Dottorato di ricerca in
"Scienze del farmaco, delle biomolecole e dei prodotti per la salute"

XXX CICLO

HDL functionality: pathophysiological role in cardiovascular disease and as a novel metric of the neurodegenerative disorder

Coordinatore:
Prof. Marco Mor

Tutore:
Prof.ssa Elda Favari

Dottoranda: Eleonora Cipollari

Triennio accademico 2014/2017
UNIVERSITA’ DEGLI STUDI DI PARMA

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Abstract

Cardiovascular diseases (CVD) remain a leading cause of morbidity and the first cause of death globally. The project builds upon the ideas that have been recently developed in the field of cardiovascular disease regarding the cholesterol efflux capacity (CEC) of high-density lipoprotein (HDL), the main acceptor of cell cholesterol in plasma. HDL CEC is thought to reflect the robustness of reverse cholesterol transport from macrophages (mRCT) to the liver for excretion with bile. Inadequate mRCT causes abnormal accumulation of cholesterol and death of macrophages, formation of lipid core in the subendothelium of coronary arteries and eventually rupture of the lesion with resultant occlusion of the artery and heart ischemia. In most recent years, HDL CEC has been identified as a strong inverse predictor of incident cardiovascular disease, independently of HDL-, LDL-cholesterol levels and other major risk factors. The definition of CEC as a biomarker of CVD may improve risk stratification in human population and help to better define the level of risk in subjects that, classified as intermediate/low risk with the currently used parameters, undergo unexpected cardiovascular events. Elucidation on the role of HDL CEC as an index of disease as well as investigations on the pathophysiological mechanisms involved in the process of cholesterol transport from cells to HDL appear as an attractive field of research and constitute the general aim of this PhD project.

Enhancement of HDL functions is a putative therapeutic strategy to treat cardiovascular disease and other human disorders. In fact, intravenous administration of synthetic HDL containing human apolipoprotein A-I (apoA-I) causes significant atherosclerosis regression in several experimental and clinical studies. The first part of my thesis project focuses on the evaluation of the effects of a new kind of reconstituted HDL (TN-rHDL) on \textit{ex vivo} CEC in relationship to atherosclerosis development in a model of hypercholesterolemic rabbit. Main finding was that TN-rHDL is effective in increasing plasma total CEC after 4 hours from a single high-dose infusion. This effect on cholesterol efflux capacity is associated with parameters of plaque stabilization and may explain, at least in part, the reduced atherosclerosis progression observed in treated rabbits.

In the second part of my thesis, I investigated the physiological role of a specific human protein involved in lipid metabolism on HDL functionality and mRCT. Phospholipid transfer protein (PLTP) is a key player in lipoprotein remodeling and both direct and inverse correlations between PLTP activity and atherosclerotic cardiovascular disease have been reported in human studies. A unique \textit{in vivo} experimental design was used in order to understand the function of
PLTP overexpression and deletion on HDL efflux capacity and macrophage reverse cholesterol transport in mice. Our results showed that PLTP overexpression and deletion reduce HDL cholesterol mass and *ex vivo* plasma cholesterol efflux capacity without adversely affecting the rate of cholesterol efflux from macrophages to plasma and *in vivo* macrophage cholesterol transport in plasma to the liver. These interesting findings imply a substantial resilience of mRCT in face of drastic changes in HDL metabolism.

In the third paragraph, CEC was evaluated in a human population of overweight individuals that underwent diet supplementation with a new kind of wheat flour enriched with bioactive components and functional probiotics. Several foods and dietary supplements have been shown to protect against the development of CVD; in this study, a slight but significant improvement of plasma ABCG1 CEC was observed in humans after three months of innovative food consumption. Despite no change in HDL concentration, a rearrangement of their composition can occur with enhancement of their function. ABCG1-mediated CEC of treated subjects inversely correlates with homocysteinemia, an independent risk factor for coronary disease, while a significant, direct relation was found with plasmatic folic acid, considered as a cardioprotective factor. It can be speculated that consumption of bioactive components within the innovative pasta act simultaneously on the amelioration of subjects inflammatory profile and on HDL functional quality thus “linking” ABCG1 CEC to the levels of such metabolic markers.

The last part of my thesis focuses on the development of a method to measure cholesterol efflux of human cerebrospinal fluid. Alterations in lipid metabolism is a common feature underlying atherosclerotic cardiovascular disease (ASCVD) and neurodegenerative disorders. Indeed, experimental evidence suggests that excessive cholesterol in neurons, astrocytes and microglia promotes Aβ accumulation and stimulates Aβ-driven inflammation in Alzheimer’s disease. In particular, we advance the hypothesis that cholesterol efflux to apolipoproteins and HDL for disposal via the cerebrospinal fluid (CSF) is an important mechanism in neurons and glia for management of intracellular cholesterol and prevention of Aβ pathology. Here, we aim to adapt the HDL CEC assay to measure CSF CEC for the purposes of early diagnosis of Alzheimer’s disease and gaining insight into pathogenesis of this condition. Therefore, I characterized cell cholesterol efflux in in vitro cellular models relevant to the pathology of AD and identified biochemical components in CSF that determine CSF CEC variability in a small human cohort. Alltoghether, these results establish a robust method to study human CSF CEC as a putative, novel metric of the neurodegenerative disease.
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Introduction
1. Atherosclerosis

Cardiovascular disease (CVD) remains a leading cause of morbidity and the first cause of death globally, as stated by the World Health Organization (WHO) in a recent report. Cardiovascular diseases are a complex group of disorders of the blood and heart vessels, which encompasses coronary heart disease, cerebrovascular disease and peripheral arterial disease. About 80% of CVD deaths are due to acute events such as heart attacks and strokes. The most common underlying condition of CVD is atherosclerosis, a silent, multifactorial, progressive pathology.

The onset of the atherosclerotic disease often develops in childhood and is characterized by deposition of fatty streaks within the wall of major arteries.

For many years, the process of atherogenesis has been described as a response of the vessel wall to an “injury” (Ross 1993) with subsequent maladaptive inflammation, accumulation of lipids and reactive intimal thickening of arteries (Libby 2006). The primary causative event in atherosclerosis is infiltration of cholesterol-rich low-density lipoprotein (LDL) through the endothelium of the intima layer of arteries in regions of low shear stress (Moore and Tabas 2011). Sub endothelial LDL triggers an immune response that, together with the dysregulation of cholesterol metabolism, leads to formation of the atheromatic plaque.

A large number of insulting agents can initiate the inflammatory activation of the endothelium: free radicals, hypertension, glycosylated molecules due to diabetes, infections. Endothelial cells start to secrete chemokines leading to recruitment of circulating monocytes, T-cells and other inflammatory cells. Thanks to the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) and selectins, these cell types adhere to the endothelium and penetrate in the sub endothelial space (Hansson 2005). Proliferation and differentiation of monocytes into macrophages is stimulated by growth factors such as the macrophage colony-stimulating factor (M-CSF) and the tumor necrosis factor-α (TNF-α) (Smith et al. 1995). At the same time, trapped LDL are exposed to a number of agents that promote their modification and oxidation. The monocyte-derived macrophages contribute to fuel inflammation within the intima and accumulate modified lipids, such as oxidized LDL (Cybulsky and Jongstra-Bilen 2010). Since animals models lacking macrophage CD36 and SR-A display less severe lesions, these two scavenger receptors are thought to be the main responsible for LDL uptake in macrophages (Moore et al 2006). Scavenger receptors activity is not down regulated by high intracellular cholesterol content, thus the uncontrolled
cholesterol uptake results in the formation of lipid-laden macrophages, also known as “foam cells” (de Villiers and Smart 1999), which represents the typical hallmark of atherosclerosis. If inflammation persists, smooth muscle cells (SMC) are recruited from the media layer and proliferate in the intima while producing extracellular matrix rich in collagen, elastin and proteoglycans. Plaque foam cells eventually die, precipitating formation of cholesterol crystals and the lipid core in advanced atheromas. A mature atheroma is considered as the typical focal lesion of the pathology and is characterized by a cholesterol-rich necrotic core enclosed in a fibrous cap that contains few smooth muscle cells and many macrophages, angiogenesis and ongoing remodeling (Falk 2006) (Fig.1).

Fig.1 Development of an atherosclerotic plaque (Skeoch and Bruce 2005)

Depending on genetic and environmental inputs, atherosclerosis may remain asymptomatic for years or evolve in a clinically significant, more advanced state. The enlargement of the plaque can cause the blockage of the circulation, preventing the blood flowing to the heart or the brain, with subsequent ischemia of the tissues. In worst cases, rupture or erosion of a vulnerable plaque leads to occlusive thrombosis and precipitates in symptomatic
cardiovascular events, such as acute coronary syndromes (ACS), which include angina and myocardial infarction (Falk 2006).

The dramatic incidence of CVD is western society has encouraged the scientific community to identify numerous risk factors of atherosclerotic cardiovascular disease (ASCVD). Among those, the so-called behavioral risk factors, such as tobacco use, physical inactivity and unhealthy diet, can be addressed to prevent or modify the disease via pharmacological treatments and by pursuing a healthy lifestyle. The effect of behavioral risk factors is particularly critical in individuals with diabetes mellitus, hypertension and obesity, all conditions that raise the cardiovascular risk. Unmodifiable risk factors for CVD include genetic predisposition, age and gender (World Health Organization 2017).
2. Lipoproteins

It is well known that high cholesterol content in the blood stream is associated with the incidence of cardiovascular events (Stamler et al. 1986). For this reason, levels of circulating cholesterol and related lipids are important predictive factors utilized to estimate cardiovascular risk in the clinical practice.

Cholesterol and triglycerides, being non-polar lipid substances, require to be transported in the circulation associated with particles, in order to be shielded from the aqueous nature of plasma; specialized agglomerates of protein and lipids accomplish this role. Plasma lipoproteins are spherical particles containing a hydrophobic core, made of unpolar lipids such as cholesterol esters (CE) and triglycerides (TG), and an outside hydrophilic surface consisting in polar lipids, mostly phospholipids (PL) and unesterified free cholesterol (FC), and proteins (apolipoproteins). Lipoproteins are heterogeneous in term of composition, density, size and functions.

Depending on the ratio between the lipid and the protein component within the micelles, circulating lipoproteins can be classified into four major groups of density and electrophoretic mobility: chylomicrons, very-low-density (preβ) lipoproteins (VLDL), low-density (β) lipoproteins (LDL) and high-density (α) lipoprotein (HDL) (Fig.2). After electrophoresis, chylomicrons remain at the origin, while VLDL, LDL, and HDL migrate in the same position as preβ-, β- and α- globulins, respectively (Rothblat and Phillips 2010). Interaction between lipoproteins and tissue elements, such as cell receptors or transporters, occurs upon recognition of apolipoproteins on the particles. Therefore, apolipoproteins not only exert important structural functions, but also act as functional markers on lipoproteins surface. The composition of the particles distinguishes each group of lipoprotein from the others and reflects separate role of lipoproteins in the trafficking of lipids in the circulation. Depending on their composition, size and density upon density-gravity ultracentrifugation, lipoproteins are assigned to six classes (Cox and Garcia-Palmieri 1990) as follows:

- Chylomicrons (δ < 0.94 g/mL): large triglyceride- and apolipoprotein B48 (apoB48)-rich particles produced by enterocytes. They are involved in the transport of dietary triglycerides and cholesterol to peripheral tissues and liver. The size of chylomicrons varies depending on the amount of fats introduced with the diet.
- **VLDL** (0.94 < δ < 1.006 g/mL): very low density lipoproteins produced in the liver from triglycerides, free and esterified cholesterol, apolipoprotein B100 (apoB100), apolipoproteinE (apoE) and apolipoprotein C (apoC). As triglycerides production in the liver increases, secreted VLDL particles are larger in size.

- **IDL** (1.006 < δ < 1.019 g/mL): intermediate density lipoproteins that take origin from VLDL, they are enriched in cholesteryl esters.

- **LDL** (1.019 < δ < 1.063 g/mL): low density lipoproteins deriving from VLDL and IDL remodeling in the plasma. They are enriched in cholesterol and apoB100 (one molecule per particle). Most abundant lipoproteins in the bloodstream, LDL particles can vary in size and density (Mahley et al. 2006).

- **HDL** (1.063 < δ < 1.21 g/mL): high density lipoproteins synthesized primarily in the liver and partially in the intestine. Marked by the presence of one or more molecules of apolipoprotein AI (apoAI), they also contain apoE, apoC, apoAII, cholesterol and phospholipids.

<table>
<thead>
<tr>
<th>Lipoprotein particle</th>
<th>Size (Å)</th>
<th>Density</th>
<th>Mobility by electrophoresis</th>
<th>Cholesterol percentage</th>
<th>Triglyceride percentage</th>
<th>Phospholipid percentage</th>
<th>Apoprotein percentage</th>
<th>Major apoproteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons</td>
<td>800–5000</td>
<td>0.95</td>
<td>Remains at origin</td>
<td>3</td>
<td>90</td>
<td>5</td>
<td>9</td>
<td>Al, All, B, Cl, CII, CIII</td>
</tr>
<tr>
<td>Very-low-density lipoprotein (VLDL)</td>
<td>300–800</td>
<td>0.95–1.006</td>
<td>Pre-beta</td>
<td>10</td>
<td>70</td>
<td>10</td>
<td>10</td>
<td>Bl, Cl, CII, CIII, E</td>
</tr>
<tr>
<td>Intermediate-density lipoprotein (IDL)</td>
<td>250–350</td>
<td>1.006–1.019</td>
<td>Slow-pre-beta</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>B, CIII, E</td>
</tr>
<tr>
<td>Low-density lipoprotein (LDL)</td>
<td>180–280</td>
<td>1.019–1.063</td>
<td>Beta</td>
<td>26</td>
<td>10</td>
<td>15</td>
<td>25</td>
<td>B</td>
</tr>
<tr>
<td>High-density lipoprotein (HDL)</td>
<td>50–120</td>
<td>1.063–1.210</td>
<td>Alpha</td>
<td>20</td>
<td>5</td>
<td>25</td>
<td>50</td>
<td>Al, All</td>
</tr>
</tbody>
</table>

Fig.2 Characteristics and classification of the lipoproteins (Cox and Garcia-Palmieri 1990)
2.1 Lipoprotein metabolism

Plasma lipoprotein modification and remodeling are usually described in three routes of metabolism: the exogenous pathway, the endogenous pathway and the reverse cholesterol transport.

The exogenous pathway is responsible for the metabolism of diet-derived lipids and involve the transport of lipids from the intestine to the liver. Lipids are solubilized by the bile in the intestinal lumen, absorbed in the enterocytes and packaged into chylomicrons with a variety of apolipoproteins, such as apoAI, AII, B-48, CI, CII, CIII, E. Chylomicrons are then secreted into the lymph and then enter the blood bloodstream via the vena cava. At the endothelial surface of vessels, triglycerides-rich particles are hydrolyzed by lipoprotein lipase (LPL) and release free fatty acids (FFA). ApoCII and apoAV are cofactors for LPL activation, while apoCIII has inhibitory proprieties. Fatty acids are taken up by peripheral tissues, particularly adipose tissue and muscles, to be stored or used as energy source. In the non-fasting state, FFA are released by lipolysis from adipose storage depots and captured by either muscles or liver cells (Hegele et al. 2009).

In the circulation, chylomicrons are remodeled into smaller-size remnants particles. The presence of ApoE on the surface of the particles enables the hepatic uptake of remnants chylomicrons through binding to LDL receptor (LDLr), LDL receptor related protein-1 (LRP1), heparin sulphate proteoglycans and Scavenger Receptor class B Type I (SRBI) (Mahley and Ji 1999). There are three different apoE isoforms: apoE2, apoE3 and apoE4 (characterized by single amino acid substitutions at positions 112 and 158) giving origin to three homozygous (E2/2, E3/3 e E4/4) and heterozygous (E2/3, E2/4, E3/4) phenotypes. ApoE2 has low affinity for binding to hepatic receptors and is not able to mediate remnants internalization (Altenburg et al. 2008).

In the endogenous pathway, VLDL are assembled in the hepatocytes by means of microsomal triglycerides transfer protein (MTTP) and contain ApoB100, ApoE and ApoC. VLDL circulate until triglycerides are hydrolyzed by LPL and phospholipids are transferred to HDL by phospholipid transfer protein (PLTP). These processes result in the formation of IDL that can be internalized in the liver or further remodeled by hepatic lipase (HL), which facilitates the conversion of IDL into LDL (Wasan et al. 2008). LDL particles are characterized by enrichment in CE and contain only apoB100 as protein component. LDL main function is to transport cholesterol to the tissues. Similarly to chylomicrons, LDL are removed from the circulation
upon interaction between ApoE and the LDLr exposed on the liver cells and, in much lower amount, in other peripheral tissue (Mahley et al. 1999).

After binding, LDL are internalized into the cells via endocytosis pathway and localize into lysosomes where CE are hydrolyzed. As a consequence, FC becomes available in the cells for different physiological functions such as cellular membrane building and synthesis of steroid hormones. Alternatively, FC can be stored intracellularly in lipid droplets, after being esterified by the acyl-coenzyme A (CoA):cholesterol acyltransferases (ACAT) enzyme.

Reverse cholesterol transport is the pathway through which cholesterol is transported from peripheral tissues on HDL lipoproteins to the liver for excretion. This aspect will be discussed in details in paragraph 5.

2.2 Lipoproteins and cardiovascular risk

Among the main well-known risk factors for CVD, elevated concentration of plasma cholesterol, in particular the one associated with the low-density fraction of lipoproteins (also called LDL-cholesterol), is unique in driving the progression of atherosclerosis. Indeed, other risk factors, such as the ones mentioned above -hypertension, diabetes, smoking, inflammatory conditions and male gender- can accelerate a process initiated by atherogenic lipoproteins but are not thought to be sufficient for ASCVD development (Falk 2006).

Elevation of low-density lipoprotein cholesterol (LDL-C) was first shown in the 1960s by the Framingham Heart Study to be a major risk factor for CVD (Kannel et al. 1961). Both the LDL- and the HDL-associated cholesterol (HDL-C) levels were then found to correlate with ASCVD-directly and inversely, respectively (Miller 1982). More precisely, a large number of epidemiological studies has showed that an increased LDL-C/HDL-C ratio in the blood is a major factor in determining the occurrence of cardiovascular events (Barter et al. 2007).

The importance of HDL-C, other than LDL-C, was also reinforced in animal studies where increased HDL levels, obtained by transgenic overexpression of human apolipoprotein A-I, the main HDL apolipoprotein, inhibited atherosclerotic lesion development (Rubin et al. 1991), or where intravenous infusions of HDL promoted lesion regression (Badimon et al. 1990). The positive outcomes of these observational and pre-clinical studies provided an incentive for the development of therapeutic agents specifically designed to increase HDL levels in humans. However, major developments in human genetics and clinical trials with pharmacological agents have led to doubt about the power of HDL-C as a risk modifier in CVD. Mendelian
randomization studies attempted to ask whether genetic variants associated with HDL-C that exist at some frequency in the general population, are associated with CVD. The work of Voight and colleagues demonstrated that common variants and most other loci associated with HDL-C have no significant association with coronary heart disease (Voight et al. 2012).

The other evidence leading to uncertainty about the HDL-C hypothesis relates to pharmacological trials with niacin and cholesteryl ester transfer protein (CETP) inhibitors. The inability of these drugs, which raise HDL levels, to improve cardiovascular outcomes definitely excluded HDL-C levels as an atheroprotective agent (Wright 2013; Rader and Tall 2012). Therefore, both the genetic and the pharmacological approach failed to mechanistically and causatively link HDL-C and CVD (Rader and Tall 2012, Rader 2014).

Among the other lipid parameters, hypertriglyceridemia associated to high levels of VLDL and chylomicrons appears to be less atherogenic than hypercholesterolemia. Because elevated triglyceride levels are often associated with low HDL levels it has been argued that low HDL-C is just a neutral player in (post-prandial) hypertriglyceridemia. Indeed, characterization of mendelian disorders as well as genome wide association studies (GWAS) of lipid metabolism have underscored that plasma HDL-C and TG can barely be considered as independent traits (Oldoni et al. 2014).

In recent years, however, a better understanding of HDL functional properties led to a new consideration of its atheroprotective quality. In particular, HDL-mediated reverse cholesterol transport (RCT) from the arterial wall to the liver, heralded by elevated HDL-C levels, relieves plaque-resident macrophages of excessive cholesterol that is acquired after internalization of oxLDL in the plaque. In this way, HDL counteracts high LDL-C detrimental effects. There are several evidences showing RCT or HDL quality, as a mRCT metric, to be implicated in CVD development and progression. First, mouse studies show that ATP binding cassette transporter A1 (ABCA1) activity as a mediator of cholesterol efflux to HDL impedes CVD development (Yvan-Charvet et al. 2010); second, correlational studies find strong inverse association between certain HDL particle species, particularly small HDL3 subpopulation, and CVD (Camont et al. 2011); third, most clinical investigation on HDL cholesterol efflux capacity (CEC) from macrophages, a surrogate for the in vivo mRCT, consistently revealed that this metric correlates with CVD inversely and independently of HDL-C (Khera et al. 2011).

Evidence from animal models of atherosclerosis also shows that HDL can ameliorate CVD by regulating immune processes, such as macrophage chemokine and cytokine production (Zhu and Parks 2012).
Therefore, while LDL-C is itself a well-established causative factor, it is concluded that HDL-C is just a surrogate marker for estimating the particle number and size of the total bulk of HDL, but it does not tell any information about the heterogeneous composition and, thus, functionality of HDL particles.

3. HDL

Human plasma HDL is a large, heterogeneous family of complex macromolecules that differ in size, shape and density (Fig.3). HDL is the smallest lipoprotein particle with a mean size of 8–10 nm and density of 1.063–1.21 g/ml (Kontush and al. 2015).

In the early 1950s Gofman and colleagues described for the first time HDL distribution by using analytic ultracentrifugation. Two HDL classes were identified: the less dense, relatively lipid-rich form, classified as HDL2 (1.063–1.125 g/ml), and the denser, relatively protein-rich form, classified as HDL3 (1.125–1.21 g/mL). HDL2 and HDL3 can be further fractionated by non-denaturing polyacrylamide gradient gel electrophoresis based on the particle size in distinct subclasses note as HDL2a, HDL2b, HDL3a, HDL3b, HDL3c (Nichols et al. 1986). HDL can also be resolved on agarose gel and classified according to surface charge and shape. Based on their electrophoretic mobility, HDL are distinguished into α-migrating particles, which have a high negative surface charge and represent the majority of circulating HDL, and preβ-migrating particles, consisting of nascent discoidal, poorly lipidated HDL which mainly contain apoAI and phospholipids (Rothblat and Phillips 2010). Agarose gels can be stained with Coomassie blue or blotted with anti-apoAI antibodies, and the relative protein content of the two HDL subclasses can be determined (Favari et al. 2004). The agarose and the electrophoresis gel can be combined into a 2D electrophoretic method, which separates HDL according to charge in the first run and to size in the second run. With this method, 12 distinct apoAI-containing HDL particles can be identified namely as pre-β (pre-β1 and pre-β2), α (α1, α2 and α3) and pre-α (pre-α1, pre-α2 and pre-α3) (Asztalos et al. 1993).

Protein components of HDL are majorly ApoAI (70%) and ApoAII (20%). Some HDL only contain ApoAI (LpAI) and others contain both apoAI and apoAII (LpAI: AII); those two groups can be distinguished by using immune-affinity methods.
3.1 HDL metabolism

In plasma, HDL are continuously interconverted into the different classes. The major structural protein of HDL is ApoAI, which is synthesized in the liver and, to a lesser extent, in the small intestine in humans. After its secretion into blood and mesenteric lymph, ApoAI associates with cholesterol and phospholipids to form discoidal complexes called nascent HDL (pre-β HDL). Initial lipidation of pre-β HDL in the interstitial fluid of tissues occurs via the ATP-binding cassette subfamily A member 1 (ABCA1) transporter-mediated efflux of unesterified cholesterol and phospholipids (Favari et al. 2009). Discoidal HDL can then acquire additional free cholesterol from other lipoproteins and from cell membranes in a process that does not require ABCA1.

