accompanied by a less pronounced Sudan staining (Fig. 6.), suggesting higher content of free cholesterol and a significant impact of LCAT inhibition with DTNB.

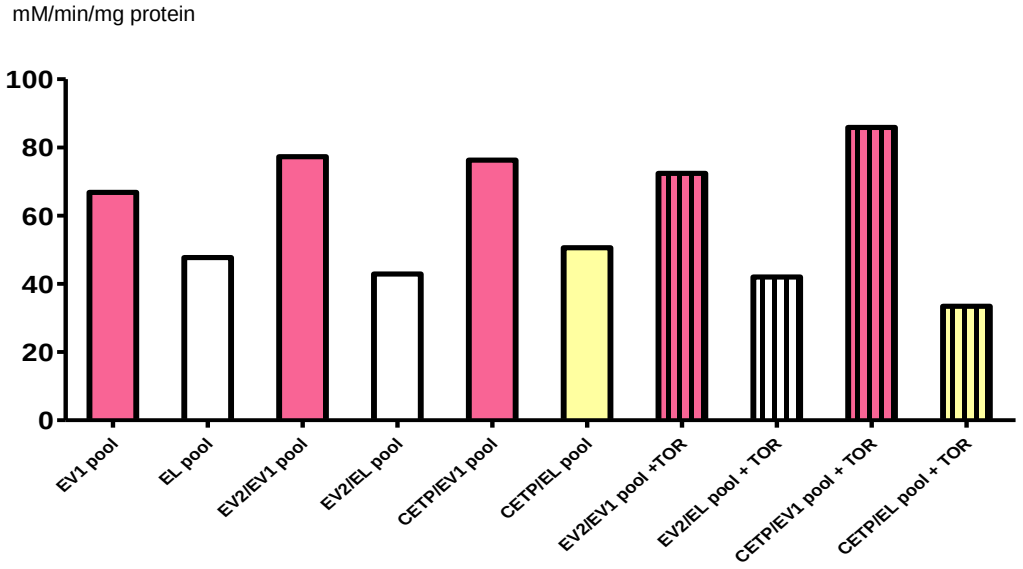


Figure 14. PON1-activity of samples without and with torcetrapib.

EL-HDL had decreased PON1-activity (Fig. 14.). Torcetrapib slightly lowered PON1-activity in CETP/EL sample (yellow). Decreased PON1-activity of EL-HDL was not improved with either torcetrapib (Fig. 14.) or DTNB (Fig. 15.).