Plasma lecithin–cholesterol acyltransferase (LCAT) is a 63kDa glycoprotein synthesized in the liver and the only enzyme capable of esterifying cholesterol in plasma. LCAT is responsible for the esterification of cholesterol on the surface of large discoidal HDL thus determining the maturation of high-density lipoproteins to spheroidal HDL3 and HDL2 particles (α-HDL) with a core of cholesteryl esters (Ossoli et al. 2016). These particles contain two to four molecules
of ApoAI, which act as a co-factor for the LCAT-mediated reaction. Although it is believed to be atheroprotective, LCAT deficiency in human as well as in animal models has not be associated with increased atherosclerosis *per se* (Calabresi et al. 2009) (Kunnen and Van Eck 2012). Large HDL can efflux cholesterol and phospholipids from peripheral cells via ABCG1; moreover, in some tissues, such as macrophages, scavenger receptor class B member 1 (SRB1) may also have a role in efflux to both HDL3 and HDL2 (Favari et al. 2009).

Further remodeling of α-HDL occurs via processes that are catalyzed by CETP, hepatic lipase, endothelial lipase and plasma phospholipid transfer protein (PLTP)

In humans, cholesterol and cholesteryl esters are transferred from HDL2 to the liver directly, after direct binding to SRB1 on hepatocytes, or indirectly, by being transferred to atherogenic lipoproteins (chylomicrons, VLDL, IDL LDL) via cholesteryl-ester transfer protein (CETP) (Fig.4). Some species, such as mice, do not express CETP, and the transfer of cholesterol and CE to the liver occurs by the direct pathway. CETP is a 476 residues hydrophobic glycoprotein that shuttles between HDL and apoB-containing lipoproteins and facilitates the bidirectional transfer of cholesteryl esters and triglycerides between them. Mutation of CETP gene results in elevation of HDL-C and moderates reduction of LDL-C and apoB levels (Matsuura et al. 2006).

Hepatic lipase (HL) is a 65kDa glycoprotein that hydrolyzes both triglycerides and phospholipids from all classes of lipoproteins (Vergeer et al. 2010). Conversely, the endothelial lipase (EL) exhibits higher affinity to phospholipids on HDL (Rader and Jaye 2000). In human plasma, EL inversely correlates with HDL-C levels and atherosclerosis. Consistent with these data, overexpression of EL in mice accelerates HDL catabolism whereas inhibition increases HDL levels (Rosenson et al. 2012).

Phospholipid transfer protein (PLTP) mediates the exchange of phospholipids between HDL and other lipoproteins (Masson et al. 2009). The transfer of PL mediated by PLTP from mature HDL results in the formation of lipid-poor HDL particles and shedding from HDL of lipid-free APOA1, which can interact with ABCA1 in the next lipidation cycle or being eliminated from the body via kidney excretion.
3.2 HDL Functions

In human HDL, more than 80 proteins have been identified by advanced proteomic technologies. Among those, apolipoproteins, enzymes, lipid transfer proteins, acute-phase response proteins, complement components and proteinase inhibitors represent the major subgroups (Hoofnagle and Heinecke 2009). Given this, it should not be surprising that HDL exert a broad range of functions thus playing important roles in human physiology and pathology.

**Anti-inflammatory and anti-oxidative activity**

HDL has been recognized to have anti-inflammatory properties on a variety of cell types, including endothelial cells and macrophages (Mineo and Shaul 2012). Oxidation has a central
role in the initiation and progression of atherosclerosis. The removal of lipids from oxLDL represents the first step of HDL-mediated protection from the oxidative damage.

The inhibitory effect of HDL on LDL oxidation was suggested to be related to metal ion chelation or to a peroxidase-like activity of HDL (Calabresi et al. 2015). The anti-inflammatory action of HDL involves hydrolysis of oxidized lipids by HDL-associated enzyme paraoxonase 1 (PON1). Other than PON1, other HDL-associated enzymes such as LCAT and platelet-activating factor acyl hydrolase (PAF-AH) are thought to be responsible for the anti-oxidative properties of HDL (Aviram et al. 1998). Moreover, HDL is a major transporter of sphingosine-1-phosphate (S1P), which activate several intracellular signals through its receptors on endothelial cells (Poti et al. 2014, Norata and Catapano 2005). The anti-oxidative activity of HDL is also related to the presence of various apolipoproteins including apoAI, apoE, apoJ, apoAII and apoAIV. In particular, apoAI was shown to prevent and delay LDL oxidation by removing long-chain palmitoyl-phosphorylcholines from trapped LDL and cells in the arterial wall (Kontush and Chapman 2006). Another plausible mechanism through which HDL exert their anti-inflammatory action is represented by direct interaction of apoAI with T lymphocytes, which can block subsequent activation of monocytes. Moreover, apoAI has been reported to decrease neutrophil activation in vitro (Burger and Dayer 2002). Furthermore, HDL can limit the expression of cytokines such as TNF-α and interleukin-1 that mediate up-regulation of leukocyte endothelial adhesion molecules. The ability of HDL to directly reduce leukocyte adhesion molecule expression on endothelial cells was then ascribed to the presence of apoAI, apoAII, apoAIV as well as S1P and other sphingolipids (Ansell et al. 2005).

**Anti-apoptotic, vasodilatory, antithrombotic and anti-Infectious activities**

HDLs promote endothelium proliferation and diminish endothelial apoptosis induced by oxLDL or TNF-α. Indeed, HDL-associated bioactive lysosphospholipids, including S1P, are potent anti-apoptotic agents, which may mediate the anti-apoptotic effect of HDL by increasing NO production. The stimulation of NO release by endothelial cells also explains HDL vasodilatory activity. HDL induce the expression and the activity of endothelial NO synthase after binding to scavenger receptor class B type I (SR-BI). Similarly, HDL can stimulate the production of prostacyclin, which displays vasorelaxing activity. Again, the vasoactive effects of HDL can be explained by S1P acting via its lysosphospholipid receptor S1P3. S1P may be equally important for HDL mitogenic effects on endothelial cells and for the HDL inhibitory action on migration of vascular smooth muscle cells (Calabresi et al. 2003). Moreover, the increased production of NO and prostacyclin are responsible for the inhibitory actions of HDL.
on platelet activation and aggregation. The antithrombotic properties of HDL was also related to the suppression of the coagulation cascade as well as to the modulation of platelet signaling via SR-BI (van der Stoep et al. 2014). HDL play a major role in the binding and biliary clearance of circulating endotoxins, thereby inhibiting endotoxin-induced cellular activation, which then results in potent anti-infectious activity. The inactivation of endotoxin by HDL occurs through a direct interaction between apoAI and endotoxin and involves the reduction of CD14 expression on monocytes as a key step (Hajduk et al. 1989).

**Reverse cholesterol transport**
One of the most important function of HDL to protect against atherosclerosis is their capacity to promote the reverse cholesterol transport (RCT). This consists in a physiological process whereby cholesterol is removed from extra hepatic tissues and delivered to the liver for final excretion into bile. In this process, HDL play a critical role as they remove cholesterol from peripheral cells, particularly macrophages and foam cells, via interaction with membrane transporters. Lipid-free apoAI, apoAII, apoE, and other HDL apolipoproteins induce fast, saturable, unidirectional and LCAT-independent removal of cholesterol and phospholipids from cells (Favari et al. 2009). Cholesterol efflux from macrophages to plasma is the first critical step of RCT and is considered a key anti-atherosclerotic function of HDL (Rohatgi et al. 2015).
4. Reverse Cholesterol transport

Most peripheral tissues, including macrophages, lack the ability to catabolize cholesterol to soluble, simple derivatives. Instead, cholesterol is released by diffusion or protein-mediated transport to HDL and other acceptors in the interstitial fluid for reverse cholesterol transport (RCT) in lymph and plasma to the liver, from where cholesterol is secreted into the gastrointestinal tract (Randolph and Miller 2014) (Fig. 5). Several recent in vivo studies established the inverse relationship between the rate of macrophage RCT and ASCVD. For this reason, the macrophage RCT has been proposed as a novel therapeutic target to reduce atherosclerotic plaque burden and subsequent cardiovascular events (Rosenson et al. 2012).

The process of RCT can be summarized in three steps:
- Cell cholesterol efflux
- HDL remodeling in plasma
- Hepatic cholesterol uptake and excretion

4.1 Cell cholesterol efflux

The removal of unesterified cholesterol and phospholipids from the cells is the first, rate-limiting step in RCT and occurs via multiple pathways (Adorni et al. 2007). The efflux process involves cholesterol localized on the plasma membrane, which may derive from intracellular sites, such as the late endosomal or lysosomal compartment, and from the Golgi apparatus (Maxfield and Tabas 2005). The transfer of FC to the blood compartment occurs via passive aqueous diffusion and by the participation of different transporters expressed on the cell membranes. Several factors, such as cell cholesterol status, lipid transporter activity and the nature of extracellular acceptors were shown to impact its efficiency (Zanotti el al. 2012).

Passive diffusion mainly involves the desorption of free cholesterol molecules from the plasma membrane of cells and their diffusion through the aqueous phase until they collide with, and are absorbed by, an acceptor (Davidson et al. 1995). The release of cholesterol and phospholipid via passive diffusion occurs in all cell types and is accelerated when the scavenger receptor class B type I (SR-BI) is present on the cell membrane. SR-BI is a bidirectional cholesterol transporter that facilitates cell cholesterol desorption down to a concentration gradient between the donor and the acceptor compartment. Both passive diffusion and SR-BI mediated efflux
occur to mature HDL, lipidaded apolipoproteins and phospholipid-enriched acceptors (Yancey et al. 2000).

ATP binding cassette A1 (ABCA1)- and G1 (ABCG1)- mediated cholesterol efflux utilizes ATP as an energy source to transport lipids across membranes. Both protein are member of the large superfamily of ABC transporters and are characterized by the presence of a nucleotide-binding domains containing two conserved peptide motifs known as Walker A and Walker B. ABC transporters are integrated into the cell membrane by domains containing six transmembrane helices. The minimum requirement for an active ABC transporter is two nucleotide-binding and two 6-helix transmembrane domains (Oram and Lawn 2001). The human ABCA1 gene has been mapped on chromosome 9q31 and is composed of 50 exons, which encode for a full size ABC transporter of 2261 amino acids. The human ABCG1 gene has been mapped on chromosome 21q22.3, it is composed of 23 exons and has multiple transcripts. Structurally, the ABCG1 protein is a half transporter where the nucleotide-binding domain at the N terminus is followed by six transmembrane domains. Therefore, ABCG1 forms homodimers to be functionally active (Schmitz et al. 2001). ABCA1 promotes unidirectional efflux of phospholipids and cholesterol to lipid-free apolipoproteins and small discoidal preβ-HDL while ABCG1 transports cell cholesterol to mature α-migrating HDL particles, as well as to discoidal preβ-HDL, but not to lipid-free apoA1 (Favari et al. 2009).

Cholesterol efflux via ABCA1 and ABCG1 depend on the protein expression levels, in turn regulated by transcriptional or post-translational mechanisms. The transcription of Abca1 and Abcg1 genes is primarily under control of the Liver X Receptor/Retinoid X Receptor (LXR/RXR) axis, which is stimulated upon intracellular cholesterol enrichment (Larrede et al. 2009). After being internalized into the cell, cholesterol is oxidized by cytochrome P-450 enzymes to form oxysterols 22-hydroxycholesterol, 24-hydroxycholesterol, and 24,25-epoxycholesterol. Subsequently, oxysterols bind the LXR/RXR heterodimer that acts as a nuclear transcription factor by binding to the promoter motif of the Abca1 gene (Costet et al. 2000). Moreover, Oram et al. showed that ABCA1 expression can be induced by analogs of cAMP (Oram et al. 2000). One more possible way to modulate Abca1 transcription involves the activation of peroxisome proliferator activating nuclear receptors (PPARs), which induces ABCA1 protein expression through a transcriptional cascade mediated by the LXR nuclear receptor (Chinetti et al. 2001).

ABCA1 protein expression is post-transcriptionally regulated via several mechanisms including: 1) the stabilization of ABCA1 protein by apoAI, and 2) the acceleration of its turnover by calpain-mediated proteolysis or polyunsaturated fatty acids (Vaughan and Oram
The relative contribution of each pathway to cholesterol export has been determined *in vitro* in both normal and cholesterol-loaded cells (Adorni et al. 2007). It is important to remember that the contribution of each single pathway of cholesterol flux is species-specific: whereas ABCA1-mediated efflux is the principal mechanism in both human and murine cultured macrophage foam cells, the role of ABCG1 is elusive in the former, but not in the latter. Conversely, SR-BI has a pivotal role in human cells, but not in the murine ones (Adorni et al. 2007).

Another mechanism of cell cholesterol export via diffusion to apolipoprotein E (ApoE), or to apoE-containing HDL, was described to have a relevant role in cholesterol efflux from macrophages and RCT (Mahley et al. 2006, Zanotti et al. 2011).

ApoE is a 34-kDa protein synthesized by many cell types, including macrophages, upon different stimuli. Once in the plasma membrane, secreted apoE can be released into the extracellular medium or stay bound to the cell surface in association with heparan sulfate proteoglycans. When apoE is secreted from cholesterol-loaded macrophages, this process can promote cholesterol efflux both in absence or in presence of exogenously added HDL as cholesterol acceptors, causing the generation of nascent HDL lipoproteins. Experimental evidence suggests that apoE can promote cholesterol release by both ABCA1-dependent and independent mechanisms, whereas ABCG1 may contribute by driving cholesterol efflux to apoE-enriched particles (Huang et al. 2006).

### 4.2 HDL remodeling in plasma

Nascent HDL (n-HDL) particle are spheroidal and contain more than 40% of their total lipids as free cholesterol. The dynamic remodeling of these n-HDL in plasma results in the formation of a variety of HDL particles with different density, size, and composition (Rader and Hovingh 2014). Lipid-free apolipoprotein A1 (pre-β HDL) promotes the cell cholesterol efflux via ABCA1 (Favari et al. 2009) while mature HDL2 and HDL3 are the typical acceptors for cell cholesterol provided by the ABCG1 and SR-BI transporters (Santos-Gallego et al. 2011). It is important to note that under physiologic conditions ABCA1 and ABCG1 can act in a sequential fashion, with ABCA1 generating nascent HDL, which then promote lipid release via ABCG1 (Gelissen et al. 2006). However, this concept has changed recently, since ABCG1, unlike ABCA1, seems to efflux cell cholesterol by a process that is not dependent upon interaction with an extracellular protein (Tarling and Edward 2012). Once accumulated into HDL, cholesterol is esterified by the plasma enzyme lecithin:cholesterol acyltransferase (LCAT),
yielding to mature, lipid rich-HDL with a core of cholesteryl esters, the so-called HDL3. HDL3 release phospholipids and cholesteryl esters to VLDL and chylomicrons thus allowing, in exchange, the acquisition of more apolipoproteins addressing to the formation of mature HDL2 particles (Dieplinger et al. 1985). The majority of the cholesteryl esters are transferred by plasma CETP, in exchange for triglycerides, to the apoB-containing lipoproteins, which will then be catabolized by the liver through their receptors (Barter et al. 2003). Triglyceride-enriched HDL are then susceptible to lipolytic modification by HL and EL. Upon modification by these enzymes, a smaller HDL particle is formed, which undergoes faster catabolism.

4.3 Hepatic cholesterol uptake and excretion

A minor fraction of cholesteryl esters are directly delivered to the liver by HDL through interaction with SR-BI, an integral membrane protein that can also uptake HDL unesterified cholesterol. SR-BI, an 82-kDa protein belonging to the CD36 family, is a key regulator in HDL metabolism: it facilitates the efflux of cholesterol from cells in peripheral tissues to HDL and mediates the selective uptake of cholesteryl esters from HDL in the liver upon interaction with ApoAI. In particular, hepatic SR-BI has a role of major importance in determining the plasma levels of HDL lipoproteins (Trigatti et al. 2000).

Additionally, HDL2 can be recognized by LDL receptor on hepatocytes surface, which then mediates the whole lipoprotein uptake in the liver (van der Velde et al. 2010). After hepatic uptake, cholesteryl esters first need to be hydrolyzed into free cholesterol molecules that can be then excreted in the bile via ABCG5/ABCG8 as neutral sterols (Yu et al. 2005) or via ABCA11 after conversion to bile acids (Wakabayashi et al. 2004). Either way, bile salts are secreted from the gallbladder in the duodenum region of the intestine where they act as detergent to emulsify and digest dietary fats and are ultimately eliminated into feces.
Fig.5 The reverse cholesterol transport (RCT) pathway (van der Velde & Groen, 2005)
5. Cholesterol Efflux Capacity (CEC)

RCT was first described by Glomset as the physiological pathway whereby cholesterol is removed from peripherals tissue, delivered by HDL into blood and finally uptaken by liver for disposal into feces. However, cholesterol efflux from macrophage cells differs from cholesterol efflux from other peripheral cells.

This is most evident in the phenotype of Tangier disease, a condition caused by null mutations in the gene for ATP-binding cassette transporter A1 (ABCA1), which is essential and sufficient for nascent HDL formation (Rust et al. 1999). When ABCA1 is present in the cell plasma membrane, apoAI binds to ABCA1/plasma membrane lipids and forms a lipoprotein particle that consists of plasma membrane-derived phospholipid and cholesterol and 2 or more molecules of apoAI. Cholesterol efflux by the ABCA1/apoAI pathway occurs to the extent that cell cholesterol departs the cell in the newly formed HDL particles. Tangier disease patients do not form HDL and exhibit accumulation of cholesterol in many tissue macrophage (Tall and Wang 2000). Beside macrophages, Schwann cells, muscle cells and certain fibroblasts have been reported to accumulate cholesterol, but the rest of peripheral cells that normally express ABCA1 (hepatocytes, adipocytes, enterocytes, skin fibroblasts, etc) appear at least superficially normal in these subjects. It was concluded that macrophages are unique among all the cell types in the overwhelming reliance on nascent HDL particles as a means to remove excessive intracellular cholesterol. This dependence of macrophages, but not other cells, on HDL for cholesterol release led to the concept of macrophage RCT (mRCT) as a unique branch of the reverse cholesterol transport.

It is thought that accumulation of foam cells in the coronary arteries is caused at least in part by deficient mRCT, because when mRCT is compromised, arterial macrophages cannot readily release scavenged cholesterol. A radio-isotopic assay was developed to measure specifically mRCT using murine animal models (Zhang et al. 2003). In this method macrophages cells are radiolabeled with cholesterol and then injected into recipient mice. Afterwards, macrophage-derived cholesterol is directly measured in plasma, liver and feces, which are the main compartments involved in RCT.

mRCT can be assessed in vivo in mice, but not in human subjects. This necessitated the development of an ex vivo assay that measures HDL cholesterol efflux capacity (HDL CEC) as a marker of the RCT efficiency. HDL cholesterol efflux capacity (CEC) measures the ability of human plasma HDL to accept cholesterol specifically from macrophages: higher CEC signifies a better ability of HDL to accept macrophage cholesterol and thus a lower probability that
plaque macrophages will become foam cells. (It should be noted here that plaque macrophages release cholesterol to HDL in the interstitial fluid, while we measure CEC with plasma HDL lieu of interstitial fluid).

Epidemiological studies define HDL as the most powerful plasmatic factor with atheroprotective activity in humans (Di Angelantonio et al. 2009): a 1 mg/dl increase of plasma HDL-C corresponds to a 3-4% reduction in mortality for cardiovascular events (Assmann et al. 2002). As described in paragraph 3, since the genetically-determined and the pharmacologically-induced raising of HDL levels failed to be proven beneficial for cardiovascular outcomes (Degoma and Rader 2011, Voight et al. 2012), the concept of HDL quality, as a more pertinent measure than HDL quantity for CVD, emerged. In other words, if HDL anti-atherogenic functions is, at least partially, independent of their plasma levels, a new metric of HDL efficiency must be used to describe HDL protective effects in cardiovascular diseases. HDL cholesterol efflux capacity (CEC) is the sole measure of HDL functionality with respect to reverse cholesterol transport in humans. Therefore, evaluation of HDL cholesterol efflux capacity, as a metric of HDL functionality, may offer a better prediction of CVD than HDL-C levels alone (Bhatt and Rohatgi 2016).

As said before, cell cholesterol efflux is mediated by a number of pathways, with the various HDL subclasses displaying different capacity to promote the cholesterol export via each of these pathways. The efficiency of plasma or serum CEC can be measured in a single individual ex vivo by using a widely standardized, radio-based technique that allows to distinguish among the various mechanisms involved (Adorni et al. 2007).

The “HDL quality” hypothesis brings together the concept of HDL functionality with that of HDL structural composition. Subjects affected by CVD not only have low HDL, but also major rearrangements of their composition (Campos et al. 1995; Sweetnam et al. 1994) and consequently function. All HDL particles are able to promote cholesterol efflux from cells by interacting with specific cell membrane proteins, but the removal of cholesterol through ABCA1 promoted by small preβ-HDL appears as the most important mechanism to explain the variability in cholesterol efflux among individuals. For instance, subjects with LCAT deficiency or the apo-AI Milano mutation have high levels of small, lipid-poor ApoAI particles and efficient serum ability to induce macrophage cholesterol depletion despite very low plasma levels of HDL (Favari et al. 2007; Calabresi et al. 2009). Moreover, it was shown that apolipoprotein B-depleted sera from subjects with similar HDL-C can have higher CEC due to
significantly higher ABCA1-mediated efflux, and this efflux is significantly correlated with the levels of preβ-HDL (de la Llera-Moya et al. 2010; Calabresi et al. 2009).

A large body of recent data reinforced the idea that the ability to transfer cholesterol from macrophage cells to HDL is inversely related to CVD risk. In two distinct cohort of subjects, the capacity of serum HDL to promote cholesterol efflux from macrophages was shown to have a strong, inverse association with both carotid intima–media thickness, an index of subclinical atherosclerosis, and the likelihood of angiographic coronary artery disease (Khera et al. 2011). This results was then confirmed in a population of healthy subjects where ABCA1-mediated CEC inversely correlated with pulse wave velocity (PWV), an index of arterial stiffness and atherosclerosis, independently of HDL-C serum levels (Favari et al. 2013). Additionally, the role of HDL CEC as a marker of cardiovascular protection has been strongly supported by a prospective study showing that serum CEC had a powerful predictive power of incident cardiovascular events in a population-based cohort free of cardiovascular disease at baseline, which was followed over a period of 9 years (Rohatgi et al. 2014).

In particular, Rohatgi and colleagues found that CEC, in contrast to HDL-C, had minimal association with multiple traditional risk factors. In a fully adjusted model that included traditional risk factors and HDL-C there was a 67% reduction in CVD risk in the highest quartile of CEC vs the lowest. Furthermore, adding CEC to traditional risk factors was associated with improvement in discrimination and reclassification indices.

A study measuring a parameter of endothelial function note as flow-mediated dilation, showed a positive correlation with the ABCA1- and ABCG1-mediated efflux pathway confirming the idea that the functional measures of HDL might perform as a more accurate marker for CVD risk rather than HDL-C levels (Vazquez et al. 2012, Vigna et al 2014). Recently, Li and colleagues provided data indicating that individuals in the top tertile of CEC was paradoxically associated with moderate increased prospective risk for cardiovascular end point of myocardial infarction, stroke, and death during three years of follow-up (Li et al. 2013). In the same study, however, CEC was inversely associated with coronary artery disease; the lack of a dose-response relationship across increasing tertiles of CEC suggests a threshold effect. The reasons for this apparently contradictory findings require further evaluation, but may be related to the characteristics of the population being studied (Khera and Rader 2013).

The amelioration of HDL function, in terms of CEC, might become an important tool to improve atheroprotection and prevention strategies as well as pharmacological treatment.

Plasma/serum cholesterol efflux capacity (CEC) is the sole measure of HDL functionality with respect to RCT in humans and is a potent marker of atherosclerotic cardiovascular disease
Numerous findings have reinforced the idea that changes in HDL-C levels do not always impact CVD risk and are an inadequate surrogate for therapeutic use. Instead, the present data suggest that evaluation of CEC may better correlate with coronary artery diseases than HDL-C, however further studies are required to demonstrate its suggested role as strong, independent predictor of incident ASCVD. Altogether, the available evidences lead to the conclusion that HDL CEC may represent in the future a useful biomarker of ASCVD risk (Rohatgi 2015).
6. Therapeutic strategies for the treatment of CVD

The most common therapeutic strategy presently used to prevent CVDs aims to reduce its well-known risk factors, in particular lowering lipid levels. Both randomized controlled trials of LDL-C-reducing drugs as well as studies of the human genetics of LDL-C and their relationship to ASCVD have unequivocally established LDL as a causal risk factor. For this reason, the cholesterol carried in LDL has been the major focus of therapeutic intervention for more than three decades. Statin therapy focuses on reducing cholesterol inflow into the sub-endothelium by lowering serum LDL levels. In particular, statins inhibit HMG-CoA reductase, an enzyme that catalyzes the rate-limiting step of cholesterol biosynthesis, and aim to reduce hepatic cholesterol content to enhance transcriptional upregulation of the LDL receptor leading to reduction of plasma LDL-C. Among drugs for the treatment of CVD, statins have been the most successful and widely used since their introduction on the market. However, despite their ability in reducing LDL levels and thus cardiovascular risk, statin drugs seem to have reached the limits of efficacy leaving 50 to 70% of CVD risk unaddressed (Mehra et al. 2012).

Because of the large “residual risk” with common lipids-lowering treatments new therapeutic strategies need to be evaluated. The cholesterol absorption inhibitor ezetimibe, was recently proven to reduce CV events when added to a statin compared with statin alone (Cannon et al. 2015). Ezetimibe can be particularly useful in class of patients at high cardiovascular risk who are not able to reach LDL-C treatment goals on high-intensity statin therapy. In combination with statins, ezetimibe further lowers serum LDL and improves cardiovascular outcomes without altering the side effect profile (Ip et al. 2015).

Bile acid sequestrants (cholestyramine, colestipol and colesevelam) work as bile acid–binding exchange resins: they are not absorbed systemically, but remain in the gut and are excreted along with the bile containing cholesterol. The sequestrants are safe drugs but are used relatively infrequently since they are not as effective as statins and have adverse side effects (constipation and bloating) that result in poor compliance. However, they are useful in combination with a statin for patients with severe LDL-C elevations who fail to meet target levels and in statins-intolerant patients (2016 ESC/EAS Guidelines for the Management of Dyslipidaemias).

Recently, a new important class of drugs has become available to manage CVD: the proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors. Elevated levels and/or functions of the PCSK9 protein in plasma reduce the LDL receptor expression by promoting, upon binding, the
LDLr lysosomal catabolism, with consequent increase of plasma LDL-C concentration (Norata et al. 2014). Therapeutic strategies have been developed mainly using monoclonal antibodies (evolocumab, alirocumab) that reduce LDL-C levels by 60% independently from the presence of a background lipid-lowering therapy. The mechanism of action relates to the reduction of plasma levels of PCSK9, which in turn is not available to bind the LDLr. Higher expression of LDLr at the cell surface leads to a reduction of circulating LDL levels. According to current European guidelines, people at very high CVD risk such as people with Homozygous Familial Hypercholesterolemia (HoFH) on maximally tolerated statin doses and/or with persistent high levels of LDL-C are optimal candidates for this antiPCSK9-based therapy (2016 ESC/EAS Guidelines for the Management of Dyslipidaemias). PCSK9 inhibitors appear to have a good safety and side effect profile; however, they have the disadvantage of requiring injection for administration. Mipomersen inhibits production of apolipoprotein B, the major structural component of LDL particles. It is given in patients with FH by subcutaneous injections. Lomitapide, a molecule that works by inhibiting the microsomal triglyceride transfer protein (MTP) and thus the assembling of VLDL in the liver, is another agent that is used chiefly in patients with HoFH or in adults with very severe HeFH. Unlike PCSK9 inhibitors and mipomersen, it is given orally and hence does not require injections. However, in comparison with these other two novel agents, lomitapide has a greater risk of hepatotoxicity (Cuchel et al. 2013).

World Health Organization (WHO) has estimated that 38% of patients on short term treatment and 43% of patients on long term treatment do not adhere to medical therapy. There is strong evidence showing that dietary factors may influence atherogenesis directly or by acting on traditional risk factors such as plasma lipids and glucose or blood pressure. The role of nutrition in the prevention of CVD has been extensively reviewed (Dalen et al. 2014). Moreover, innovative nutritional strategies have been developed to improve dyslipidaemia; these so-called “nutraceuticals” can be used either as alternatives or as support to pharmacological approach (2016 ESC/EAS Guidelines for the Management of Dyslipidaemias). Nutraceutical therapies include molecular complexes and microorganisms, also called probiotics, which can be found in plants, milk and functional foods. Nutritional evaluation of functional foods not only includes the search for clinical evidence of health benefits but also the demonstration of good tolerability and the absence of major undesirable effects. As future perspectives, drugs that act by lowering the inflamed environment of the lesions or impairing foam cell formation through promotion of the HDL atheroprotective quality, could be of use for additional therapeutic intervention.
Part I

Effect of Tetranectin-ApoAI infusion on HDL-mediated cholesterol efflux capacity in hypercholesterolemic rabbits
Aim
One of the main independent risk factor for CVD is a low level of circulating high-density lipoprotein (HDL-C) (Barter et al. 2007). However, all pharmacological treatments aiming to increase the HDL-C concentration failed to reduce cardiovascular risk so far.

The concept of a “HDL-based therapy” to treat CVD is recently evolving to a more dynamic approach that emphasizes the cholesterol efflux capacity of HDL, a metric of HDL functionality, for atherosclerosis prevention or regression rather than HDL plasma level (Zheng and Stroes 2016). Among the therapies that aim to raise HDL plasma levels and possibly HDL functionality, intravenous administration of synthetic HDL (s-HDL) containing human apoAI demonstrated to induce a significant atherosclerosis regression (Zheng and Stroes 2016).

HDL infusion therapies are a physiological-like approach for raising HDL particle number and enhancing HDL anti-atherotrombotic functions. HDL infusions induce an elevation in apoAI and pre-β HDL particles; these formulations mime endogenous HDL particles and they are rapidly remodeled into mature spherical HDL after infusion (Kingwell et al. 2014). However, one of the main issue concerning this therapeutic approach may be a rapid apoAI catabolic rate due to fast renal clearance.

Based on this approach, a recombinant high-molecular mass variant of human apoAI, named Tetranectin-apoAI, has been engineered by fusing three apoAI molecules with the trimerization domain of human tetranectin, a plasminogen-binding plasma protein. This trimeric apoAI does not pass the glomerular filters and hence shows slower renal clearance and a prolonged half-life as compared to the natural variant of human apoAI. Moreover, it was previously shown that sHDL containing trimeric apoAI (TN-sHDL) maintains the biological functions of monomeric apoAI by promoting cell cholesterol efflux, stimulating LCAT-mediated cholesterol esterification, and exerting anti-inflammatory effects (Graversen et al. 2008).

The aim of this study was to evaluate the effect of infusion of a novel reconstituted HDL containing recombinant human trimeric apoAI complexed with 1-palmitoyl-2-oleoyl phosphatidylcholine and di-palmitoyl phosphatidylcholine (TN-rHDL) on CEC in relationship to atherosclerosis development in a model of hypercholesterolemic rabbit.

18 New Zealand male rabbits were induced to develop the atherosclerotic lesion. To this aim, animal in study were anesthetized and common carotid arteries were perivascularly injured. All animals were then fed a 1.5% cholesterol diet for the entire duration of the study. 90 days after lesion induction, rabbits were anesthetized and a complete scan of right carotids, including the area of plaque formation, was recorded by intravascular ultrasound (IVUS). Cross sectional area at the point of maximal stenosis was measured, and 16 animals with stenosis between 25-50% (excluding calcific and fibrotic lesions) were selected for the study.
All animals were fasted overnight and the right jugular vein of the selected rabbits was isolated and cannulated for treatment. Rabbits were then randomly divided into 2 groups, containing 8 animals each, and treated with a single infusion of 200 mg/Kg TN-sHDL or with placebo (5mM Sodium phosphate and 240mM sucrose, pH 7.3). Furthermore, other 15 rabbits were randomly divided into 3 groups and treated with multiple infusions (five in total) of 100 mg/Kg TN-sHDL, 8 mg/Kg TN-sHDL or with placebo.

Plasma samples were withdrawn from rabbits before infusion (baseline), and at 4h and 72h after the end of the infusions.

ABCA1-mediated, aqueous diffusion (AD)-dependent and total cholesterol efflux capacity (CEC) of rabbit plasma was evaluated at baseline (0h) and at the different time points of plasma sampling after the treatment (4h and 72h). CEC was measured ex vivo by a well-established radio-isotopic assay using J774 murine macrophages as cellular model.
Materials and methods
Cell lines
Murine macrophage J774 cells were used as the reference cell line in the cholesterol efflux capacity assay. Cells were grown in DMEM containing 10% FCS (Sigma-Aldrich) supplemented with 1% geneticin (Gibco, Life technologies) and 1% penicillin/streptomycin (Gibco). Cells were maintained in sterile flasks and incubated at 37°C with 90-95% humidity and 5% CO2. When adherent cells reached about 100% confluency, they were washed with sterile PBS (Lonza) and detached mechanically with the use of a sterile scraper, in order to be used in experiments. Cells were seeded in 12 wells plate (Corning) at approximately a 50-60% of confluency in 10% FCS/DMEM.

Reagents
All culture media were stored at 4°C. Plasma of the infused rabbits were kindly provided by Professor Giulia Chiesa from Dipartimento di Scienze Farmacologiche e Biomolecolari, Università di Milano and immediately stored at -80°C upon arrival. Acyl CoA-acyltransferase (ACAT) inhibitor (Sandoz) was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C; in order to avoid toxic effects, the concentration of this solvent did not exceed 0.5% of the total volume of the medium used for treating the cells. Purified apolipoprotein AI and chlorophenylthio-cyclic adenosine monophosphate (cAMP) were provided by Sigma-Aldrich; ApoA-1 solution was kept at -20°C while cAMP was dissolved in distilled water and kept at at -20°C.

Measurement of Aqueous Diffusion-dependent CEC (AD CEC)
After 24h from the seeding, cell monolayers were washed with PBS and incubated for 24h in DMEM containing 2μCi/ml of [1,2-3H]-cholesterol (Perkin-Elmer). The labeling medium also contained 1% FCS and 2μg/ml of an ACAT inhibitor (Sandoz) to ensure the intracellular cholesterol being in the non-esterified form. Following the labeling period, cells were washed and incubated in DMEM with 0.2% BSA (Sigma-Aldrich) for 18h. After this incubation, some wells were washed with PBS, dried, and extracted with isopropanol; the radioactivity in these cell extracts provide baseline (T0) values for total [3H]cholesterol content. The other cells containing [3H]cholesterol were washed with PBS and incubated in the presence of 0.5% (v/v) whole rabbit plasma in FCS-free medium. Cholesterol efflux was allowed to proceed for 4hours. Cell medium was then filtered through a
0.45μm filter to remove floating cells, and the radioactivity in the supernatant was determined by liquid scintillation counting (Optifluor for aqueous solvents and Instafluor for organic solvents, Packard Instrument International S.A.).

Cholesterol efflux capacity is calculated as the percentage ratio between the radioactivity of cell medium and the total radioactivity present in the cell extracts at time zero. The negative control is represented by the cholesterol efflux capacity of medium without acceptors, the efflux percentage of this condition is subtracted from the cholesterol efflux capacity of each serum sample, in order to discard the unspecific contribution of medium to cholesterol efflux capacity of serum samples. The positive control is represented by the cholesterol efflux capacity value of a 0.5% standard serum (SN) that is included in every plate.

**Measurement of Total and ABCA1-mediated CEC (TOTAL CEC and ABCA1 CEC)**

Stimulation of J774 murine macrophages with cAMP up-regulates the expression and activity of the ABCA1 protein. In such conditions, total release of cholesterol mainly occurs by both the ABCA1-mediated pathway and the aqueous diffusion route. Indeed, the release of cholesterol to serum from J774 macrophages treated with cAMP is noted as total cholesterol efflux. Cells were grown and labeled for 24h as previously described for the AD assay. Afterwards, cells were washed and incubated with 0.3mM of cAMP in DMEM/0.2% BSA for 18h, to upregulate ABCA1. Cell monolayers were then washed with PBS and incubated with 0.5% (v/v) of whole rabbit plasma; efflux was allowed to proceed for 4h. As positive controls, both 0.5% SN and 10 μg/ml of purified ApoAI, the preferential acceptor for ABCA1, were used. The negative control was given by the cholesterol efflux of wells treated with only medium. Cholesterol efflux capacity was calculated as described above. The ABCA1-mediated cholesterol efflux was calculated as the difference between the percentage of efflux from cAMP-stimulated J774 macrophages (total efflux) and the percentage of efflux from unstimulated cells (AD-dependent efflux).

**Statistical analysis**

Each condition was run in triplicate in order to optimize the reproducibility of data. The data are expressed as the average of each triple and its relative standard deviation (SD). The statistical significance between groups was determined by the 2-way ANOVA test using GraphPad Prism 5.0 software. The changes observed were considered significant for values of
P <0.05. In each single experiment, a standard serum (SN) was included as internal control and used to normalize the sample values in order to correct for the inter-assay variability.
Results
Total CEC of rabbits treated with a single infusion of 200 mg/kg TN-sHDL

Total CEC of rabbit plasma collected 4h after the end of TN-sHDL infusion (blue bars) was significantly increased compared to total CEC of rabbit plasma in the placebo group (white bars) at the same time point (p<0.0001). Instead, no differences in total CEC were observed between the two groups at baseline (0h) and at the 72h sampling point. Moreover, for TN-sHDL treated rabbits, plasma total CEC at 4h was significantly higher than that measured before infusion or 72h after the end of the infusion (p<0.0001) (Fig.I.1).

In the placebo group, no differences in total CEC were observed between the different time points.

Fig.I.1 Effect of a single infusion of 200 mg/kg TN-sHDL on total CEC. Total CEC was measured as described in the Materials and methods section. Total CEC was evaluated in plasma of rabbits treated with a single infusion of 200 mg/kg TN-sHDL (n=8, blue bars) or placebo (n=8, white bars) at baseline (0h) or 4 hours (4h) and 72 hours (72h) after the treatment. Mean ± SD. Statistical analysis - 2-way ANOVA to compare differences between the two treatment groups (TN-sHDL or placebo) at each time point and to compare differences within group between the different time points (**** p<0.0001 vs placebo; § p<0.0001 vs TN-sHDL at 0h and 72h).
AD-dependent CEC of rabbits treated with a single infusion of 200 mg/kg TN-sHDL

Similarly to what observed for total CEC, 4 hours after the end of the infusion the AD-dependent CEC of TN-sHDL-treated rabbit plasma (blue bars) was significantly increased as compared to that of placebo (white bars) (p<0.01). Again, AD-mediated CEC at 4h after TN-sHDL infusion was also significantly higher than AD CEC measured at baseline (0h) and 72h after infusion (p<0.05) (Fig.I.2).

In the placebo group, no differences in AD CEC were observed between the different time points.

![Fig.I.2 Effect of a single infusion of 200 mg/kg TN-sHDL on AD-dependent CEC. AD-dependent CEC was measured as described in the Materials and methods section. AD CEC was evaluated in plasma of rabbits treated with a single infusion of 200 mg/kg TN-sHDL (n=8, blue bars) or placebo (n=8, white bars) at baseline (0h) or 4 hours (4h) and 72 hours (72h) after the treatment. Mean ± SD. Statistical analysis - 2-way ANOVA to compare differences between the two treatment groups (TN-sHDL or placebo) at each time point and to compare differences within group between the different time points (** p<0.01 vs placebo; § p<0.05 vs TN-sHDL at 0h and 72h).](image)
ABCA1-mediated CEC of rabbits treated with a single infusion of 200 mg/kg TN-sHDL

In TN-sHDL-treated rabbits (blue bars), the ABCA1-mediated CEC significantly increased 4 hours after the infusion as compared to pre-treatment values (0h) and returned to baseline values 72h after the end of the infusion (Fig.I.3). In the placebo group (white bars), no differences in ABCA1-mediated CEC were observed between the different time points.

No significant differences in ABCA1-mediated CEC were observed between TN-sHDL treated rabbits and placebo ones at any analyzed time point (Fig.I.3).

Fig.I.3 Effect of a single infusion of 200 mg/kg TN-sHDL on ABCA1-dependent CEC. ABCA1-mediated CEC was measured as described in the Materials and methods section. ABCA1 CEC was evaluated in plasma of rabbits treated with a single infusion of 200 mg/kg TN-sHDL (n=8, blue bars) or placebo (n=8, white bars) at baseline (0h) or 4 hours (4h) and 72 hours (72h) after the treatment. Mean ± SD. Statistical analysis - 2-way ANOVA to compare differences between the two treatment groups (TN-sHDL or placebo) at each time point and to compare differences within group between the different time points (§ p<0.0001 vs TN-sHDL at 0h and 72h).
CEC of rabbits treated with infusion of 100 mg/kg TN-sHDL

We also investigated rabbit plasma CEC after multiple infusions of two different doses of TN-sHDL (100 mg/kg and 8 mg/kg). First, we evaluated the effect of 100 mg/kg TN-sHDL on plasma CEC at the end of the first infusion.

4 hours after the end of the infusion, AD-dependent CEC of TN-sHDL-treated rabbit (green bars) was significantly higher than that measured at baseline (0h) and 72h after infusion (p<0.05). Differently, total CEC and ABCA1-mediated CEC did not significantly change as compared to CEC measured at baseline (0h) and 72h after infusion in treated rabbits (Fig.I.4). In the placebo group (white bars), no differences were observed in total, AD- or ABCA1-mediated CEC between the different time points. No significant differences in total, AD- or ABCA1-mediated CEC were observed between TN-sHDL treated rabbits and placebo ones at any analyzed time point (Fig.I.4).

![Fig.I.4 Effect of a single infusion of 100 mg/kg TN-sHDL on total, AD-dependent and ABCA1-mediated CEC. Total, AD-dependent and ABCA1-mediated CEC was measured as described in the Materials and methods section. CEC was evaluated in plasma of rabbits treated with a single infusion of 100 mg/kg TN-sHDL (n=5, green bars) or placebo (n=5, white bars) at baseline (0h) or 4 hours (4h) and 72 hours (72h) after the treatment. Mean ± SD. Statistical analysis - 2-way ANOVA to compare differences between the two treatment groups (TN-sHDL or placebo) at each time point and to compare differences within group between the different time points (§ p<0.05 vs TN-sHDL at 0h and 72h).](image-url)
CEC of rabbits treated with infusion of 8 mg/kg TN-sHDL

Similarly, we evaluated the effect of 8 mg/kg TN-sHDL on plasma CEC at the end of the first infusion. 4 hours after the end of the infusion total, AD- or ABCA1-mediated CEC of TN-sHDL-treated rabbit (purple bars) did not significantly change as compared to CEC measured at baseline (0h) and 72h after the infusion (Fig.I.5).

No significant differences in total, AD- or ABCA1-mediated CEC were observed between TN-sHDL treated rabbits and placebo ones at any analyzed time point.

In the placebo group (white bars), no differences were observed in total, AD- or ABCA1-mediated CEC between the different time points (Fig.I.5).

![Bar chart showing CEC of rabbits treated with infusion of 8 mg/kg TN-sHDL](image1.png)

**Fig.I.5 Effect of a single infusion of 8 mg/kg TN-sHDL on total, AD-dependent and ABCA1-mediated CEC.**

Total, AD-dependent and ABCA1-mediated CEC was measured as described in the Materials and methods section. CEC was evaluated in plasma of rabbits treated with a single infusion of 8 mg/kg TN-sHDL (n=5, purple bars) or placebo (n=5, white bars) at baseline (0h) or 4 hours (4h) and 72 hours (72h) after the treatment. Mean ± SD. Statistical analysis - 2-way ANOVA to compare differences between the two treatment groups (TN-sHDL or placebo) at each time point and to compare differences within group between the different time points. No significant differences were observed.
CEC of rabbits treated with multiple infusions of 100 mg/kg and 8 mg/kg TN-sHDL

Subsequently, we evaluated the effect of TN-sHDL treatment on plasma CEC at the end of the fifth infusion. Total, AD- or ABCA1-mediated CEC did not significantly change 4 hours after the infusion of both 8 mg/kg TN-sHDL (purple bars) and 100 mg/kg (green bars) of TN-sHDL as compared to CEC measured at baseline (0h) and 72h after the infusion. No differences in total, AD- or ABCA1-mediated CEC were observed between TN-sHDL treated rabbits and placebo ones at any analyzed time point. In the placebo group (white bars), no differences were observed in total, AD- or ABCA1-mediated CEC between the different time points (Fig.1.6).

![Fig.1.6 Effect of a multiple infusions of 100 mg/kg and 8 mg/kg TN-sHDL on total, AD-dependent and ABCA1-mediated CEC.](image)

Total, AD-dependent and ABCA1-mediated CEC was measured as described in the Materials and methods section. CEC was evaluated in plasma of rabbits treated with multiple infusions (5 infusions) of 100 mg/kg TN-sHDL (n=5, purple bars) and 8 mg/kg TN-sHDL (n=5, green bars) or placebo (n=5, white bars) at baseline (0h) or 4 hours (4h) and 72 hours (72h) after the treatment. Mean ± SD. Statistical analysis - 2-way ANOVA to compare differences between the two treatment groups (TN-sHDL or placebo) at each time point and to compare differences within group between the different time points. No significant differences were observed.
Discussion
In this study we have evaluated cholesterol efflux capacity (CEC) of HDL reconstituted with trimeric apoA-I (TN-sHDL) using a well established in vitro cell-based assay. A 200 mg/kg single infusion of TN-sHDL caused a marked increase of plasma total-CEC in hypercholesterolemic rabbit at 4h after the end of the infusion as compared to baseline (4.13±1.17% vs 8.63±0.72%, p<0.0001). In agreement with these data, a significant increase in plasma free cholesterol levels that peaked at 4h after the end of the high-dose single infusion was observed in the same animals. A similar sharp rise in free cholesterol concentration was observed in both animal and human study after the infusion of the HDL mimetic Commonwealth Serum Laboratories (CSL)-112, a HDL-like particle containing full length, plasma-derived human apoAI, reconstituted with phosphatidylcholine (Diditchenko et al. 2013).

Plasma CEC via both AD and ABCA1 was significantly increased at 4h after infusion with TN-rHDL compared to baseline, whereas no changes in CEC were observed at 72h after the infusion, for both efflux pathways. Interestingly, results from the current study revealed that 200 mg/kg TN-sHDL single infusion predominantly affected the aqueous diffusion-dependent cholesterol efflux pathway. A physical explanation for the preferential stimulation of the AD pathway may be related to the size of TN-sHDL. In fact, trimeric apoAI complexed with 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) forms discoidal HDL-like particles with the longer axes of about 15 nm. This hypothesis is in line with previous cholesterol efflux studies showing that human HDL particles with a diameter greater than 9 nm act as preferred acceptors of cholesterol that is removed from the cells via the aqueous diffusion pathway (Rosenson et al. 2012).

The effect of TN-sHDL on plasma CEC amelioration was associated with a reduction in the plaque macrophage content and with a regression in the atheroma volume, as measured by IVUS, which are both features of atherosclerotic plaques stabilization. Therefore, carotid plaque stabilization that was observed in hypercholesterolemic rabbits after a single infusion of 200 mg/kg TN-sHDL may be related, at least in part, to the increased CEC that reached the peak 4 hours after the infusion treatment. Indeed, this hypothesis is in line with previous studies indicating a strong and inverse association between HDL-mediated CEC from macrophages and both obstructive coronary artery disease and incident cardiovascular events (Rohatgi et al. 2014).

Encouraged by these first data, we thought that lower-dose but multiple infusions of TN-sHDL could result in a more significant increase of CEC. After five infusions, TN-sHDL does not show any effect in treated rabbits as compared to placebo ones on the evaluated CEC pathways.
There is no dose-effect and no difference can be observed between the 100mg/kg and the 8mg/kg TN-sHDL. In both cases, repeated infusions of the reconstituted HDL might have generated an immune response with production of anti-drug antibodies (ADA) or immune-complexes directed against circulating exogenous particles (previously observed in Cynomolgus monkeys by Regenass-Lechner et al. 2016). Indeed, even the effect of the 100mg/kg TN-sHDL on AD CEC that was noted at 4h after a single infusion was lost after five multiple infusions. The presence of such ADAs against TN-HDL could be detected by ELISA. Other that being immunogenic, repeated infusion of TN-HDL have been shown to accelerate its own clearance thus rendering the treatment less effective (Regenass-Lechner et al. 2016).