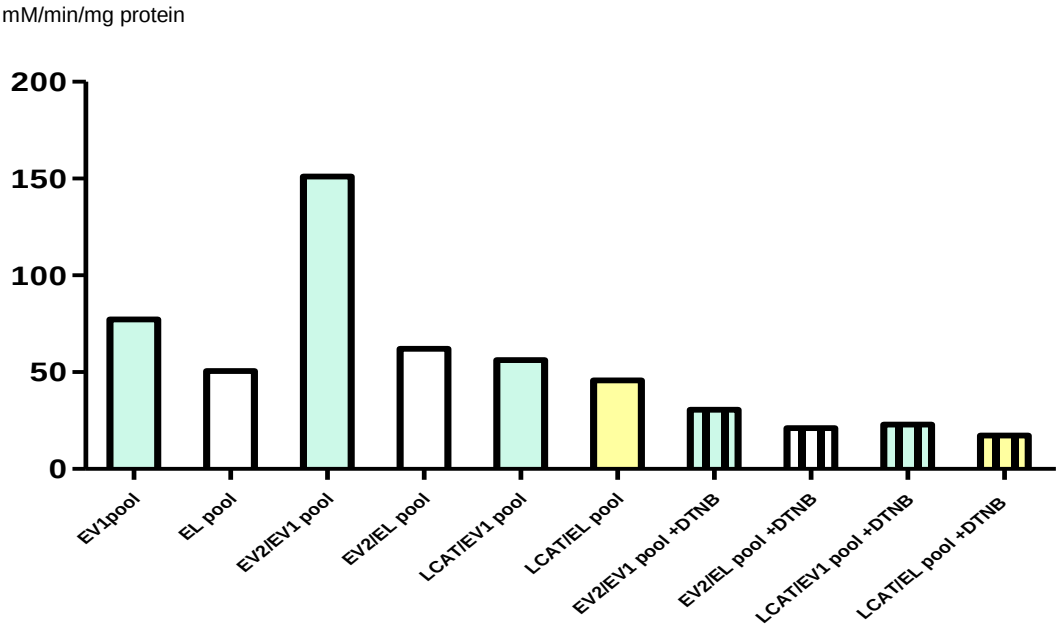


Figure 15. PON1-activity of samples without and with DTNB.

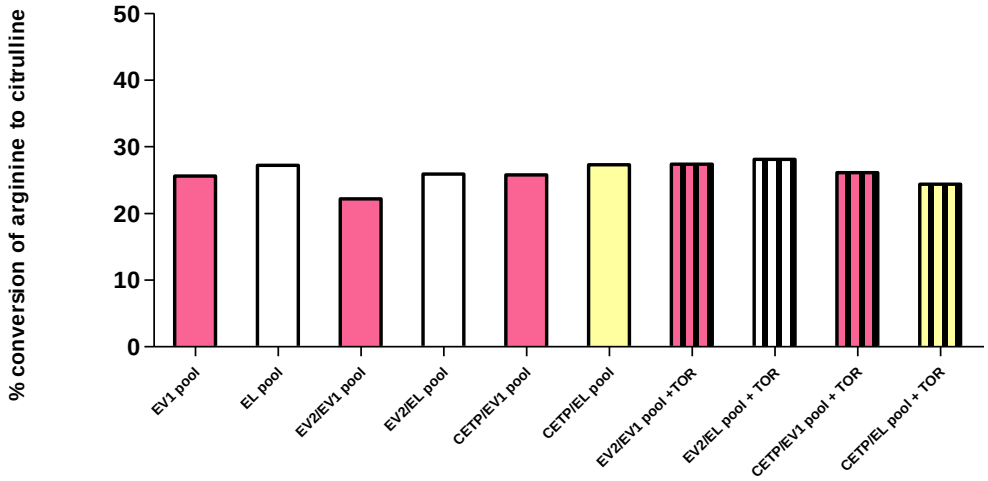


Figure 16. eNOS-inducing activity of HDL prepared in the absence or presence of torcetrapib.

Compared to EV-HDL, EL-HDL exhibited increased capacity to induce eNOS-inducing activity in cultured endothelial cells. The eNOS-inducing capacity of both EV-HDL and EL-HDL was increased with CETP and with torcetrapib (Fig. 16.). A combination of CETP and torcetrapib attenuated eNOS-inducing activity of EL-HDL but not of EV-HDL. (Fig. 16.).

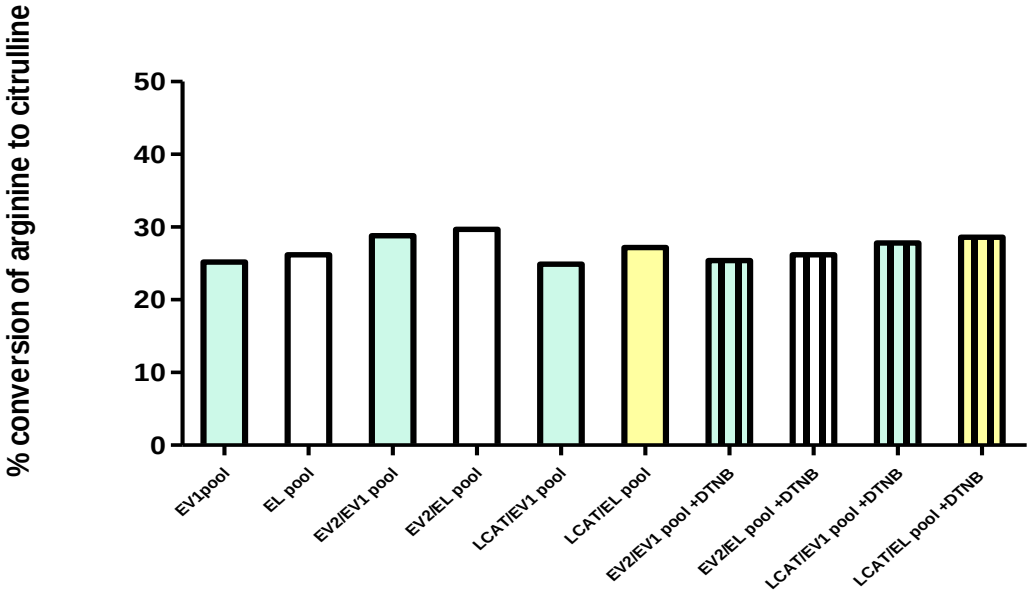


Figure 17. eNOS-inducing activity HDL prepared in the absence or presence of DTNB.

LCAT attenuated eNOS-inducing activity of EV-HDL and EL-HDL. DTNB only slightly affected eNOS-inducing capacity of EV-HDL and EL-HDL (Fig. 17.).

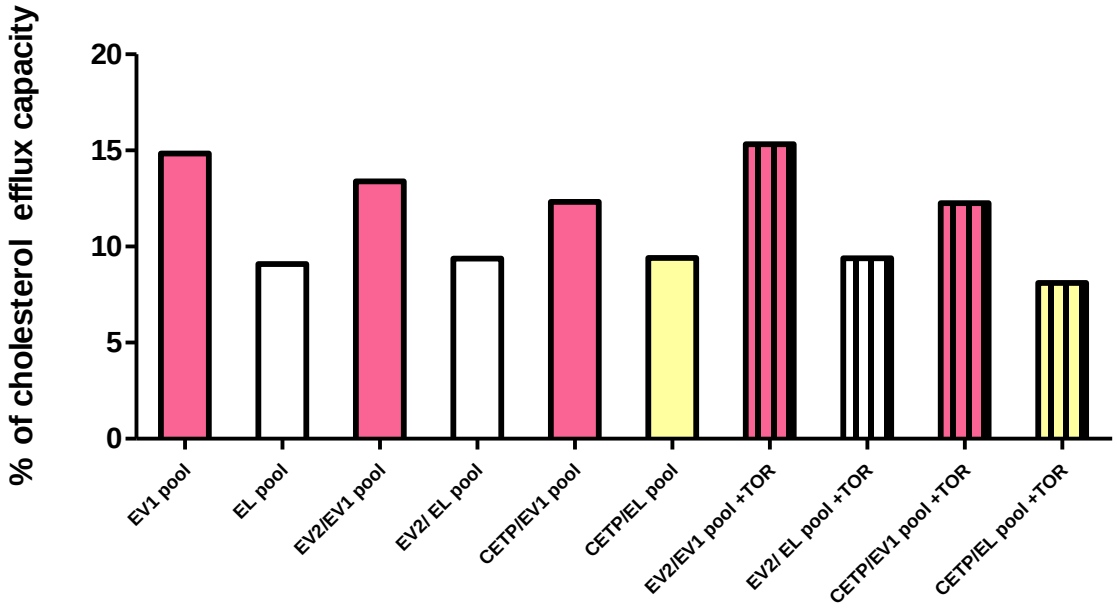


Figure 18. Cholesterol efflux capacity of samples without and with torcetrapib.

Cholesterol efflux capacity of EL-HDL was significantly lower compared to EV-HDL (Fig. 18.). CETP, torcetrapib, LCAT and DTNB did not affect cholesterol efflux capacity of EV-HDL and EL-HDL (Fig. 18, 19.).