Therefore, we measured rabbit plasma CEC after the first of five infusions, considering it as a single dose infusion to be compared with the 200 mg/kg TN-sHDL single infusion. In this case, AD-dependent CEC measured at 4h after 100 mg/kg single infusion of TN-SHDL was significantly higher than that measured before infusion and 72 hours after infusion (p <0.05), probably for the same reasons connected with the infused particle size explained above. However, the single infusion of 100 mg/kg TN-sHDL seems not to ameliorate the ABCA1-mediated cholesterol efflux capacity as it was noted with the 200 mg/kg TN-sHDL, while the positive effect on AD efflux was maintained. One possible explanation is that the concentration of free apoAI in plasma of treated rabbits due to the remodeling of the infused HDL particles at 100 mg/kg is not sufficient to induce a relevant enhancement of ABCA1 CEC as it was with the higher (double) dose at 4h after the infusion. A possible way to demonstrate this hypothesis would be to measure the concentration of free apoAI in pooled plasma of treated animals for each time. Since no effects on plasma CEC were noted after a single infusion of 8 mg/kg TN-sHDL, it is possible that this dose is too low for observing any effects in those hypercholesterolemic animals, either at a single or multiple infusion.

In conclusion, data of 200 mg/kg TN-sHDL single infusion corroborate the HDL hypothesis as a therapeutic strategy able to positively modify plaque biology and possibly reduce CVD risk (Feig et al. 2014). This study demonstrates that TN-rHDL is effective in increasing plasma CEC after 4 hours from a single, high-dose infusion in a rabbit model of hypercholesterolemia. Importantly, this effect on CEC was associated with parameters of plaque stabilization and may explain the reduced atherosclerosis progression that was observed in treated rabbits.

The PhD candidate gave her contribution to this work by performing the efflux experiments, and by acquiring and analyzing in vitro experimental data.
Part II

Role of Phospholipid transfer protein (PLTP) on HDL functionality in macrophage reverse cholesterol transport
Aim
Phospholipid transfer protein (PLTP) facilitates bidirectional exchange of lipid between lipoproteins and performs many extra- and intra-cellular functions (Albers et al. 2012). In the plasma, PLTP mediates a net transfer of phospholipids from triglyceride-rich apoB-containing lipoproteins to HDL and induces fusion of small-sized HDL particles into larger-sized HDL particles with concurrent release of lipid-poor apolipoprotein AI (apoAI) (Albers et al. 2012). In humans, common variants at the PLTP locus are strongly associated with plasma HDL-cholesterol (HDL-C) levels (Kathiresan et al. 2009). Interestingly, both overexpression and deletion of PLTP in the mouse lead to a decrease in HDL-C (Foger et al. 1997, Jiang et al. 1999).

PLTP is therefore a key regulator in the metabolism of high-density lipoprotein (HDL), a major factor in macrophage reverse cholesterol transport (mRCT).

Reverse cholesterol transport from macrophages (mRCT) to the liver for excretion in feces is thought to reduce atherosclerosis independently of HDL-C mass (Rader et al. 2009, Rader and Hoving 2014). The ex vivo HDL cholesterol efflux capacity and in vivo mRCT assays have been used to assess how various factors of interest affect mRCT (Rader et al. 2009, deGoma et al. 2008).

Little data are available regarding the effect of PLTP on these measures of HDL function. Whole-body overexpression of PLTP in the mouse has been reported to reduce mRCT by possibly increasing cholesterol uptake in the intestine (Samyn et al. 2009). To gain a better understanding of the role of PLTP with respect to cardiovascular disease, aim of this study was to understand the function of PLTP overexpression and deletion on HDL functionality in macrophage reverse cholesterol transport. Cholesterol efflux capacity and in vivo mRCT were assessed in the contexts of PLTP liver-specific overexpression and whole-body deletion.

To directly compare the effects of higher and lower PLTP activity on metrics of mRCT, wild-type mice were injected with adeno-associated viral (AAV) vector carrying human PLTP while control wild-type animals and PLTP deletion animals were injected with AAV-null. The results show that plasma PLTP affects cholesterol efflux capacity and but not in vivo mRCT.
Materials and methods
Mice
C57BL/6J and B6.129P2-Plptpm1Jia/J mice were acquired from The Jackson Laboratory and bred to derive wild-type controls, animals heterozygous for the Pltp deletion allele (PLTP-Het) and animal homozygous for the Pltp deletion allele (PLTP-KO). For each experiment, animal genders, ages and numbers are indicated in Results. All animal procedures were conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (Kuwano et al. 2017).

AAV vectors
The adeno-associated virus (AAV) vector AAV.2/8.TBG.hPLTP was used to overexpress human PLTP (AAV-hPLTP) in PLTP overexpression (hPLTP) mice. AAV.2/8TBG.PI.null.bGH (AAV-null) was employed as a null vector. All vectors were produced at the University of Pennsylvania Vector Core (Kuwano et al. 2017).

PLTP activity, plasma lipids and size exclusion chromatography
PLTP activity was measured using the Roar PLTP Activity Assay Kits (Roar Biomedical). HDL-C and plasma phospholipid were measured on a Cobas Mira biochemistry analyzer (Roche) using EZ HDL Cholesterol (Trinity Biotech) and Phospholipids C (Wako) kits, respectively (Kuwano et al. 2017).

Plasma cholesterol efflux capacity from macrophages
J774 macrophage cells were obtained from American Type Culture Collection and maintained in RPMI/10% FBS at 37 °C in 5% CO2. To measure cholesterol efflux capacity, J774 cells were seeded, allowed to attach for 6-24 hours, incubated with 2 μCi/mL [1,2-3H(N)]cholesterol or 0.12 μCi/mL [4-14C]cholesterol (both from PerkinElmer) in RPMI/0.2% BSA (fatty acid free) or RPMI/2.5% FBS for 24 hours. In most cases, the labeling medium also contained 25 mg/mL acetylated LDL (acLDL) to load the cells with cholesterol. The cells were then treated with 0.3 mM 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate sodium salt (cAMP; Sigma-Aldrich) in RPMI/0.2% BSA for 16 hours to upregulate ATP-binding cassette transporter A1 (ABCA1) expression and exposed to mouse plasma (0.5-5% in RPMI/0.1% BSA/+- 0.15 mM cAMP or RPMI/+-0.3 mM cAMP medium) or medium without plasma for 4 hours. Mouse
plasma was not apoB-depleted because it contains little apoB-lipoprotein in comparison with human plasma. Cell medium was centrifuged to remove floating cells; cell lipids were extracted with isopropanol or hexane/isopropanol (3:2, v/v), and both medium and cell lipids were read in a scintillation counter. Cell cholesterol efflux was quantified as a percentage of counts in the medium relative to the total counts in the medium and cells. The average percent efflux to cell medium without plasma was subtracted from percent efflux values to medium with plasma (Kuwano et al. 2017).

**In vivo mRCT assays**

In vivo mRCT assays were conducted as previously described (Zhang et al. 2003). J774 cells were incubated with 3-5 µCi/mL [3H]cholesterol and 25-100 µg/mL acLDL in RPMI/0.2% BSA or RPMI/5% FBS for 48 hours, washed with PBS and further kept in RPMI/0.2% BSA for 4-6 hours. The cells were then scraped, centrifuged, re-suspended in RPMI and injected into the mouse peritoneum. Each mouse received, 4 x 10^6 cells and 2.0 x 10^6 cpm in 300 µL of medium. In the direct comparison experiment, 25 µL of blood was collected from the tail vein at 0.5, 1, 1.5, 2, 4 and 24 hours after the cell injection; the terminal 48-hour bleed was conducted from the retro-orbital plexus. Blood was centrifuged at 10,000 g, 4ºC for 10 minutes; plasma was collected and read in a scintillation counter. The red blood cells (formed element of the blood) fraction was solubilized with SOLVABLE (PerkinElmer) as recommended by the manufacturer and then read in a scintillation counter. Mice were housed individually in wire-bottom cages during the 48-hour period after the cell injection to collect feces. Feces were weighed, soaked in water (at 100 mg/mL) overnight at 4 ºC, combined with an equal volume of ethanol and homogenized. Aliquots of the homogenate were either diluted 2X with ethanol or solubilized with SOLVABLE (PerkinElmer) and then read in a scintillation counter. Mice were euthanized, the liver was perfused with cold PBS and collected. Liver lipids were extracted by the Bligh and Dyer method and read in a scintillation counter; or liver tissue was homogenized in PBS with a steel bead using TissueLyser II (Qiagen) and read in a scintillation counter. Bile was collected and read in a scintillation counter without processing. Radiocholesterol counts in plasma, formed elements, liver, bile and feces were expressed as a percent of the counts injected with the cells (Kuwano et al. 2017).
Agarose and polyacrylamide gel electrophoresis

Plasma was analyzed on 0.7% agarose gels in 0.05 M barbital buffer (pH 8.6)/0.33% albumin (w/v) as previously described (Bi et al. 2013), and on native 4-20% polyacrylamide gels in Tris-glycine buffer (WedgeWell precast gels from Thermo Fisher). Agarose and polyacrylamide gels were blotted and probed with an anti-apoAI antibody (K23001R; Meridian Life Science, formerly BioDesign) (Kuwano et al. 2017).

Statistical analysis

GraphPad Prism (GraphPad Software) was used to graph and analyze data as indicated in figure legends.
Results
Direct comparison of PLTP overexpression and deletion with respect to HDL functionality in mRCT

Effect of PLTP overexpression and deletion on HDL mass

Wild-type mice (n=7) were injected with $3 \times 10^{10}$ genome copies (GC) of AAV-hPLTP to overexpress human PLTP in the liver. The reference group of animals (n=7) consisted of wild-type mice injected with $3 \times 10^{10}$ GC of a control AAV-null vector; PLTP-KO mice (n=6) were also injected with $3 \times 10^{10}$ GC of the AAV-null control vector to allow direct comparison to the PLTP overexpression animals (all males, 8-16 week-old, age-matched among the groups). HDL-C and plasma phospholipid were measured before and 14 and 28 days after the virus injection on fasting plasma (4 hours). At the 28-day mark, PLTP overexpression and deletion plasmas contained, respectively, 81 ± 9% and 73 ± 9% less HDL-C and 68 ± 8% and 51 ± 5% less phospholipid than wild-type plasma (Fig.II.1 A). The 28-day plasma was pooled by genotype/AAV type and used to measure PLTP activity. PLTP activity in the plasma of PTLP overexpression mice was higher by 27 ± 6% ($p=0.0002$) than in wild-type plasma; PLTP activity in PLTP deletion plasma was negligible (Fig.II.1 B) (Kuwano et al. 2017).

![Fig.II.1 Direct comparison of PLTP overexpression (hPLTP) and deletion (PLTP-KO) with respect to HDL mass. A: HDL-C and plasma phospholipid levels in wild-type, PLTP overexpression and PLTP-KO animals at the indicated time points after the AAV-hPLTP injection. Mean ± SD. Statistical analysis – one-way ANOVA by time point with Bonferroni’s multiple comparisons test to compare wild-type values with hPLTP and PLTP-KO values (***$P < 0.001$, ns not significant). B: PLTP activity in plasma of wild-type, PLTP overexpression and PLTP deletion mice 28 days after the AAV-hPLTP vector injection. Mean ± SD. Statistical analysis – one-way ANOVA with Bonferroni’s multiple comparisons test to compare wild-type values with hPLTP and PLTP-KO values (***$P < 0.001$).]
**Effect of PLTP overexpression and deletion on cholesterol efflux capacity**

Cholesterol efflux capacity was measured on the 28-day pooled plasma. Total cholesterol efflux to PLTP overexpression and deletion plasmas was reduced by 41 ± 7% and 41 ± 13%, respectively; ABCA1-independent efflux was reduced by 67 ± 2% and 63 ± 6%, respectively; and ABCA1-dependent efflux was elevated by 30 ± 12% (ns) and remained unchanged, respectively (Fig.II.2 A-C). To determine whether the decrease in cholesterol efflux capacity resulted from a loss of HDL mass, the amount of plasma added to cells in a second set of cholesterol efflux assays was adjusted to the same amount of HDL-C (1.6 µg of HDL-C per well of a 24-well plate or 0.5, 2.6 and 1.9% of wild-type, PLTP overexpression or PLTP deletion plasma, in cell medium). HDL-C-normalized ABCA1-independent efflux to PLTP overexpression and deletion plasma was elevated modestly by 33 ± 4% and 21 ± 7% (ns), respectively, while HDL-C-normalized ABCA1-dependent efflux increased more dramatically by 93 ± 15% and 83 ± 7%, respectively, and HDL-C-normalized cholesterol efflux capacity (total efflux) was elevated by 60 ± 7% and 49 ± 2%, respectively (Fig.II.2 D-F). These observations suggest that PLTP overexpression and deletion reduce cholesterol efflux capacity by lowering the ability of plasma to accept cholesterol by ABCA1-independent, diffusional efflux, while the ability of plasma to accept cholesterol by the ABCA1-dependent pathway remains unaltered or trends toward an increase (Kuwano et al. 2017).
Fig.II.2 Direct comparison of PLTP overexpression (hPLTP) and deletion (PLTP-KO) with respect to plasma CEC. Mouse plasma was exposed to J774 macrophage cells treated with either cAMP to upregulated ABCA1 or vehicle. A, B, C: The same volume of plasma (1% of cell medium) was added to cells regardless of the plasma HDL-C concentration. D, E, F: The amount of plasma added to cells was adjusted to the same HDL-C amount. A, D: Total cholesterol efflux and HDL-C-normalized total cholesterol efflux. B, E: ABCA1-independent (diffusional) efflux and HDL-C-normalized ABCA1-independent efflux. C, F: ABCA1-dependent and HDL-C-normalized ABCA1-dependent efflux. Mean ± SD. Statistical analysis – one-way ANOVA with the wild-type values set as the control for Bonferroni's multiple comparisons test (***P < 0.001).
Effect of PLTP overexpression and deletion on HDL particles assortment

Pre-β HDL is a key acceptor of cell cholesterol released by the ABCA1-dependedent pathway (de la Llera-Moya et al. 2010). To ascertain whether PLTP activity affected pre-β HDL levels, plasma was resolved on an agarose gel, blotted and probed with an anti-apoAI antibody. PLTP overexpression and deletion dramatically reduced the amounts of pre-β HDL and α-HDL (Fig.II.3 A). Because HDL was detected using an anti-apoAI antibody, this outcome implies a dramatic loss of apoAI. Plasma analysis using native polyacrylamide gel electrophoresis confirmed the loss of HDL/apoAI in the PLTP overexpression and deletion mice (Fig.II.3 B). In addition to the main species prominently present in HDL from all three groups of mice, PLTP deletion HDL also contained two smaller species (one very prominent and the other near the limit of detection) that were absent in the wild-type and PLTP overexpression HDL. These results show that pre-β HDL was reduced together with α-HDL and thus cannot account for the preservation of ABCA1-dependent efflux in PLTP overexpression and deletion plasma (Kuwano et al. 2017).

Fig.II.3 HDL particle species assortment in PLTP overexpression (hPLTP) and deletion (PLTP-KO) animals. A: Agarose gel electrophoresis analysis of pooled plasma. Plasma from ABCA1-KO mice was included as a control to show specificity of the anti-apoAI antibody and to identify the location of pre-β HDL on the gel. B: Native polyacrylamide gel electrophoresis analysis of pooled plasma for HDL particle species assortment. Two new particle species are highlighted in PLTP deletion plasma, but not in wild-type or PLTP overexpression plasma.
**Effect of PLTP overexpression and deletion on mRCT**

On day 32 after the vector administration, the same mice were injected with acLDL-loaded, \(^{3}H\)cholesterol-labeled J774 cells into the peritoneum. \(^{3}H\)Cholesterol counts were significantly lower in plasma from PLTP overexpression mice by the 1.5-h sampling point and in plasma from PLTP deletion mice by the 4-h sampling point (Fig.II.4 A). At the 24 and 48-h marks after the cell injection \(^{3}H\)cholesterol counts were reduced by 60 ± 9% and 70 ± 10%, respectively, in PLTP overexpression plasma and by 37 ± 20% and 58 ± 11%, respectively, in PLTP deletion plasma (Fig.II.4 A). Notwithstanding the reduced plasma radiolabel counts, the liver, bile and feces \(^{3}H\)cholesterol counts remained unchanged (Fig.II.4 B-D), indicating that the total amount of cholesterol transported to the liver was unaffected. \(^{3}H\)Cholesterol was read in the 48-h formed elements fraction. Wild-type, PLTP overexpression and PLTP deletion formed elements fractions contained the same amount of the radiolabel (Fig.II.4 E). Interestingly, the wild-type, PLTP overexpression and PLTP deletion formed elements fractions contained the same percentage of the injected \(^{3}H\)cholesterol as the wild-type plasma (1.4 ± 0.5%, 1.3 ± 0.5 and 1.6 ± 0.5%, respectively, in the formed elements versus 1.4 ± 0.3% in the wild-type plasma). These findings show that PLTP activity does not affect mRCT or cholesterol levels in the blood cells (the formed elements fraction).
Fig.II.4 mRCT in PLTP overexpression (hPLTP) and deletion (PLTP-KO) mice. A: After the injection of acLDL-loaded, radiocholesterol-labeled J774 cells, blood of wild-type, PLTP overexpression and PLTP deletion mice was sampled via the tail vein at the indicated time points (the small panel in the upper-right corner shows the early blood sampling time points in detail). Mean ± SD. Statistical analysis – one-way ANOVA by time point with Bonferroni’s multiple comparisons test to compare wild-type values with hPLTP and PLTP-KO values (*P < 0.05, **P < 0.01, ***P < 0.001, ns not significant). B, C, D: [3H]Cholesterol counts in liver, bile and feces from wild-type, PLTP overexpression and PLTP deletion mice. E: The 48-h formed elements fraction was solubilized and read for [3H]cholesterol, and the counts were expressed as a percent of the total counts injected with cells. Mean ± SD. Statistical analysis – one-way ANOVA with the wild-type values set as the control for Bonferroni’s multiple comparisons test (ns not significant).
To determine whether mouse manipulation during the mRCT assay affected PLTP activity or plasma properties, the 48-h plasma was pooled by genotype/AAV type and assessed for PLTP activity, HDL particle composition and cholesterol efflux capacity. PLTP activity in the wild-type 48-h plasma was essentially the same as in the wild-type 28-day plasma (229 ± 5 nmoles mL⁻¹ h⁻¹ for 48-h plasma vs 213 ± 1 nmoles mL⁻¹ h⁻¹ for 28-day plasma). However, PLTP activity in the PLTP overexpression 48-h plasma was higher than in the PLTP overexpression 28-day plasma (333 ± 16 nmoles mL⁻¹ h⁻¹ vs 271 ± 13 nmoles mL⁻¹ h⁻¹, respectively) (data not shown). The 48-h values for PLTP activity in the PLTP overexpression plasma were 45 ± 7% higher than in wild-type plasma (versus 27 ± 6% higher for the 28-day plasmas; see above). The 48-h values for PLTP activity in the PLTP deletion plasma were negligible. The 48-h plasma was analyzed using native polyacrylamide gel electrophoresis (Fig.II.5). PLTP overexpression 48-h plasma appeared to contain even less HDL/apoAI than PLTP overexpression 28-day plasma. However, in terms of HDL particle assortment, 48-h and 28-day plasmas of the same genotype/AAV type were similar (note that 28-day plasma had already been thawed once).

Fig.II.5 Native polyacrylamide gel analysis of pooled plasma from wild-type, PLTP overexpression (hPLTP) and PLTP deletion (PLTP-KO) mice. 48-h and 28-day plasma were loaded side-by-side. Arrows on the right and numbers on the images indicate the four HDL particle species that formed distinct bands. Species/band 2 is the most prominent HDL species present in all plasmas. Species/band 1 was present in wild-type and PLTP overexpression, but not PLTP deletion plasmas, while species/bands 3 and 4 were present only in PLTP deletion
plasma. Band 4 is not pre-β HDL, because a corresponding band is absent in ABCA1-KO plasma (not shown). 48-h plasma was not fasting, while 28-day plasma was fasting. Two exposures are shown for greater clarity.

Total and ABCA1-independent cholesterol efflux from cAMP-treated, acLDL-loaded J774 cells to pooled PLTP overexpression 48-h plasma were reduced by 25 ± 4% and 55 ± 1%, respectively (Fig.II.6 A,B); ABCA1-dependent efflux to the same plasma was unaffected (Fig.II.6 C). HDL-C-normalized ABCA1-independent cholesterol efflux to PLTP overexpression and deletion 48-h plasma was elevated by 37 ± 2% and 26 ± 6%, respectively (Fig.II.6 B), while HDL-C-normalized ABCA1-dependent efflux was increased by 52 ± 10% and 44 ± 16%, respectively (Fig.II.6 C), total cholesterol efflux rose by 47 ± 5% and 40 ± 8%, respectively (Fig.II.6 A). Thus, PLTP activity was elevated and HDL/apoAI was reduced at the conclusion of mRCT assay relative to several days prior to the assay onset in PLTP overexpression mice, but these changes failed to dramatically change plasma cholesterol efflux capacity (Kuwano et al. 2017).

Fig.II.6 Cholesterol efflux capacity and HDL-C-normalized cholesterol efflux capacity in pooled wild-type, PLTP overexpression (hPLTP) and PLTP deletion (PLTP-KO) 48-h plasma. These plasmas were from the 48-h terminal bleed. A: Total cholesterol efflux from cAMP-treated, acLDL-loaded J774 cells. B: ABCA1-independent efflux. C: ABCA1-dependent efflux. Total and ABCA1-dependent efflux were not measured in PLTP overexpression plasma. Mean ± SD. Statistical analysis – one-way ANOVA by time point with Bonferroni’s multiple comparisons test to compare wild-type values with hPLTP and PLTP-KO values (**P < 0.01, ***P < 0.001, ns not significant).
Discussion
In this study, a unique experimental design was employed to directly compare the effects of increased and decreased PLTP activity on plasma HDL metabolism and on mRCT. The relationship between PLTP activity and HDL mass is parabolic (\(\cap\)-shaped). The level of PLTP activity in wild-type mice is finely regulated to support the highest HDL mass (the highest HDL-C, plasma phospholipid, apoAI, pre-\(\beta\) HDL and \(\alpha\)-HDL levels). Deviations above or below this optimal PLTP activity cause reductions in HDL components. These results are in line with previous publications where deviations above or below the WT PLTP activity in mice caused reductions in HDL-C. The reduced HDL levels in PLTP-KO mice can reflect both a decreased transfer of components (phospholipids, cholesterol) from triglyceride-rich particles into HDL and altered stability of HDL particles (Jiang et al. 1999) due to the absence of PLTP. On the other hand, the hypercatabolism (enhanced hepatic uptake and clearance) of HDL components and apoAI due to the higher activity of PLTP are thought to be the cause of the reduced HDL-C levels found in hPLTP mice (Foger et al. 1997).

The relationship between PLTP activity and plasma cholesterol efflux capacity (CEC), an \textit{ex vivo} metric of mRCT, is also parabolic, with the wild-type PLTP activity ensuring the highest CEC. PLTP overexpression and deletion-induced reductions in cholesterol efflux capacity occur because of weaker ABCA1-independent (diffusional) cholesterol efflux, while ABCA1-dependent cholesterol efflux remains intact or even increases. Weak ABCA1-independent efflux results from the loss of HDL mass (Fournier et al. 1997).