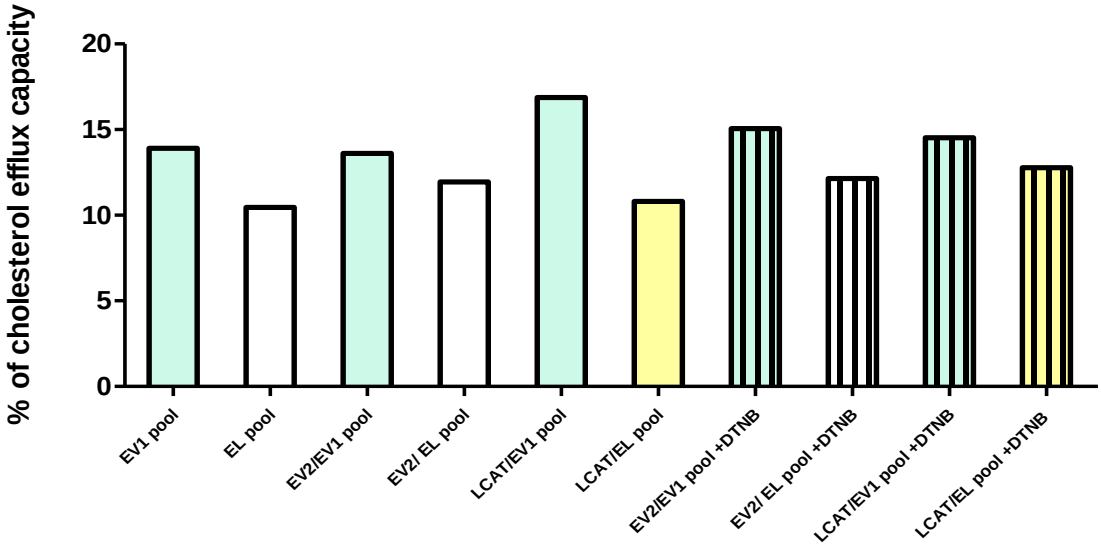


Figure 19. Cholesterol efflux capacity of samples without and with DTNB.

5. Discussion

Native gels in all figures 4., 5., 6. and 7. showed that HDL modified by EL is smaller in size in the presence or absence of inhibitors (Torcetrapib and DTNB). In figure 7. there are significant shifts in bands when control samples are compared with EL-HDL samples. Control samples are always approx. between 12.5 and 8.2 nm, while EL-HDL samples are approx. between 12 and 7.2 nm. Therefore, inhibitors do not modulate the total size of EL-HDL particles after CETP and LCAT overexpression.

Sudan black staining (Fig. 4.) revealed that with torcetrapib, bands of samples were weaker when compared to those samples without inhibitor. When comparing bands from CETP/EV1 and CETP/EL (with torcetrapib), decrease in lipid content is more visible in the latter. Considering that Sudan has higher affinity for cholesterol esters (than free cholesterol) follows that torcetrapib had more effect on the control samples when compared to CETP-samples. Therefore, it seems that EL-HDL is less affected by CETP inhibition then control sample (CETP/EV1).

Results in figure 5. showed an altered protein pattern in the sample CETP/EL with torcetrapib, especially around 7.2 nm (last two bands), possibly a consequence of torcetrapib-induced alterations in HDL-particle conformation.

Results shown in figure 6. showed increased lipid content of cholesterol esters in LCAT/EV1 with DTNB, compared to LCAT/EL with DTNB suggests more pronounced inhibitory effect of DTNB on LCAT during acting on EL-HDL than on EV-HDL.

Furthermore, protein bands in figure 7. showed some protein conformation changes in the presence of DTNB.

Lipid profile for CETP overexpression and inhibition, in figures 11. and 12., for samples of control HDL (sample EV1) and EL-HDL (sample EL) showed increase in total cholesterol and decrease in triglycerides and phospholipids for all EL modified samples. Only EL-HDL from CETP overexpression, without torcetrapib, had decreased total cholesterol (samples CETP/EV1 and CETP/EL in figure 10). Also, when torcetrapib was present in sample EV2/EL (Fig. 11.), decrease of total cholesterol was noticed. Sudan staining bands of CETP/EL with torcetrapib were also less prominent in figure 4 (sample CETP/EL), confirming that control sample was more prone for inhibition. Combining data from native gels and kit measurements, suggest a negligible effect of torcetrapib on the size and composition of CETP-modified EL-HDL.