It is less clear how ABCA1-dependent efflux is preserved. In fact, contrary to our expectations we found less smaller-size pre-\(\beta\) HDL in PLTP overexpression and deletion plasma as compared to WT plasma (likely because pre-\(\beta\) HDL was catabolized in the kidney). One possibility is that ABCA1-dependent efflux to mature \(\alpha\)-HDL substitutes for ABCA1-dependent efflux to pre-\(\beta\) HDL. PLTP has been shown to transfer cholesterol from J774 cells with upregulated ABCA1 expression to mature HDL (Oram et al. 2003). Higher concentrations of PLTP in PLTP overexpression plasma could increase cholesterol efflux to mature HDL to compensate for cholesterol efflux to pre-\(\beta\) HDL. Denser, smaller-sized mature HDL species (HDL3b and HDL3c) have been shown to accept cholesterol by the ABCA1-dependent pathway directly (Du et al. 2015). PLTP deletion plasma contains two new smaller-sized HDL particle species. ABCA1-dependent efflux to these new HDL species could account for the preservation of the ABCA1-dependent efflux pathway in PLTP deletion plasma. Given these large effects of PLTP activity on HDL mass and HDL particle assortment, we then evaluated PLTP overexpression and deletion effects on macrophage RCT.
mRCT involves release of cholesterol from macrophages to plasma, residence of cholesterol in plasma and uptake of plasma cholesterol by the liver, followed by cholesterol metabolism in the liver and elimination with feces (Rader and Hoving 2014). The cholesterol efflux capacity and in vivo mRCT assays estimate the amount of cholesterol that is transported from macrophages to plasma and from macrophages to feces, respectively, over a period of time (during the first 4 hours after plasma addition to macrophage cells in the cholesterol efflux capacity assay and during 48 hours after macrophage cell injection into mice in the in vivo mRCT assay). For robust results, it is critical that cholesterol efflux from macrophages exceeds cholesterol influx into macrophages throughout the assay duration (because otherwise the assays will measure the bidirectional cholesterol exchange between macrophages and plasma instead of net cholesterol flux from macrophages to plasma). To ensure this, cholesterol efflux is upregulated in the macrophage cells by treatment with cAMP and/or loading with acLDL (Weibel et al. 2014). However, cholesterol efflux from macrophages at a high rate (cholesterol mass released to plasma per unit of time) may quickly saturate the cholesterol holding capacity of plasma (amount of cholesterol that plasma HDL can accept per plasma volume) in the CEC assay. In this case, cholesterol efflux capacity will estimate cholesterol holding capacity, and because cholesterol holding capacity is proportional to HDL mass, cholesterol efflux capacity will be also proportional to HDL mass. Similarly, strong cholesterol efflux from macrophages may exceed cholesterol uptake by the liver and other tissues in the in vivo mRCT assay. In this case, cholesterol released from macrophages will accumulate in plasma, and plasma steady-state counts of macrophage-derived radiocholesterol will give an estimate of plasma cholesterol holding capacity.

In our study, the standard in vivo mRCT protocol was modified to evaluate the rate of macrophage-derived radiocholesterol levels in plasma at 0, ½, 1, 2, 4, 24 and 48h. Early time points after the assay onset should reflect the rate of cholesterol efflux more closely. Radiolabel levels in plasma of PLTP deletion mice increased at the same rate as in wild-type plasma for the first 2 hours and were only slightly lower at the 4h time point after the assay start. Radiocholesterol levels in PLTP overexpression plasma were the same as in wild-type plasma at the 1-h time point, but substantially reduced at the 1.5-h time point. The 24h and 48h sampling reflect steady state levels of macrophage-derived radiocholesterol, which is proportional to HDL holding capacity. At both time points, radioactive counts of PLTP overexpression and deletion plasma were significantly lower than that of WT plasma.
However, when the amount of radioactivity in liver and feces was measured at the 48h time point (at sacrifice), no differences were found between PLTP overexpression and deletion mice as compared to WT mice, meaning that both PLTP overexpression and deletion do not affect *in vivo* mRCT.

Together, the above observations suggest that PLTP overexpression and deletion reduce plasma cholesterol holding capacity (which is evident in the standard *ex vivo* cholesterol efflux capacity assay as well as in the *in vivo* steady state levels of macrophage derived radiocholesterol) by reducing mature and pre-β HDL levels, but do not adversely affect the rate of *in vivo* mRCT.

Moreover, these findings affirm the conclusion that HDL mass is a poor predictor of HDL functionality, and further suggest that plasma cholesterol efflux capacity may in certain instances closely follow HDL mass and thus fail to reveal the true state of *in vivo* mRCT.

Red blood cells have been shown to contribute to mRCT (Temel and Brown 2015). Neither PLTP overexpression nor deletion affected the amount of macrophage-derived radiocholesterol in the formed elements fraction, which consists overwhelmingly of red blood cells. This suggests that PLTP activity does not affect macrophage cholesterol transport in red blood cells, and furthermore red blood cells do not carry more macrophage cholesterol when HDL mass and plasma cholesterol holding capacity are reduced.

Reduced HDL mass and cholesterol efflux capacity and normal mRCT have been reported for animals with liver deletion of ABCA1 (Bi et al. 2013), probucol-mediated suppression of ABCA1 activity (Yamamoto et al. 2011) and whole-body deletion of one LCAT allele (Tanigawa et al. 2009). Increased HDL mass and cholesterol efflux capacity and normal mRCT have been reported for animals with whole-body deletion of hepatic lipase, endothelial lipase or both (Brown et al. 2010). A mechanism must exist that ensures the same amount of cholesterol is taken up by the liver when HDL mass is lower or higher than normal. One possibility is that HDL particles in plasma with low HDL mass have higher affinities for the cognate receptors on the liver. Another is that the assortment of the liver HDL receptors changes to increase or decrease the cumulative affinity for HDL particles. Overexpression of SR-BI in the liver of normolipidemic mice increases mRCT, while whole-body deletion of it decreases mRCT (Zhang et al. 2005). Feeding probucol to SR-BI-deficient animals reverses the negative effect of the receptor deletion and promotes mRCT (Yamamoto et al. 2011). Probucol may induce a change in the liver HDL receptor assortment, eliminating the need for SR-BI and increasing the contribution of other receptors. The ecto-F1-ATPase/P2Y13 purinergic receptor pathway mediates HDL holoparticle uptake by the liver and was recently shown to promote...
mRCT (Lichtenstein et al. 2015). This pathway and SR-BI likely share the burden of cholesterol uptake in the liver in a coordinate manner.

Findings of this study regarding reduced levels of plasma HDL-C, phospholipid and apoAI in PLTP overexpression and deletion mice agree with the previous reports. Samyn and colleagues reported that hPLTP-tg animals had reduced radiocholesterol counts in plasma, unchanged counts in liver and bile and reduced counts in feces (in contrast to our finding of no difference) in comparison with wild-type controls in vivo mRCT assays (Samyn et al. 2008). The authors attributed the low fecal counts to increased cholesterol absorption in the intestine. The AAV vector used in the present study contains a hepatocyte-specific thyroxine binding globulin promoter to express PLTP and the promoter is not active in other liver tissues, therefore the intestine phenotype was not involved.

In the human studies that have found the association between PLTP and coronary heart disease, PLTP activity was measured in plasma (Kim et al. 2015). For this reason, our primary interest was to increase plasma PLTP activity, and PLTP overexpression via a liver-specific vector was appropriate for this purpose.

A recent study showed that PLTP is required for mRCT on a high-fat high-cholesterol diet (Si et al. 2016). This finding suggests a new hypothesis for the role of PLTP in mRCT: optimum PLTP activity may be required to facilitate mRCT in diet-induced hyperlipidemia (Escolà-Gil et al. 2011), while failure to keep PLTP activity at this optimum level may promote atherosclerosis.

Higher and lower PLTP activities are associated with lower HDL-C in different human populations (Chen et al. 2009, Cheung et al. 2009). Both direct and inverse correlations between PLTP activity and atherosclerotic cardiovascular disease (ASCVD) have been reported in human studies (Kim et al. 2015). It is well-established that higher PLTP activity promotes atherosclerosis in mouse models (Albers et al. 2012). Whole-body deletion of PLTP in mice has been reported to reduce atherosclerosis (Jiang et al. 2001), but deletion of macrophage PLTP has been shown to increase atherosclerosis (Liu et al. 2007). It is imaginable that the relationship between PLTP and ASCVD is parabolic, with both high and low levels of PLTP activity promoting atherosclerosis by different mechanisms.

In summary, PLTP overexpression and deletion reduce HDL mass and plasma cholesterol holding capacity without affecting the rate of cholesterol efflux from macrophages to plasma and macrophage cholesterol transport in plasma to the liver. These findings imply a substantial resilience of mRCT in face of drastic changes in HDL metabolism.
This project has been conducted at the Division of Translational Medicine and Human Genetics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, USA. Results have been included in the publication: “Overexpression and deletion of phospholipid transfer protein reduce HDL mass and cholesterol efflux capacity but not macrophage reverse cholesterol transport” J Lipid Res. 2017 Apr;58(4):731-741. doi: 10.1194/jlr.M074625. Epub 2017 Jan 30. The PhD candidate significantly contributed to the in vivo mRCT experiment and conduction of sample analysis and acquired the in vitro data (all effluxes, lipoprotein electrophoresis, PLTP activity). In collaboration with other authors, the PhD candidate analyzed and interpreted the data. Moreover, the PhD candidate substantially contributed to the manuscript revision and re-submission.
Part III

Evaluation of ABCG1 CEC after consumption of an innovative food enriched with bioactive components and functional probiotics in overweight individuals
Aim
In recent years, the amelioration of the quality of life and the increased life expectancy in the general population of western society have led to a less fatal and more prevalent diffusion of cardiovascular diseases (CVD) than in past decades (Heidenreich et al. 2011). Without novel therapies to supplement LDL-C lowering treatment, the incidence of CVD may dramatically increase as the population ages in developed countries. Indeed, despite advances in medical therapy, cardiovascular diseases remain the leading cause of morbidity and mortality worldwide (WHO).

The use of drugs is not always effective in lowering CVD burden either in terms of costs or in terms of management of potential side effects (Promoting Cardiovascular Health in the Developing World: A Critical Challenge to Achieve Global Health). Moreover, large part of cardiovascular events occur in un-treated subjects with subclinical disease such as individuals with moderately increased blood pressure and/or LDL-C (Preiss and Sattar 2009, Toth 2008).

In this scenario, widespread prevention strategies becomes increasingly necessary to contain CVD and its underlying pathology, atherosclerosis. Prevention can be particularly beneficial in the population at low cardiovascular risk, for which a pharmacological treatment is usually not advisable nor cost-effective. Beside circulating-cholesterol levels, a parameter of high-density lipoprotein (HDL) functionality, the so-called cholesterol efflux capacity (CEC), has recently been proposed as a cardiovascular risk marker (Rohatgi et al. 2014) and emerged as a putative, promising target in CVD.

Since the improvement of lifestyle is not always sufficient to reach desirable cholesterol levels, the implementation of healthy food choice is considered as the primary cost-effective, preventive approach in both adults (Weintraub et al. 2011) and children (Expert Panel on Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children and Adolescents 2011).

Nutraceuticals are defined as foods or dietary components, which provide health benefits beyond basic nutrition, thereby acting as borderline products between nutrients and drugs (DeFelice 2002). The implementation of nutraceutical components in everyday foods and their possible synergistic effect represent the rationale for the design of a functional food aimed at the prevention of metabolic disorders. Several foods and dietary supplements have been shown to protect against the development of CVD (Sosnowska et al. 2017).

Whole-wheat foodstuff in the form of bread and/or pasta hold a central position on the dietary habits of the population of Mediterranean countries (Pauwels EK 2011). Epidemiological studies indicate that high intake of whole grains cereals, rather than refined, is associated to the improvement of numerous traditional CVD risk factors such as blood pressure level (Tighe et
al. 2010), visceral adipose fat (McKeown et al. 2009), glycemia, inflammation index and serum lipid levels (Jensen et al. 2006).

In the present study, we evaluated the effect of a new functional pasta on index of high-density lipoprotein (HDL) functionality in healthy overweight individuals. The innovative pasta was formulated *ad hoc* in the context of the SFLABPASTA-PASSWORLD project. In more details, pasta was industrially developed after the identification of cultivars of wheat rich in carotenoids, un-soluble fibers and antioxidants, and fortified with barley β-glucans and spores of Bacillus coagulans. Conversely, a control pasta was made from the same cultivar of grain and by the same technological process, but without any pre- or pro-biotic supplementation.

The relevance of a functional parameter such as CEC in metabolic diseases is illustrated by the evidence that cell cholesterol efflux not only acts as a mechanism limiting intracellular cholesterol content but also is coupled with regulatory intracellular signaling; the resulting modulation of several cell functions leads to the inhibition of inflammatory and immune reactions in both macrophages and endothelial cells (Prosser et al 2012, Yvan-Charvet L 2010). In particular, several studies have shown a role for ABCG1 CEC in both dyslipidemia disorders (Bellanger at al. 2011, Zimetti et al. 2015) and inflammatory-based diseases (Ronda et al. 2014, Zimetti et al. 2017). In the present study, the cohort was composed by a population of subjects with high BMI and obesity, a condition characterized by a baseline inflammatory status. For this reason, the effects of the novel nutraceutical preparation on HDL functionality (i.e, CEC) were evaluated by looking at the ABCG1 mediated pathway.
Materials and methods
Protocol of functional pasta administration

Study participants were the subjects selected for the SFLABPASTA - PASSWORLD (Healthy Effects of an Innovative Probiotic Pasta) study. The protocol of the study was performed in accordance with the ethical principles set in the Declaration of Helsinki. All study participants gave their written informed consent, and the study protocol received approval from the local ethical committee. The SFLABPASTA-PASSWORLD is a parallel, controlled intervention trial of 12 weeks duration conducted by the Department of Food Science at the University of Parma, aiming to evaluate the healthy effects of an innovative whole grain pasta in overweight volunteers (Pellegrini, Angelino et al. unpublished).

Enrolled participants were assigned to two treatments in randomized order: 1. Whole-wheat pasta containing phenolic acids (50.3mg/100g) and not-soluble fibers (12.5g/100mg), taken as control or 2. Innovative pasta with phenolic acids (42.4mg/100mg) and fibers (13.6g/100mg) enriched in β-glucans from barley (2.3g/100g) and supplemented with spores of Bacillus coagulans GBI-30, 6086© (108-109 CFU/100g) (Pellegrini, Angelino et al. unpublished).

The POFI (POliphenols and FIbers) control pasta was produced with the same technological process and with the same variety of whole grain flour as the innovative POFI-SB (POliphenols and FIbers enriched in Spores and Barley β-glucans) pasta.

The main difference between the two pasta preparations was the absence or the presence of a combination of prebiotics (β-glucans) and probiotics (Bacillus coagulans spores), which makes the innovative POFI-SB pasta “functional” in providing healthy, anti-inflammatory and antioxidant effects in human hosts (Pellegrini, Angelino et al. unpublished).

During the study, enrolled volunteers in each group were asked to eat a portion of the provided pasta on a daily basis by replacing the portion of pasta they usually consumed. Moreover, they were asked to maintain their eating habits and their usual level of physical activity. Extensive clinical and laboratory profiles of subjects were assessed according to a standardized protocol that has been described in details elsewhere (Pellegrini, Angelino et al. unpublished).

Parameters that were evaluated before and at the end of the intervention study included body mass index (BMI), total cholesterol, HDL-C, LDL-C, glucose, homocysteine (HCY) and folic acids (FOL).

Beyond the standard procedures established in the protocol, participants of the study were evaluated for their plasma cholesterol efflux capacity (CEC) at baseline and after 3 months of POFI or POFI-SB pasta consumption.
Characteristics of study participants

The enrolled population of volunteers consisted of 41 healthy overweight subjects (20 females and 21 males aged 30 to 65 years, among which 20 were smokers) with a sedentary lifestyle. Selected subjects for the trial had a BMI value greater than 25 kg/m² and dietary habits characterized by the absence of whole grain cereals and by a low intake of vegetables and fruits fibers. For admission in the trial, subjects must be habitual consumer of pasta. Enrolled volunteers must not having taken antibiotics over the three months prior to the study and must not being under particular diet regimen. Additional exclusion criteria were the presence of any chronic disease or pregnancy status in women.

After being matched for age, sex, smoking habits and BMI values at baseline, the POFI and POFI-SB groups consisted of 20 subjects (40% males) and 21 subjects (38% males), respectively. Characteristics of recruited subjects in the two study populations are shown in Table.III.1.

<table>
<thead>
<tr>
<th>Subjects parameter</th>
<th>POFI Mean</th>
<th>SD</th>
<th>POFI-SB Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>55.60</td>
<td>8.73</td>
<td>52.52</td>
<td>13.49</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>31.27</td>
<td>6.11</td>
<td>30.61</td>
<td>3.41</td>
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<tr>
<td>Weight (kg)</td>
<td>86.53</td>
<td>15.64</td>
<td>88.20</td>
<td>14.29</td>
</tr>
<tr>
<td>TOT CHOL (mg/dL)</td>
<td>218.45</td>
<td>37.89</td>
<td>214.62</td>
<td>30.87</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>115.85</td>
<td>53.77</td>
<td>109.95</td>
<td>42.22</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>59.25</td>
<td>11.57</td>
<td>55.82</td>
<td>7.10</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>136.03</td>
<td>32.11</td>
<td>137.01</td>
<td>25.15</td>
</tr>
</tbody>
</table>

Table.III.1 Mean and SD of anthropometrics and metabolic characteristics of the two subject populations involved in the study. BMI: body mass index; TOT CHOL: total cholesterol; TG: triglycerides; LDL-C: LDL cholesterol; HDL-C: HDL cholesterol.
**Ex vivo plasma CEC**

CEC measurement was performed *ex vivo* on whole plasma collected from subjects enrolled in the trial before (T0) and after three months of feeding (T3) with the POFI or the innovative POFI-SB pasta. In order to prevent lipoprotein modifications, plasma samples were frozen at -80°C immediately after collection and thawed in ice just before use.

Chinese hamster ovary cells not transfected (CHO) or transfected with the human ABCG1 gene (CHO-G1) were used to evaluate ABCG1-mediated efflux as previously described (Ronda et al. 2014). Briefly, the cells were cultured in 10% FCS medium, labeled with [1,2-3H] cholesterol (Perkin Elmer) for 24 hours and incubated with 0.2% bovine serum albumin (BSA, Sigma Aldrich) in FBS-free medium for 18 hours. Cholesterol efflux from cells was then promoted to 1% (volume/volume) subject whole plasma for 6 hours (Ronda et al. 2014). To minimize the intra-assay variability, every subject plasma was tested in triplicates and for each triplicate, the mean and the standard deviation were calculated. For each plasma sample, ABCG1-mediated cholesterol efflux capacity (ABCG1 CEC) was expressed as the percentage of cholesterol counts that were released in the medium over the total radioactivity incorporated by cells. In order to correct for the inter-assay variability two different pools of human normocholesterolemic sera (Ref St1 and Ref St2) were run in each assay, and ABCG1 CEC from these sera pools was used to normalize sample values from the different experiments (Zanotti, Favari et al. 2012).

**Statistical analysis**

Mean and standard deviation were used to describe the continuous variables. To test distribution of values we applied the Shapiro-Wilk test for normality and we assessed differences between groups by Student’s t-test. To investigate correlations between CEC and metabolic markers we used Pearson’s test or Spearman’s test when appropriate. All reported p values are two-tailed, with a p value < 0.05 indicating statistical significance. For each plasma sample, cholesterol efflux experiment were performed in triplicate and, except where indicated differently, data were expressed as the mean ± standard deviation. Grubb test for outliers was performed with GraphPad software. Subjects for which CEC had outlier values were excluded from statistical analysis Statistical analysis were performed with IBM SPSS 22.0 Statistics.
Results
Plasma lipid, glucose, homocysteine and folic acid levels

Data on serum levels of lipids (total cholesterol, LDL-cholesterol, HDL-cholesterol), glucose, homocysteine, folic acid in POFI and POFI-SB groups before (T0) and after three months of pasta consumption (T3) are shown in Table. III.2. At T0, no significant differences were observed between the two groups for any of the parameters. No significant change in the serum levels of lipids, glucose, homocysteine and folic acid levels was found at T3 as compared to T0 in both POFI and POFI-SB groups.

<table>
<thead>
<tr>
<th></th>
<th>POFI T0</th>
<th>POFI T3</th>
<th>POFI-SB T0</th>
<th>POFI-SB T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOT CHOL (mg/dL)</td>
<td>218.45 ±37.89</td>
<td>220.10 ±40.44</td>
<td>214.62 ±30.87</td>
<td>211.86 ±30.78</td>
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<tr>
<td>LDL-C (mg/dL)</td>
<td>136.03 ±32.11</td>
<td>137.83 ±31.56</td>
<td>137.01 ±25.15</td>
<td>132.39 ±28.68</td>
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<tr>
<td>HDL-C (mg/dL)</td>
<td>59.25 ±11.57</td>
<td>57.79 ±11.45</td>
<td>55.62 ±7.10</td>
<td>55.48 ±10.04</td>
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<td>GLU (mg/dL)</td>
<td>101.7 ±11.08</td>
<td>101.05 ±13.86</td>
<td>101.71 ±13.11</td>
<td>97.86 ±10.95</td>
</tr>
<tr>
<td>HCY (umol/L)</td>
<td>11.45 ±3.62</td>
<td>12.55 ±5.25</td>
<td>11.14 ±3.84</td>
<td>13.58 ±7.92</td>
</tr>
<tr>
<td>FOL (ng/mL)</td>
<td>5.76 ±2.23</td>
<td>5.91 ±2.48</td>
<td>7.08 ±3.29</td>
<td>6.23 ±3.44</td>
</tr>
</tbody>
</table>

Table. III.2 Serum lipids, glucose, homocysteine and folic acid in POFI (n=20) and POFI-SB (n=21) subject groups before (T0) and after three months of pasta consumption (T3). Values are expressed as means ± SD. At baseline, metabolic parameters did not differ between the two groups. POFI or POFI-SB pasta feeding did not result in significant changes on parameters here described. TOT CHOL: total cholesterol, LDL-C: LDL cholesterol, HDL-C: HDL cholesterol, GLU: glucose, HCY: homocysteine, FOL: folic acid.
Effect of innovative pasta consumption on ABCG1-mediated CEC

Serum capacity to promote cholesterol efflux via ABCG1 was measured in subjects enrolled in the study before (T0) and after three months of POFI or POFI-SB pasta consumption (T3). ABCG1-mediated CEC expressed as a ratio between T3 ABCG1 CEC and T0 ABCG1 CEC, to highlight the change occurring in each subject for the considered parameter. A T3/T0 ABCG1-CEC equal to 1 describes no changes in plasma capacity to promote cholesterol efflux. As higher than 1 the T3/T0 ABCG1 CEC is, the more the plasma capacity of volunteer to promote cholesterol efflux via ABCG1 has improved between the two blood sampling time points. As shown in figure III.1, serum ABCG1-mediated CEC as T3/T0 ratio was significantly higher in the POFI-SB group than in the POFI group (Student's t-test p < 0.05).

Fig. III.1 Effect of POFI and POFI-SB pasta consumption on plasma ABCG1-mediated CEC. For each subject ABCG1-dependent CEC is expressed as a ratio between ABCG1-CEC after three month of pasta consumption (T3) and ABCG1-CEC at baseline (T0). ABCG1-mediated CEC was the difference in efflux between hABCG1 overexpressing CHO and control CHO cells and it is calculated as the percentage of cholesterol efflux over the total cell cholesterol content. Differences between groups were assessed by using Student's t-test, p < 0.05.
Relationship between ABCG1-mediated CEC and plasma glucose levels

A significant inverse correlation was found between ABCG1-mediated CEC values and plasma glucose levels (mg/dL) in POFI group at baseline (T0) (Pearson r=-0.586, p=0.007) and after three months of pasta consumption (T3) (r=-0.650, p=0.003) (Fig.III.2 A). Similarly, an inverse correlation was found between ABCG1-mediated CEC and plasma glucose levels in POFI-SB group at T0 (r=-0.440, p=0.046) while no correlation was found between ABCG1-mediated CEC and glucose levels in POFI-SB group at T3 (r=-0.70, p=0.764 NS) (Fig.III.2 B).

**Fig.III.2 Relationship between ABCG1-mediated CEC and plasma glucose levels (mg/dL).** Scatter plots refer to volunteers fed with POFI (A) or POFI-SB (B) pasta at baseline (T0) and after three months of pasta consumption (T3). ABCG1-CEC is expressed as the percentage of cholesterol efflux of total cholesterol content of cells. Pearson’s correlations were calculated and bold values highlight significant correlations between parameters (p < 0.05).
To investigate more deeply the relationship between ABCG1 CEC and plasma glucose and to understand why after three months of innovative POFI-SB pasta consumption the correlation between the two parameters is lost, we examined the ratio between ABCG1 CEC and glucose levels in each POFI-SB subject at baseline (T0) and after pasta intake (T3). As shown in figure III.3, the ABCG1 CEC/glucose ratio increases (n=13) or remains stable (n=3) in the majority of subjects after three months of POFI-SB pasta administration, while it decreases (n=4) in a few cases. An increased ABCG1 CEC/glucose ratio indicates that each glycemia level is associated to higher ABCG1 CEC; as ABCG1 CEC/glucose ratio increase is variable and absent in some cases, the correlation between the two parameters is lost at T3.

![Graph](image.png)

**Fig.III.3 Analysis of subject ABCG1-mediated CEC/glucose ratio in POFI-SB group.** For every subject the ratio between ABCG1-mediated CEC values and plasma glucose levels (mg/dL) has been calculated at baseline (T0) and after three months of pasta administration (T3). As compared to T0, the ABCG1-CEC/glucose ratio at T3 is increased in 13 subjects, it remains unchanged in 3 subjects and it decreases in 4 subjects.
To clarify if pasta consumption influences ABCG1 CEC/glucose ratio differently according to body weight, we stratified each group by the BMI threshold of 30 kg/m². The significant, inverse correlation between ABCG1-mediated CEC and serum glucose levels in the POFI group is confirmed in both BMI populations (BMI <30 r=-0.605, p=0.001; BMI ≥30 r=-0.731, p=0.005 by Pearson’s test) (Fig.III.4 A). Similarly to what seen in the whole POFI-SB group, we found a lack of correlation between ABCG1-mediated CEC and serum glucose levels (r=-0.068, p=0.775) in the POFI-SB subjects with BMI <30. Instead, an inverse correlation between ABCG1-mediated CEC and glucose levels was found in the population with BMI ≥30 (r=-0.447, p=0.035) (Fig.III.4 B).

Fig.III.4 Relationship between ABCG- mediated CEC and plasma glucose level (mg/dL): BMI stratification. Volunteers in POFI and POFI-SB groups were stratified according to the BMI threshold of 30 kg/m². The correlation between ABCG1 CEC and plasmatic glucose has been evaluated in the populations of subjects with BMI <30 and with BMI ≥30 in both groups. Scatter plots refers to volunteers fed with POFI (A) or POFI-SB (B). Pearson’s correlations were calculated and bold values highlight significant correlations between parameters (p < 0.05).
**Relationship between ABCG1-mediated CEC and plasma homocysteine levels**

No significant relationship was found between ABCG1-mediated CEC and plasma homocysteine levels (µmol/L) in the POFI group neither at baseline (T0) (Spearman $r=-0.007$, $p=0.976$ NS) nor after three months of pasta consumption (T3) ($r=-0.276$, $p=0.253$ NS) (Fig.III.5 A). In the POFI-SB group no correlation was observed at T0 ($r=-0.332$, $p=141$ NS) while an inverse relationship close to statistical significance between ABCG1-mediated CEC and plasmatic homocysteine was evidenced at T3 ($r=-0.420$, $p=0.058$) (Fig.III.5 B).

![Fig.III.5](image)

**Fig.III.5 Relationship between ABCG1 mediated CEC and plasma homocysteine levels (µmol/L).** Scatter plots refer to volunteers fed with POFI (A) or POFI-SB (B) before (T0) and after three months of pasta administration (T3). Spearman’s correlations were calculated and bold values highlight significant correlations between parameters ($p < 0.05$).
**Relationship between ABCG1-mediated CEC and plasma folic acid levels**

We did not find any significant relationship between ABCG1-mediated CEC and plasma folic acid levels (ng/mL) in the POFI group neither at baseline (T0) (Spearman r=-0.98, p=0.682 NS) nor after three months of pasta consumption (T3) (r=0.131, p=0.594 NS) (Fig.III.6 A). Conversely, a positive correlation between ABCG1-mediated CEC and plasma folic acid was found at T0 (r=0.440, p=0.046) and, stronger, at T3 (r=0.606, p=0.004) in the group of volunteers fed with the POFI-SB pasta (Fig.III.6 B).

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**Fig.III.6 Relationship between ABCG1 mediated CEC and plasma folic acid levels (ng/mL).** Scatter plot refers to volunteers fed with POFI (A) or POFI-SB (B) before (T0) and after three months of pasta administration (T3). Spearman’s correlations were calculated and bold values highlight significant correlations between parameters (p < 0.05).
Discussion
Some early observational studies investigated the effect of non-pharmacological interventions on human cholesterol efflux capacity. For example, physical exercise was shown to improve CEC. However, the association between CEC and exercise was related directly to higher levels of apoAI and HDL particles rather than to an improved ability of HDL to accept cholesterol (Brownell and Rohatgi 2016). A certain number of studies present in literature evaluated the effects of isolated nutritional components supplementation on HDL in various patient populations (RESVERATROL: Voloshyna et al. 2016, ANTHOCYANIN: Zhu et al. 2014, ISOFLAVONE: Badeau et al. 2007, COENZYME Q10: Xiao Yan et al. 2015). Only few examples can be found where consumption of complex food (OLIVE OIL: Hernaez et al. 2014 and Helal et al. 2013, WALNUTS: Berryman et al. 2013) or diet (MEDITERRANEAN DIET: Hernaez et al. 2017) was tested for its capacity to modify HDL functionality.

In the present study, we found that plasma capacity to promote cholesterol efflux via the ABCG1 transporter (ABCG1-mediated CEC) was modestly improved after three months of feeding with an innovative food in healthy overweight individuals. Functional food was a novel kind of wheat-flour pasta enriched with bioactive components and functional probiotics. Conversely, a control pasta was made from the same cultivar of grain and by the same technological process, but without any supplementation. This investigation was started by the research group of Professor Pellegrini, Department of Food Science at the University of Parma, which conducted the intervention trial. The characterization of bioactive components present in food consumed on a daily basis represents an essential step in the validation process of nutritional strategies and is central for specific “functional” food definition (Leoncini et al. 2012). Our results show that plasma ABCG1 CEC was slightly but significantly higher in subjects fed on a daily basis for three months with the innovative pasta as compared to subjects fed with the control one.

Among the fibers in pasta, β-glucans are worth to exert several health properties in relation to their activity of increasing viscosity of the meal bolus and delaying the rate of absorption of nutrients (D. El Khoury et al. 2012). Moreover, a randomized trial showed that β-glucans are able to enhance the probiotic effects of wheat on gut microbiota, thus exhibiting a prebiotic effect (Costabile et al. 2008). Further, pasta was fortified with spores of Bacillus coagulans, a microorganism belonging to Lactobacillus family. Probiotics are microorganisms that are believed to provide health benefits when consumed. It is well demonstrated the ability of this microorganism to survive the technological processes and the gastrointestinal transit, in order to exert beneficial effects in the lower gut through its anti-microbial, immunomodulatory activity, and to improve intestinal motility (Kimmel et al. 2010). As literature suggests, there
are several studies demonstrating the cholesterol lowering effects of Lactobacilli in both animal models and human clinical trials (Gilliland et al. 1985, Jones et al. 2012); however, none of these reported modification of HDL functionality.

In our study, despite LDL-C and plasma glucose tended towards a decrease in the group of subject fed the innovative food, the 3-months feeding with either the innovative or the control pasta did not induce any significant change in serum lipid levels. However, we did observe an enhancement of HDL function, as measured by the increased ABCG1 CEC.

It is well established that ABCG1-dependent efflux preferentially involves mature HDL particles and occurs in a phospholipid-dependent manner (Camont et al. 2001). Therefore, despite no change in plasma HDL-C concentration, a rearrangement of their composition promoted by the feeding with the bioactive components can occur. This may imply variation in the protein or phospholipid cargo of such HDL, with consequent enhancement of their function. After pasta intake, no significant changes in the plasma levels of glucose, homocysteine or folic acid were observed as compared to baseline values in both subjects fed with the innovative pasta or the control one. However, we found that supplementation of bioactive components within the innovative pasta can modulate the relationship between ABCG1-mediated CEC and these serum metabolic markers.

First, we found that ABCG1-mediated CEC inversely correlates with plasma glucose levels and that this correlation is lost in subjects that ate the innovative pasta. In order to better explain this observation, we looked at the ABCG1 CEC/glucose ratio in this group of subjects, before and after pasta consumption. After three months of pasta intake, the ABCG1 CEC/glucose ratio was increased in the majority of subjects that received the innovative food, as compared to baseline, indicating that each glycemia level is associated to higher ABCG1 CEC; as ABCG1 CEC/glucose ratio increase is variable and absent in some cases, the correlation between the two parameters is lost. Moreover, we observed that the lack of correlation between ABCG1 CEC and plasma glucose levels occurred in subjects with BMI <30. It is plausible that the higher the glucose level is, the less HDL are efficient in promoting removal of cholesterol via ABCG1, as it was previously documented (Duell et al. 1991, Hedrick et al. 2000), and that feeding with bioactive components alters this relation in vivo.

Blood concentrations of homocysteine are usually inversely related to that of folic acid, which is in line with our observation of ABCG1 CEC being inversely or directly related to one or the other factor, respectively. Interestingly, we found that ABCG1-mediated CEC of subjects fed the innovative pasta significantly and positively correlates with plasma folic acid, considered a cardioprotective factor, while an almost significant inverse relation was found in the same group
of subjects between ABCG1 CEC and plasma homocysteine, an independent risk factor for coronary disease (Marcus et al. 2007). Such correlations were absent in subjects fed the placebo pasta.

It can be speculated that consumption of bioactive components within the innovative pasta act simultaneously on the amelioration of subjects inflammatory profile and on HDL functional quality thus linking ABCG1 CEC to the levels of some metabolic markers. The results of the SFLABPASTA-PASSWORLD trial will provide important insights on this point.

Other than being one of the major known pathway for cell cholesterol removal, the ABCG1-mediated cholesterol transport was found to play an important role in inflammatory disorder by recent studies. Functional impairment of sera HDL with respect to the ABCG1-mediated efflux capacity was found in patients with inflammatory- based diseases such as lupus erythematosus (SLE), rheumatoid arthritis (RA) and acute phase reaction (APR) independently of serum HDL levels (Ronda et al. 2014, Zimetti et al. 2017). The impact of inflammation on the ABCG1 pathway of efflux seems to be confirmed by the observation that methotrexate, an anti-inflammatory agent widely used in rheumatoid arthritis patients, improves ABCG1-mediated CEC (Ronda et al. 2015). Given the emerging role of CEC as novel marker of CVD risk (Rohatgi et al. 2014), the amelioration of ABCG1 CEC may become an important target to improve atheroprotection.

The use of nutraceuticals is a useful preventive approach, because of its safety, high tolerability and the opportunity to address more risk factors within one product (Leoncini et al. 2012). With the focus on prevention strategies, the design and the study of functional food with nutraceutical supplements appear as an attractive therapeutic tool in the field of chronic multifactorial disorders, such as cardiovascular and metabolic diseases. Our study, despite preliminary, provides evidence that consumption of an ad hoc-formulated functional food can modulate HDL functionality. This possibly occurs through the synergic effects that its probiotic and probiotic components have on the gut microbiota as well as on the absorption and metabolism of other nutritional components.

The PhD candidate performed the described in vitro experiments and gave her significant contribution to the analysis and interpretation of data. The PhD candidate will draft a paper on presented results.
Part IV

Cholesterol efflux capacity of cerebrospinal fluid: development and characterization of a novel metric of the neurodegenerative disease
Aim
Cholesterol metabolism plays a critical role in many complex human diseases such as atherosclerotic cardiovascular disease (ASCVD) and several neurodegenerative disorders (Vance 2012). Since the inheritance of the apolipoprotein E ε4 (APOE ε4) allele was identified as the strongest genetic risk factor for the common, sporadic form of Alzheimer’s disease (AD), different lines of research addressed to study lipid metabolism in the brain and its implication in the amyloid-β (Aβ) pathology (Di Paolo and Kim 2011).

In human brain, different cells work in synergy to maintain cholesterol homeostasis for optimal nervous functions. Neurons, astrocytes and microglia co-localize at amyloid-β deposits in senile plaques of the grey matter of the brain, which are considered as the hallmark of Alzheimer’s disease (Itagaki et al. 1989). Moreover, several experimental studies have shown that excessive cholesterol in neurons, astrocytes and microglia promotes Aβ accumulation and Aβ-driven inflammation in AD. In neurons, β- and γ-secretase, the two enzymes that process amyloid precursor protein (APP) into the Aβ peptide, are associated with lipid rafts of the plasma membrane and their activity specifically depend on cholesterol content (Yang et al. 2014). High intracellular cholesterol levels enhance amyloid-β secretion while a decrease in cholesterol esters suppresses Aβ production in cultured neurons (Puglielli et al. 2001). Taking into consideration that cholesterol synthesis varies among different neuronal types (Ko et al. 2005), experimental studies provided evidence that the majority of adult neurons rely on cholesterol derived from glial cells to maintain healthy synaptic functions (Vance et al. 2000). Glial cells are thought to be the major source of cholesterol in the brain and among these, astrocytes act as pivotal cholesterol exporters. In brain parenchyma, glial cells synthesize cholesterol and, by assembling it within apolipoprotein E (apoE) and apolipoprotein J (apoJ), secrete lipoproteins that are responsible for cholesterol supply to neurons and other cell types (Nieweg et al. 2009, Hirsch-Reinshagen et al. 2004).

Astrogliosis, a second pathological hallmark of neurodegenerative disorders (Colangelo et al. 2014), include a set of morphological, molecular and functional changes of glial cells, some of which are exacerbated by high cholesterol (Osborn et al. 2016). High membrane cholesterol renders astrocytes and neurons susceptible to Aβ- induced cell death (Abramov et al. 2011) whereas the removal of intracellular cholesterol mediated by apoE promotes intracellular Aβ degradation in primary microglia cells (Lee et al. 2011).

Neurons can metabolize cholesterol to 24-S-hydroxycholesterol, which readily diffuses through the brain-blood barrier (BBB) into the systemic circulation (Lütjohann et al. 1996). Moreover, intriguing evidences suggest that an important route in both neurons and glial cells for
management of intracellular cholesterol levels is cholesterol efflux to lipoproteins for disposal via the cerebrospinal fluid (CSF) (Pitas et al. 1987).

CSF, which originates from both drainage of brain perivascular fluid and blood filtration at the choroid plexuses, contains brain-derived apoE and apoJ as well as apoAI, which enters the fluid from blood (Liu et al. 2012, Stukas et al. 2014). Apolipoproteins accept cell cholesterol via ATP-binding cassette transporter A1 (ABCA1) and form high-density lipoproteins (HDL) that accept diffusional cell cholesterol released by different transporters, namely ATP cassette transporters G1 and G4 (ABCG1, ABCG4) and scavenger receptor B type1 (SR-BI) (Vitali et al. 2014).

In the circulation, HDL-mediated cholesterol efflux reflects the first step of macrophage cholesterol transport (mRCT) in plasma to liver for excretion with feces. The mRCT pathway is critical to the etiology of ASCVD (Rosenson et al. 2012) and the efficiency of plasma HDL to promote cholesterol efflux from reference macrophages can be measured ex vivo. This metric, called HDL cholesterol efflux capacity (HDL CEC), is a strong predictor of incident ASCVD inversely and independently of other risk factors (Khera et al. 2011, Rohatgi et al. 2014).

It is possible that, as well as the plasma ability to remove cholesterol from macrophage prevents progression of ASCVD, the capacity of CSF to remove cholesterol from neurons, astrocytes and microglia facilitates optimal intracellular cholesterol turnover and alleviates the Aβ burden in AD. In this study, we aimed to adapt a well-established methodology that quantitate human HDL CEC to measure CSF CEC. Moreover, we propose to assess the extent of variability in CSF CEC and to identify CSF factors that account for CSF CEC in a small cohort of anonymized individuals.
Materials and methods
Cell lines

Human neuronal SH-SY5Y cells, human astrocyte A172 cells and murine microglial N9 cells were selected to serve as reference cells for the CSF CEC assay. In every step of this study, these cell lines were compared with murine macrophage J774 cells, the reference cell line in the HDL CEC assay. Neuroblastoma SH-SY5Y and immortalized microglia N9 cells are extensively used in the scientific community and are thought to reflect the molecular biology of the corresponding primary cells most closely among all the available immortalized cell lines of the same type (Kovalevich et al. 2013, Stansley et al. 2012). Human astrocytoma A172 is a well documented cell line in neurochemistry studies and it was taken as a model of astrocytic cell line (Davis et al. 2004). SH-SY5Y cells, N9 cells, A172 cells and J774 cells were purchased from American Type Culture Collection. SH-SY5Y were grown in DMEM (DMEM with 4.5 g/L D-glucose, L-glutamine and 110 mg/L Sodium Pyruvate, Life Technologies) supplemented with 10% FBS (Sigma-Aldrich) while A172, N9 and J774 cells were maintained in RPMI (RPMI 1640 with L-glutamine, Life Technologies)/10% FBS; all cells were incubated at 37°C and 5% CO2.

CSF samples

The study was performed in accordance with ethical standards indicated by the Helsinki Declaration in 1964. All subjects provided their written informed consent for use of the samples in research. CSF samples (1 ml each) from 20 anonymous individuals were collected for analysis following lumbar punctures conducted at clinical sites of the Hospital at the University of Pennsylvania (HUP). CSF tubes were received frozen and sequentially labeled (1, 2, 3, etc.), and contained no patient identifying information when provided to the study staff. A pooled CSF sample (CSF Pool 2) assembled from hundreds different subjects was obtained from the same clinical laboratory to serve as CSF sample for preliminary experiments. Contamination by blood elements or plasma proteins was assessed by visual examination of CSF and by measuring apolipoprotein B expression in the samples. All CSF specimens that were suspected being contaminated by blood or by apoB-containing lipoproteins were considered as outliers in all analysis of the study.

Moreover, 6 CSF samples (1 ml each, approximately) were purchased from a commercial source (Discovery Life Sciences). CSF was collected from 6 individuals of different age and gender (2 caucasian females and 2 caucasian males aged < and > 65 years, 1 african american female aged 50 years, 1 african american male aged 57 years) under IRB/EC approval. All
commercial samples had physiological protein (provided for 5 samples, mean±SD: 36±5.4 mg/dl) or glucose content (provided for 1 sample: 92 mg/dl), as diagnosed by standard laboratory methods. Portions of the commercial samples were homogeneously combined to make a pooled CSF sample (CSF Pool 1) to be used as internal standard for the CSF CEC assay. After collection, all CSF samples were thawed on ice, divided in 75 µl aliquots and stored at -80°C until usage.

**Western blotting**

Cells were seeded in 12-well plates in DMEM/10% FBS or RPMI/10% FBS and grown in fresh medium/2.5% FBS for 24 hours. Subsequently, ATP-binding cassette transporter A1 (ABCA1) expression was induced in human SH-SY5Y and A172 by treatment with 2 µM of the Liver X receptor (LXR) agonist T0901317 (Sigma-Aldrich) in DMEM/0.2% BSA or in RPMI/0.2% BSA (fatty acid free), respectively, for 18 hours. In murine N9 and J774 cells, ABCA1 expression was induced for 18 hours by treatment with 0.3 mM 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate sodium salt (cAMP, Sigma-Aldrich) in RPMI/0.2% BSA. In parallel, a set of cells per cell type were treated with inducer-free medium/0.2% BSA for 18 hours to evaluate protein expression at basal level. Cells were then washed with cold PBS and lysed on ice by adding RIPA buffer (Cell Signaling) containing a protease inhibitor cocktail (Sigma-Aldrich). Whole cell lysates at a fixed protein content (20 µgr), as measured by the BCA protein assay (Thermo Fischer Scientific), were loaded on a 4–12% SDS-PAGE (NuPAGE Bis-Tris protein gel, Thermo Fischer Scientific). The gel was blotted, and the membrane was probed with an anti-ABCA1 antibody (NB400-105, NOVUS Biologicals). The same cell lysates were used to evaluate the expression of other cholesterol transporters in absence or presence of ABCA1 induction: primary antibodies specific for ABCG1 (EP1366Y, Abcam), SR-BI (NB400-113, NOVUS Biologicals) and ABCG4 (NBP2-15229, NOVUS Biologicals) were employed. The antibodies were chosen for their ability to recognize both the human and the murine isoform of proteins of interest, as stated by the manufacturer. An anti-GAPDH antibody (NB300-221, NOVUS Biologicals) was used to visualize GAPDH as control protein.

Cell endogenous apoE expression and secretion was evaluated in medium of cultured cells. SH-SY5Y, A172, N9 and J774 cells were grown in regular medium/10% FBS for 3 days or in medium/0.2%BSA/0.3 mM cAMP for 4 hours. 12 µl of 3-days cell-conditioned medium or 4-
hours cell-conditioned medium/cAMP was run on a 4–12% SDS-PAGE gel. An anti-human or -mouse apoE antibody (NB100-1530 and NB100-2040, respectively, both NOVUS Biologicals) were used to incubate the blots. In each gel, human or mouse plasma (1 µl) was included as a positive control to trace the presence of apoE.

To detect the presence of apolipoprotein B (apoB), 10 µl of undiluted individual CSF were analyzed in sample buffer containing 2% SDS, 5% β-mercaptoethanol and 10% glycerol. The mixture was heated at 80°C for 10 minutes and run on a 3-8% Tris-Acetate gel (NuPAGE Tris-Acetate gel, Thermo Fischer Scientific). The gel was blotted and the membrane was probed with an anti-apoB antibody (Abcam, ab20737).

**Cell cholesterol efflux to purified acceptors**

To measure cholesterol efflux, SH-SY5Y, A172, N9 and J774 cells were seeded in 24-well plates in regular medium/10% FBS, allowed to attach for 24 hours, and incubated with 2 µCi/mL [1,2-3H(N)]cholesterol (Perkin Elmer) in DMEM/2.5% FBS or RPMI/2.5% FBS for 24 hours. The cells were then treated with either 2 µM of LXR agonist T0901317 or 0.3 mM of cAMP in FBS-free medium/0.2% BSA for 18 hours to upregulate ABCA1 expression. Simultaneously, a set of cells were treated with vehicle-containing medium/0.2% BSA for 18 hours. Cells were exposed to medium, 10 µgr/ml purified apoAI or 50 µg/ml isolated HDL in FBS-free medium/+/- 2 µM T0901317 or /+/- 0.3 mM cAMP and cholesterol efflux was allowed to proceed for 4 hours. Cell medium was filtered using 0.45µM filter plates to eliminate floating cells, cell lipids were extracted with isopropanol for 30 minutes and an aliquot of both medium and isopropanol was read in a scintillation counter. Cell cholesterol efflux was expressed as the percent of [3H]cholesterol counts released in medium over the total [3H]cholesterol counts in medium and cells. Each efflux condition was conducted in triplicate, and the average of percent efflux was calculated.

**CSF cholesterol efflux capacity assay**

To measure CSF cholesterol efflux capacity SH-SY5Y (12.8*10⁴ cells/well), A172 (3.75*10⁴ cells/well), N9 (6*10⁴ cells/well) and J774 (6*10⁴ cells/well) cells were seeded in 96 wells plate in DMEM/10% FBS or RPMI/10% FBS, allowed to attach for 6 hours and labeled with 2 µCi/mL [1,2-3H(N)]cholesterol in DMEM/2.5% FBS or RPMI/2.5% FBS for 18 hours. Human cell lines SH-SY5Y and A172 were treated with 2 µM of the LXR agonist T0901317 in
DMEM/0.2% BSA or RPMI/0.2% BSA, respectively, for 6 hours to upregulate ABCA1 expression. Murine N9 and J774 cells were incubated with 0.3 mM of cAMP in RPMI/0.2% BSA for 6 hours to upregulate ABCA1. All cells were subsequently exposed to 33 µl of individual CSF (corresponding to 44% CSF in cell medium) in FBS-free MEM-HEPES/2 µM T0901317 or FBS-free MEM-HEPES/0.3 mM cAMP for 2.5 hours to promote cholesterol efflux. Cell medium was centrifuged at 10,000 RPM for 3 minutes to remove floating cells; cell lipids were extracted with isopropanol (200 µl/well) overnight and both cell medium and lipids were read in a scintillation counter. Cell cholesterol efflux was quantified as the percent [3H]cholesterol counts in the medium relative to the total counts in medium and cells. Efflux to each CSF sample was conducted in duplicates, and an average of the two replicas was calculated. If the individual sample readings deviated from the average by >10%, then efflux to this CSF was measured again. In each plate, two wells were used to measure efflux to CSF Pool 1. In all the experiments, this CSF was included to serve as the reference sample for normalization. The percent efflux to each individual CSF was divided by the percent efflux to CSF Pool 1 to derive a ratio normalized for the inter-assay variability. This ratio is CSF cholesterol efflux capacity (CSF CEC) from every cell line.