Lipid profile for LCAT overexpression and inhibition, in figures 12., and 13., for samples of control HDL (EV1) and EL-HDL (sample EL) was similar as shown in figures 10. and 11; increased total cholesterol and decreased triglycerides and phospholipids were observed in all EL modified samples. Furthermore, EL-HDL modified with LCAT (LCAT/EL in Fig. 12.) had decreased total cholesterol when compared to control without inhibitor. This pattern also fits with Sudan gel in figure 6. In the presence of LCAT inhibitor the levels of total cholesterol were decreased in EV2/EL sample (Fig. 13.), but increased in EL-HDL from LCAT overexpressing HepG2 cells, likely due to inhibition of cholesterol esterification and accumulation of unesterified cholesterol in these particles (sample LCAT/EL in Fig. 13.). Sudan staining of those samples was somewhat different (Fig. 6., samples with inhibitors) with sample LCAT/EV1 having higher cholesterol ester content compared to

LCAT/EL which, as suggested above might be enriched with free unesterifeid cholesterol poorly detectable with Sudan staining. Combining data from native gels and kit measurements, it seems that DTNB affects more profoundly the impact of LCAT on the composition of EL- HDL, than EV-HDL

In conclusion, EL-HDL particles were substrates for CETP and LCAT. Furthermore, structural and functional properties of EL-HDL were affected by both LCAT and CETP. While torcetrapib more profoundly affected CETP-modification of EV-HDL compared to EL-HDL, situation was opposite with DTNB, which more profoundly affected LCAT-modification of EL-HDL compared to EV-HDL.

PON1-content was decreased in EL-modified HDL. PON1-content did not change in the presence of torcetrapib or DTNB presence. In conclusion, both inhibitors did not show any positive effect regarding PON1-content of EL-HDL.

PON1-activity was decreased in EL-HDL. Torcetrapib slightly lowered PON1-activity in CETP/EL-HDL. DTNB effect on PON1-activity followed the same pattern.

In figures 16. and 17., eNOS-inducing activity was higher in cells treated with EL-HDL without inhibitors. EL-HDL modified with CETP in the presence of, torcetrapib exhibited decreased eNOS-inducing activity, compared to CETP/EV1 HDL sample. Hence, torcetrapib negatively affects only eNOS-inducing activity of EL-HDL modified by CETP, while in other particles from EL pool eNOS-inducing activity remains higher, compared with control HDLs.

DTNB effects on eNOS-inducing activity of HDL activity were insignificant.

Cholesterol efflux capacity of EL-HDL was decreased regardless of inhibitors or CETP or LCAT overexpression (Fig. 18 and 19).

6. Conclusion

EL-HDL is suitable substrate for CETP and LCAT. Overexpression of CETP and LCAT altered lipid composition without affecting size of EL-HDL Pharmacological modulation showed that inhibitors, torcetrapib and DTNB are able to affect lipid composition of EL-HDL.

- Torcetrapib has more pronounced effect on EL-HDL without additional CETP modification, while DTNB effects on EL-HDL are dependent on LCAT overexpression.

PON1-content, PON1-activity and cholesterol efflux capacity are decreased in all EL-HDL samples, regardless of CETP, or LCAT, overexpression and inhibition. In contrast, eNOS-inducing activity of all EL-HDL samples, except those with torcetrapib, were higher when compared to control HDL.

In conclusion, composition but not functionality of EL-HDL is affected by CETP and LCAT. When inhibitors, torcetrapib and DTNB, are present - result is the same. In vitro pharmacological inhibition of CETP and LCAT, failed to affect their impact on the functionality of the EL-HDL.

7. References

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