**CSF protein, apolipoproteins, cholesterol and phospholipid levels determination**

CSF protein concentration was measured by the turbidimetric Benzethonium Chloride method (Luxton et al. 1989, Kay et al. 2003) using a commercial kit (Microprotein Reagent, Thermo Fischer Scientific) with the following modifications. The assay was manually performed in a 96-well plates and an albumin solution from 0 to 1000 µg/ml was included in the plate as internal protein standard. 6.5 µl of each CSF sample were mixed with 244 µl of Reagent 1 for 30 seconds and 48 µl Reagent 2 were subsequently added. After 6 minutes of incubation, turbidity was quantitated at 405 nm with a spectrophotometer held at the constant temperature of 37°C.

CSF apoAI, apoJ and apoE content was measured by commercial enzyme-linked immunoassays (Human Apolipoprotein AI and Human Clusterine Quantikine® ELISA kit, R&D SYSTEMS; Human Apolipoprotein E ELISA kit, Abcam). The commercial kits display high sensitivity (0.275 and 0.189 ng/ml for the apoAI and apoJ ELISA kit; 0.03 µg/ml for the apoE ELISA kit) and relevant intra- and inter-assay precision (reported coefficients of variation <10%). To measure apolipoprotein levels, CSF samples were diluted 1:20 or 1:100 and the
assays were performed following manufacturer instructions.

CSF total cholesterol was enzymatically determined by the Amplex® Red Cholesterol Assay Kit (Thermo Fisher Scientific). Briefly, 50 µl of cholesterol reference in Reaction Buffer 1X was used to make a standard curve ranging from 0 to 0.004 µg/µl and 30 µl of CSF in 50 µl Reaction Buffer 1X were tested in duplicate. The assay was run as recommended by the manufacturer and the fluorescence of the reaction product (measured at 560 nm excitation/590 nm emission) was detected in a fluorescence plate reader.

CSF phospholipid levels were enzymatically assessed with the commercial Phospholipid Assay kit (Sigma Aldrich). Following manufacturer’s instructions, 20 µl of 0, 6, 12, 20 µM choline standard and CSF samples were tested in duplicate in a 96 wells-plate. Phospholipids in the sample were hydrolyzed to release choline by incubation with the reaction mix and the fluorescence intensity of reaction products (measured at 530 nm excitation/585 nm emission) was read in a fluorimeter. Free choline content of the samples, measured in 20 µl of each CSF by incubating the samples in the reaction mix without the key hydrolytic enzyme, was below the lowest detectable concentration of the standard curve. Consequently, the concentration of choline obtained in the samples was assumed to directly reflect phospholipid concentration.

**Statistical analysis**

In cell cholesterol efflux to purified acceptors experimental data are expressed as mean ± SD, as resulted from three determinations. In CSF cholesterol efflux capacity experiments, data are expressed as mean ± range, as resulted from duplicate measurements of CEC. CSF protein, cholesterol, apolipoprotein and phospholipid quantification was determined in duplicate and expressed as mean ± range. GraphPad Prism was used to graph and analyze data as indicated in figure legends. Linear regression analysis (GraphPad Prism software version 7.0) and Pearson’s correlation test (R software version 3.2.2) were used to assess the relationships between CSF CEC from different cell lines and between CSF CEC and each CSF biochemical component.

Machine learning tools were used to perform clustering analysis. In particular, unsupervised hierarchical clustering (R software version 3.2.2) was used as a method to cluster CSF specimens according to their similarity in terms of CEC and levels of biochemical components.
Results
Characterization of cell cholesterol efflux pathways in neuronal, astrocytic and microglial cell lines

Cholesterol transporter expression in neuronal, astrocytic and microglial cell lines

The efflux of cholesterol and phospholipid to lipid-poor apoAI is promoted by the activity of the ATP-binding cassette transporter A1 (ABCA1), which is essential and sufficient for HDL formation. Further cell cholesterol release depends on cholesterol diffusion to newly assembled HDL. The desorption of cell cholesterol from plasma membrane onto HDL occurs via active transport mediated by ABC transporters G1 (ABCG1) and G4 (ABCG4) or via scavenger receptor class B, type 1 (SR-BI) -facilitated diffusion (Adorni et al. 2007, Wang et al. 2004). J774 and many other cell lines do not express high level of ABCA1 and therefore its expression must be upregulated during the CEC assay. ABCA1 induction is usually achieved by treating murine cells with cAMP. Since the cAMP response element in the promoter of the human ABCA1 gene is mutated, human cells are commonly treated with a synthetic agonist of the Liver X receptor (LXR) T0901317 to induce ABCA1 transcription. Protein expression of cholesterol transporters was detected in each cell line by Western blotting analysis. As shown in figure IV.1, astrocytic A172 and neuronal SH-SY5Y express low detectable levels of ABCA1 at basal (un-induced) level while microglial N9, similarly to macrophage J774, showed higher levels of ABCA1 basal expression. Treatment with 0.3 mM of cAMP or 2 µM of the LXR agonist T0901317 up-regulated ABCA1 protein expression in all three cells line, albeit at lower levels when compared to J774 macrophages. ABCG1, SR-BI and ABCG4 were constitutively expressed in all cells with some peculiarities among the cell types. SH-SY5Y showed higher levels of ABCG1 as compared to other cell lines, A172 cells showed higher variability in ABCG1 protein expression in comparison with other cells. SH-SY5Y cells did not show a detectable band corresponding to the SR-BI protein, while a unique distinct band was observed for this cell line. Because a corresponding band is absent in other cell lines, the antibody may have recognized a modified isoform of the SR-BI protein or a protein with a similar structure and lower molecular weight to that of SR-BI, which is specifically expressed in neuronal cells. In the brain, SR-BI has been found to be expressed in glial cells but not in neurons (Rigotti et al. 2003). For all the cell lines, the expression of ABCG4 was detected by two separate bands. Treatment with either 0.3 mM of cAMP or 2 µM of the LXR agonist T0901317 did not significantly induce the expression of ABCG1, ABCG4 or SR-BI over a
basal level in any of the three cell lines. The same result was observed in J774 macrophages (Fig.IV.1).

**Fig.IV.1** Cell cholesterol transporters expression. Western blotting. ABCA1, ABCG1, SR-BI and ABCG4 protein expression in A172 astrocytes, SH-SY5Y neurons and N9 microglia as compared to J774 macrophages. Protein levels were evaluated in absence (-) or presence (+) of 0.3 mM cAMP (in the case of murine cell lines, J774 and N9) or 2 µM of the LXR agonist T0901317 (in the case of human cell lines, A172 and SH-SY5Y) as ABCA1 inducers. In each separate gel, measurement of GAPDH expression was included as a control for protein loading. Each condition is shown in duplicate.
Apolipoprotein E expression in neuronal, astrocytic and microglial cell lines

ApoE is the most abundant lipoprotein expressed in the brain. A western blot analysis was performed to investigate the endogenous apoE expression and secretion in selected cell lines. As shown in figure IV.2, A172 and SH-SY5Y cells did not express apoE while N9 cells did express low levels of this apolipoprotein. As expected, J774 did not show any expression of apoE. The apoE band was prominent in the medium collected from N9 cells after 3 days of culture and detectable when apoE expression was measured in medium collected after 4 hours of culture in presence of cAMP (Fig.IV.2). The latter observation suggests that apoE expression in N9 cells is weak and thus may not affect cholesterol efflux in the 2.5 hours assay. In line with this hypothesis, low levels of apoE gene expression were previously detected in the N9 cell line by qPCR analysis (Butovsky et al. 2014).

Fig.IV.2  Cell endogenous apolipoprotein E expression. Western blotting. ApoE secretion in 3-days cell-conditioned medium from A172 and SH-SY5Y cells (left panel). ApoE secretion in 3-days cell-conditioned medium (3-d) or in 4-hours cell-conditioned medium in presence of cAMP (4-h) from N9 and J774 cells (right panel). In each gel, human or mouse plasma was run side-by-side to cell medium as control to identify the location of human or mouse apoE, respectively.
**Cell cholesterol efflux to purified acceptors**

In order to functionally characterize the ability of the selected cell lines to release cholesterol via the main known ABCA1-mediated and un-mediated pathways, we conducted cholesterol efflux assay to medium, purified lipid-free apoAI and isolated mature HDL. Background cholesterol release (a) derives from plasma membrane vesicles shedding and from basal or upregulated ABCA1-mediated microparticle release to medium and cell endogenous apoE. Treatment with the LXR agonist T0901317 did not induce a significant increase in background cholesterol release in SH-SY5Y and A172 cells. On the contrary, background cholesterol release was significantly improved in N9 and J774 cells upon induction with cAMP, as compared to un-induced cholesterol release (**P < 0.01, for both cell lines) (Fig.IV.3 a). Although N9 expressed apoE, cholesterol efflux from these cells was lower than cholesterol release from J774.

ABCA1-mediated cholesterol efflux (b) reflects the expression and activity of basal or up-regulated ABCA1, which drives cholesterol from plasma membrane to lipid-free apoAI. Exogenous apoAI was added at saturating concentration so that cell endogenous ABCA1 expression was the limiting factor in the assay. Treatment with the LXR agonist T0901317 or cAMP determined a significant increase in ABCA1-mediated efflux to apoAI in SHSY5Y, A172 and N9 cells (**P < 0.001, for all cell lines), albeit at much lower levels as compared to J774 macrophage (Fig.IV.3 b). These results were in accord with the levels of ABCA1 protein expression, as shown in the Western blot analysis. Cholesterol efflux to lipiddated particles reflects ABCG1-, ABCG4- and SR-BI -facilitated desorption of free cholesterol in the plasma membrane for diffusion to lipid-enriched acceptors (c). In order to evaluate the contribution of these pathways in selected cell lines, cholesterol efflux was promoted to mature HDL and compared to efflux to medium without acceptors (**P < 0.001, for all cell lines). Desorption efflux from J774 cells was the highest followed by that from N9 cells, while SH-SY5Y and A172 cells had a similar, weaker capability to release cholesterol to HDL (Fig.IV.3 c).
Fig.IV.3 Cell cholesterol efflux characterization of the ABCA1-mediated and -unmediated pathways in SH-SY5Y neurons, A172 astrocytes and N9 microglia cells, as compared to J774 macrophages. Cholesterol efflux to medium (a) or to purified ApoAI (b) represents background cholesterol release or specific ABCA1-mediated cholesterol efflux, respectively. Cholesterol efflux to medium and to 10 µg/µl apoAI was run in absence (white bars) or presence of 2 µM of the LXR agonist T0901317 or 0.3 mM of cAMP to upregulate ABCA1 activity (black bars). c. Cholesterol efflux to 50 µg/µl HDL (black bars) was compared to cholesterol efflux to medium (white bars) to evaluate cholesterol efflux by desorption. Experimental conditions were as described in Materials and Methods. Data are expressed as mean ± SD, as resulted from three determinations. Statistical analysis, Student’s t test for unpaired samples (*P < 0.1, **P < 0.01, ***P < 0.001, ns not significant) to compare cholesterol efflux in absence or presence of ABCA1 induction (a, b) or in absence or presence of cholesterol acceptors (c) in each cell line.
CSF CEC method development

The standard HDL cholesterol efflux capacity assay (Khera et al. 2011) required several modifications and considerations to be adapted to the study of CSF CEC. First, CSF does not contain apoB-containing lipoproteins and therefore, differently from plasma, it does not require apoB depletion via treatment with polyethylene glycol. In this study, the presence of apoB in CSF was assayed by Western blotting and those samples (2 samples, namely CSF 12 and 18) with detectable levels of apoB were excluded from statistical analysis (Fig.IV.4).

The absence of apoB-containing particle in CSF is generally ascribed to its high molecular weight and impossibility to permeate the blood brain barrier (the blood/CSF concentration ratio of apoB is estimated as about 6000) (Roheim et al. 1979). However, several studies report the presence of apoB in the cerebrospinal fluid of subjects with severe brain injury and defective BBB (Salen et al. 1987, Kay et al. 2003) as well as in the brain of subjects with AD (Namba et al. 1992). Therefore, whether the presence of apoB in CSF is the consequence of an advanced neuropathological status or derived from blood contamination that eventually occurred during the CSF drainage procedure, it is difficult to establish.

Fig.IV.4 Apolipoprotein B expression in CSF samples. Western Blotting. The 20 CSF specimens were tested simultaneously and diluted human plasma was included on the first gel as a positive control. At the same condition, low density-lipoproteins (LDL) were run on a third gel as a control to identify the location of apoB-100 and as a reference to make a standard curve of apoB-100 mass. LDL were isolated from plasma by density gradient ultracentrifugation, and therefore are purified from the lipoprotein fraction containing apoB-48 which is present on chylomicrons assembled in the intestine after dietary lipids absorption. In particular, 10 µl of LDL containing 5.4,1.8, 0.6, 0.2, 0.067, 0.022 µg of protein were loaded in each well. The sensitivity of the anti apoB-100 antibody was estimated as about 0.067 µg of mass protein.
Second, the concentration of HDL and lipoproteins in CSF ranges from 0.5 to 15% of their corresponding plasma levels. This necessitated the application of larger amounts of CSF to cells as compared to plasma, in order to obtain robust data. Given that CSF is scarce in the amount and difficult to obtain, CSF cholesterol efflux assay was conducted in 96-well plates and 15-70 µL of CSF per well were tested in preliminary studies. Employing CSF Pool 1 and CSF 7 from the samples cohort, we ascertained that cholesterol efflux was in the linear range at the CSF volume and efflux duration that we selected for the assay.

Increasing volumes of CSF were tested for cholesterol efflux from J774 cells (Fig.IV.5 a, left panel) and SH-SY5Y cells (Fig.IV.5 a, right panel). J774 and SH-SY5Y cells were taken as cell models with different (high and low, respectively) capability to release radioactive cholesterol in the efflux assay, as resulted from the characterization of the cell cholesterol efflux to purified acceptors. CSF 7 and CSF Pool 1 were employed as test samples and incubated at 16.5, 33 and 66 µl/well with cells for 2 hours. Cholesterol efflux from J774 and SHSY5Y cells increased linearly with increasing volumes of both CSF 7 (R²= 0.97 p=0.0002 and R²= 0.91 p=0.0028, respectively) and CSF Pool 1 (R²= 0.99 p=0.0001 and R²= 0.95 p=0.0008, respectively) (Fig.IV.5). 33 µl of CSF/well was the volume selected to run the CSF CEC assay. First, it falls in the middle of the cholesterol efflux versus CSF volume linear curve. Second, [³H]cholesterol counts transferred to medium without CSF were low as compared to counts transferred to medium with 33 µl of CSF as cholesterol acceptor. In particular, background efflux to medium from J774 and from SHSY5Y cells was 13 or 30% and 17 or 28% of cholesterol efflux to CSF 7 or CSF Pool 1, respectively (data not shown). Therefore, this volume was selected as the minimum amount of CSF/well that was necessary to have robust efflux values in the CSF CEC assay.

To test whether CSF cholesterol efflux capacity changed with different incubation times, cholesterol efflux to 33 µl CSF was measured for 30 min, 1, 2, 3 and 4 hours from J774 cells (Fig.IV.5 b). Cholesterol efflux from J774 cells to both CSF Pool 1 and CSF Pool 2 was linear for 3 hours. From 3 hours on, cholesterol efflux to CSF Pool 1 tended to plateau and the second order polynomial-least squares curve (R²= 0.90) best described the cholesterol efflux versus time relation. More robustly, cholesterol efflux to CSF Pool 2 maintained linearity from 30 min to 4 hours (R²= 0.96 p<0.0001) (Fig.IV.5 b). In order to run the assay in the linear range of cell cholesterol efflux with a diverse array of CSF samples, 2.5 hours was selected as the optimal duration to measure CSF cholesterol efflux capacity.
Fig.IV.5 Optimization of CSF cholesterol efflux capacity assay parameters: CSF volume and assay duration. 

**a.** Cholesterol efflux to increasing volumes of CSF from J774 cells (left panel) and SH-SY5Y cells (right panel). CSF 7 and CSF Pool 1 were incubated at 16.5, 33 and 66 µl/well with cells for 2 hours. Cell cholesterol efflux was quantified as the percentage of cholesterol counts released in the medium over the total counts in medium and cells. Statistical analysis, linear regression analysis to show the relation between cell cholesterol efflux and CSF volumes.

**b.** Cholesterol efflux to 33 µl of CSF, the sample volume selected for the assay, for 30 min, 1, 2, 3 and 4 hours from J774 cells. Statistical analysis, linear regression analysis or second order polynomial analysis to show linearity of cell cholesterol efflux to CSF Pool 1 and CSF Pool 2, respectively, during the assay duration. The CSF cholesterol efflux assay was performed as described in Materials and Methods. Each condition was run in duplicate, data are expressed as mean ± range.
CSF CEC from neuronal, astrocytic and microglial cell lines

A panel of 20 CSF samples were tested for their capacity to promote cholesterol efflux following the CEC protocol optimized as described above. The variability of CSF CEC was assessed in SH-SY5Y, A172 and N9 cells and compared to CSF CEC from J774 macrophages, which are in standard use for plasma HDL CEC quantitation. Linear regression analysis was employed to describe the relationship between CSF CEC from different cell lines, as shown in figure IV.6. CSF CEC from all three selected cell types correlated well with CSF CEC from J774 macrophages. In particular, CSF CEC from N9 microglia and CSF CEC from J774 macrophages were nearly identical (R² = 0.94 p<0.0001) (Fig.IV.6 c). CSF CEC from SH-SY5Y neurons and CSF CEC from A172 astrocytes were significantly correlated with CSF CEC from J774 cells (R² = 0.77 p<0.0001 and R² = 0.78 p<0.0001, respectively) (Fig.IV.6 a, b) and less strongly correlated with each other (R² = 0.66 p<0.0001) (Fig.IV.6 d).

![Graphs showing correlation between CSF CEC from different cell lines](image)

Fig.IV.6 Relationship between CSF cholesterol efflux capacity (CSF CEC) from J774 cells and CSF CEC from SH-SY5Y cells (a), A172 cells (b) and N9 cells (c). Relationship between CSF CEC from SH-SY5Y cells and CSF CEC from A172 cells (d). CSF CEC from every cell line was measured and normalized for the inter-assay variability as described in Materials and Methods. Efflux to each individual CSF from every cell line was conducted in duplicate, data are expressed as mean ± range. Statistical analysis, linear regression analysis to describe the association between CSF CEC measured from different cell lines. R² values and significance of the regression coefficient are depicted in each graph.
Biochemical parameters of CSF

Protein, apoAI, apoJ, apoE, cholesterol and phospholipid content of each CSF sample included in the study was established by specific techniques as described in Materials and Methods. CSF specimens enrolled in the cohort study were blindly analyzed with regard to their health status, race, gender and age. CSF total protein content was in normal range, generally reported as about 0.2-0.75% of blood total protein (Mahley et al. 2016), and varied from 0.09 to 0.79 mg/ml (mean±SD 0.33±0.19 mg/ml). Mean concentration of CSF cholesterol and phospholipid was 2.79±1.28 µg/ml and 2.83±1.04 µg/ml, respectively. ApoAI, apoJ and apoE are most abundant lipoproteins in cerebrospinal fluid (Koch et al. 2017) and they have found to be present at 0.3-15% of their corresponding plasma concentration (Vitali et al. 2014, Mahley et al. 2016). In the study cohort, CSF ApoAI (mean±SD 4.34±1.99 µg/ml), apoJ (mean±SD 6.76±3.37 µg/ml) and apoE (mean±SD 8.66±2.15 µg/ml) levels were comparable with concentration ranges previously reported in literature.

The relationship among the measured CSF parameters was evaluated by linear regression analysis. As shown in the table IV.1, we found a strong correlation between CSF total protein and CSF apoAI (R²= 0.91 p<0.0001), as well as between CSF total protein and CSF cholesterol (R²= 0.85 p<0.0001) or phospholipid (R²= 0.84 p<0.0001) levels, while a less strong significant relation was found between CSF total protein content and CSF apoJ levels (R²= 0.67 p<0.0001). To a similar extent, CSF apoAI, cholesterol and phospholipid levels were highly correlated among each other (apoAI vs cholesterol R²= 0.80 p<0.0001; apoAI vs phospholipid R²= 0.75 p<0.0001; cholesterol vs phospholipid R²= 0.77 p<0.0001) and less strongly but significantly correlated with CSF apoJ levels (apoJ vs apoAI R²= 0.60 p=0.0004; apoJ vs cholesterol R²= 0.62 p=0.0003; apoJ vs phospholipid R²=0.58 p=0.0006). As compared to other CSF components, CSF ApoE was found to display a much weaker linear regression with CSF total protein (R²= 0.31 p=0.0295) or phospholipid (R²= 0.43 p=0.0081) levels. No significant relationship was found between CSF ApoE levels and CSF apoAI (R²= 0.19 p=0.0986), apoJ (R²= 0.13 p=0.1722) or cholesterol (R²= 0.20 p=0.0890) levels (Table.IV.1).
<table>
<thead>
<tr>
<th></th>
<th>Protein, mg/ml</th>
<th>ApoA1, µg/ml</th>
<th>ApoA1, µg/ml</th>
<th>ApoE, µg/ml</th>
<th>Cholesterol, µg/ml</th>
<th>Phospholipid, µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, mg/ml</td>
<td>-</td>
<td>0.91 (&lt;0.0001)</td>
<td>0.67 (&lt;0.0001)</td>
<td>0.31 (0.0295)</td>
<td>0.85 (&lt;0.0001)</td>
<td>0.84 (&lt;0.0001)</td>
</tr>
<tr>
<td>ApoA1, µg/ml</td>
<td>0.91 (&lt;0.0001)</td>
<td>-</td>
<td>0.60 (0.0004)</td>
<td>0.19 (0.0986 ns)</td>
<td>0.80 (&lt;0.0001)</td>
<td>0.75 (&lt;0.0001)</td>
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<tr>
<td>ApoA1, µg/ml</td>
<td>0.57 (&lt;0.0001)</td>
<td>0.60 (0.0004)</td>
<td>-</td>
<td>0.13 (0.1722 ns)</td>
<td>0.62 (0.0003)</td>
<td>0.58 (0.0006)</td>
</tr>
<tr>
<td>ApoE, µg/ml</td>
<td>0.31 (0.0295)</td>
<td>0.19 (0.0986 ns)</td>
<td>0.13 (0.1722 ns)</td>
<td>-</td>
<td>0.20 (0.0890 ns)</td>
<td>0.43 (0.0081)</td>
</tr>
<tr>
<td>Cholesterol, µg/ml</td>
<td>0.85 (&lt;0.0001)</td>
<td>0.80 (&lt;0.0001)</td>
<td>0.62 (0.0003)</td>
<td>0.20 (0.0890 ns)</td>
<td>-</td>
<td>0.77 (&lt;0.0001)</td>
</tr>
<tr>
<td>Phospholipid, µg/ml</td>
<td>0.84 (&lt;0.0001)</td>
<td>0.75 (0.0001)</td>
<td>0.58 (0.0006)</td>
<td>0.43 (0.0081)</td>
<td>0.77 (&lt;0.0001)</td>
<td>-</td>
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</table>

ApoA1 = apolipoprotein A1; ApoA1 = apolipoprotein A1; ApoE = apolipoprotein E

Table IV.1 Relationship between each measured CSF biochemical component in the study cohort. Statistical analysis, linear regression analysis; R2 values and significance of the regression coefficient (p value) are shown for each correlation. Ns, not significant linear regression.
Prediction of CEC from biochemical components of CSF by statistical analysis

In order to investigate which CSF biochemical component is the best predictor for CSF CEC, we used linear regression analysis to correlate CSF protein, apoAI, apoJ, apoE, cholesterol and phospholipid levels (taken as independent variables) with CSF CEC measured from every cell line (taken as dependent variable) (Table.IV.2). A strong, significant correlation was found between CSF total protein and CSF CEC from SH-SY5Y (R² = 0.78 p<0.0001), N9 (R² = 0.82 p<0.0001) and J774 cells (R² = 0.75 p<0.0001). CSF CEC from A172 cells was found to be moderately correlated with CSF total protein (R² = 0.54 p=0.0007). Moreover, both CSF cholesterol and phospholipid levels correlated significantly with CSF CEC from SH-SY5Y (R² = 0.65 p= 0.0002 and R² = 0.80 p<0.0001), A172 (R² = 0.43 p=0.0053 and R² = 0.50 p=0.0013), N9 (R² = 0.62 p= 0.0003 and R² = 0.58 p= 0.0004) and J774 cells (R² = 0.58 p= 0.0006 and R² = 0.57 p= 0.0002). CSF apoAI was highly correlated with CSF CEC from SH-SY5Y (R² = 0.60 p= 0.0001), N9 (R² = 0.70 p<0.0001) and J774 cells (R² = 0.65 p<0.0001) and less strongly correlated with CSF CEC from A172 cells (R² = 0.42 p=0.0049).

<table>
<thead>
<tr>
<th></th>
<th>Protein, mg/ml</th>
<th>ApoAI, µg/ml</th>
<th>ApoJ, µg/ml</th>
<th>ApoE, µg/ml</th>
<th>Cholesterol, µg/ml</th>
<th>Phospholipid, µg/ml</th>
</tr>
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<tbody>
<tr>
<td>CSF CEC SH-SY5Y</td>
<td>0.78 (&lt;0.0001)</td>
<td>0.60 (0.0001)</td>
<td>0.30 (&lt;0.0001)</td>
<td>0.39 (0.0135)</td>
<td>0.65 (0.0002)</td>
<td>0.80 (&lt;0.0001)</td>
</tr>
<tr>
<td>CSF CEC A172</td>
<td>0.54 (0.0007)</td>
<td>0.42 (0.0049)</td>
<td>0.73 (&lt;0.0001)</td>
<td>0.29 (0.0333)</td>
<td>0.43 (0.0053)</td>
<td>0.50 (0.0013)</td>
</tr>
<tr>
<td>CSF CEC N9</td>
<td>0.82 (&lt;0.0001)</td>
<td>0.70 (&lt;0.0001)</td>
<td>0.85 (&lt;0.0001)</td>
<td>0.28 (0.0407)</td>
<td>0.62 (0.0003)</td>
<td>0.58 (0.0004)</td>
</tr>
<tr>
<td>CSF CEC J774</td>
<td>0.75 (&lt;0.0001)</td>
<td>0.65 (&lt;0.0001)</td>
<td>0.90 (&lt;0.0001)</td>
<td>0.26 (0.0503 na)</td>
<td>0.58 (0.0006)</td>
<td>0.57 (0.0002)</td>
</tr>
</tbody>
</table>

CSF= cerebral spinal fluid; CEC= cholesterol efflux capacity; ApoAI= apolipoprotein AI; ApoJ= apolipoprotein J; ApoE= apolipoprotein E

Table.IV.2 Relationship between CSF CEC from SH-SY5Y cells, A172 cells, N9 cells, J774 cells and measured CSF total protein or CSF apoAI in the study cohort. Statistical analysis, linear regression analysis to describe the association between CSF CEC from every cell line and each CSF variable; R² values and significance of the regression coefficient (p value) are shown for each correlation. Ns, not significant linear regression.
Fig. IV.7 Relationship between CSF CEC from SH-SY5Y cells (a), A172 cells (b), N9 cells (c), J774 cells (d) and measured CSF total protein and apoAI in the study cohort. Statistical analysis, linear regression analysis.

A strong linear regression was observed between CSF apoJ and CSF CEC from SH-SY5Y ($R^2 = 0.80 \ p < 0.0001$), N9 ($R^2 = 0.85 \ p < 0.0001$), J774 ($R^2 = 0.90 \ p < 0.0001$) and, to a lesser extent, A172 cells ($R^2 = 0.73 \ p < 0.0001$). The relation between CSF apoE levels and CSF CEC tended towards a weak linear regression and reached statistical significance in SH-SY5Y ($R^2 = 0.39 \ p = 0.0135$), A172 ($R^2 = 0.29 \ p = 0.0353$) and N9 cells ($R^2 = 0.28 \ p = 0.0407$), while this association was not statistically significant in J774 cells ($R^2 = 0.26 \ p = 0.0503$).

Fig. IV.8 Relationship between CSF CEC from SH-SY5Y cells (a), A172 cells (b), N9 cells (c), J774 cells (d) and measured CSF apoJ and apoE in the study cohort. Statistical analysis, linear regression analysis.
Simple linear regression analysis was performed on row data. Logarithmic transformation was then applied to those variables that were not normally distributed (CSF CEC from all cell lines, CSF protein content and CSF apoJ) and Pearson’s correlation test was performed on normally distributed data. The coefficient and the statistical significance of Pearson’s correlation between CSF CEC and each CSF biochemical factor reflected the significance of the relationship obtained with simple linear regression were as shown in Table.IV.3. Overall, CSF protein, apoAI and apoJ levels were identified as the best predictors of CSF CEC from N9 and J774 cells with similar statistical significance, while CSF protein, phospholipid and apoJ were the CSF components that best predicted CSF CEC from SH-SY5Y and A172 cells with distinctive regression coefficients.

<table>
<thead>
<tr>
<th></th>
<th>log Protein.</th>
<th>ApoAI μg/ml</th>
<th>ApoJ μg/ml</th>
<th>ApoE μg/ml</th>
<th>Cholesterol μg/ml</th>
<th>Phospholipid μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>log CSF CEC SH-SY5Y</td>
<td>0.84</td>
<td>0.76</td>
<td>0.80</td>
<td>0.63</td>
<td>0.78</td>
<td>0.92</td>
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<td></td>
<td>(3.38e-06)</td>
<td>(0.0001)</td>
<td>(0.0003)</td>
<td>(0.9112)</td>
<td>(0.0003)</td>
<td>(1.95e-07)</td>
</tr>
<tr>
<td>log CSF CEC A172</td>
<td>0.69</td>
<td>0.63</td>
<td>0.72</td>
<td>0.58</td>
<td>0.67</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>(0.0020)</td>
<td>(0.0066)</td>
<td>(0.0023)</td>
<td>(0.0216)</td>
<td>(0.0047)</td>
<td>(0.0007)</td>
</tr>
<tr>
<td>log CSF CEC N9</td>
<td>0.88</td>
<td>0.89</td>
<td>0.85</td>
<td>0.53</td>
<td>0.83</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>(2.83e-07)</td>
<td>(4.22e-07)</td>
<td>(4.54e-05)</td>
<td>(7.64e-05)</td>
<td>(0.0001)</td>
<td></td>
</tr>
<tr>
<td>log CSF CEC J774</td>
<td>0.81</td>
<td>0.84</td>
<td>0.90</td>
<td>0.50</td>
<td>0.79</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>(5.30e-06)</td>
<td>(2.81e-06)</td>
<td>(4.04e-06)</td>
<td>(0.9564)</td>
<td>(0.0002)</td>
<td>(0.0001)</td>
</tr>
</tbody>
</table>

**Table.IV.3** Correlation between CSF CEC from SH-SY5Y cells, A172 cells, N9 cells, J774 cells and each measured CSF biochemical component in the study cohort. Those variables that were not normally distributed were log-transformed and statistical significance of the correlations was evaluated with Pearson’s test. Pearson’s r and p value are shown for each correlation.
CSF specimens clustering

CSF specimens were clustered with a bottom-up approach according to the similarity with respect to their CEC and composition in terms of total protein, apoAI, apoJ, apoE, total cholesterol and phospholipid content. Results of samples clustering analysis were as presented in the dendogram in the figure IV.9. Interestingly, the study cohort was clustered into two separate main groups, one of which is the group of samples with the highest CEC, as measured from all cell lines, that overall also showed the highest concentration of biochemical components (sample n. 9, 10, 14 and 16). Because of the lack of information about the health status of study subjects, whether the separation observed in our cohort is of some bio-pathological significance remains unknown and this question need to be addressed in a separate study.

![Hierarchichal structure of specimens clustering](image)

**Fig.IV.9 Hierarchichal structure of specimens clustering.** The dendogram schematized in figure represents the process of bottom-up clustering of CSF samples in the study cohort (20 CSF samples + CSF commercial samples) according to similarity of their cholesterol efflux capacity and level of biochemical components (total protein, apoAI, apoJ, apoE, total cholesterol and phospholipid levels).
Discussion
Alzheimer’s disease (AD) is a complex, unremitting neurodegenerative disorder accounting for 60% cases of dementia worldwide (Querfurth and LaFerla 2010). Currently, no curative or preventive treatments are available for use in the general population and discovery of new targets as well as validation of early biomarkers for the disease remain urgent unmet needs (Masters et al. 2015).

Although some epidemiological studies addressed to highlight a connection between plasma levels of cholesterol and severity of dementia, a relation between circulating lipids and risk of developing AD remains nowadays controversial (Wood et al. 2014). Nevertheless, increasing experimental observations strongly suggest that excessive cholesterol induces a shift in APP processing to the amyloidogenic pathway in neurons and impairs function and survival in astrocytes and microglia (Di Paolo and Kim 2011, Abramov et al 2011, Lee et al. 2011). Therefore, impeded cholesterol transport out of brain cells may promote Aβ accumulation and AD development.

In the present study, we characterized the process of cell cholesterol efflux in in vitro cellular models relevant to the pathology of AD and we adapted a well-established technique that measures plasma HDL cholesterol efflux capacity (HDL CEC) to measure cerebrospinal fluid cholesterol efflux capacity (CSF CEC) in humans.

In the standard HDL CEC assay, the reference immortalized cell line is labeled with exogenously added [3H]cholesterol and is then exposed to medium containing 1-2% of human plasma from which apoB-lipoprotein is removed and which contains only HDL. The choice of the reference line is absolutely critical. For atherosclerosis research, it has to be a macrophage cell line. Macrophages play a central role in the etiology of ASCVD and specifically depend on HDL functional quality for cholesterol release (de la Llera-Moya et al. 2010, Favari et al 2009).

While the key role of macrophages in atherosclerosis is well-established, which cell type is most relevant to AD is unclear. Aβ is synthesized by neurons and astrocytes and is removed by microglia, and thus any one – or even all – of these three cell types could be important to AD. We characterized human neural cells SH-SY5Y, human astrocyte cells A172 and mouse microglial cells N9 as reference cell line for the CSF CEC assay and compared them to murine macrophage J774 cells, which are in standard use for quantitating HDL CEC.

Cell expression of key cell cholesterol transporters was detected by Western blotting. Cell ability to release cholesterol by the ABCA1-dependent and -independent pathways was determined by exposing [3H]-labeled cells to purified apoAI and HDL. The three cell lines expressed ABCA1 in an inducible manner, even though at significant different levels, and released cholesterol to exogenously added apoAI, albeit at lower levels than J774 cells. The
selected cells constitutively expressed ABCG1, SR-B1 and ABCG4, mediators of cell cholesterol release by desorption, and robustly released cholesterol to HDL. Thus, it can be concluded that, even though to different extents, the selected neuronal and glial cell lines express all major cholesterol transporters and can access the ABCA1/apoAI/apoE and the ABCG1/ABCG4/SR-BI/desorption pathways of cell cholesterol efflux. Importantly, the cells did not secrete endogenous apolipoproteins, except for N9 cells, which expressed apoE at low levels.

The standard HDL cholesterol efflux assay required several modifications to be applied to CSF CEC measurement. First, CSF does not contain apoB and thus apoB precipitation with polyethylene glycol is not required. Second, the concentration of HDL and its components (apolipoproteins, phospholipid and cholesterol) in CSF is much lower than in plasma. Moreover, CSF is a scarce resource and the quantity to use for research purpose is limited. Therefore, we scaled-down the HDL CEC assay, which is normally performed in 24-well plates, to 96-well plates. We further determined that cholesterol efflux to 33 µl of CSF (diluted to 44% CSF in cell medium) for 2.5 hours is in a linear range and is optimal in terms of efflux magnitude, background counts as a percent of total efflux and CSF usage.

Among the pathways for cell cholesterol removal, the ABCA1-mediated efflux is considered the most relevant pathway in macrophages. In the brain, ABCA1 is ubiquitously expressed in various cell types (Koldamova et al. 2003) and is thought to have a pivotal role in the formation of brain particles by mediating the flux of cell cholesterol and lipids (Wahrle et al. 2004, Kim et al. 2007). During the efflux assay the ABCA1 expression was upregulated with 2 µM T0901317 (an LXR agonist) or with 0.3 mM of cAMP, in human or murine cell lines, respectively. To assess the variability of CSF CEC, we measured CSF cholesterol efflux capacity of 20 anonymized individuals from every cell type and we correlated CSF CEC from each cell type with one another. CSF CEC from N9 cells and CSF CEC from J774 cells were virtually identical (R²=0.94). However, CSF CECs from SH-SY5Y, A172 and J774 cells were correlated with one another much less (R²=0.66-0.78). There was a 1-2 fold difference between the samples with the lowest and highest CECs.

CSF, which originates from both drainage of brain perivascular fluid and blood filtration at the choroid plexuses, contains a diverse array of apolipoproteins complexed with cholesterol and phospholipids into lipoprotein particles that have similar density and size to plasma HDL (Koch et al. 2001). While apoAI is not synthesized by brain cells and access the CSF through filtration of blood at the choroid plexuses (Stukas et al. 2014), CSF apoE is thought to derive from the brain where it is produced by glial cells (Liu et al. 2012). Other than apoE, apoJ is highly
expressed in astrocytes (Saura et al. 2003) and is a major brain lipoprotein found in CSF. In order to determine which biochemical components of the CSF contribute the most to its CEC, we measured the levels of major cholesterol acceptors (apoAI, apoJ and apoE) and the amounts of protein, choline phospholipid and cholesterol in CSF samples. CSF CECs from the 4 cell types were correlated with the biochemical parameters of CSF to varying extents. In particular, statistical analysis show that CSF protein, apoAI and apoJ levels were the best predictors of CSF CEC from microglial N9 and macrophage J774 cells while CSF protein, phospholipid and apoJ levels were the best predictors of CSF CEC from neuronal SH-SY5Y and, to a lesser extent, astrocytic A172.

Our results together indicate that microglial N9 and macrophage J774 cells use the same cholesterol efflux transporters and pathways to similar extents while neuronal SH-SY5Y and astrocyte A172 cells differ from each other and from microglia and macrophages in the utilization of cholesterol efflux transporters and pathways. Since cholesterol efflux from cell types is substantially different, we can not use one representative cell type in lieu of the remaining two to measure CSF CEC. Interestingly, we also found that CSF CEC strongly correlates with CSF apoJ content, while weak or no association was found with CSF apoE levels. Despite some cells to cells peculiarity, this result was consistent among the four cellular models. Thus, it can be concluded that apoJ, rather than apoE, must be major acceptors of cholesterol in CSF together with apoAI. Given that apoJ variants are associated with AD in GWAS studies, this latter finding is particularly important and must be confirmed in further studies.

Cholesterol efflux capacity is a functional metric of plasma HDL with respect to atherosclerotic cardiovascular disease (ASCVD) (Rader and Hovingh 2014). In particular, plasma CEC measures the ex vivo ability of human HDL to accept cholesterol from macrophage foam cells, considered as the hallmark of atherosclerosis. It is demonstrated that plasma HDL CEC is a predictor of incident ASCVD inversely and independently of other major HDL metrics and ASCVD risk factors (Khera et al. 2011, Rohatgi et al. 2014).

A large body of experimental evidences strongly suggest that excessive intracellular cholesterol in neurons, astrocytes and microglia promotes Aβ accumulation and Aβ-driven inflammation in Alzheimer’s disease (AD) (Simons et al 1998, Lee et al. 2011, Abramov et al. 2011). Neurons, astrocytes and microglia co-localize at amyloid beta (Aβ) deposits in senile plaques of the grey matter of brain, considered as the hallmark of Alzheimer’s disease (Mori et al. 2001). It is plausible that just as the ability to readily release cholesterol to plasma helps macrophages to avoid becoming foam cells in ASCVD, the ability to readily release cholesterol to CSF fluids
helps neurons, astrocytes and microglia to overcome Aβ toxicity and maintain proper homeostasis. If this is indeed the case, the study of CSF CEC may be a useful tool to provide an insight into the etiology and pathogenesis of Alzheimer’s disease.

To test the hypothesis that cholesterol efflux capacity of CSF (the ability of CSF to accept large amounts of cholesterol from cells) can serve as diagnostic biomarker of the neurodegenerative Alzheimer’s disease we further suggest to evaluate CSF CEC in neurons, astrocytes and microglia in a broad human cohort of case and control subjects. Interestingly, the study cohort was clustered into two separate main groups when CSF specimens were clustered with an bottom-up approach in classes of similarity with respect to their CEC, total protein, apoAI, apoJ, apoE, total cholesterol and phospholipid content. Since no informations regarding the health status of these study subjects were available, the bio-pathological significance of this separation in our cohort remains unknown. Further insights on this question should be addressed in a separate study where controls, mild-demented and AD subjects will be compared.

In conclusion, we characterized cell cholesterol efflux in *in vitro* cellular models relevant to the pathology of AD. Moreover, we identified biochemical components in CSF that determine its CEC and we established a robust method to study human CSF CEC as a putative, novel metric of the neurodegenerative disease.

This project has been conducted at the Division of Translational Medicine and Human Genetics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, USA. The PhD candidate significantly and critically contributed to experiments design; the PhD candidate performed all experiments presented. The PhD candidate analyzed and interpreted the data in collaboration with other research team members.
Conclusion
Both alterations of lipid/lipoprotein metabolism and inflammatory events contribute to the formation of the atherosclerotic plaque, characterized by the accumulation of abnormal amounts of cholesterol-loaded macrophages in the artery wall. Reverse cholesterol transport (RCT) may counteract the pathogenic events leading to the formation and development of atheroma, by promoting the high density-lipoprotein (HDL)-mediated remodeling and removal of cholesterol from the artery wall. Although the inverse association between HDL plasma levels and CVD risk has been postulated for years, recently this concept has been challenged by studies reporting that HDL functions may be independent of their plasma levels. In fact, the failure of therapies targeting HDL-C levels has led to significant interest in directly evaluating HDL’s main antiatherosclerotic function-the promotion of macrophage RCT.

Cholesterol efflux capacity (CEC) is the most widely accepted method to assess HDL function, as it represents the first step of the RCT, and has recently been demonstrated to be more important than HDL-C levels for CV risk. CEC is studied not only as a prognostic parameter of CVD but also as a target to be addressed with a pharmacological intervention to reduce or slow progression of atherosclerosis and thus achieve cardiovascular protection. In the first part of this thesis, three different dosages of a reconstituted HDL were evaluated in an animal model of hypercholesterolemia. Even though a significant increase in CEC was found with infusion of the highest dose of this TN-HDL, the same results was not achieved with multiple lower dose of the treatment. Importantly, the increase in CEC observed at 4h after infusion of 200 mg/kg TN-HDL was associated with parameters of plaque stabilization, supporting the idea that enhancing CEC is a promising strategy to regress atherosclerosis. As future perspective, kinetic studies should be planned to support the evaluation of pharmacological effects of those HDL preparation and better characterize the dynamics of HDL particle remodeling and the generation of lipid-free apoA-I/pre-B HDL.

A variety of pharmacologic agents have been show to affect CEC in vivo. In the second part of thesis, I moved to the evaluation of CEC in overweight individuals before and following the consumption of a novel functional food. The relevance of a functional parameter such as CEC in dyslipidemia and inflammatory disorders is illustrated by the evidence that cell cholesterol efflux not only acts as a mechanism limiting intracellular cholesterol content but also is coupled with regulatory intracellular signaling in both macrophages and endothelial cells. A slight improvement of CEC was observed after 3 months of the new functional food enriched in probiotics and prebiotics consumption. Despite innovative, this study remains preliminary and the observed effects on CEC are more likely due to the synergic effects of its probiotic and probiotic components on the gut microbiota or on the absorption and metabolism of other
nutrients than to a direct effect on HDL metabolism. However, since the market of nutraceutical products is successfully spreading to address metabolic dysfunctions in the healthy population, the formulation of an ad hoc functional food that modulates HDL functionality may represent the rationale for the design of a nutraceutical aimed at the prevention of such disorders (Adorni et al. 2017).

A number of published literature demonstrated that CEC can predict the atherosclerosis extent in humans. However, this must be further confirmed in populations at different risk of CVD and various genetic background. When evaluating CEC, it is worth to take into account the concentration of HDL-C. As it was observed in the PLTP overexpression and deletion studies, changes in HDL-C levels can easily unmask changes in the cholesterol efflux capacity. In this case, the evaluation of the entire mRCT process together with the HDL particles assortment, become necessary to uncover the role of genetic variations of implicated genes on HDL metabolism. Other than CEC, the number of HDL particles may represent an important surrogate for HDL functions. HDL particle number reflects the concentration of nascent (lipid-poor, small) HDL particles, which are more active at accepting cholesterol esters and thus responsible for RCT. That is why a recent analysis of the JUPITER trial has shown that, even though HDL-C did not predict CVD risk in statin-treated patients, HDL-P did predict CVD risk in all patients (both placebo and statin-treated), even after adjusting for HDL-C levels. Furthermore, a recent work by Khera and colleagues demonstrates that CEC was associated with incident CVD in individuals on potent statin therapy but not at baseline, while HDL particle number was the strongest of all HDL-related biomarkers as an inverse predictor of incident events and biomarker of residual risk.

Other than in atherosclerosis and CVD, HDL functions have been implicated in several other diseases both as vascular and non-vascular factors, yet not so well characterized. In particular, large evidence suggests that defective cholesterol metabolism in neurons, astrocytes and microglia promotes amyloid accumulation and stimulates Aβ-driven inflammation in Alzheimer’s disease. Therefore, CEC may become a useful tool to test the hypothesis that alterations in cholesterol transport is a common feature underlying both cardiovascular and neurodegenerative disorders and is now being studied in different labs. The development and characterization of a method to evaluate the cholesterol efflux capacity of CSF from neurons, glia and astrocytes as potential metric of Alzheimer’s dementia was the aim of the last part of this thesis.

It is largely accepted that HDL-C per se does not represent a causal mediator of cardiovascular protection and thus fails as a therapeutic target. HDL CEC, taken as a metric of HDL
functionality, was recently shown to predict incident CVD and emerged as a novel target. Therefore, strategies for identifying efficacy must move beyond the idea of simply raising static HDL-C levels and towards methods of measuring the dynamics of HDL particle remodeling. To pursue in that direction, the number of assays and methodologies measuring CEC should further be harmonized among laboratories. Moreover, the functional assessment of CEC might be accompanied with the evaluation of nascent particle number and assortment to further confirm the role of HDL CEC as a reliable parameter in predicting risk of developing disease and response to therapies.


de la Llera-Moya D, Drazul-Schrader D, Asztalos BF, Cuchel M, Rader DJ, Rothblat GH. The ability to promote efflux via ABCA1 determines the capacity of serum specimens with similar high-density lipoprotein cholesterol to remove cholesterol from macrophages. Arterioscler Thromb Vasc Biol. 2010 Apr;30(4):796-801.


deGoma EM, deGoma RL, Rader DJ. Beyond high-density lipoprotein cholesterol levels evaluating high-density lipoprotein function as influenced by novel therapeutic approaches. J Am Coll Cardiol. 2008 Jun 10;51(23):2199-211.


A polygenic basis for four classical Fredrickson hyperlipoproteinemia phenotypes that are characterized by hypertriglyceridemia. Hum Mol Genet. 2009 Nov 1;18(21):4189-94


Impaired serum cholesterol efflux capacity in rheumatoid arthritis and Roheim PS Cardiovasc Dis. Rohatgi PW. Endocrine and other tissues. Endocr Rev. 2003;24:357


Rothblat GH, Phillips MC. High-density lipoprotein heterogeneity and function in reverse cholesterol transport. Curr Opin Lipidol. 2010 Jun;21(3):229-38


Skeoch S1, Bruce IN2. Atherosclerosis in rheumatoid arthritis: is it all about inflammation? Nat Rev Rheumatol. 2015 Jul;11(7):390-400


Tarling EJ, Edwards PA. Dancing with the sterols: critical roles for ABCG1, ABCA1, miRNAs, and nuclear and cell surface receptors in controlling cellular sterol homeostasis. Biochim Biophys Acta. 2012 Mar;1821(3):386-95.


Vaughan AM, Oram JF. ABCA1 and ABCG1 or ABCG4 act sequentially to remove cellular cholesterol and generate cholesterol-rich HDL. J Lipid Res. 2006 Nov;47(11):2433-43.


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